

FILLING THE LIMPET GAP: MOLECULAR CHARACTERIZATION OF THE GENUS *PATELLA* (PATELLIDAE, GASTROPODA) IN THE ALGERIAN COASTS OF ORAN

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Several molecular studies have been conducted on northeastern Atlantic and Mediterranean patellid limpets, but Algerian specimens have never been included in these studies. This work intends to fill this gap and characterize populations of different *Patella* species inhabiting the Algerian coasts of Oran, clarifying the presence of *Patella ferruginea* that is considered as endangered. Moreover, comparison of their intraspecific variation with that of other areas might enlighten about populations connectivity and the conservation status of the analysed species.

The molecular analyses performed on the samples from Oran's coasts, confirmed the presence of *Patella ferruginea*, *P. rustica* and *P. caerulea*, all of which shared haplotypes with other Mediterranean localities previously analysed. The intraspecific differentiation was limited, with the exception of *P. rustica*, which showed the greatest diversity, while *P. ferruginea*, the most endangered species, showed the lowest.

Key words: population diversity, invertebrates, biodiversity, genetics, endangered species.

INTRODUCTION

Proper conservation strategies require knowing species' distribution and population differentiation to better preserve its genetic pool, i.e. the entire genetic richness of the species (WEEKS *et al.* 2016). Local adaptations can lead to a certain level of genetic differentiation, while still maintaining a cohesive general structure in a metapopulation framework. Gene flow among populations determines the level of differentiation or homogeneity. Distance between populations, habitat discontinuity, and species dispersal capabilities are some factors that can affect gene flow. Thus, knowledge of the entire distribution of a given species is necessary for better understanding their status and, consequently, ensuring their adequate protection and management.

In the case of western Mediterranean patellids, until more recently, little was known of their taxonomic status and distribution along the Algerian coast with the exception of PALLARY (1900), who first cited the presence of

patellids in the area. Since then, FRENKIEL (1975), FRENKIEL and MOUËZA (1982), SEMROUD and BOUMAZA (1998), BOUMAZA and SEMROUD (2001), KALLOUCHE *et al.* (2011, 2012, 2014a), and BELDI *et al.* (2012) studied different aspect about their ecology and biology, while MAATALLAH and DJEBAR (2014) performed ecotoxicological analyses. However, molecular phylogenetic studies of patellids in this area have not been conducted.

Limpets are abundant and familiar inhabitants of intertidal rocky shores worldwide from tropical to polar regions, playing important roles in littoral marine ecosystems (BRANCH 1985). Limpets of the genus *Patella* Linnaeus, 1758 are widespread along the north-eastern Atlantic intertidal rocky shores and have been extensively studied in terms of population dynamics, ecology, and phylogeography (e.g., LEWIS & BOWMAN 1975, SÀ-PINTO *et al.* 2005, CASU *et al.* 2006, CABRAL 2007, RIVERA-INGRAHAM *et al.* 2011a, COSSU *et al.* 2015, 2017). Patellid species have similar life cycles with a short planktonic larval stage during which current-driven dispersal occurs. Because their supposedly limited dispersal ability, and therefore theoretical small-scale differentiation, population level genetic analyses are required to understand phylogeographic and microevolutionary processes. Assessing genetic structure among populations along the species range and across boundaries is key for understanding the mechanisms that shape a species' current distribution. Moreover, they may serve as a model for addressing specific conservation-related questions as some limpet species are included in red lists and others have been massively overexploited. Currently 12 valid species are recognized along the Mediterranean and north-eastern Atlantic coasts: *Patella aspera* Röding, 1798, *P. caerulea* Linnaeus, 1758, *P. candei* d'Orbigny, 1840, *P. depressa* Pennant, 1777, *P. ferruginea* Gmelin, 1791, *P. lugubris* Gmelin, 1791, *P. natalensis* Krauss, 1848, *P. pellucida* Linnaeus, 1758, *P. piperata* Gould, 1846, *P. rustica* Linnaeus, 1758, *P. ulyssiponensis* Gmelin, 1791, and *P. vulgata* Linnaeus, 1758) (GOFAS 2015). However, a recent study (MMONWA *et al.* 2017) undoubtedly identified *P. natalensis* as belonging to the genus *Scutellastra* H. Adams et A. Adams, 1854. Moreover, SÀ-PINTO *et al.* (2005, 2008, 2010, 2012) proposed to reconsider the species *P. orientalis* (Pallary, 1938), before synonymised as *P. rustica*, following the genetic differentiation found between these two species.

In the Mediterranean Sea, the genus *Patella* is represented by four species: *P. caerulea*, *P. ulyssiponensis*, *P. rustica*, and *P. ferruginea* (SELLA *et al.* 1993, MAURO *et al.* 2003, TEMPLADO 2011). Additionally, SÀ-PINTO *et al.* (2010) suggested that one of the three differentiated *P. rustica* lineages they found around the Adriatic and Aegean seas could represent a cryptic species: *P. orientalis*. While *P. ferruginea* is restricted to the southwestern part of the Mediterranean, the other species are distributed and commonly found throughout the entire basin. These species occur sympatrically along the Mediterranean rocky shores, but inhabit different vertical zones. *Patella rustica* inhabits the upper intertidal

zone, while *P. caerulea* and *P. ulyssiponensis* inhabit the lower zone on sheltered rocks or rocks exposed to wave action, respectively (SELLA *et al.* 1993).

Along the Algerian coasts, the presence of three species, *P. ferruginea*, *P. caerulea* and *P. rustica*, was previously cited (KALLOUCHE 2014a). Other patellids such as *P. vulgata* (KALLOUCHE 2014a), *P. intermedia* (= *P. depressa*) and *P. ulyssiponensis* (BELDI *et al.* 2012), were also reported. However, some of these specimens were probably misidentified and likely represent *P. ulyssiponensis*, since *P. vulgata* and *P. depressa* are exclusively distributed in the Atlantic Ocean.

Significant variability in limpets was previously reported (MAURO *et al.* 2003). Indeed, highly variable *Patella* haplotypes were found over short distances, while others were present over long distances (SÁ-PINTO *et al.* 2005). Therefore, the purpose of this study is to compare Algerian coastal limpets with other Mediterranean populations, assessing genetic variation and verifying whether there are local adaptations or, on the contrary, certain genetic homogeneity.

Patella ferruginea is considered endangered (BOUDOURESQUE *et al.* 1996) and is included in the annexes of endangered or threatened species of the Barcelona and Bern Conventions and in the European Habitat Directive (TEMPLADO *et al.* 2004). Thus, its disappearance from several areas (such as most mainland European coasts) has led to a variety of protection protocols that have been implemented in an effort to preserve remaining populations (TEMPLADO *et al.* 2004, GUALLART & TEMPLADO 2012, GUALLART *et al.* 2013a). Therefore, the study of this species is particularly important. Although currently distributed mainly along the coast of North Africa, from north-eastern Morocco to Algeria and Tunisia, some populations remain in Corsica (LABOREL-DEGUEN & LABOREL 1991), Sardinia (DONEDDU & MANUNZA 1992, PORCHEDDU & MILELLA 1991), and the southern coast of Spain (ESPINOSA 2006, GUALLART & TEMPLADO 2012). As a broadcast spawner, the patchy distribution and small effective population size of *P. ferruginea* will likely lead to poor local reproductive success (RIVERA-INGRAHAM *et al.* 2011b, GUALLART *et al.* 2013b). A patchy distribution combined with a lack of contact among populations and continued pressure from human activities are certain to impact local survival and genetic diversity (MACHORDOM *et al.* 2010). Thus, knowledge of a given species' exact distribution and population status is essential to properly undertake conservation and management measures.

Thus, our overall aim is to molecularly characterize Algerian *Patella* populations and examine potential population differentiation, thus providing more molecular data of the species inhabiting the Maghrebian coasts and contributing to the successful management of such species, particularly of the critically endangered *P. ferruginea*. Hence, we combined phylogenetic and phylogeographic tools to better characterise the genetic structure of *Patella* populations and the potential factors determining the patterns (or lack of) gene flow among populations.

MATERIAL AND METHODS

Sampling and data collection

Samples were collected in three sites (Madagh II, Oran Port and Kristel), with the corresponding permission from western Algeria (Fig. 1) along the Oran and Ain Temouchent coasts. To avoid populations disturbance, and taking into account the endangered condition of some of them, a minimum number of individuals were sacrificed and included in the collections of our institutions, as references. Samples were morphologically identified, some measures taken and external features recorded, mostly for the differentiation of the two previously described forms of *Patella ferruginea*: "lamarcki" and "rouxi" (PAYRAUDEAU 1826). Unfortunately, none of the sampled specimens were identified as *P. ulyssiponensis*. Thus, a total of 28 specimens were collected and classified as 6 *P. caerulea*, 8 *P. rustica*, and 14 of both forms of *P. ferruginea* (Table 1).

Additionally, 82 mitochondrial cytochrome oxidase subunit I (COI) sequences available from GenBank were included in our dataset. For each *Patella* species haplotype pre-

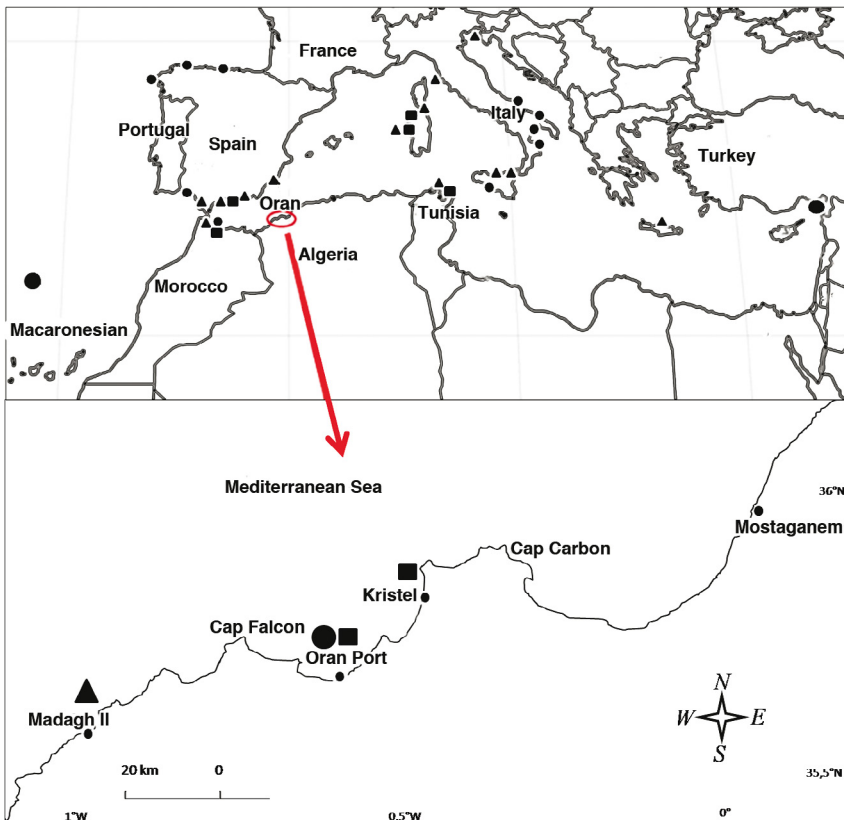


Fig. 1. Map of the geographic locations of the *Patella* sequences downloaded from the GenBank and the studied zone (western coast of Algeria). Legend: ▲ = *P. caerulea*, ■ = *P. ferruginea*, ● = *P. rustica*

Table 1. Localization and GenBank accession numbers of *Patella* species collected from Algeria.

Region	Localization	Species	Reference	GenBank acc. number
Madagh II	35.632936N, 1.071474W	<i>P. caerulea</i>	Pc1	KX792528
			Pc2	KX792529
			Pc3	KX792530
			Pc4	KX792531
			Pc5	KX792532
			Pc6	KX792533
Oran Port	35.42811 N, 0.393591W	<i>P. rustica</i>	Prus1	KX792534
			Prus2	KX792535
			Prus3	KX792536
			Prus4	KX792537
			Prus5	KX792538
			Prus6	KX792539
			Prus7	KX792540
			Prus8	KX792541
		<i>P. ferruginea</i>	Pf537	KX792542
			Pf539	KX792543
			Pf541	KX792544
			Pf545	KX792545
			Pf550	KX792546
			Pf562	KX792547
Kristel	35.877224N, 0.296423W	<i>P. ferruginea</i>	Pf569	KX792548
			Pf504	KX792549
			Pf505	KX792550
			Pf506	KX792551
			Pf507	KX792552
			Pf508	KX792553
			Pf509	KX792554
Pf510	KX792555			

sent in the Mediterranean and close Atlantic, only one specimen was selected. Table 1 lists the Algerian specimens; Table 2 lists the additional limpet specimens analysed.

For the phylogenetic reconstruction, *Cymbula safiana* (Lamarck, 1819) was selected as an outgroup. This limpet was previously considered as belonging to *Patella* but, based on molecular analysis, is now included in a different genus of the family Patellidae (SÁ-PINTO *et al.* 2005, NAKANO & OZAWA 2007).

Table 2. Additional COI sequences included in the final data matrix, and areas where identical haplotypes were found.

Species	# GenBank	Author	Localization	Other localities
<i>Cymbula saffiana</i>	AB445098	Espinosa <i>et al.</i> (2010)	Estepona (Spain)	
	DQ089596	Sá-Pinto <i>et al.</i> (2005)	Macaronesian Is.	
	DQ089599		Canary Is. (Spain)	Selvagem, Madeira (Portugal), Canary Is. (Spain)
<i>P. caerulea</i>	EU073932	Sá-Pinto <i>et al.</i> (2008)	Madeira (Portugal)	
	AJ291549	Mauro <i>et al.</i> (2003)	Gallo cape (Italy)	
	AJ291552		Ustica Is. (Italy)	
	AY996045	Espinosa and Ozawa (2006)	Alboran West (Hap 2) (Spain)	Malaga (Spain)
	AY996046		Alboran West (Hap 3) (Spain)	Ceuta, Melilla, Alboran Is. (Spain), Gibraltar
	AY996047		Alboran West (Hap 4) (Spain)	Chafarinas (Spain)
	DQ089583	Sá-Pinto <i>et al.</i> (2005)	Bizert (Tunisia)	
	DQ089584		Bizert (Tunisia)	
	DQ089585		Valencia (Spain)	Ceuta (Spain)
	DQ089586		Bizert (Tunisia)	Valencia, Ceuta, Alboran, Melilla (Spain), Gibraltar
	DQ089587		Vrysi (Greece)	
	GQ469862	Casu <i>et al.</i> (2010)	Ceuta (Spain)	
	GQ469863		Cadiz (Spain)	
	GQ469865		Argentiera, N.W. Sardinia (Italy)	Carloforte, S.W. Sardinia (Italy), Melilla, Alboran, Ceuta, Valencia (Spain), Bizert (Tunisia).
	GQ469866		Costa Paradiso, N. Sardinia (Italy)	Molaroto, N.E. Sardinia (Italy)
	GQ469868		Punta Ala, Tuscany (Italy)	
	GQ469869		Trieste, Nord Adriatic Sea (Italy)	
	JN105785	Sanna <i>et al.</i> (2012)	Aghero, W. Sardinia (Italy)	Valencia (Spain), Bizert (Tunisia)

Table 2. (continued)

Species	# GenBank	Author	Localization	Other localities
<i>P. canidae</i>	DQ089564	Sá-Pinto <i>et al.</i> (2005)	Azores (Portugal)	Sao Jorge, Santa Maria, Sao Miguel (Portugal)
	DQ089567		Desertas, Madeira (Portugal)	
	DQ089573		Selvagem Grande, Madeira (Portugal)	La Gomera, Canary Is. (Spain)
<i>P. depressa</i>	EU073864	Sá-Pinto <i>et al.</i> (2008)	Azores (Portugal)	
	EU073875		Madeira (Portugal)	
	JN105797	Sanna <i>et al.</i> (2012)	Las Galletas, Tenerife (Spain)	Canary Is. (Spain), Selvagem, Madeira (Portugal)
	DQ089614	Sá-Pinto <i>et al.</i> (2005)	Vila Nova de Milfontes (Portugal)	Asturias (Spain)
	DQ089615		Moledo (Portugal)	
	JF937113	Muñoz-Colmenero <i>et al.</i> (2012)	Cuevas Del Mar, Asturias (Spain)	San Pedro (Spain)
<i>P. ferruginea</i>	JF937120		La Atalaya, Asturias (Spain)	
	AY996039	Espinosa and Ozawa (2006)	Alboran West (Hap 2) (Spain)	Chafarinas and Alboran Is. (Spain)
	AY996040		Alboran West (Hap 3) (Spain)	Chafarinas (Spain)
	AY996041		Alboran West (Hap 4) (Spain)	Gibraltar
	AY996042		Alboran West (Hap 5) (Spain)	Ceuta (Spain)
	AY996043		Alboran West (Hap 6) (Spain)	Ceuta (Spain)
	DQ089622	Sá-Pinto <i>et al.</i> (2005)	Estopena (Spain)	
	DQ089623		Korbus (Tunisia)	Ceuta, Melilla, Chafarinas (Spain), Gibraltar
<i>P. lugubris</i>	HQ639201	Casu <i>et al.</i> (2011)	Mal di Ventre (Italy)	
	HQ639202		Argentiera (Italy)	
	HQ639203		Coscia di Donna (Italy)	
	DQ089577	Sá-Pinto <i>et al.</i> (2005)	Fogo Is. (Cape Verde)	
	EU073889	Sá-Pinto <i>et al.</i> (2008)	Fogo Is. (Cape Verde)	

Table 2. (continued)

Species	# GenBank	Author	Localization	Other localities
<i>Patella orientalis</i>	GU205463	Sá-Pinto <i>et al.</i> (2010)	Santa Maria di Leuca (Italy)	
	GU205464		Santa Maria di Leuca (Italy)	
	GU205465		Taranto (Italy)	
	GU205466		Crete (Greece)	
	GU205467		Savelletri (Italy)	
	GU205468		Oludeniz (Turkey)	
	GU205469		Crete (Greece)	
	DQ089620	Sá-Pinto <i>et al.</i> (2005)	Sampaio (Portugal)	
	DQ089621		Sampaio (Portugal)	
<i>P. rustica</i>	AJ291540	Mauro <i>et al.</i> (2003)	Sicily (Italy)	
	AJ291541		Sicily (Italy)	
	AJ291542		Sicily (Italy)	
	AJ291543		Sicily (Italy)	
	AJ291544		Sicily (Italy)	
	DQ089610	Sá-Pinto <i>et al.</i> (2005)	Desertas, Madeira (Portugal)	
	DQ089612		Selvagem, Madeira (Portugal)	
DQ089624		Samandag (Turkey)		
DQ089627		Samandag (Turkey)		
GQ469878	Casu <i>et al.</i> (2010)	Chorillo, Ceuta (Spain)	Punta la Cruz-Asturias (Spain)	
GU205455	Sá-Pinto <i>et al.</i> (2010)	Eastern Mediterranean Basin		
HF547502	Sá-Pinto <i>et al.</i> (2012)	Savelletri (Italy)	Peschici, Crotone, Taranto (Italy)	
HM120720	Ribeiro <i>et al.</i> (2010)	Asturian and Basque coast (Spain)	NW and SW Iberian coast	
HM120721		Asturian and Basque coast (Spain)	NW and SW Iberian coast	

Table 2. (continued)

Species	# GenBank	Author	Localization	Other localities
<i>P. rustica</i>	HM120724		Asturian and Basque coast (Spain)	NW and SW Iberian coast and Porcia (Spain)
	HM120730		Asturian and Basque coast (Spain)	NW and SW Iberian coast
<i>P. ulysipponensis</i>	JF937191	Muñoz-Colmenero <i>et al.</i> (2012)	Porcia, Asturias (Spain)	
	DQ089588	Sá-Pinto <i>et al.</i> (2005)	Castelejo (Portugal)	
	DQ089593		Vrisy (Greece)	
	GQ469887	Casu <i>et al.</i> (2010)	Molarotto Is., SW Sardinia (Italy)	
	GQ469889		Cres Is. (Croatia)	Argentiera, NW Sardinia, Punta Ala, Tuscany (Italy)
	HF547537	Sá-Pinto <i>et al.</i> (2012)	Sao Juliao (Portugal)	San Juliao, Castelejo (Portugal), Galicia (Spain)
<i>P. vulgata</i>	HF547554		Estepona (Spain)	Tarifa (Spain)
	HF547556		Estepona (Spain)	Iberian Peninsula (Atlantic)
	HF547585		Valencia (Spain)	
	AB238580	Nakano and Ozawa (2007)	Millport (UK)	
	JF937162	Muñoz-Colmenero <i>et al.</i> (2012)	La Franca, Asturias (Spain)	
	JF937165		Gijón, Asturias (Spain)	
	JN105837	Sanna <i>et al.</i> (2012)	Fort du Dellec, Plouzané (France)	Figueras (Spain), Cumbre Is. (UK)

DNA extraction and PCR amplification

Total DNA was extracted from pieces of foot muscle preserved in absolute ethanol. After digesting the tissue with proteinase K overnight, genomic extraction was performed using the DNeasy kit (Qiagen), following the manufacturer's protocol.

The polymerase chain reaction (PCR), carried out in a total volume of 50 μ l, contained 2 μ l of a 1:20 dilution of total genomic DNA, 1.25 U of Taq DNA Polymerase (Biotools), 1 \times reaction buffer, 2 mM of MgCl₂, 0.16 μ M of both LCO1490 and COI-H primers (FOLMER *et al.* 1994, MACHORDOM *et al.* 2003), and 0.2 mM of a dNTPs mix.

PCR amplifications were conducted in thermal cyclers under the following conditions: initial denaturation at 94 °C for 4 minutes, followed by 40 cycles at 94 °C for 45 seconds, 48 °C for 1 minute, and 72 °C for 1 minute. A final extension at 72 °C for 10 minutes was performed prior to cooling to 10 °C.

Both positive and negative controls were included in all amplifications. To verify the positive amplification and amplicon size, PCR products were run on 0.8% agarose gels (TBE buffer) at 90 mV for 50 minutes. PCR products were purified by ethanol precipitation. The final BigDye Terminator reaction and sequencing of both strands were performed by the SECUGEN service (Spain).

Data analysis

Cytochrome c oxidase subunit I (COI) sequences were trimmed to 658 base pairs fragments (removing primer ends); no gap was necessary for the alignment. All new haplotypes were submitted to GenBank (accession numbers KX792528 to KX792555). The matrix obtained was combined with the data downloaded from GenBank (Table 2).

To verify the species and lineage adscription of the samples collected, phylogenetic reconstructions were developed with the specimens here sequenced together with the GenBank haplotype references. Maximum parsimony (MP; PAUP v. 4.0a, SWOFFORD 2002), maximum likelihood (ML; PHYML v. 3.0, GUINDON *et al.* 2010) and Bayesian inference (BI; MrBayes v.3.2, HUELSENBECK & RONQUIST 2001, RONQUIST & HUELSENBECK 2003) methods were used to test the phylogenetic relationships of sequenced samples. To test the support of MP and ML results, 1000 bootstrap pseudoreplicates were performed. For the BI analysis, two parallel runs of 2 \times 10⁶ repetitions were done, sampling one in every 1000 trees and discarding the first 25% as burn-in; node support was evaluated by posterior probabilities.

Haplotype networks for *P. caerulea*, *P. rustica*, and *P. ferruginea* were analysed independently with Haplotype Viewer (<http://www.cibiv.at/~greg/haploviewer>), to situate the Algerian haplotypes among the biogeographical variation of each species.

RESULTS

Phylogenetic lineages recognition

The phylogenetic tree obtained (Fig. 2) shows four main clusters. The first one included specimens belonging to *P. caerulea*, *P. candei*, *P. lugubris*, and *P. depressa*. However, some samples considered as *P. candei* were differentiated

from the main group and clustered as the sister group of *P. lugubris*, instead of with the other conspecific samples. Algerian and Mediterranean samples of *P. caerulea* clustered together, showing no preferential phylogenetic association among samples from the southwestern Mediterranean Sea (Bizert, Oran, Gibraltar), Greece, Sardinia or Spanish and Italian coasts.

Another cluster consisted of haplotypes corresponding to *P. aspera*, *P. vulgata*, and *P. ulyssiponensis*. However, similar to the case of *P. candei*, *P. aspera* haplotypes did not group all together: one haplotype grouped with *P. vulgata*, while the others grouped with some *P. ulyssiponensis* specimens.

A third cluster contained haplotypes of *P. ferruginea*, *P. rustica* and *P. orientalis*. Within the clade of *P. rustica*, no apparent phylogeographic structure has been recovered, even between Atlantic and Mediterranean (eastern or western) haplotypes. *Patella ferruginea* specimens clustered in a well-supported monophyletic assemblage in which the haplotypes showed few differences. The Algerian specimens were included within this group. The specimens here named as *P. orientalis*, according to SÀ-PINTO *et al.* (2005, 2008, 2010, 2012), previously considered as *P. rustica*, formed a well differentiated sub-

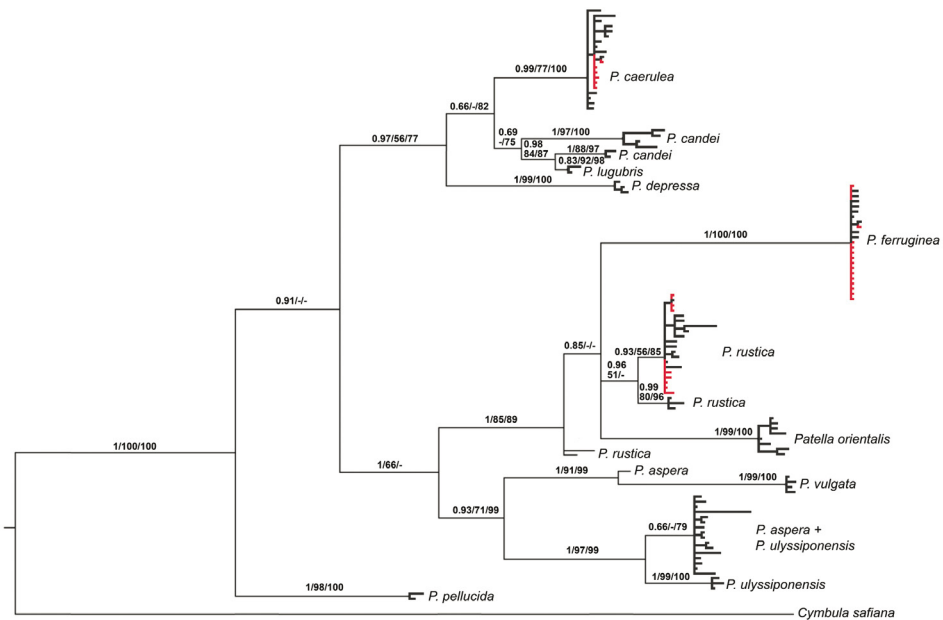


Fig. 2. Phylogenetic tree based on Bayesian inference of *Patella* species. Numbers on branches represent posterior probabilities based on Bayesian inference analysis and bootstrap values based on maximum parsimony and maximum likelihood analyses, respectively. Red branches represent Algerian samples

Table 3. Intra- (in bold) and interspecific mean uncorrected distances (in percentage) based on COI sequences.

	<i>P. aspera</i>	<i>P. caerulea</i>	<i>P. candei</i>	<i>P. depressa</i>	<i>P. ferruginea</i>	<i>P. lugubris</i>	<i>P. pellucida</i>	<i>P. rustica</i>	<i>P. ulyssiponensis</i>	<i>P. vulgata</i>	<i>C. safiانا</i>
<i>P. aspera</i>	2.57										
<i>P. caerulea</i>	11.32	0.46									
<i>P. candei</i>	11.29	7.01	4.19								
<i>P. depressa</i>	11.81	8.94	8.32	0.23							
<i>P. ferruginea</i>	11.89	11.87	11.89	11.28	0.18						
<i>P. lugubris</i>	11.19	5.41	4.34	8.00	11.09	0.34					
<i>P. pellucida</i>	11.01	11.22	12.11	11.87	11.51	11.12	0.50				
<i>P. rustica</i>	11.07	12.11	12.01	10.51	9.01	11.12	12.65	1.99			
<i>P. ulyssiponensis</i>	2.20	11.52	11.31	11.54	11.43	11.22	10.94	10.84	1.91		
<i>P. vulgata</i>	12.08	13.50	13.10	13.78	12.79	13.15	13.81	13.02	12.40	0.31	
<i>P. orientalis</i>	11.87	11.97	12.39	12.15	10.47	11.96	13.12	6.73	11.85	14.00	0.93
<i>Cymbula safiانا</i>	21.37	21.28	21.92	21.33	23.42	22.48	22.57	23.00	21.27	23.04	22.03

cluster respect to *P. rustica*. Furthermore, the phylogenetic relationship between *P. ferruginea*, *P. rustica*, and *P. orientalis* could not be resolved with high support.

A final main cluster, consisting of representatives of *P. pellucida*, was observed in a basal position relative to the other *Patella* species.

Intra- and interspecific distances

To better classify the Algerian samples within species or lineages, intra- and interspecific divergences were calculated. Intraspecific divergences reached values up to 4.19% for *P. candei* or 2.57% for *P. aspera*; in contrast, *P. caerulea* and *P. vulgata* had values between 0.3 and 0.5% (Table 3).

The lowest interspecific value found was 2.2% between *P. aspera* and *P. ulyssiponensis*. The other interspecific distances ranged from 4.34% (*P. candei*–*P. lugubris*) to 14% (*P. vulgata*–*P. orientalis*). However, a clear gap in these values existed when compared to the outgroup, which showed more than 21% divergence.

When compared to the respective nominal species, the Algerian samples always showed low divergence values.

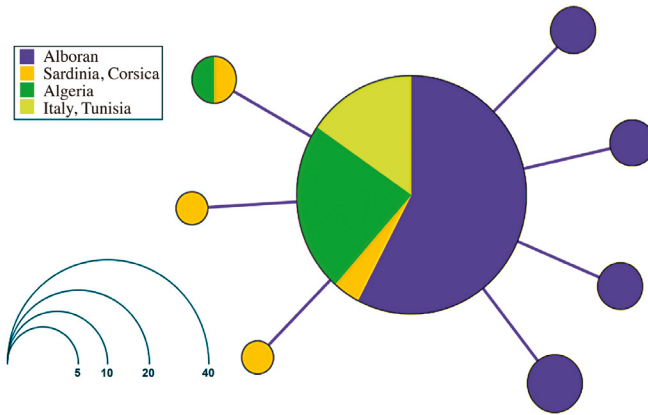


Fig. 3. *Patella ferruginea* haplotype network

Haplotypes and phylogeographic distribution

Analysis of the haplotype networks of the three *Patella* species inhabiting Algerian coasts revealed different topologies. Figure 3 shows a star-like topology for the eight *P. ferruginea* haplotypes and their frequencies. Only a single substitution differentiated the satellite haplotypes from the most frequent one, which was found in samples from all localities included in the analysis. The Algerians samples shared the most frequent haplotype.

For *P. caerulea*, which is also endemic to the Mediterranean, 31 haplotypes were recorded (Fig. 4). In this case, the most frequent haplotype was present in almost all regions, except in the eastern Mediterranean, Tunisia or Sicily. How-

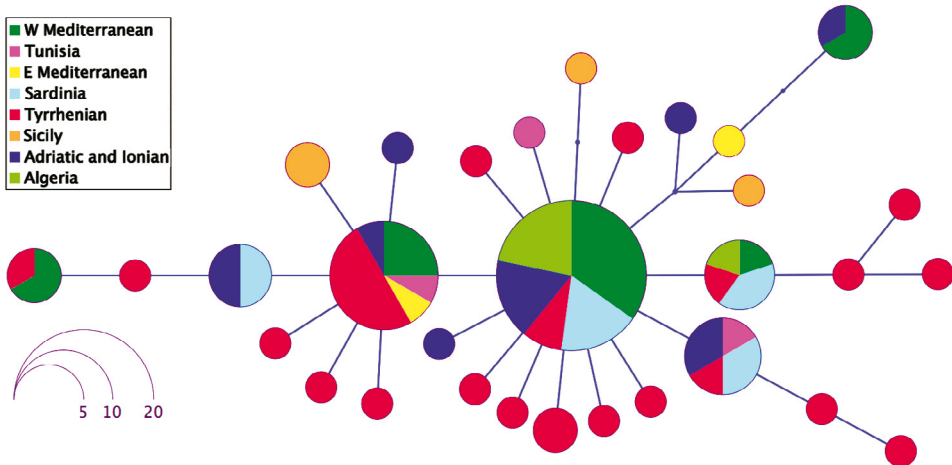


Fig. 4. *Patella caerulea* haplotype network

ever, these last localities shared the second most frequent haplotype together with samples from the western Mediterranean, and Tyrrhenian, Adriatic and Ionian Italian coasts. Differentiated by only one substitution from others but not sharing any haplotype appeared those from Sicily. Thus, except the Sicilian samples, in all the others localities shared haplotypes with at least one region were present, lacking an apparent biogeographic structure. Two different haplotypes were found for the Algerian specimens: one coincided with the most frequent haplotype, while the other was shared with specimens belonging to Sardinian, Tyrrhenian and Iberian localities.

For *P. rustica*, the haplotype network shows 64 haplotypes (Fig. 5). This network not only included the greatest number of haplotypes but also the most differentiated. The eastern and central Mediterranean and Macaronesian representatives had the highest number of substitutions compared to the rest. The Algerian haplotypes appeared in a core group, consisting of specimens primarily from Atlantic and west Mediterranean localities. In addition to the main haplotype shared by Algerian specimens, one Algerian sample has one minority haplotype in common with an Atlantic specimen. Two unique haplotypes were also detected in the Algerian samples.

DISCUSSION

Overall, our analyses of *Patella* COI haplotypes, which included both newly reported (this study) and previously obtained haplotypes (MAURO *et al.* 2003, SÁ-PINTO *et al.* 2005, 2008, 2010, 2012, ESPINOSA & OZAWA 2006, NAKANO & OZAWA 2007, BORRELL *et al.* 2010, CASU *et al.* 2010, RIBEIRO *et al.* 2010, ESPINOSA *et al.* 2010, CASU *et al.* 2011, MUÑOZ-COLMENERO *et al.* 2012, VILLAMORE *et al.* 2014) has permitted to situate the samples analysed from the Oran's coasts within the Mediterranean patellid diversity. All these samples were included

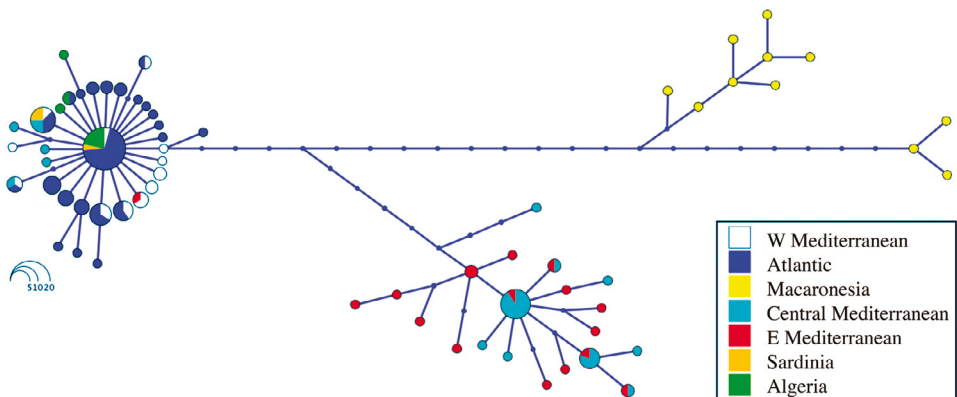


Fig. 5. *Patella rustica* haplotype network

in lineages previously defined in different phylogenetic studies (e.g. KOUFO-PANOU *et al.* 1999, SÁ-PINTO *et al.* 2005, CASU *et al.* 2010). Moreover, with the exception of the high intraspecific divergence among *P. candei* samples and the low interspecific divergence between *P. aspera* and *P. ulyssiponensis*, the three species here analysed match the principles for barcoding identification. In fact, the values found for *P. aspera* and *P. ulyssiponensis* probably reveal misidentifications of some GenBank specimens, and the high diversity inside *P. candei* could hide cryptic differentiation.

Despite these misidentifications, the presence of three *Patella* species along the Algerian coast was confirmed: *P. ferruginea*, *P. caerulea*, and *P. rustica* are distributed in the studied area. Unfortunately, no *P. ulyssiponensis* specimens were found in these localities. Moreover, in the Port of Oran dam, two of these species co-exist on the dam's artificial substrate at high densities: up to 20 individuals/square meter for *P. ferruginea* and 120 individuals/square meter for *P. rustica* (KALLOUCHE 2014a). Interestingly, the density of *P. ferruginea* in this locality, regardless of the methods used to measure it, is several times higher than that found, for instance, in Sardinian protected areas (0.02 individuals/m, COPPA *et al.* 2012) or on Alboran Island (0.06 individuals/m, PARACUELLOS *et al.* 2003). In any case, this comparison should be taken as a simple indication as differences in census methodologies can greatly bias the figures (GUALLART & TEMPLADO 2016). For instance, some studies only consider adults, while others consider all specimens, or the surface analysed could be randomly selected or chosen based on areas where a species is present in certain numbers (for a revision see GUALLART & TEMPLADO 2016).

In both the tree reconstruction and the haplotype network analysis, the Algerian specimens showed no special differentiation with respect to specimens from neighbouring areas. Samples of *P. ferruginea* grouped with the other representatives of the species in a fully-supported lineage that clustered with *P. rustica* and *P. orientalis*. Nevertheless, the relationships between *P. ferruginea* and the other two taxa were not well determined. In terms of diversity, only one haplotype, corresponding to the most frequent one, was shared among all *P. ferruginea* studied populations. Only seven other haplotypes, differing in a single substitution from the central one, were reported, resulting in a clear star-like shape. This topology usually indicates non-structured populations that suffered a selective sweep or reduction of effective size and that could currently be in expansion. Poor diversity is a general feature for this species in all its remaining populations, as is a lack of population structure, with the exception of slight differentiation within the Corsican-Sardinian populations (ESPINOSA & OZAWA, 2006, CASU *et al.* 2011, COSSU *et al.* 2017).

In contrast to *P. ferruginea*, within the *P. rustica* cluster, different lineages appeared. Moreover, the topology of the network indicates great differentiation, mostly for specimens distributed in the central and eastern Mediterra-

nean and the Macaronesian islands. In the latter case, none of the haplotypes were shared with any of the other *P. rustica* representatives. SÁ-PINTO *et al.* (2010, 2012) previously suggested the existence of different clusters in the Mediterranean and Atlantic, two of which are in the eastern Mediterranean. The Algerian samples analysed here were included in the western Mediterranean and close Atlantic waters group. *Patella rustica* appears to be the most diverse of the three species analysed, not only because of the number of haplotypes observed, but also because the Algerian samples presented unique haplotypes, while samples of the other two species always shared haplotypes with other areas.

Algerian populations of *P. caerulea* shared haplotypes with north- and southwestern Mediterranean populations. The most differentiated and diverse populations are those from Tunisia and Italy; however, they do not form a structured group as the different haplotypes are derived from the most common ones. Moreover, the Almeria-Oran Front, considered a potential gene flow barrier for patellids (SÁ-PINTO *et al.* 2012), does not appear to biogeographically limit *P. caerulea* populations dispersion or connectivity.

Despite belonging to the same area and even the same localities, the three species from the Algerian coast showed different degrees of genetic diversity and population structure. A similar number of specimens were analysed for *P. caerulea* and *P. rustica* and almost double the number for *P. ferruginea*. Even though greater sampling effort was made for the endangered *P. ferruginea*, this species presented the lowest number of haplotypes.

In the past, *P. ferruginea* populations were widely present at high densities throughout the western Mediterranean basin (LOZET & DEJEAN-ARREGROS 1977, BEAUFORT *et al.* 1987, LABOREL-DEGUEN & LABOREL 1990). Nowadays, this species is experiencing an alarming regression, having disappeared in many areas (LABOREL-DEGUEN *et al.* 1993, CULIOLI 2002). Indeed, only a few populations, which are also in decline, remain thus resulting in its status as an endangered species (TEMPLADO *et al.* 2004). Although two significantly different morphotypes have been described, *lamarcki* and *rouxi*, according to PORCHEDDU and MILELLA (1991), genetic evidences for such differentiation were not observed by ESPINOSA and OZAWA (2006), nor in this study. Dense populations of *P. ferruginea* have been reported on the Algerian coasts, especially in inaccessible or isolated places such as Plana Island, Habibas Island (ESPINOSA, 2009), and Rechgoun Island (TAIBI *et al.* 2014) and in prohibited/restricted areas (Oran Port, the cited Habibas Island, and militarized zones). Therefore, these populations have not been overconsumed or stressed, except for large specimens sacrificed for their shells for trophies, souvenirs, and decoration. However, in recent years, limpets have been increasingly used as fish bait and in some areas where fishing occurs throughout the year, we note the complete absence of limpets. Thus, even if the Algerian coasts harbour ones of

the few and more abundant *P. ferruginea* populations, our molecular analyses also show a lack of differentiation, thus indicating an impoverished situation, similar to other extant populations.

However, the contrasting results of *P. ferruginea* compared to the other two limpet species in the area (*P. caerulea* and *P. rustica*) raise questions about the factors influencing the low genetic diversity in *P. ferruginea*. CASU *et al.* (2011) hypothesized that selection could explain the patterns observed in this species. Furthermore, by analysing a large dataset that included different animal groups, BAZIN *et al.* (2006) concluded that mtDNA variation in invertebrates would not reflect population size, body size or ecology, claiming that positive selection is, in fact, acting on this putative neutral marker (GILLEPSIE 2001). Differences in the timing of the last selective sweep event could be key for explaining mitochondrial diversity (BAZIN *et al.* 2006), such that the species analysed here, despite having the same life cycle pattern and habitat, are or were subjected to different evolutionary forces. Even if these differences are reflected in the mitochondrial gene analysed (i.e. COI), complementary nuclear data analyses are necessary to better understand the processes that led to their current situations. The existence of Atlantic populations of *P. rustica* could be part of the response of the differential diversity patterns. The variation provided by gene flow with the Atlantic populations, which likely experience different phenomena than those in the Mediterranean (e.g. changes in temperature or water currents), might explain the greater diversity found in Mediterranean populations of *P. rustica*.

Abiotic factors such as temperature, surface currents, and hydrodynamics affect reproduction, occasional long-term dispersion, and oxygenation and organismal respiration in coastal areas, respectively. The three *Patella* species analysed here all share the same Mediterranean habitat, and therefore, should experience the same abiotic factors. However, biotic factors such as predation e.g. by seabirds (gulls), crabs (*Eriphia verrucosa* and *Pachygrapsus marmoratus*), or another gastropod (*Stramonita haemastoma*) (GUALLART & TEMPLADO 2012), can differentially impact the three species. According to MUÑOZ-COLMENERO *et al.* (2012), rapid climate change or natural catastrophes can also affect patterns of distribution. It is currently unknown if the temperature increase in the Mediterranean (VARGAS-YÁÑEZ *et al.* 2010) partially explains the results observed in this study. However, the reduction of *P. ferruginea* populations seems to precede such increases as the species was widely distributed until the Palaeolithic (TEMPLADO & CALVO 2004) when it started to decline.

In addition to environmental changes, the influence of anthropogenic activities should be taken into account, having played a very important role in recent decades: fishing, shell harvesting, pollution and coastal development have all caused havoc on different coastal ecosystems (KALLOUCHE *et al.* 2014b). Actually, MARRA *et al.* (2016) highlighted how human activity is detrimentally

impacting *P. ferruginea* since the best Sardinian populations were just those most inaccessible. Thus, the human mediated effect of population bottleneck cannot be neglected to explain the loss of haplotypic diversity.

The slight genetic differentiation between western and eastern Mediterranean basin samples (Fig. 2) and the lack of differentiation among western Mediterranean samples suggest that *P. caerulea* forms a very large unique population. It also suggests that Mediterranean currents provide *P. caerulea* planktonic larvae sufficient dispersion abilities to cause genetic homogeneity across the Mediterranean Sea. Unlike the life histories of other limpets, such as *P. vulgata*, *P. candei*, or *P. rustica* (CÔRTE-REAL *et al.* 1996, SÁ-PINTO *et al.* 2008), for which structured genetic variation has been observed at similar spatial scales, *P. caerulea* does not show such genetic variability.

During the Last Glacial Maximum (18,000 years ago), the sea level was about 100 m below the current mean water level. Only in the past 10,000 years has the Mediterranean flooded again, thus leading to recent colonization by certain marine flora and fauna from the Atlantic (THIEDE 1978, HEWITT 2000). Conversely, other species' distributions might be the consequence of much older events, such as the existence of refugia during the Messinian salinity crisis (CALVO *et al.* 2015).

Genetic similarities in Mediterranean *P. caerulea* populations may reflect past founder effects linked with colonization after the Pleistocene glaciations (FAUVELOT *et al.* 2009). Indeed, several studies have recently stressed the relevance of palaeoecological events in determining genetic patterns in marine populations (FAUVELOT *et al.* 2003, IMRON *et al.* 2007, VIRGILIO *et al.* 2009, WILSON 2006, FAUVELOT *et al.* 2009).

In addition to possible historical palaeogeographic signals, as FAUVELOT *et al.* (2009) suggested, gene flow is modulated by factors affecting life histories and ocean dynamics. The differences found for the three limpet species in the Oran area indicate that some factors, such as timing of gamete release, could affect dispersion and thus, the genetic structure or diversity observed in a species.

Overall, this study has verified the presence of three *Patella* species along the Oran's coasts (*P. caerulea*, *P. rustica*, and the endangered *P. ferruginea*). Although having a shared habitat, molecular and network analyses show distinct patterns of genetic structure and diversity for the different species. In the case of *P. ferruginea*, while Algerian populations do not differ from other Mediterranean and Atlantic populations, they represent ones of the few remnant populations; therefore, strict surveillance and conservation plans must to be implemented to safeguard their survival. The existence of endemic haplotypes in the Algerian *P. rustica* populations is also noteworthy. Moreover, the geographic situation of the Algerian populations in the Maghrebic coast assures the continuity of populations; for species with restricted larval dispersion, this continuity can be crucial for survival.

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Acknowledgements – Our gratitude to Dr. Templado and Dr. Buckley for greatly improving this manuscript. We thank the Algerian “Commissariat National du Littoral” for the sampling permit granted. Melinda Modrell carefully reviewed the language. This study was funded by the project of the Ministry of Economy and Competitiveness REF. CTM2014-57949-R.

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Received July 27, 2017, accepted February 27, 2018, published May 31, 2018