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Original Research Article

Application of RAPD-PCR in Taxonomy of Two Morphs of Genus *Patella* from the Egyptian Rocky Shores of the Mediterranean Sea

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Abstract: Adult specimens of the common edible limpet; *Patella vulgata* were collected from rocky shores of Abu-Quir, Alexandria, Egypt. There are two morphs of this species; the first morph has shell with projecting ribs from its aboral surface while, the second one with soft-surfaced shell. The authors used one of the most recent techniques; random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) to determine if these morphs represent two separate species or only one species. The current result was depending on the estimation of the genetic distance (D) that was based on the proportion of shared alleles of the two morphs. Four random primers were used and the estimated genetic distance was slightly above (0.5). According to the current result, the two morphs of *Patella vulgata* can be considered as two subspecies.
Keywords: RAPD-PCR, genetic distance, Egyptian limpets, *Patella vulgata*, Alexandria, Egypt.

INTRODUCTION

Marine limpets occurring in Egyptian shores represent a neglected animal group among molecular taxonomy or diversity. The limpets of the order patellogastropods have certain evolutionary interest since this group near to all the other living gastropods, according to morphological and molecular analysis [1-4].

Patellogastropods are considered as cosmopolitan and common among members of many marine intertidal rocky communities around the world. The extant species of patellogastspods are grouped into five families: *Lottiidae, Lepetidae, Acmaeidae, Patellidae and Nacellidae* [5]. In spite of many studies have been carried out on the anatomical, morphological and morph-metrical features of limpets, few studies using the recent molecular techniques were applied. The molecular analysis was used [6] to study the giant Mediterranean limpet; *patella ferruginea* which is an endangered marine limpet due to deep human exploitation [7].

Molecular studies were used by some authors to solve problems of the taxonomic identification and the phylogenetic relationships and also, to complement all other morphological characters. The sequence of the mitochondrial cytochrome oxidase I gene (COI) was used [8] in three species of *Patella* – with overlapping geographical distributions – that were hard to identify on the basis of shell morphology. Other researchers [9] found two forms of *Patella ferruginea* correspond to phenotypes and not to different species. They used two genes; mitochondrial COI and 16s ribosomal RNA.

Some authors [10] found four species of *Patelloida* that had been synonymized and considered as one species with two ecological forms. The mitochondrial DNA sequence and the inter-simple sequence repeat polymerase chain reaction (ISSR-PCR) were used in taxonomy of several phenotypes of genus *Nacella* [11]. The genetic gene flow by mitochondrial DNA sequence in population of the South-American Limpet; *Nacella mytilina* was also, recently investigated [12].

The authors of the present work aimed to apply one of the most recent molecular techniques (RAPD-PCR) – usually used in molecular cell biology – as an applicable analysis tool for studying the genetic variation of two morphs of *Patella vulgata* which taxonomically were considered as one species.

MATERIALS AND METHODS

Biological Material

Adult limpets of two forms of *Patella vulgata* were collected from rocky shore of Abu-Quir, Alexandria, Egypt. Specimens were placed in Absolute Alcohol for further molecular analysis in the laboratory at Zoology Department, Faculty of Science, Ain Shams

University, and Cairo, Egypt. Samples were dissected and their soft parts carefully isolated and preserved in absolute ethyl alcohol at - 20°C until use.

DNA Extraction

The preserved foot samples were cut into small pieces of about 0.5 cm in length and kept in ddH_2O in the refrigerator (5°C) overnight for rehydration. These samples were centrifuged for one minute at 1400 rpm. QIAgen Dneasy tissue kit (QIAgen, Hilden, Germany) was used to isolate and purify the DNA. The procedure used according to the protocol provided by QIAgen catalogue. This kit contained proprietary DNA purification buffers that were used for lysis (ATL and AL buffers), washing (AW1 and AW2 buffers) and elution (AE buffer). 180 µl of lysis buffer (ATL) and 20 µl of proteinase K were added to each 1.5 ml tube containing the tissue samples and vortexed for 1 min. Another 20 µl of proteinase K were added and the samples were centrifuged for 20 seconds. Then, they were incubated at 55°C for about 3 hours. Following incubation, 200 µl of (AL) lysis buffer were added and the samples were vortexed for 10 seconds. 200 µl of ethyl alcohol, were then added, and vortexed for 10 seconds. Samples were spun for 1 min, then the DNA was washed with (AW1) and (AW2) buffers; 500 µl each. Finally, 200 µl of (AE) buffer were added to elute DNA samples.

RAPD-PCR

10 primers were used in the present work but only four of them worked properly (Table 1). Amplifications were performed by modifying the protocol reported by Williams *et al.* [13]. The 25 µl mixture contained 25 ng of template DNA, 1.5 unit *Taq* Polymerase, 10mM dNTPs (4 types of deoxyribonucleotide triphosphate; dATP, dCTP, dGTP, and dTTP), 10 pM primer, and 25 µl of 10x PCR buffer. T-personal thermal cycler (Biometra; Gottingen, Germany) programmed for 45 cycles of 94°C, 35°C, and 72°C; 1 minute for each temperature. An initial denaturation step (for 3 minutes at 94°C) and a final extension holding (for 10 minutes at 72°C) were included in the first and last cycles, respectively. Reaction products (10 µl) were resolved by 2 % agarose gel electrophoresis at 85 volt in 1xTAE buffer. The gel was stained with ethidium bromide and photographed by Polaroid camera under UV trans-illuminator. From the comparison of the amplified products, population-specific fragments were detected. Two replicas per individual and primers were carried out. Only primers producing similar patterns in the two replicas were considered. The sizes of the DNA fragments of the PCR products were measured according to the DNA (1 kb) marker (Promega, GE Healthcare Bio-science, UK) which was used in the same experiment.

Calculations of the genetic distance

The present work applied the calculation according to one of the simplest estimation methods of genetic distance that was based on proportion of shared alleles [14]. For individual pairwise comparisons, the proportion of common shared alleles ($\boldsymbol{P_{SA1}}$) was estimated, as follows.

$$\boldsymbol{P_{SA1} = \Sigma u\ S\ /\ 2u}$$

i.e. The number of shared alleles (S) summed the overall loci or bands (u) divided by doubled number of total bands (2u).

Then, the genetic distance between individuals (D_{SA1}) is estimated by,

$$\boldsymbol{D_{SA1} = 1 - P_{SA1}}$$

RESULTS

In the current study, RAPD analysis was performed on samples of DNA that were extracted from two forms of *Patella vulgata* gastropod; the first morph (Fig. 1) had shell with projecting ribs from its aboral surface while, the second morph (Fig. 2) possessed soft-surfaced shell. The results of individual amplifications of DNA from both forms were analysed using the agarose gel electrophoresis (Fig. 3) with four primers; each with 2 replicas for morphs 1 and 2, respectively (Table 2). All bands were ranging between 400 and 800 base pairs (bp). Each primer of the four primers revealed two to four bands on the agarose gel with the two morphs (P1 and P2) of *Patella vulgata*. The first primer showed three bands with the first morph (P1); (580, 650, and 800 bp) and four bands with the second morph (P2); (500, 580, 650, and 750 bp). The second primer resulted in four DNA bands with P1; (500, 580, 650, and 750 bp) and three bands with P2; (640, 600, and 700 bp). The third primer gave four bands with P1; (400, 500, 600, and 700 bp) and only two bands with P2; (650, and 750 bp). The last primer produced three loci of DNA bands with both P1 and P2; (520, 600, and 700 bp) and (500, 600, and 700 bp), respectively. On comparison of the produced bands, there were all together 26 bands or loci at similar and different base pairs measured by the base pairs scale according to the DNA marker that was used in the current study. The similar loci of all bands for the two morphs of *Patella vulgata* were 22 in number, while the different DNA fragments were only four; 800 bp (primer 1, P1); 460 bp (primer 3, P2); 400 bp (primer 3, P1); and 520 bp (primer 4, P1). See (Table 2) for the values of the bands obtained and the calculation of the genetic distance between the two morphs of *Patella vulgata.* The estimated genetic distance (D_{SA1}) equaled 0.58 which was slightly more than (0.50), so that the two morphs of *Patella vulgata* may be two subspecies.

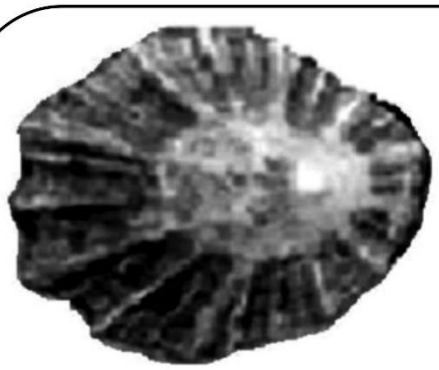

Fig. 1: Morph "1" of *Patella vulgata* showing the aboral surface of the shell with its projecting ribs.

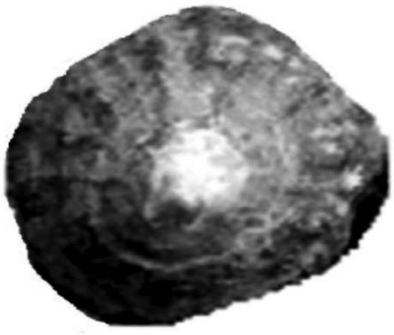

Fig. 2: Morph "2" of *P*atella *vulgata* showing the smooth aboral surface of the shell (without projecting ribs).

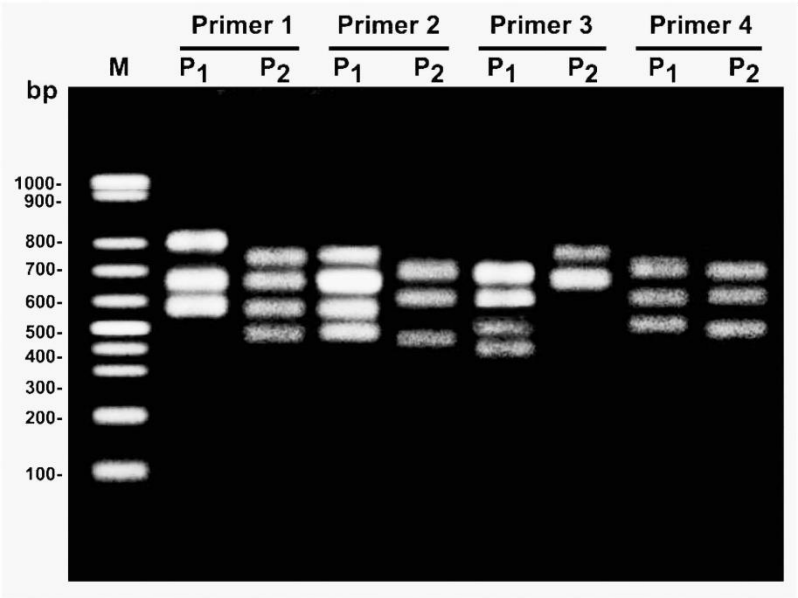

Fig. 3: A photo of agarose gel showing RAPD-PCR profiles produced from the two morphs of *Patella vulgata*, using primers 1, 2, 3 and 4, M; DNA marker, P1; Morph (1), P2; Morph (2) and bp; base pairs' scale of bands.

Table-1: The four primers (out of 10) that worked for amplification of DNA extracted from the two morphs of *Patella vulgata.*

Primer number	Primer name	Primer sequence	Mwt	GC%
Primer 1	OpA2	5`-TGCCGAGCTG-3`	3108	60
Primer 2	OpA 7	5`-ACGCATCGCA-3`	2997	60
Primer 3	OpA 9	5`-CTTCCGCAGT-3`	2979	60
Primer 4	OpA11	5`-GACGCCACA-3`	2982	70

Table-2: The values of amplified DNA bands of the two morphs of *Patella vulgata* revealing overall similar loci (S = 22) and 4 unique bands (bp*).

Primers and Morphs	base pairs (bp) of bands			
Primer 1 – Morph 1 (P1)	580 bp	650 bp	**800 bp***	--------
Primer 1 – Morph 2 (P2)	500 bp	580 bp	650 bp	750 bp
Primer 2 – Morph 1 (P1)	500 bp	580 bp	650 bp	750 bp
Primer 2 – Morph 2 (P2)	**460 bp***	600 bp	700 bp	--------
Primer 3 – Morph 1 (P1)	**400 bp***	500 bp	600 bp	700 bp
Primer 3 – Morph 2 (P2)	650 bp	750 bp	--------	--------
Primer 4 – Morph 1 (P1)	**520 bp***	600 bp	700 bp	--------
Primer 4 – Morph 2 (P2)	500 bp	600 bp	700 bp	--------
The number of shared alleles; S = 22				
The total number of loci or bands (u) = 26				
$\boldsymbol{P_{SA1}}$ **= Σu S / 2u = 22 / (2x26) = 22 / 52 = 0.42**				
$\boldsymbol{D_{SA1}}$ **= 1 –** $\boldsymbol{P_{SA1}}$ **= 1 – 0.42 = 0.58**				

DISCUSSION

ISSR-PCR molecular techniques were more useful in revealing genetic differences between closely related species of *Nacella* than mitochondrial DNA sequence [15], and great morphological than molecular evolution rates and incongruence between nuclear and mitochondrial DNA have been suggested in other gastropod groups [16]. The phenotypic plasticity has been argued as responsible for the morphological variation of limpets, supporting that morphological differences in shell shape could be environmentally determined [8].

In the present work, shell shape and its differences reflected genetic differences. Other studies, [15] considered *Nacella delicatissiina* not to be a separate species but an infrequent morphotype of both *Nacella magellanica* and *Nacella deaurata*, characterized by simple and low shell with a frequent incidence of epizoic algae; *Lithothamnia* type. Our findings proved that the two morphs of *Patella vulgata* can be considered as two separate subspecies.

Levels of mitochondrial DNA diversity in *N. mytilina* were lower than those recorded in tropical and temperate patellogastropods [17] and in other Patagonian limpets [15]. In fact, levels of genetic polymorphism in *N. mytilina* were lower than those registered in the limpet *Nacella concinna* [18]. Traditional genetic models of postglacial recolonization and refugia include the prediction that formerly ice-covered area should show low levels of genetic diversity and a small number of haplotype dominating large area [19]. While in the current study, the authors claimed that the two morphs of Mediterranean Sea; *Patella vulgata* as Middle East Fauna represented two separate subspecies and there were a significant genetic diversity between them.

CONCLUSION AND RECOMMENDATION

Application of the recent RAPD-PCR technique on the two morphs of *Patella vulgata*, gave results that each morph of them may be a separate subspecies. The authors of the present work recommended to use the most recent and applicable molecular techniques to differentiate between similar morphs but with little differences in morphology, especially with the edible living organisms that used as non-traditional food sources.

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