

DNA Barcoding and Phylogeny of Patellids from Asturias (Northern Spain)

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Abstract — The main role for genetics in marine invertebrates is the identification of species and groups of interbreeding individuals. In Asturias exists an ancient culinary tradition of consumption for limpets (*Patella* s.s.) but there is a lack of studies about these species. We have sampled Asturian marine *Patella* s.s. specimens and conducted sequencing of the mtDNA COI gene. We have confirmed the presence of four *Patella* s.s. in the Asturian coasts (*P. vulgata*, *P. depressa*, *P. aspera*, and *P. rustica*). Our work raises concerns about the current state of the *P. vulgata* populations in Asturias, where it is exploited, due to its low levels of genetic variation. Our phylogenetic analyses using Bayesian approaches confirmed that patellids belong to four different clades but gives a new version about how these clades are related inside the genus aiming for the necessity of more work to address this issue.

Index Terms — Patellids, COI gene, Bayes phylogenies, Asturias.



1 INTRODUCTION

The family *Patellidae* contains most of the common limpets on the temperate rocky shores of Europe. It contains around 37 species distributed in four morphological clades: *Helcion*, *Cymbula*, *Scutellastra* and *Patella* [1]. The phylogeny and taxonomy of this family has been revised and modified many times and it is not completely resolved yet [1], [2], [3]. Several morphological characters have been used to differentiate these species (e.g. radular morphology, headfoot, sperm, etc); however, the external form of the shell had been the principal character used in the species-level taxonomy of patellids [1], [4]. Despite this, it is known that shells are highly variable and usually lead to taxonomy confusions (see Mauro et al. [4]).

The main role for genetics in marine invertebrates is the identification of species and groups of interbreeding individuals [5]. A public library of sequences linked to named species, and the promotion of portable devices for DNA barcoding, will

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also considerably help in the management and conservation of marine species [6]. The cytochrome c oxidase I gene (COI) seems to be a useful genetic tool to be used in patellids molecular genetics. The universal primers for this gene are very robust and COI appears to possess a greater range of phylogenetic signal than any other mitochondrial gene [7], [8]. Kouffopanou et al. [2] used 12S and 16S genes for constructing the first molecular phylogeny of patellids. More recently Sá-Pinto et al. [3] used also COI and pointed to *Patella* s.s. showing five strongly supported clades (I: *P. candei*, *P. lugubris*, *P. caerulea*, *P. depressa*; II: *P. ulyssiponensis* (*P. aspera*); III: *P. vulgata*. IV: *P. rustica*, *P. ferruginea*; V: *P. pellucida*).

The *Patella* s.s. show problems for their conservation and management today. Conservation of declining stocks of *P. candei* and *P. aspera* has become a concern on the Atlantic islands [9], while in the Mediterranean, *P. ferruginea* is seriously endangered [10] and in the eastern Pacific *P. mexicana* may be locally extinct on parts of the mainland coast of Mexico [1]. In Asturias, northern Spain, exists an ancient culinary tradition of consumption for limpets (*Patella* s.s.). Even when they have been under commercial exploitation for decades, there is not any previous genetic data about them. There is only a few data about the composition of the genus and the more recent morphological studies dates from 1980 [11], [12], [13]. It had been reported four species (*P. vulgata*, *P. depressa*, *P. aspera* and *P. rustica*) of which the principally harvested is *P. vulgata*. We have sampled Asturian marine *Patella* s.s. specimens and conducted sequencing of the mtDNA COI gene. Our aims were to identify/confirm the patellids species present in our coasts using molecular methods and to analyze the phylogenetic status of the genus using a Bayesian approach.

2 MATERIAL AND METHODS

Forty five *Patella* s.s. specimens were collected in four areas (western Asturias: Punta La Cruz (10 inds), central Asturias: Moniello (8 inds), Antromero (18 inds) and eastern Asturias: Tereñes (9 inds)) (Fig. 1). Patellid individuals were morphologically identified by an experienced zoologist. DNA extractions were carried out using the ZR Genomic DNA Kit™. We used the universal primers described by Folmer et al. [7] for the amplification of a 658 bp fragment of the Cytochrome Oxidase subunit I gene (COI). Sequences were aligned in a multiple alignment with the BioEdit software package. After alignment and trimming, the final sequence length used was 642 bp. We used Collapse 1.2 for collapsing sequences to haplotypes. A Global search in the Barcoding of Life system (CBOL) resource for the identification of species using the COI sequences was carried out. Nucleotide (π) and haplotype (h) diversities were calculated using Arlequin 3.11. For Bayesian phylogenetic inferences MrBayes 3.1.2 was used evaluating tree topologies and models of nucleotide substitution for the aligned dataset. The model of nucleotide evolution was the general time-reversible model (GTR+G) with gamma-distributed rate variation across sites but not proportion of invariable sites.

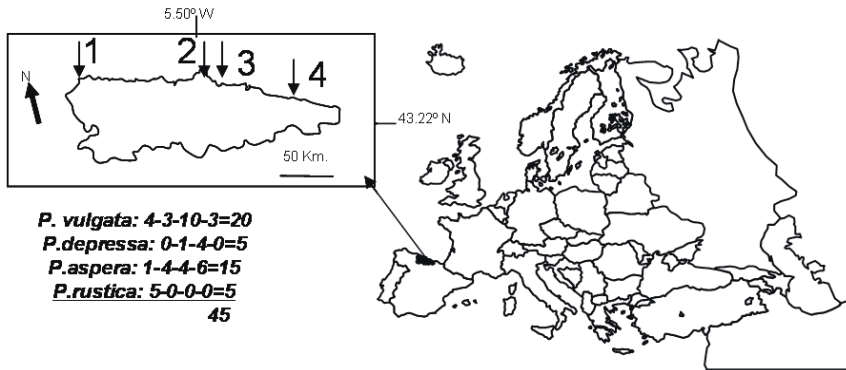


Fig. 1 – Map showing the geographic localization for patellids samples in Asturian coastal area (northern Spain) 1- Punta la Cruz. 2- Moniello. 3- Antromero 4- Tereñes. Limpet individuals assigned to each species of the *Patella* genus after *COI* identification are indicated by areas in the same order.

3 RESULTS

The *COI* sequence data have been deposited in the GenBank nucleotide sequence database with accession numbers EF462952 to EF462975 (23 patellids haplotypes). The 45 limpets (*Patella* s.s.) collected in the Asturian coasts were morphologically assigned to the species *P. vulgata* (20), *P. depressa* (5), *P. aspera* (15) and *P. rustica* (5). This was concordant with assignments using the molecular method and the CBOL resource. However, two individuals showed more than a 2% of sequence divergence with its species sequences (PA-1205 and PA-3105 assigned to *P. aspera* with a 94.4% and 97.8% of similarity, respectively).

The levels of *COI* genetic variation for each of the mentioned above species are shown in Tab. 1. Inside the *Patella* genus we found an 8.8% and 88.1% of nucleotide (π) and haplotype (h) diversity, respectively. The most variable species was *P. aspera* (π = 1.60%) while *P. vulgata* appeared as the less variable one (π = 0.07%) (Tab. 1).

The *COI* phylogenetic tree using the Bayesian approach showed two main nodes: one contains the *P. aspera* individuals and another contains the *P. vulgata* ones and also a group of *P. depressa* and *P. rustica* individuals (Fig. 2). One individual (PA-1205), morphologically classified as *P. aspera*, was located inside the *P. vulgata* branch although it showed only a 93.6% of similarity with the available *P. vulgata* sequences (CBOL).

4 DISCUSSION

Our sampling and the identification methods used here revealed four *Patella* species in Asturias (*P. vulgata*, *P. depressa*, *P. aspera*, and *P. rustica*) confirming

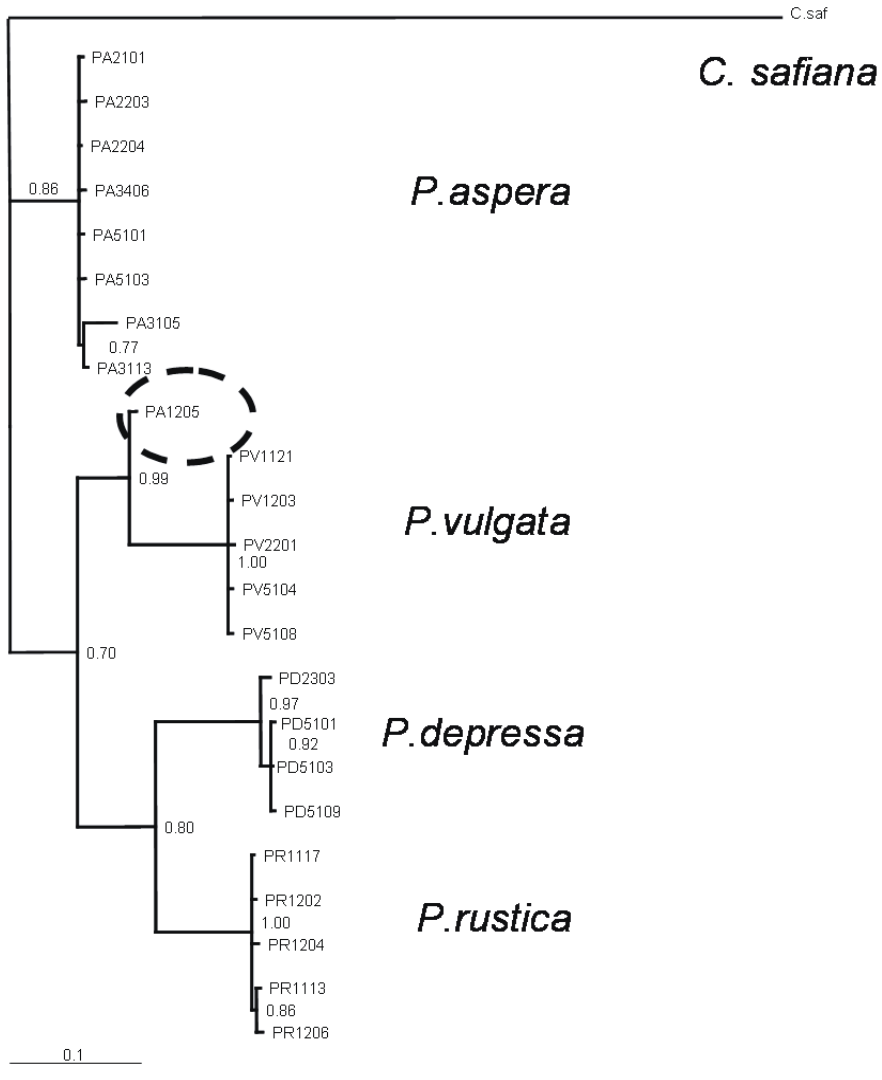


Fig. 2 – Consense Phylogenetic tree after Bayesian analysis using Cytochrome Oxidase I (COI) sequences in the genus *Patella*. *C.safiana* as output group. Numbers represent more than a 70% of branch support. A *P.aspera* individual grouping with the *P.vulgata* group is indicated by a discontinuous circle.

Patella Species	Gene	bp	n	Haplotypes numbers	Genbank AN	Polymorphic sites	Ts	Tv	Sb	Id	π	h
		COI	642	45	23	EF462952-75	156	139	38	177	0	0.0885
<i>P.vulgata</i>			20	5	EF462957-62	5	4	1	5	0	0.0007	0.4474
<i>P.depressa</i>			5	4	EF462972-75	8	5	3	8	0	0.0049	0.9000
<i>P.rustica</i>			5	5	EF462952-56	7	5	2	7	0	0.0046	1.0000
<i>P.aspera</i>			15	9	EF462963-71	68	53	16	69	0	0.0160	0.8857

Tab. 1 – Genetic variation in patellids from the Asturian Coasts.

bp: base pairs. n: number of samples. Genbank AN: Genbank accession numbers for haplotypes. Ts: transitions. Tv: transversions. Sb: substitutions. Id: indels. π : Nucleotide diversity. h: Haplotype diversity. COI: Cytochrome Oxidase I.

previous morphological studies from the 80s [12], [13]. Two individuals fall apart from the criterion of less than a 2% of divergence for correct species classifications (following the CBOL recommendations) and the PA-1205 haplotype is clearly out of its putative species branch in the phylogenetic COI-trees. This could point to cases of genetic introgression and to the necessity to clarified and revise the taxonomic classifications in *Patella* species [3].

The patellids found showed a dissimilar species distribution in the Asturian coastal area with *P. rustica* being only present in the Asturian/Galician frontier. Possibly, this species is the most sensible to changes in the sea surface temperature that determine its reproductive success and hence its dispersal potential [14]. The commercially exploited species (*P. vulgata*) is the genetically

less variable. This raises concerns about the health of this species in Asturias.

The *Patella* genus is monophyletic inside the *Patellidae* family. Sá-Pinto et al. [3] showed five strongly supported clades for patellids although relationships between them were not well supported. Working with four species included in the Sá-Pinto et al. [3] study we have recovered the expected four clades phylogenetic tree. However, we have well supported branches (more than 80%) indicating different relationships among these clades. Our results revealed two main nodes: one included *P. aspera* (*P. ulyssiponensis*) as an entity and another included *P. vulgata*, *P. depressa* and *P. rustica* species. Our results differ from the closeness between *P. depressa* and *P. vulgata* species proposed by Koufopanou et al. [2] (*P. depressa* is more close to *P. rustica* in our work) and also from the relationships between clades showed by Sá-Pinto et al [3]. They showed *P. depressa* (clade I) together with *P. aspera* (Clade II) and these two grouped to *P. vulgata* (Clade III), while *P. rustica* (Clade IV) was an independent entity. It will be necessary much more work and different approaches to ascertain which the relationship between clades inside the genus *Patella* is. This will help to clarify taxonomy and will give us clues about speciation patterns and origins inside the genus. All this information will be vital for its adequate conservation and management.

5 CONCLUSION

Molecular methods are useful tools for species identification when morphological analyses lead to taxonomic confusions. Using COI gene sequences we have confirmed the presence of four patellids in the Asturian coasts (*P. vulgata*, *P. depressa*, *P. aspera*, and *P. rustica*). Our work raises concerns about the current state of the *P. vulgata* populations in Asturias, where it is exploited, due to its low levels of genetic variation. Our phylogenetic analyses confirmed that patellids belong to four different clades; however our work gives a new version about how these clades are related inside the genus aiming for the necessity of more work to address this issue.

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