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ORIGINAL ARTICLE

Evolution and Pattern of connectivity in some species of the genus Patella from the Algerian coast

Zoheir BOUZAZA[1, 2], Karim MEZALI[2,]
[1]Departement of Biology, [2]Protection, Valorization of Littoral Marine Resources and Molecular Systematic Laboratory. Department of Marine Sciences and Aquaculture, Faculty of Natural and Life Sciences, Mostaganem University. BP 227, Mostaganem, Algeria.
Corresponding author: Zoheir BOUZAZA
E-Mail: zoheir.bouzaza@univ-mosta.dz

ABSTRACT
The aim of this work is to study the phylogeny and the phylogeography of three species of limpets (Patella rustica, Patella ferruginea and Patella caerulea) living in the Algerian shore to understand the pattern of connectivity between populations by explaining the causes of their differences, their distribution and their responses to environmental change using the paleontological data. After identifying our species morphologically, we performed the molecular work (DNA extraction, PCR, DNA sequencing ...) using the mitochondrial marker Cytochrome Oxydase I (COI). Around 45 sequences of 584 bp of the COI portion were treated using some software (FinchTV, BioEdit, MEGA 5…). We noted that the genetic divergence and geographic distribution among species were consistent with some paleontological changes, which could be the cause of differences of the studied species.
Keywords: *Phylogeny, Phylogeography, Mitochondrial DNA, Paleontological changes, Limpets, Algerian coast.*

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INTRODUCTION

Marine organisms challenge the most common evolutionary and speciation models as they live in an environment devoid of dispersal barriers, and often have large populations and well-developed dispersal capabilities [1]. Mollusks of the genus *Patella* are very common marine gastropods living on rocky shores of the North East Atlantic and the Mediterranean Sea [2, 3]. Four species of the genus *Patella* could be found in the Algerian coast: *Patella caerulea* (Linnaeus, 1758); *Patella ferruginea* (Gmelin, 1791); *Patella rustica* (Linnaeus, 1758); *Patella ulyssiponensis* (Gmelin, 1791) [4].

The systematic distinction between all limpet species is mainly based on the morphology of their shell. But the different evolutionary changes, the morphological plasticity of its individuals lead to a considerable profusion in species and disagreement between the authors concerning their morphological diversity and their geographical distribution [5]. However, phylogeny can go further taxonomic boundaries based on morphological distinction [6]. Several studies have been carried out on the phylogeny of limpets in the Mediterranean and the Atlantic [7-11]. In the Algerian coast, we note only few studies realized by Mezali in 2007 [12] on the phylogeny of some inviduals of *Patella ferruginea* and Bouzaza in 2010 [13] on the phylogeny of some invertebrates including limpets.

The aim of this work is to study the phylogeny and phylogeography of three Prosobranch gastropods (*Patella rustica*, *Patella ferruginea* and *Patella caerulea*) in order to understand the pattern of connectivity between their populations, explaining, through paleontological data, the causes of their differences, their distribution and their responses to environmental changes.

MATERIAL AND METHODS

Data collection

The samples were collected from 10 stations located on the Algerian coast (Fig. 1, Tab. 1) by collecting the species using a penknife and stored in 99% ethanol.

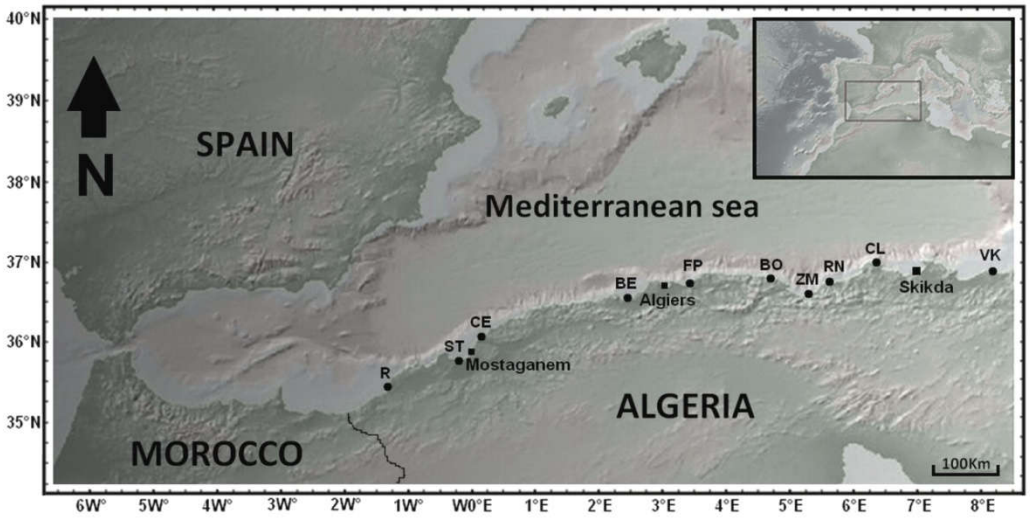


Figure 1. Map showing sampling stations of the species of limpet studied along the Algerian coast. The abbreviations of sampling stations are shown in **Table 1**. The yellow circles represent the stations where *P. rustica* was collected; the red circles represent the stations where the species *P. ferruginea* was collected and green circles represent the locations where *Patella caerulea* was collected.

Table 1. Coordinates and abbreviations of sampling stations.

Station	Abbreviation	Coordinates
Vieille Calle (El Kala)	VK	36.9° N, 8.45° E
Collo	CO	36.9° N, 6.53° E
Rocher Noir	RN	36.78° N, 5.63° E
Ziama Mensouria	ZM	36.74° N, 5.56° E
Boulimate	BJ	36.87° N, 4.84° E
Figuier Plage	FP	36.78° N, 3.53° E
Bérard	BE	36.6° N, 2.6° E
Cap Evi	CE	36.12° N, 0.23° E
Stidia	ST	35.83° N, 0° E
Rechgoun	R	35.30° N, 1.46° O

Molecular study

The DNA extraction was realized on a portion of foot muscle dissected from each individual, following the method of Botwell [14] and then used for amplification of the mitochondrial DNA portion Cytochrome Oxidase I (COI) using universal primers HCO (5'-CATGGATGACCACGACACTC-3 ') and LCO (5'GGTCAACAAATCATAAAGATATATTGG-3 ') [15]. The PCR volume was 20 µl consisting of 2 µl of DNA, 1.2 mM MgCl 2, 1.6 mM dNTPs, 0.16 µM of each primer, 0.1 unit / µl of Taq DNA polymerase, and 4 µl of loading dye and distilled water for the remainder. The PCR Thermal Protocol was a single cycle of 94 ° C / 2 min followed by 14 cycles of 94 ° C / 1 min, 58 ° C / 1 min (reducing 1 ° C / cycle until 45 ° C), 72 ° C / 1 min. This is followed by 25 cycles of 94 ° C / 30 sec, 58 ° / 45 sec, 72 ° C / 45 sec and finally one cycle of 72 ° C / 3 min for the final elongation.

Amplifiers deemed suitable are sent to the Department of Genomic Sciences of the University of Washington "High-Throughput Genomics Unit" (HTGU) for sequencing.

Data analysis

The DNA sequences were corrected using FinchTV software [16], the automatic alignment is performed with BioEdit software [17] and then manually. The program "jModeltest 0.1.1" [18] was used to find the best phylogenic model that can represent the relationship between the studied species. The phylogenic tree was realized with "Mega 5.0" software [19] using the Maximum Likelihood "ML"method [20].

The relationships between haplotypes was made using "NetWork 4.6" software [21] (available at: www.fluxus-technology.com) following the "Median Joining" method [22].
The haplotypic diversity (Hd) and nucleotide diversity (π) are estimated for each population with DNA_{SP} 5.0 software [23]. Genetic structuring are also investigated by an Analysis of Molecular Variance (AMOVA) using the software ARLEQUIN 3.52 [24]. The software DNA_{SP} 5.0 is also used to realize Tajima's D [25] and McDonald & Kreitman [26] neutrality tests.

RESULTS

We sequenced 584 bp of the COI portion of the mitochondrial DNA of 24 individuals of *Patella rustica* (Pr), 16 individuals of *Patella ferruginea* (Pf) and 4 individuals of *Patella caerulea* (Pc) (Tab. 2).

Phylogenetic and Phylogeographic study

Using "jModeltest 0.1.1" software [18], the "Tamura 3-parameter" model was considered as the most suitable with the Gamma distribution parameter "G". Using a number of bootstrap replicas of 100, the Phylogenetic tree of all sequenced species in our study was established using the "ML" method.
Sequence analysis revealed, within the individuals studied, the existence of three very strongly divergent groups as shown by the phylogenetic tree in the figure 2 based on the mean distances between individuals in pairs of populations with Log (L). = -1349.54 (L, being the likelihood). The species *Patella vulgata* (*P. vulgata*), taken from GenBank, is included as an "out group" in order to have a good topology.
The average distance between the groups of the three species studied is captured and mentioned in Figure 3.
Note that the average divergence within each group is less than 1%. Sequencing of a 584-bp long fragment of the mitochondrial COI gene revealed the existence of 94 polymorphic sites, making it possible to define 13 haplotypes on all 44 sequenced individuals. Of these 94 polymorphic sites, 98.9% of sites are parsimoniously informative and 1.1% are singletons.
Using the DNA_{SP} 5.0 program [23] we obtained average values of Hd = 0.743 and π = 0.0555. Levels of genetic diversity are shown in Table 3.
We used the "clock-relaxed" model with a log-normal distribution of the substitution rate, with two different average values [0.6% and 1.2% million year (Myr)] published for mollusks [27, 28]. The time to the most common ancestor (TMRCA) of both *P. rustica* and *P. ferruginea* could be dated from 8 to 4 Myr. However, TMRCA of *P. rustica* and *P. caerulea* could be situated between 11.08 and 5.54 Myr. Finally, the TMRCA of *P. ferruginea* and *P. caerulea* could be dated from 11.41 to 5.7 Myr.
The results of AMOVA are captured and reported in Figure 4 for a number of 584 active loci. At the inter/intra-group level our results are comparable, but at the inter-population level within each group they are not comparable.
No significant variation in nucleotide diversity (π = 0.05) and neutral evolution difference (Tajima's D = 1.69539, $P > 0.10$) is observed along the sequences.
The results of McDonald & Kreitman test are shown in Table 4 and indicate that in all three relationships; at least one of the taxa is under positive Darwinian selection without significant influence on evolution of species.
In the absence of selective effect, these features suggest a recent increase in population size. However, the representation of the minimal relations between the different haplotypes (Fig. 5) show that there are three distinct groups representing each studied taxon.
We note also that there are two undetected haplotypes mv1 (between *P. rustica* and *P. ferruginea*) and mv2 (between *P. caerulea* and *P. rustica*) (Fig. 5).

Table 2. Table showing the number of species sequenced for each study station.

Stations	*Patella rustica* (Pr)	*Patella ferruginea* (Pf)	*Patella caerulea* (Pc)
VK	6	-	1
RN	3	1	-
ZM	1	-	-
FP	5	5	-
BE	1	2	-
CE	2	4	-
ST	6	4	-
R	-	-	3

Table 3. Genetic diversity for all species of sequenced limpets. NS: number of sequences; S: number of segregation site; h: haplotype number; Hd: haplotypic diversity; π: nucleotide diversity.

Species	*NS*	*S*	*H*	*Hd*	*Π*
P. ferruginea	16	1	2	0.125	0.00021
P. rustica	24	7	7	0.504	0.00113
P. caerulea	4	2	3	0.833	0.00171

Table 4. Results of McDonald's and Kreitman's Test

Relationship between the studied species	K_N/K_S	θ_N/θ_S	G-Test	*P*-value
P. ferruginea - P. rustica	0,3	0,14	0.531	0.46
P. ferruginea - P. caerulea	0,46	0	-	-
P. rustica - P. caeulea	0,35	0,145	0.553	0.29

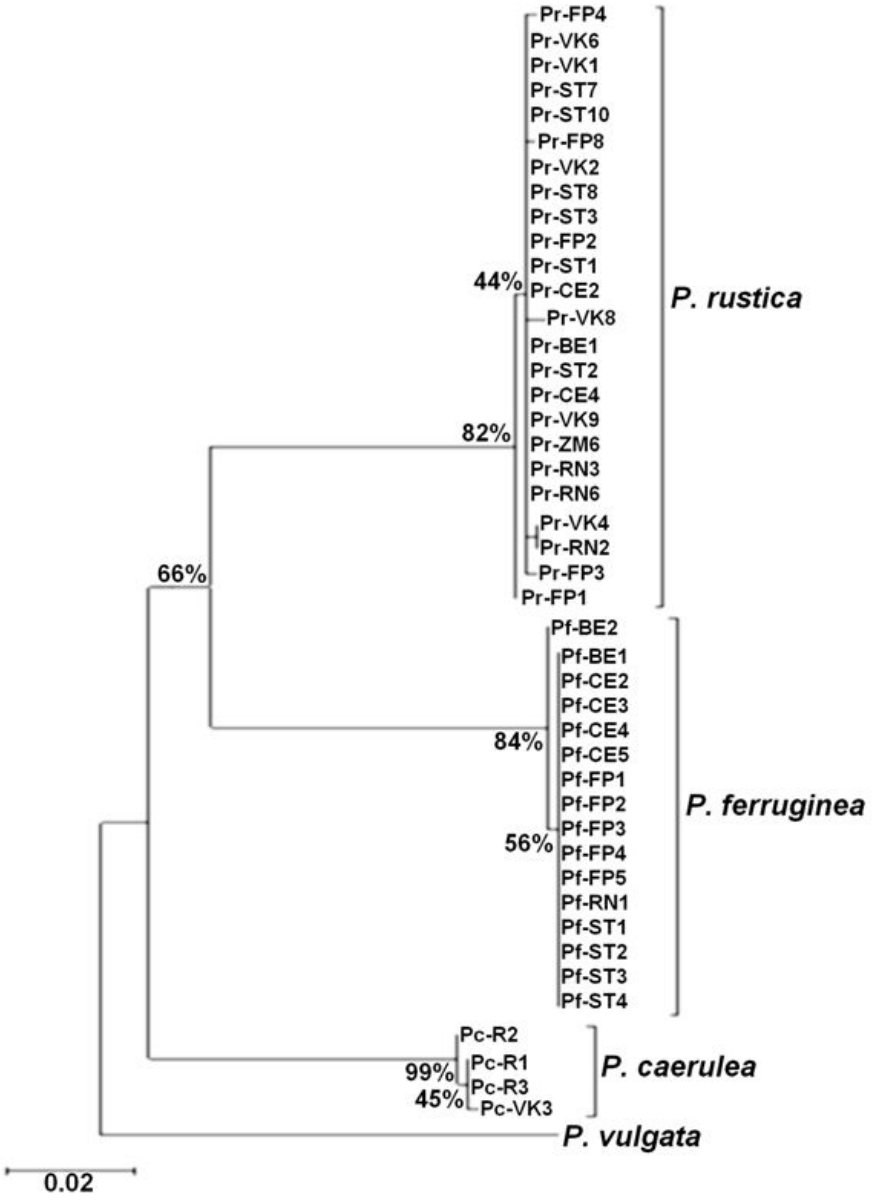
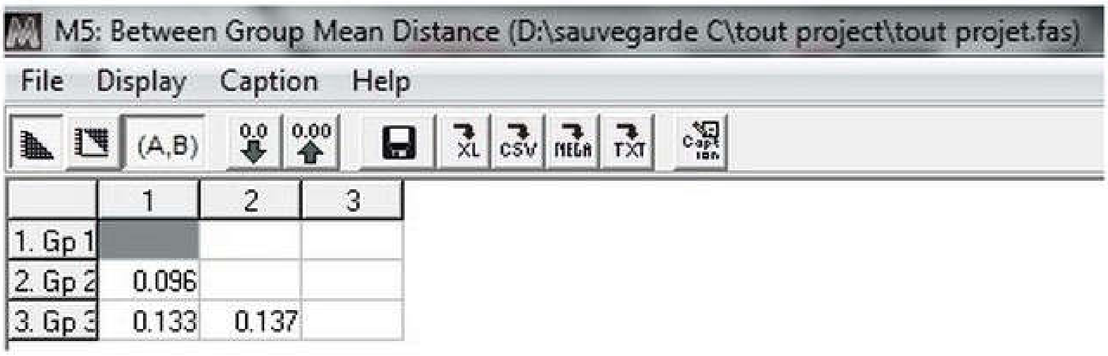


Figure 2. Phylogenetic tree of sequenced species obtained by the "ML" method with a number of bootstrap replicas of 100.



	1	2	3
1. Gp 1			
2. Gp 2	0.096		
3. Gp 3	0.133	0.137	

Figure 3. Mean distance between groups obtained by MEGA Software. G1, *P. ferrugiea* ; G2, *P. rustica* ; G3, *P. caerulea.*

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------------------------------------------------------------------------
 Source of                      Sum of       Variance         Percentage
 variation        d.f.          squares      components       of variation
------------------------------------------------------------------------
 Among
 groups             2          687.320       27.77803 Va           99.13

 Among
 populations
 within
 groups            11            2.487       -0.00834 Vb           -0.03

 Within
 populations       30            7.533        0.25111 Vc            0.90
------------------------------------------------------------------------
 Total             43          697.341       28.02081
------------------------------------------------------------------------
 Fixation Indices
      FSC :     -0.03433
      FST :      0.99104
      FCT :      0.99134
------------------------------------------------------------------------

Significance tests (1023 permutations)
------------------

Vc and FST : P(rand. value < obs. value)  =  0.00000
             P(rand. value = obs. value)  =  0.00000
                                 P-value  =  0.00000+-0.00000

Vb and FSC : P(rand. value > obs. value)  =  0.56989
             P(rand. value = obs. value)  =  0.01075
                                 P-value  =  0.58065+-0.01461

Va and FCT : P(rand. value > obs. value)  =  0.00000
             P(rand. value = obs. value)  =  0.00000
                                 P-value  =  0.00000+-0.00000
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Figure 4. The Results of the AMOVA obtained by "ARLEQUIN" v 3.52 ".

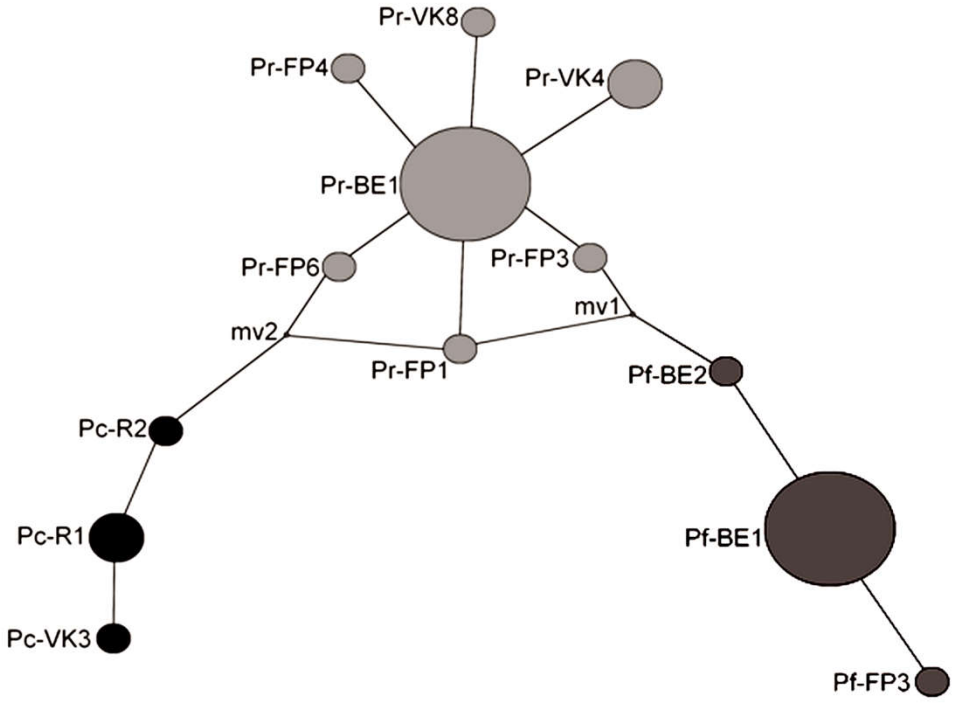

Figure 5. Haplotype networks representing the minimal relationships between the different haplotypes of the 3 species of limpets present in our study.

DISCUSSION

This study shown us that the three group studied (*P. ferruginea*, *P. rustica* and *P. caerulea*) are well separated (Fig. 2). Following the average distance between groups of the three species studied shown in figure 3, the divergence between *P. ferruginea* and *P. caerulea* group is the most important, between 11.41 and 5.7 Myr, the period is situated on the upper Miocene (between 11.608 ± 0.005 and 5.332 ± 0.005 Myr). This period also includes the period of divergence between *P. rustica* and *P. caerulea* (Fig. 3). Concerning *P. rustica* and *P. ferruginea*, the divergence is the shortest (9.6%), between 8 and 4 Myr (Fig. 3), it could have started during the Messinian by paleontological effects. The sea level in the Mediterranean had dropped to dryness, an episode that marks the Messinian salinity crisis. This period has created new biotopes with sporadic distribution, generating allopatric speciation between the Mediterranean and the Atlantic and the disappearance of many species [29]. Also, the arrival of the first periods of glaciation called Gelasian (last subdivision of the Pliocene epoch between 2,588 and 1,806 M years) which could induce a punctuated distribution inducing vicariance in relation with the studied limpets.

A possible genetic isolation began during a period of sea-level decline: a group of species could have been isolated under cold conditions while other groups could have found refuge in more temperate waters **[1]**.

This phenomenon is also reported to explain similar levels of divergence found in two genera of Northeast Atlantic polychaetes, associated with fine silted sands [**30]**. However, a genetic divergence between species close to *Pectinaria* and between the intertidal and subtidal lineages of *Owenia fusiformis* (25.9% and 19.2%, respectively) has been found as well as between two species of Polychaetes from genus *Sabellaria*, one (*S. spinulosa*) living in subtidal medium and the other (*S. alveolata*) in intertidal medium (20.6%) [31].

The Messinian crisis could be a major effect to the creation of a divergence of species *P. rustica* and *P. ferruginea* by vicariance phenomenon.

The haplotype network (Fig. 5) shows that there is a star distribution. This would explain a recent population explosion; this one could be due to the colonization of new territories after the end of the last glacial maximum (20-19 K years) which induced the increase of the level of the sea and thus the formation of the passage Atlantic waters to the Mediterranean by the Strait of Gibraltar.

The node "mv1" (Fig. 5) shows that there must be an intermediate historical taxon between *P. ferruginea* and *P. rustica*, not detected in the present work, which may have existed during the Messinian crisis. On the other hand, the node "mv2" (Fig. 5) shows that there must have been an ancestral common taxon of *P.*

caerulea and *P. rustica*. We can also see that the taxon PRFIG1 is an intermediate taxon linking *P. rustica* to the other two species by both mv1 nodes for *P. ferruginea* and mv2 for *P. caerulea*.
We can also note that the divergence between the three species was consistent with the known paleontological phenomena, which could be the cause of the divergences of the studied species.

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