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FULL LENGTH ARTICLE

Phylogenetic characterization of two echinoid species of the southeastern Mediterranean, off Egypt

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Abstract In this study we investigated the phylogenetics of two sea urchin species, *Arbacia lixula* and *Paracentrotus lividus* from the Mediterranean Sea. Specimens were collected from the east coast of Alexandria City, Egypt. Pigmentation examination showed four sympatric color morphotypes (black, purple, reddish brown, and olive green). Mitochondrial DNA was extracted from specimens and mitochondrial cytochrome oxidase subunit I (COI) and 16S ribosomal RNA (16S) were sequenced. The results showed that all black specimens constituted the species *A. lixula*. All other colors belonged to *P. lividus*, with no apparent differentiation between color morphotypes. Moreover, *P. lividus* showed high haplotype diversity (COI; $H = 0.9500$ and 16S; $H = 0.8580$) and low values of nucleotide diversity (COI; $\pi = 0.0075$ and 16S; $\pi = 0.0049$), indicating a high degree of polymorphism within this species. This study represents the first attempt at DNA barcoding of echinoid species in the southeast Mediterranean off the Egyptian coast, and will provide a base for future phylogenetic analyses.

Introduction

Measuring marine biodiversity is one of the main tools for effective management of the resources in the Mediterranean basin (Penant et al., 2013). Within marine resources, the sea urchins (Echinodermata: Echinoidea) are keystone animals in coastal areas due to their ability to alter the composition and

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the dynamics of algal resources by their grazing (Elmasry et al., 2013). Studies on the status of echinoid populations of the southeastern Mediterranean basin off Egypt are scarce including such basic data as their phylogenetics and population structure, distribution and dynamics, and fisheries status (Elmasry et al., 2013).

Echinodermata are characterized by polychromatism. Such color polymorphism is displayed in the sea cucumber *Apostichopus japonicus* (Kanno et al., 2006), the sea star (Asteroidae) *Pisaster ochraceus* (Calderon et al., 2010), the brittle star (Ophiuroidea) *Ophiothrix spiculata* (Ebert, 1996), and in sea urchins (Echinoidea) such as *Echinometra* sp., *Paracentrotus* sp. (Calderon et al., 2010), and *Heterocentrotus mammillatus* (Ebert, 1996). Calderon et al. (2010) stated that the evolution of color morphotypes within sea urchin species may be recent, and that species with polychromatism may be on their way toward becoming new species through sympatric speciation.

In this study, we investigated two species, *Paracentrotus lividus* (Lamarck, 1816) and *Arbacia lixula* (Linnaeus, 1758). The purple sea urchin *P. lividus* inhabits the entire Mediterranean basin and the eastern Atlantic from the British Isles to Morocco (west coast of Africa), including the Macaronesian Islands (Azores, Canary, Madeira and Cape Verde Islands) (Calderon et al., 2010). Similarly, the black sea urchin *A. lixula* is also widespread, and has been reported from the Mediterranean Sea and Macaronesian Islands, as well as from the Atlantic coasts of western Africa and Brazil (Wangensteen et al., 2012).

There is a high demand for commercially harvested sea urchins in Egypt, particularly during the summer season from the part of the Egyptian local consumers, tourists, recreational divers and restaurants serving seafood and sushi. *P. lividus* is the most desirable sea urchin in Egypt, and fishing workers consider it female in gender, calling it "netaya", while the black urchin *A. lixula* (Linnaeus, 1758) is considered as non-edible male "dakkar" due to the bitterness of their gonads, which are quite inferior in their taste compared to those of *P. lividus*. Unfortunately, there are no fishery records or statistics available for these two species, or for any other Egyptian commercial echinoid species. However, the collapse of *P. lividus* populations from the eastern Mediterranean has been recently reported, and this may have occurred within the last 15 years (Yeruham et al., 2015). Therefore, there is a critical need for genetic information of Egyptian populations of echinoids to help more effectively manage and conserve populations.

Thus, the aims of this study were to obtain information on the phylogenetic variation of *P. lividus* and *A. lixula* in the southeast Mediterranean off Egypt. Additionally, we examined if phenotypic coloration of different sympatric color morphotypes of *P. lividus* could be correlated with any underlying genetic structure.

Materials and methods

Sampling of sea urchins

Sampling of *P. lividus* and *A. lixula* sea urchins from different locations off the coast of Alexandria City, Egypt, was performed between January 2013 and October 2014. Specimens were collected from eastward coastline of Alexandria City (Sidi Bishr) (Fig. 1). Specimens were collected by SCUBA diving at depths ranging between 3 and 17 m. Specimens were transported alive to the laboratory for further analyses.

DNA extraction, PCR amplification, phylogenetic analyses

Twenty individuals were selected from Sidi Bishr (16 specimens of *P. lividus* and 4 specimens of *A. lixula*), dissected and the gut and gonads from samples were preserved in absolute ethanol (99.5%). Genomic DNA was extracted from 20 individual sea urchins from ~0.1 g of gonads using a DNeasy Tissue Kit (Qiagen), following the manufacturer's protocol. Two DNA markers were used in this study. Mitochondrial cytochrome oxidase subunit I (COI) was amplified using the forward primer COIe-F 5′-ATA ATG ATA GGA GGR TTT GG-3′ and the reverse primer COIe-R 5′-GCT CGT GTR TCT ACR TCC AT-3′ (Arndt et al., 1996). 16S ribosomal RNA (16S) was amplified using the forward primer 16SA-R 5′-CGC CTG TTT ATC AAA AAC AT-3′ and the reverse primer 16SB-R 5′-GCC GGT CTG AAC TCA GAT CAC GT-3′ (Palumbi et al., 1991). PCR reactions were carried out in a 20 µl total volume containing 5–20 ng of template DNA, 0.5 µM of each primer, and 10 µl of HotStarTaq™ Master Mix (Qiagen, Tokyo, Japan), in RNase-free distilled water. The PCR conditions for both markers consisted of an initial denaturing step at 95 °C for 15 min, 35 cycles (94 °C for 1 min, 46 °C for 1 min and 72 °C for 1 min) and a final step at 72 °C for 10 min for both DNA markers. PCR product sizes were checked by gel electrophoresis on 1.5% agarose gel. The amplified products were purified with Exonuclease I and Alkaline Phosphatase Shrimp (Takara) by being incubated at 37 °C for 20 min, followed by deactivation at 83 °C for 30 min. Purified PCR products were sequenced using an ABI Prism automated sequencer at Fasmac Co., Kanagawa, Japan (http://www.fasmac.co.jp/index.html), in both in forward and reverse directions.

Phylogenetic analyses

The sequences for both sea urchin species were edited and aligned using the software Geneious version 8.1 (http://www.geneious.com; Kearse et al., 2012). Novel sequences obtained in this study were deposited in GenBank (Accession Numbers KU172482–KU172520). New sequences obtained in this study for *P. lividus* were aligned with previously reported sequences from the eastern Atlantic and western Mediterranean (16S sequences from Calderon et al., 2009; COI sequences from Duran et al., 2004), as well as outgroup sequences from *Psammechinus miliaris*. As *P. lividus* sequences were shown to form a well-supported monophyly to the exclusion of the outgroup, we subsequently generated and used unrooted trees in our analyses (without outgroup sequences) to improve their resolution. Sequence data of *A. lixula* were aligned with previous reported sequences in GenBank from the Atlantic Ocean and Mediterranean Sea (16S sequences from Chenuil et al., unpulished; COI sequences from Wangensteen et al. (2012)). Maximum likelihood (ML) and Neighbor-Joining (NJ) phylogenetic trees were constructed in MEGA 6 (Tamura et al., 2013) with 1000 bootstraps using a Tamura 3-parameter model (Tamura et al., 2013) as the best-calculated model for both markers without outgroups.

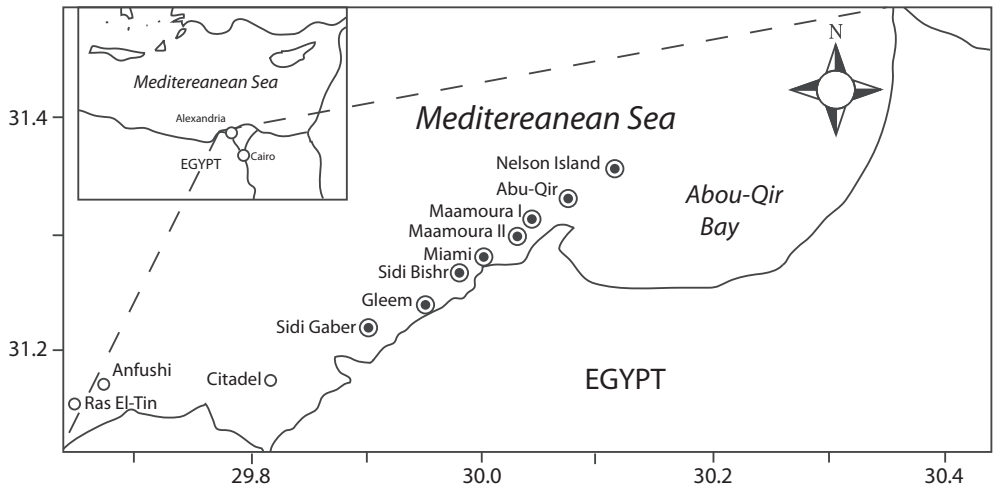

Figure 1 Map of the southeastern Mediterranean region (inset) with locations investigated in this study around Alexandria, Egypt (main map). Open circles are sites where no sea urchins were observed, while sea urchins were collected from remaining sites (filled-in circles).

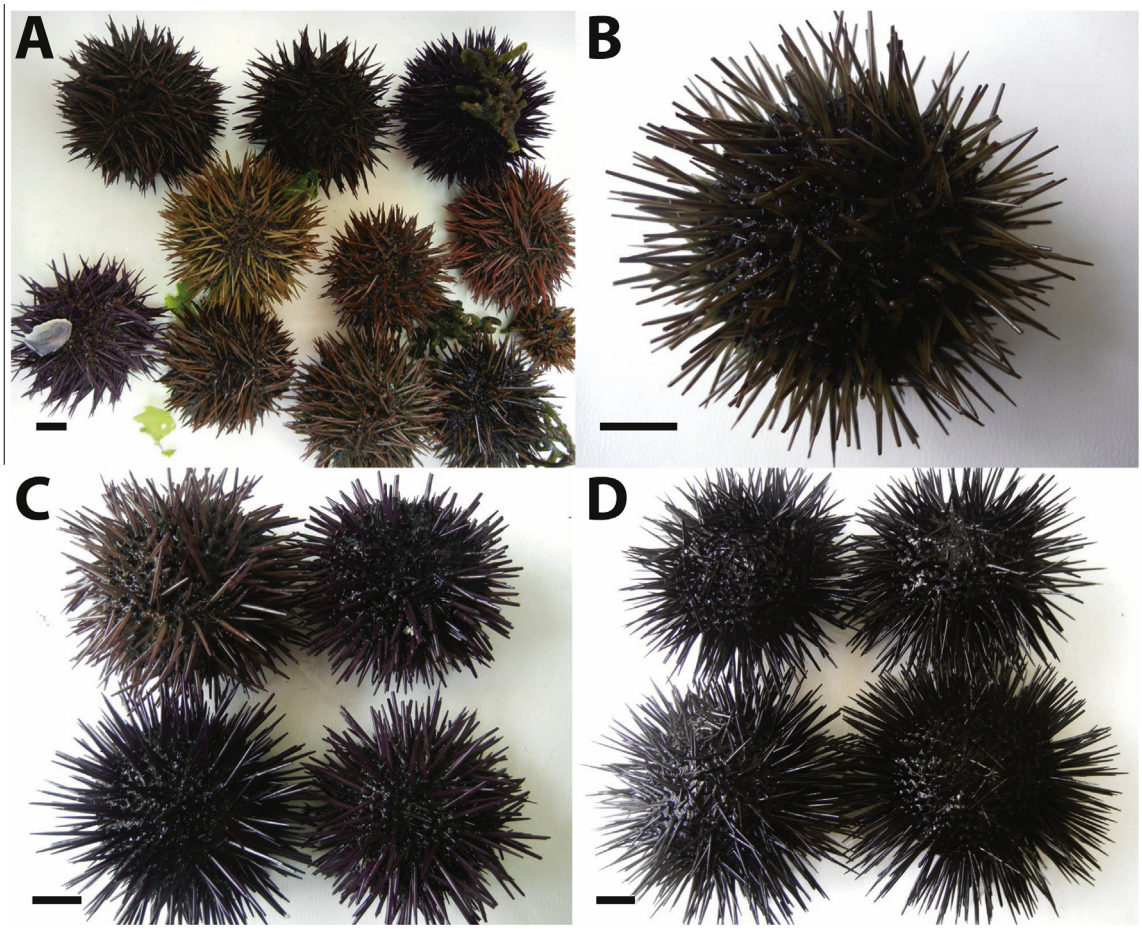

Figure 2 The two species of sea urchins investigated in this study along the coast of Alexandria, Egypt. A, B, C = *Paracentrotus lividus*, and D = black *Arbacia lixula*. A = red-brown morphotypes of *P. lividus*; B = olive-green morphotype of *P. lividus*; and C = purple morphotypes of *P. lividus*. Scale bars = 1 cm.

There are no data available for these markers in congeners of either species in GenBank. In addition, phylogenetic trees were constructed using Bayesian inference (MrBayes 3.2.2; Huelsenbeck and Ronquist, 2001), with 1,000,000 cycles of Markov Chain Monte Carlo (MCMC), 4 heated chains and a burn-in of 100,000. For *P. lividus* specimens from Egyptian

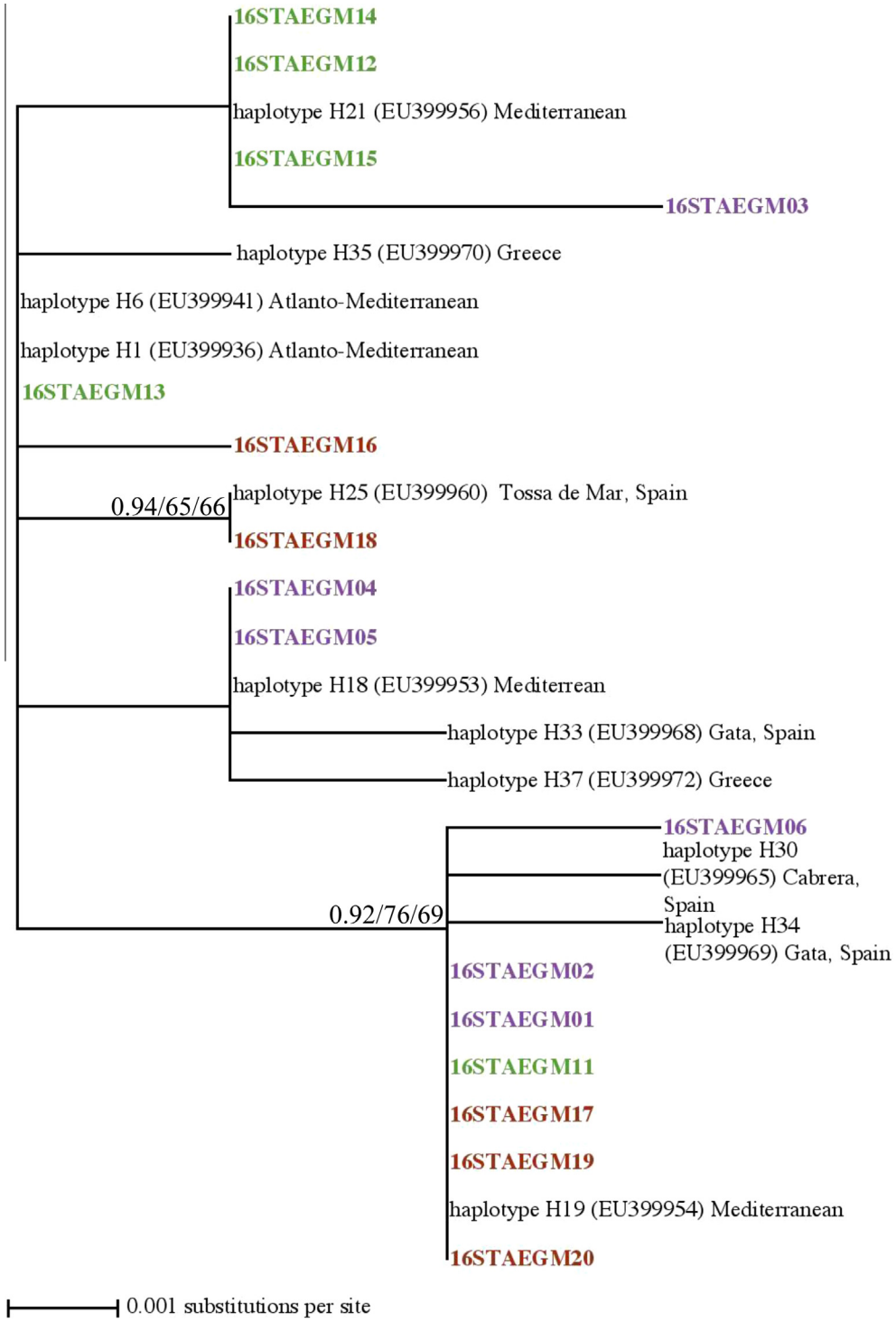

Figure 3 Unrooted maximum-likelihood (ML) phylogenetic tree generated (bootstrap values < 50% not shown) from an alignment of mitochondrial 16S sequences of *Paracentrotus lividus*. Sequences generated in this study are in bold and colored based on the morphotype of specimens, as purple, olive-green, and reddish-brown. Sequences from previous studies with haplotype number (given in original study), GenBank Accession Number, and origin(s) of specimens. Numbers at nodes represent Bayesian posterior probability values, ML bootstrap supports and neighbor-joining, respectively.

waters (n = 16), the number of haplotypes (H), haplotype diversity (Hd), and nucleated diversity (π) was estimated using DNaSP version 5.1 (Librado and Rozas, 2009).

As only 3 (COI) and 4 (16S) sequences were obtained for *A. lixula*, and there was no variation in morphology observed, we did not construct phylogenetic trees for *A. lixula* sequences,

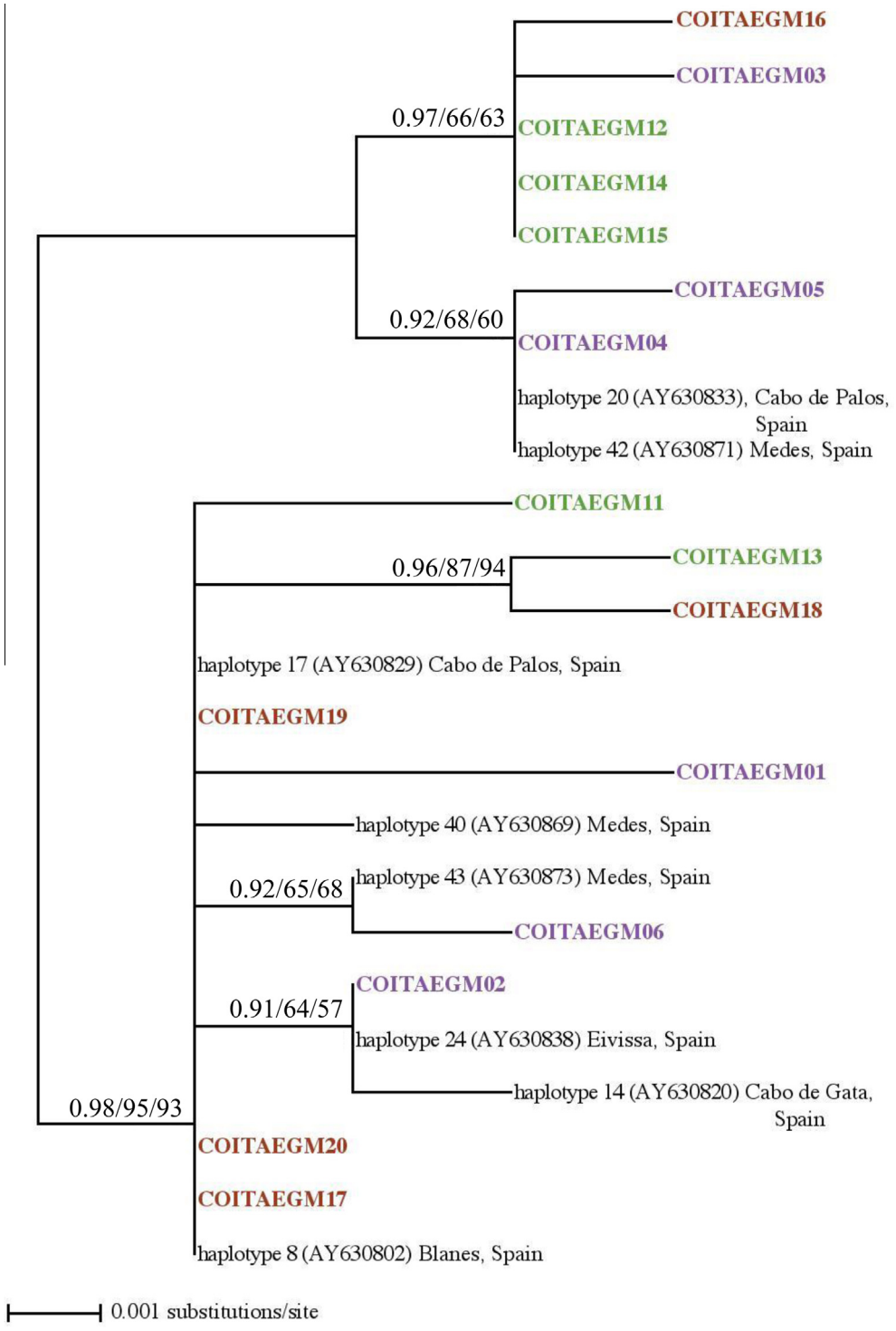

Figure 4 Unrooted maximum-likelihood phylogenetic tree generated (bootstrap values < 50 not shown) from an alignment of mitochondrial cytochrome oxidase subunit I (COI) sequences of *Paracentrotus lividus*. Sequences generated in this study are in bold and colored based on the morphotype of specimens, as purple, olive-green, and reddish-brown. Sequences from previous studies with haplotype number (given in original study), GenBank Accession Number, and origin(s) of specimens. Numbers at nodes represent Bayesian posterior probability values, ML bootstrap supports and neighbor-joining, respectively.

and simply compared sequences generated in this study with previously reported sequences in GenBank.

Results

For the COI and 16S markers, fragments of 602 bp and 515 bp, respectively, were sequenced from specimens of *P. lividus* and *A. lixula*, respectively (see Fig. 2). Alignments showed clear patterns of differences between each species. A phylogenetic tree constructed with outgroups using the maximum likelihood method showed each species as a monophyly, one clade per species (data not shown). From 16 individuals of *P. lividus* a total of 12 and 9 haplotypes were found in COI and 16S, respectively, indicating a high degree of polymorphism within this species (Figs. 3 and 4). *P. lividus* showed high values of haplotype diversity (COI; $H = 0.9500$ and 16S; $H = 0.8580$) and low values of nucleotide diversity (COI; $\pi = 0.0075$ and 16S; $\pi = 0.0049$). Genotyping results using 16S and COI sequences showed no relation between genetic differentiation and color morphotypes for *P. lividus* (Figs. 3 and 4). Sequences of both mtDNA markers of *P. lividus* matched closely or were identical to previously reported sequences (Figs. 3 and 4).

For *A. lixula*, only three and four sequences were generated using COI (602 bp) and 16S (515 bp) markers, respectively. Aligned sequences of COI ($\pi = 0.0033$) showed three haplotypes and haplotype diversity 0.9005, while 16S ($\pi = 0.0025$) showed the same numbers of haplotype and haplotypes diversity as COI. Accordingly, due to the limited number of sequences of *A. lixula*, we could not perform meaningful comparisons with previous reports.

Discussion

Recently, the attention of researchers has been drawn to temporal and spatial genetic variation in many marine organisms (Calderon et al., 2009). Harley et al. (2006) discussed the difficulty to establish patterns of genetic structure in many marine organisms that exhibit polychromatism. In some cases, color morphotypes have underlying genetic variation and may even represent separate species (Harley et al., 2006). However, in other cases, there may be no genetic distinction between phenotypic variation and genetic structure. Moreover, Harley et al. (2006) suggested that the environmental and historical factors that could affect the distribution of color morphotypes are even more challenging to distinguish.

In this study, genetic analyses of sequences of two mitochondrial markers, COI and 16S ribosomal RNA (16S), confirmed that the populations of echinoids of four color morphotypes in the study areas were composed of two species. All black specimens corresponded to the black sea urchin *A. lixula* (Wangensteen et al., 2012); while the other morphotypes belonged to the edible common purple sea urchin *P. lividus* (Lamarck, 1816). Within *P. lividus* no clear patterns were observed between phylogenies and coloration.

P. lividus populations sampled showed high values of haplotype diversity and haplotype richness, but relatively low values of nucleotide diversity. These results were similar to the work of Duran et al. (2004), who also used COI and found high haplotype diversity and low nucleotide diversity in populations of *P. lividus* in the western Mediterranean (mean $H = 0.961$ and $\pi = 0.0071$). The close match between previously reported Mediterranean *P. lividus* sequences and new sequences generated in this study confirms the assertion by Calderon et al. (2008, 2009, 2010) that populations of this species are highly connected within the Mediterranean, and also demonstrates that the Egyptian populations are apparently well-connected to both northeastern Greek populations and western Spanish populations.

It has previously been found that COI may be more efficient than sequences of either mitochondrial 16S rRNA or the nuclear ribosomal DNA region spacer ITS-2 to detect population structures on a small geographic scale in echinoids (Calderon et al., 2008; Wangensteen et al., 2012). The use of microsatellite and/or nuclear DNA markers in the future should yield considerably more accurate results than our results here with a single marker (Calderon et al., 2008).

Due to the limited number of specimens of *A. lixula*, meaningful comparisons of haplotype diversity and nucleotide diversity could not be performed. However, Wangensteen et al. (2012) examined *A. lixula* from the northwestern Mediterranean and showed that this species also has high haplotype diversity and low nucleotide diversity. More research is needed to confirm this for the *A. lixula* populations of Egypt and the southeastern Mediterranean.

Finally, further studies are needed to thoroughly assess the statuses of the populations of these two echinoid species in order to implement and accurately enforce regulatory measures to conserve endangered wild stocks of sea urchins in the southeastern Mediterranean. This is particularly important given the recent reported collapse of *P. lividus* populations in the eastern Mediterranean (Yeruham et al., 2015). As shown in other marine regions, recovery from collapse may be possible, and genetic information can help inform conservation and potential population recovery decisions (Casilagan et al., 2013). Additionally, to obtain more information on color morphotypes of sea urchins, more molecular investigations with higher resolution such as microsatellites or nDNA regions are needed to understand the diversity and evolution of sea urchins in the eastern Mediterranean in more detail.

Conclusions

The current study molecularly confirms the identity of *A. lixula* and *P. lividus* in Egyptian waters in the southeastern Mediterranean, and their close connectivity to previously reported populations in other regions of the Mediterranean. No significant phylogenetic patterns corresponded to the different observed color morphotypes of *P. lividus*. Further research is needed to understand the link between phenotypic variations in echinoid species, temporally and spatially, and their underlying genetic structure variation. Research is also needed to investigate how the genetic structure of echinoid species is affected by underlying physical and biological processes, and by anthropogenic pressures.

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