

# Effects of Pleistocene marine transgressions on the evolutionary history of the genus *Squalius* (Actinopterygii, Cyprinidae) in Southern Iberia.

Marta Cobo-Simón, Silvia Perea and Ignacio Doadrio

Museo Nacional de Ciencias Naturales. José Gutiérrez Abascal 2, 28006 Madrid. España. E-mail: marta.cobo@mncn.csic.es

## RESUMEN/ABSTRACT

Hemos realizado un estudio filogeográfico y demográfico de dos especies de peces de agua dulce (*Squalius malacitanus* y *Squalius pyrenaicus*) para estudiar los efectos que pudieron haber tenido los eventos geológicos y climáticos ocurridos durante el Cenozoico en la región del Estrecho de Gibraltar. Para ello se utilizaron genes mitocondriales y nucleares. En el estudio filogenético, los resultados mostraron dos grupos bien diferenciados para cada especie reflejando una distribución alopatrica, especialmente para *Squalius malacitanus*. Por ello, se realizó un estudio morfológico de las dos poblaciones para caracterizarlas taxonómicamente. En ambas especies, las poblaciones mediterráneas presentaron menor diversidad genética que las atlánticas y se observó una correlación entre el área de la cuenca y su diversidad genética. Además, se realizó un estudio de disponibilidad de hábitat para mejorar las estrategias de conservación de la especie endémica amenazada *Squalius malacitanus*.

To study the effects that these geological and climatic events could have had above populations of freshwater fishes inhabiting Southern Spain during Cenozoic period in Gibraltar Strait region, we carried out a phylogeographical and demographic approach of two species of primary freshwater fishes (*Squalius malacitanus* and *Squalius pyrenaicus*) using nuclear and mitochondrial genes as molecular markers. In the phylogenetic study our results showed four well-differentiated groups, divided into two main phylogroups, belonging to each studied species and reflecting an allopatric distribution. The two groups found in the species *Squalius malacitanus* have higher divergences than *S. pyrenaicus*. Therefore, we conducted a molecular and morphometric study of Atlantic and Mediterranean populations to characterize the two populations from a taxonomic point of view. In both species, Mediterranean populations showed a lower genetic diversity than Atlantic populations and we observed a correlation between area basin and genetic diversity. Moreover, we carried out a study of endemic and endangered species *S. malacitanus* habitat availability to improve its conservation status.

## INTRODUCTION

The Gibraltar Strait area is one of the most complex biogeographical regions during the Cenozoic due to tectonical and climatic processes, which must severely affect to populations of organisms inhabiting this area. (Calvo *et al.*, 1993). Among them are the species *Squalius malacitanus* (Doadrio and Carmona, 2006) and *Squalius pyrenaicus* (Günter, 1868), primary freshwater fish belonging to Cyprinidae family and endemic from the Iberian Peninsula. The species *S. malacitanus* has a more restricted distribution area, is only present in southern Spain, and has been listed as endangered (EN) in IUCN red list and in danger of extinction (E) in Spanish Catalogue of Endangered Species (Real Decreto 139/2011 of February 4th) (Doadrio *et al.*, 2011).

To study the effects that these geological and climatic events could have had above populations of freshwater fishes inhabiting Southern Spain, we realized a phylogeographical and demographic approach of the species *Squalius malacitanus* and *Squalius pyrenaicus*, using cytochrome *b* (mtDNA) and RAG-1 (nDNA) as molecular markers and a morphometric study of Atlantic and Mediterranean populations of the species *S. malacitanus* to characterize the two populations from a morphological point of view. Moreover, we carried out a study of *S. malacitanus* habitat availability to predict its future distribution and the most important variables to improve the conservation policy.

## MATERIAL AND METHODS

•9 localities and 56 individuals of *Squalius malacitanus*, which represent all their distribution area and all river basins where the species is present and 42 individuals from 8 places of *Squalius pyrenaicus* of southern Spain basins near the distribution of *Squalius malacitanus* were studied (Figure 1).

•A phylogenetic reconstruction was performed by a Bayesian Inference and Maximum Likelihood, with *Squalius laietanus* (Doadrio, Kottelat and Sostoa, 2007) and *Squalius torgalensis* (Coelho, Bogutskaya, Rodrigues and Collares-Pereira, 1998) as outgroup using MrBayes v.3.2 (Ronquist *et al.*, 2011) and PhyML 3.0 (Guindon *et al.*, 2010) software with TIM1+G model to cytochrome *b* and TPM2+G to RAG1 as provide jModelTest v0.1.1 (Posada, 2008)

•An haplotypes network was performed for the phylogeographic analysis using NETWORK v 4.5 and a batimetric study using ArcView (GIS software).

•There were obtained the genetic distances between groups based on the phylogenetic reconstruction and the divergence times were estimated using BEAST v1.7 (Drummond & Rambaut, 2007).

•We conducted a morphometric study of *S. malacitanus* to characterize the genetically differentiated populations of *Squalius malacitanus*. Nineteen morphometric measurements and five meristic variables were recorded (Figure 2), carrying out a Principal Components Analysis (PCA) and a Hotelling's test with Burnaby's correction using PAST (Hammer *et al.*, 2001).

•To study the relationship between the conspicuous genetic diversity found in the *Squalius* genus and paleoclimatic factors during the Quaternary, we built a species distribution model based on the known-presence localities of *Squalius malacitanus* and *Squalius pyrenaicus* using Maxent v3.3.3 (Phillips *et al.*, 2006).

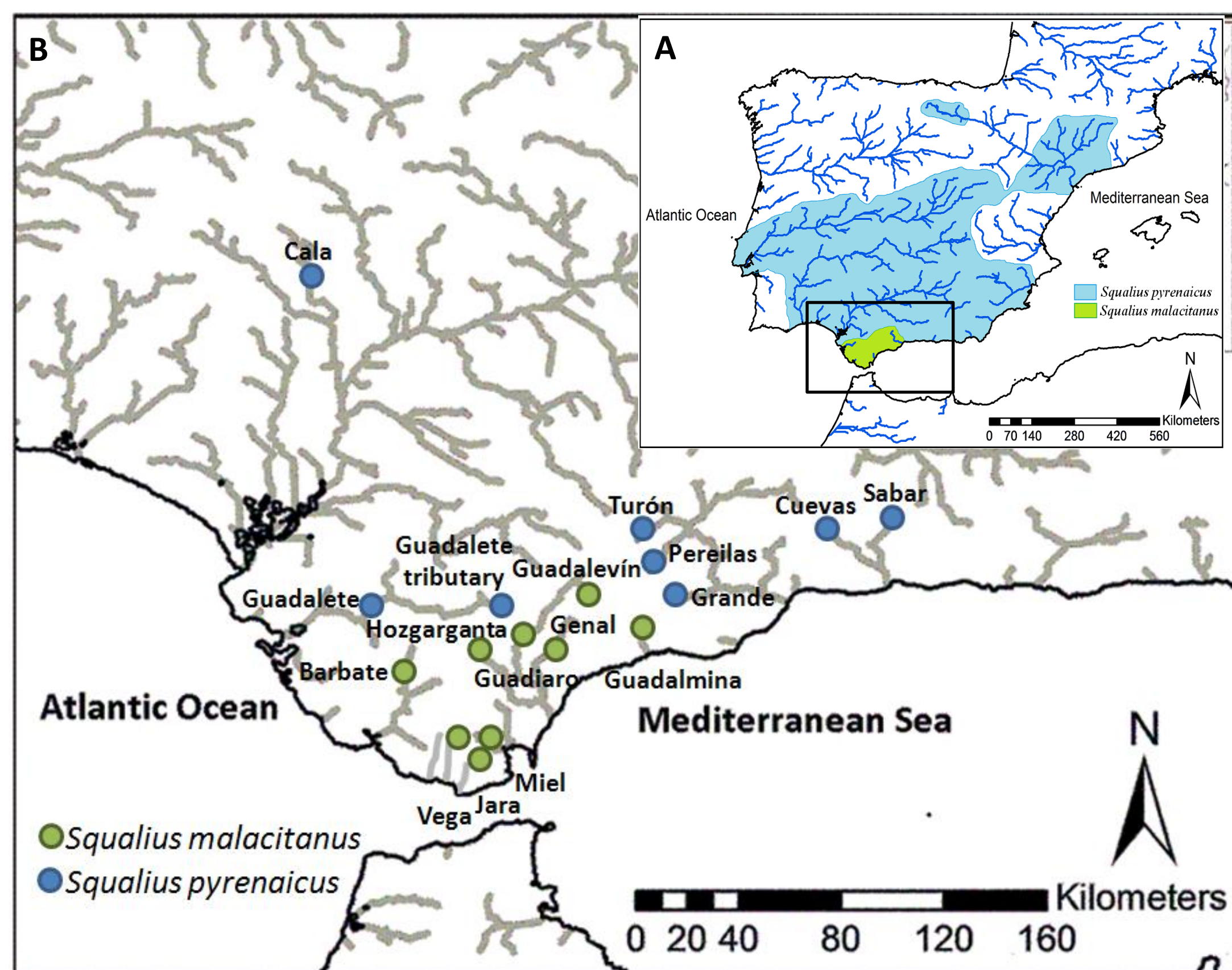


Figure 1. (A) Rango de distribución de las especies *Squalius malacitanus* (verde) y *Squalius pyrenaicus* (azul) en la Península Ibérica. (B) Sitios de muestreo de las especies *Squalius malacitanus* (verde) y *Squalius pyrenaicus* (azul).  
Figure 1. (A) Range of distribution of the species *Squalius malacitanus* (green) and *Squalius pyrenaicus* (blue) on Iberian Peninsula. (B) Sample sites of the species *Squalius malacitanus* (green) and *Squalius pyrenaicus* (blue).

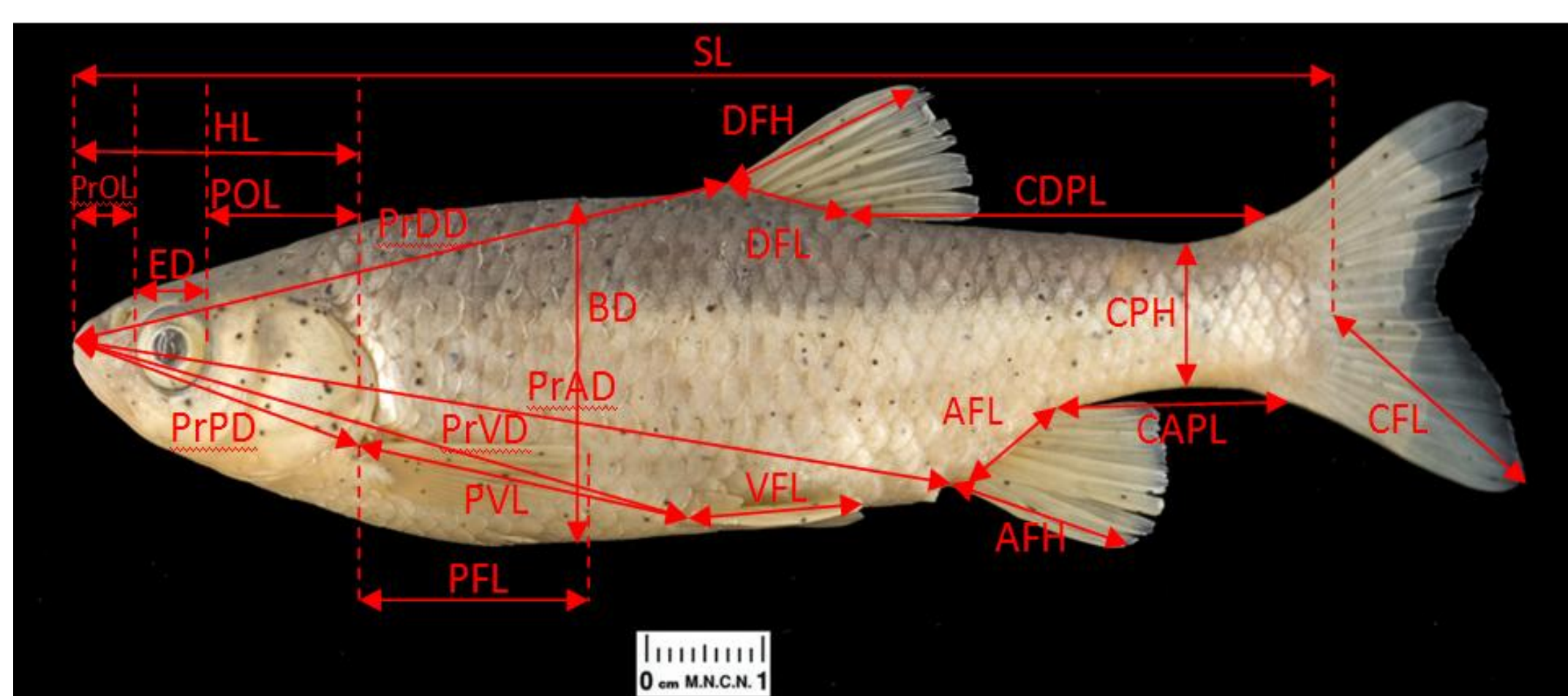


Figure 2. Medidas morfométricas. Abreviaturas: SL, longitud estándar; PrDD, distancia predorsal; PrVD, distancia preventral; PrAD, distancia preanal; PrPD, distancia prepectoral; PVL, longitud pectoral-ventral; CDPL, longitud del pedúnculo caudal-dorsal; CAPL, longitud del pedúnculo caudal-anal; BD, anchura corporal; CPH, altura del pedúnculo caudal; HL, longitud de la cabeza; ED, diámetro del ojo; PrOL, longitud preorbital; POL, longitud postorbital; DFL, longitud de la aleta dorsal; DFH, altura de la aleta dorsal; PFL, longitud de la aleta pectoral; VFL, longitud de la aleta ventral; AFL, longitud de la aleta anal; AFH, altura de la aleta anal; CFL, longitud de la aleta caudal.  
Figure 2. Morphometric measurements. Abbreviations: SL, standard length; PrDD, pre-dorsal distance; PrVD, pre-ventral distance; PrAD, pre-anal distance; PrPD, prepectoral distance; PVL, pectoral-ventral length; CDPL, caudal-dorsal peduncle length; CAPL, caudal-anal peduncle length; BD, body depth; CPH, caudal peduncle height; HL, head length; ED, eye diameter; PrOL, pre-orbital length; POL, post-orbital length; DFL, dorsal fin length; DFH, dorsal fin height; PFL, pectoral fin length; VFL, ventral fin length; AFL, anal fin length; AFH, anal fin height; CFL, caudal fin length.

## DISCUSION

Mediterranean and Atlantic populations belonging to the species *Squalius malacitanus* present high molecular differentiation, which indicates an ancient split (5.3 MYA). However, only some quantitative morphological traits showed differentiation between Atlantic and Mediterranean populations. Divergences derived of their allopatric distribution in Atlantic and Mediterranean rivers since the Early Pliocene have given rise to a high genetic divergence but lower morphological differentiation, which facilitates the taxonomic studies. Populations of the Atlantic slope of *Squalius malacitanus* probably constitute a new species that should be formally described.

In the phylogenetic study our results showed four well-differentiated groups, divided into two main phylogroups, belonging to each studied species and reflecting an allopatric distribution. The two groups found in the species *Squalius malacitanus* have higher divergences than *S. pyrenaicus* and are distributed one by the Atlantic slope of Gibraltar Strait and the other by the Mediterranean one. In both species, Mediterranean populations showed a lower genetic diversity than Atlantic populations and we observed a correlation between area basin and genetic diversity.

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## RESULTS

The phylogenetic reconstruction and phylogeographic analysis (Figs. 2 and 3) show two divergent groups for each species. In order to *Squalius malacitanus*, we observed two groups of Mediterranean and Atlantic populations, whose divergence time was 5.3 MY. However, *Squalius pyrenaicus* did not show a clear divergence and Atlantic and Mediterranean populations split up 620,000 years. The potential species-distribution study showed a small variation in habitat availability in the future. Morphological analyses did not reflect significant differences between both Mediterranean and Atlantic populations to meristic character, as number of scales or fin rays. On the other hand, morphometric characters showed differences between populations of *S. malacitanus* placed to both slopes of Gibraltar Strait.

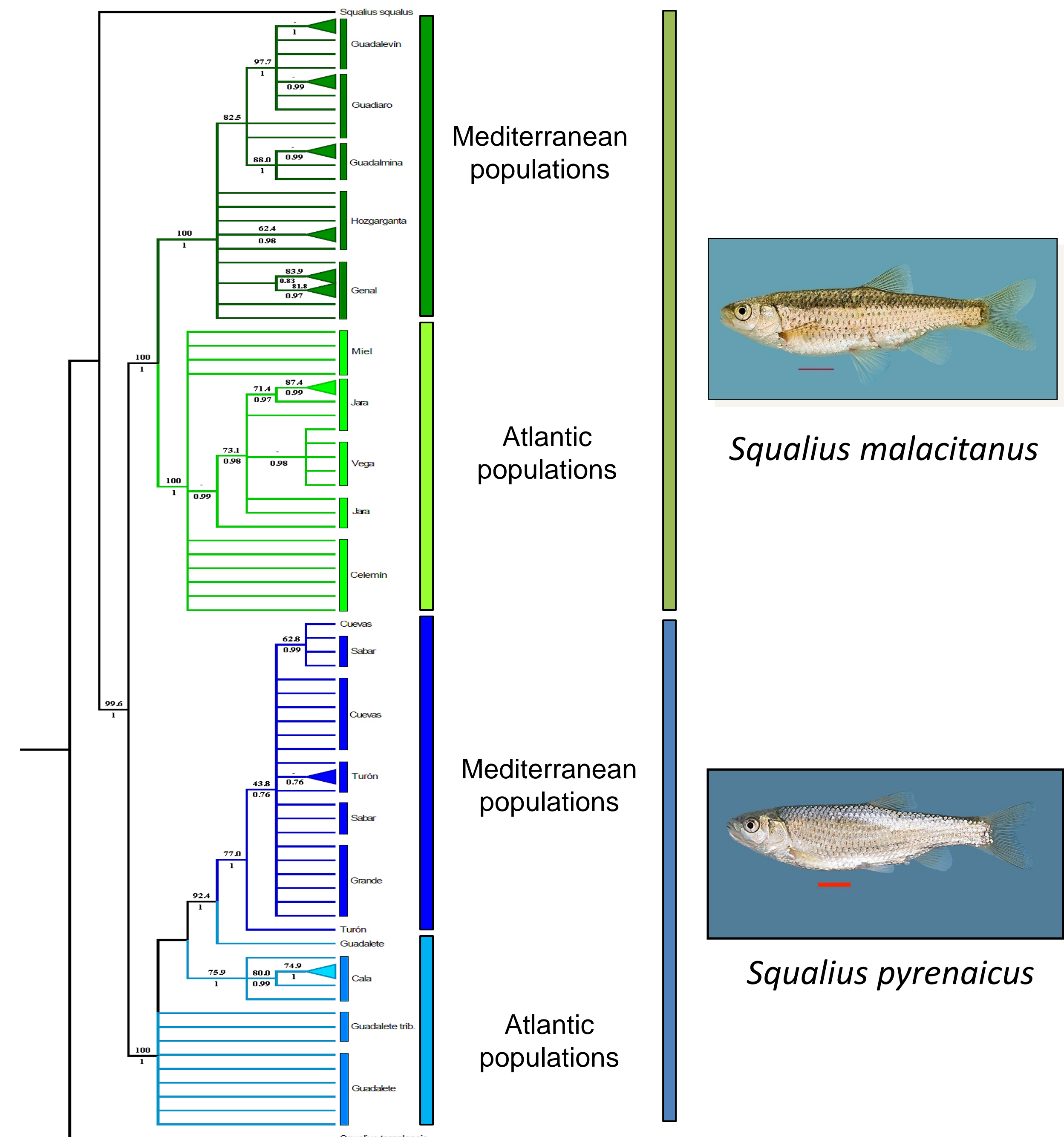


Figure 3. Reconstrucción filogenética de *Squalius malacitanus* y *Squalius pyrenaicus* basada en secuencias de citocromo *b* y RAG-1 mediante Máxima Verosimilitud (valores de bootstrap sobre los nodos) e Inferencia Bayesiana (probabilidades posteriores bajo los nodos).

Figure 3. Phylogenetic reconstruction of *Squalius malacitanus* and *Squalius pyrenaicus* based on sequences of Cytochrome *b* and RAG-1 by Maximum Likelihood (bootstrap values above nodes) and Bayesian Inference (posterior probability values below nodes).

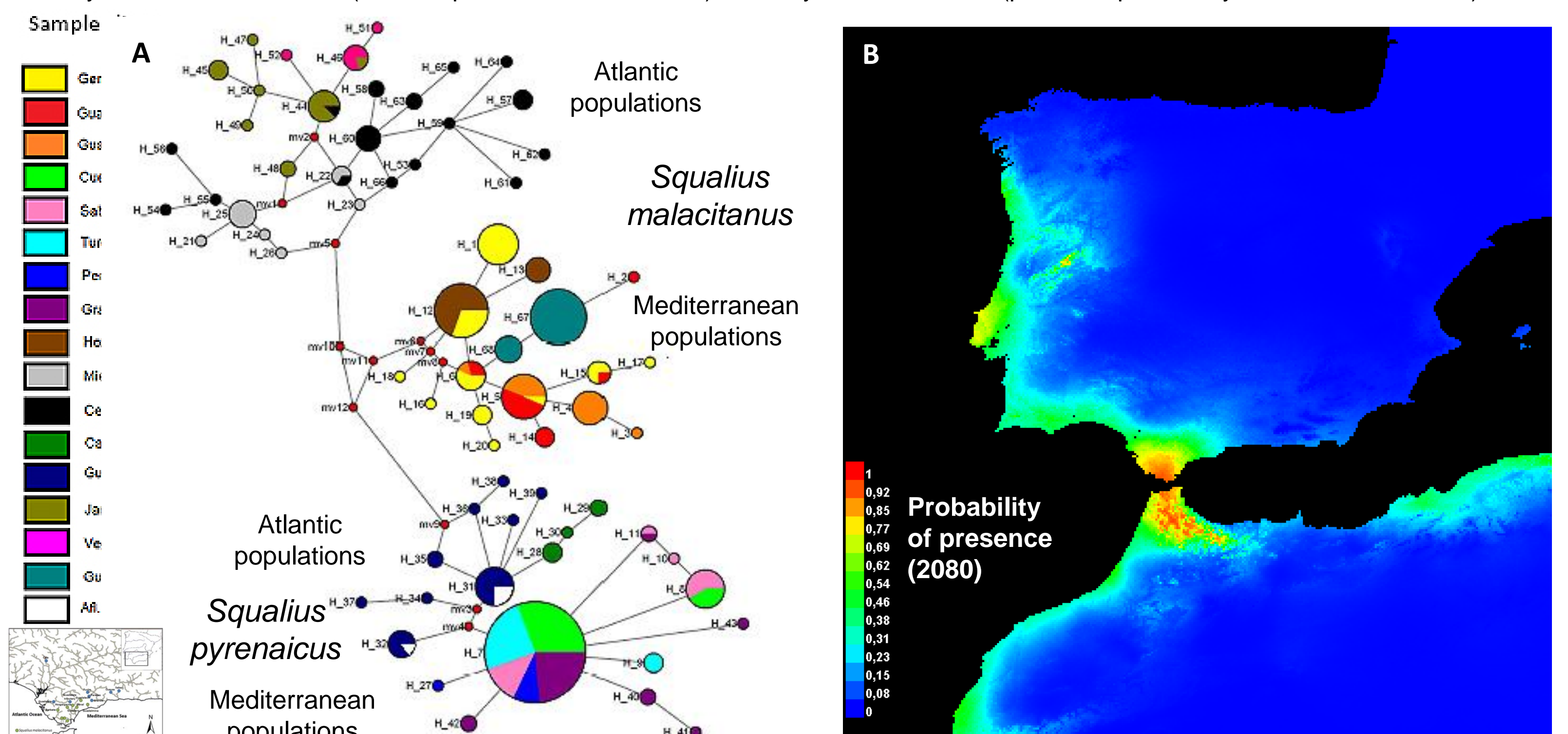


Figure 4. A. Red de haplotipos basada en el gen del citocromo *b* de las muestras de *Squalius malacitanus* y *Squalius pyrenaicus* obtenidas en este trabajo. B. Distribución potencial de *S. malacitanus* para condiciones futuras (2080).

Figure 4. A. Haplotypes Network based on Cytochrome *b* gene of *Squalius malacitanus* and *Squalius pyrenaicus* samples obtained in this work. B. Potential distribution of *S. malacitanus* for future conditions (2080).

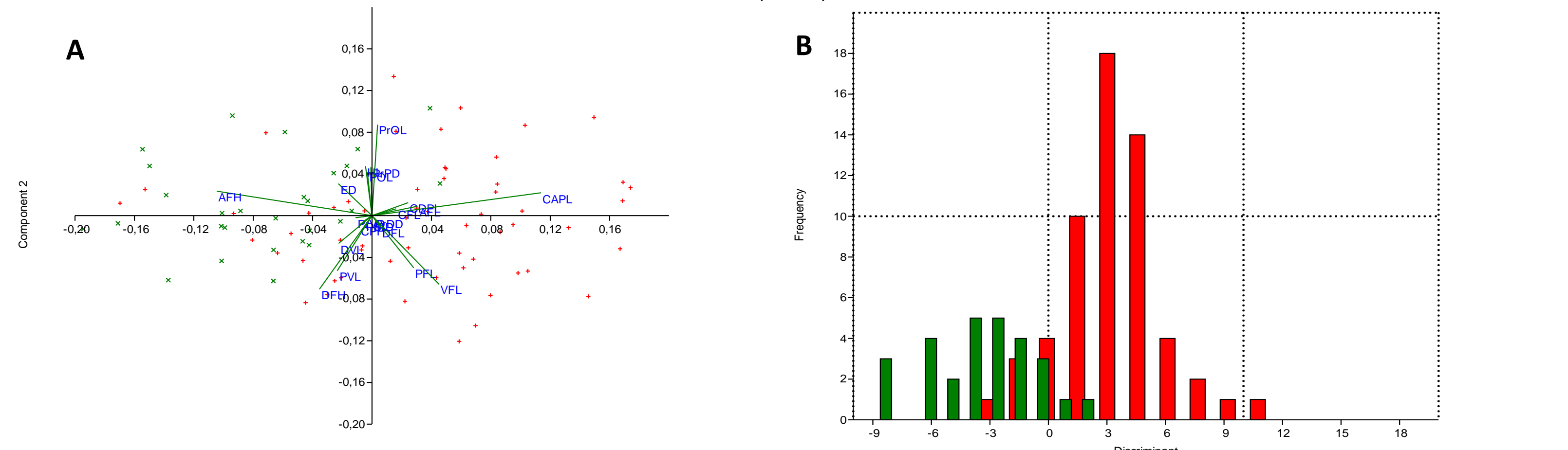


Figure 5. A. Variables que más contribuyeron en el Análisis de Componentes Principales. Las abreviaturas están descritas en la Figura 2. B.  $T^*$  del test de Hotelling para dos muestras de los caracteres morfométricos. Rojo, *Squalius malacitanus*. Verde, *Squalius* sp. Nov.

Figure 5. A. Variables that most contributed to the PCA analysis. Abbreviations are described in Figure 2. B. Two sample Hotelling's  $T^*$  test from morphometric characters. Red, *Squalius malacitanus*. Green, *Squalius* sp. nov.