

A cryptic species of freshwater fishes in Southern Spain is found by way of molecular analysis of the genus *Squalius* Bonaparte, 1837 (Actinopterygii, Cyprinidae).

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

ABSTRACT

The taxonomy of the Spanish freshwater fishes was mainly precluded for the monumental work of Lozano Rey (1935). This work considered the same origin to the Iberian and the rest of European fish fauna. Differences in morphology many times were assigned to small local variations and Spanish populations were attributed taxonomically to subspecies of well recognized central and north European species. This vision of Spanish freshwater fishes has changed because of molecular systematics, which found a high diversity and different origins to this fish fauna. The number of species was increasing since 1980 and an endemic cyprinid, *Squalius malacitanus* (Doadrio and Carmona, 2006) was currently described. As consequence of a phylogeographical approach to all populations of *Squalius malacitanus* using nuclear and mitochondrial molecular markers, we found two deep divergent groups. These two groups have higher divergences than other Spanish species of same genus.

INTRODUCTION

The Gibraltar Strait area is one of the most complex biogeographical regions during the Cenozoic, due to tectonic 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 morphologic 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 PartitionFinder v1.1.1 (Lanfear *et al.*, 2012)

•An haplotypes network was performed for the phylogeographical 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 Hottelling'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).

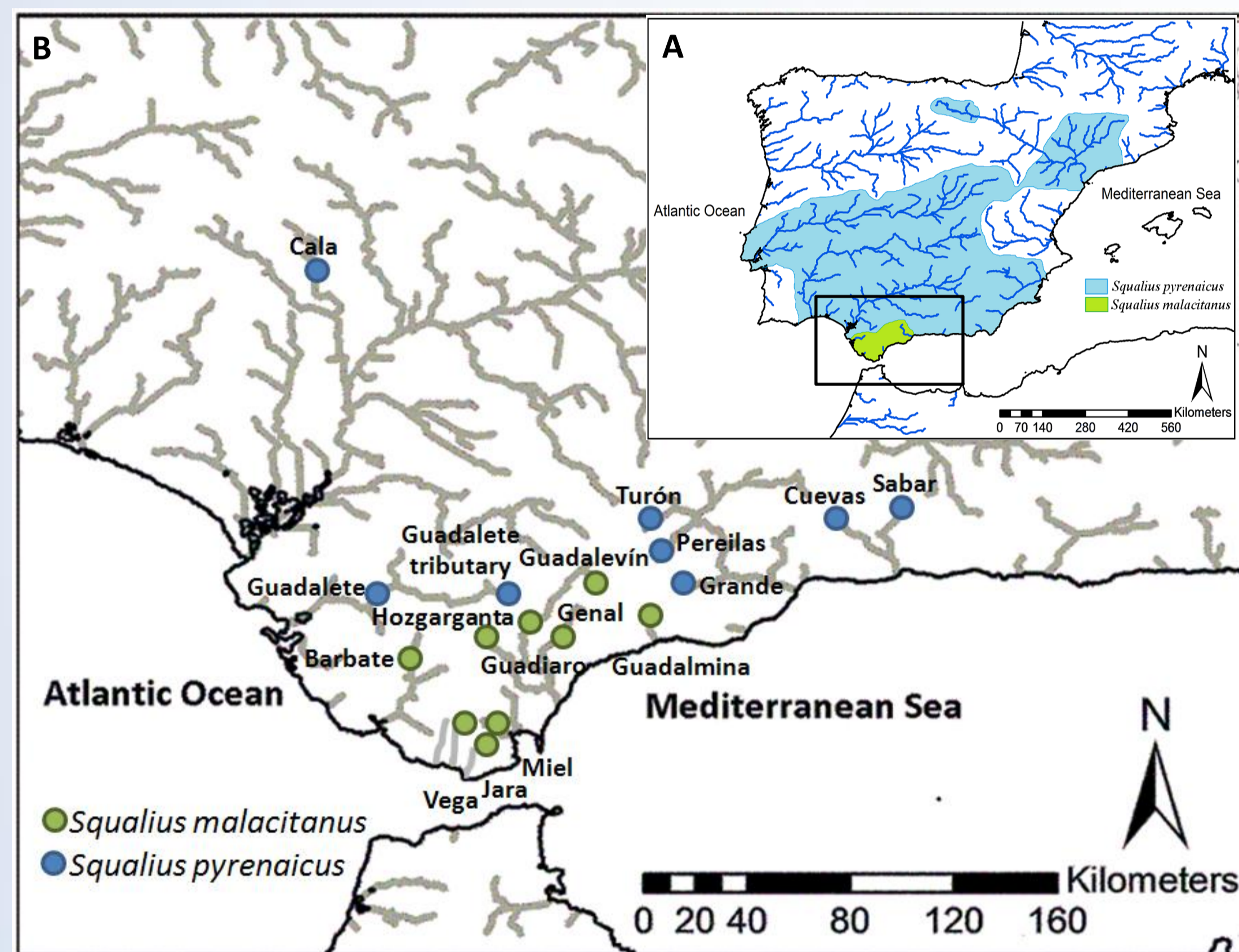


Figure 1. (A) Range of distribution of the species *Squalius malacitanus* (green) and *Squalius pyrenaicus* (blue) on Iberian Peninsula. (B) Sample sites of the species *Squalius malacitanus* (green) and *Squalius pyrenaicus* (blue).

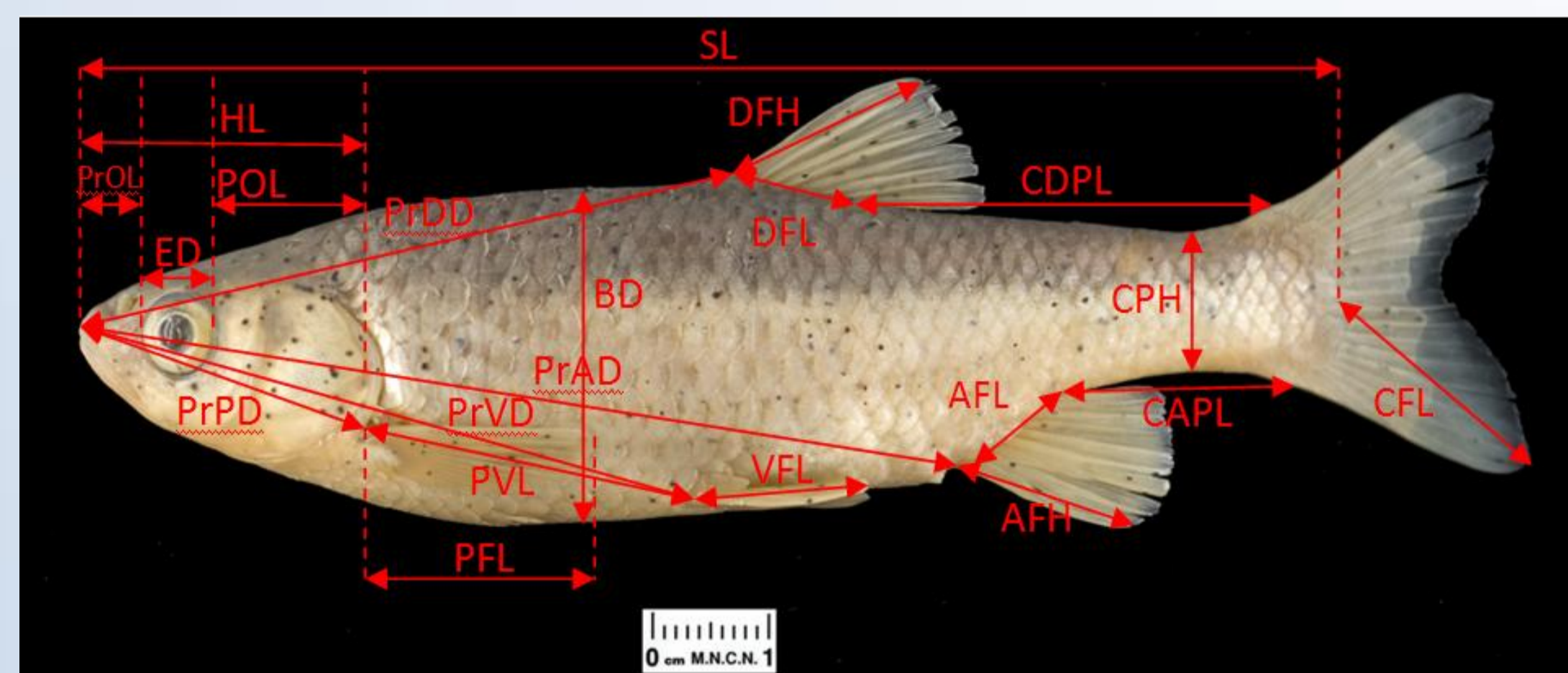


Figure 2. Morphometric measurements. Abbreviations: SL, standard length; PrDD, pre-dorsal distance; PrVD, pre-ventral distance; PrAD, pre-anal distance; PrPD, prepectoral distance; PVL, pectoral-ventral length; CDPL, caudal-dorsal peduncle length; CAPL, caudal-anal peduncle length; BD, body depth; CPH, 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.

DISCUSSION

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 complicates 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-differented 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.

ACKNOWLEDGEMENTS

The authors thank L. Alcaraz for her help in laboratory work. We are also grateful to M. Casal, I. Cobo, D. Corona, M. Esteve and E. G. González for their interest and suggestions. This study was funded by project CGL2010-15231BOS.

REFERENCES

- AVISE, J.C. (2000). *Phylogeography: The History and Formation of Species*. Harvard University Press, Cambridge.
- BERNATCHEZ, L., WILSON, C. C. (1998). Comparative phylogeography of Neartic and Palearctic fishes. *Mol Ecol* 7: 431-452.
- CALVO, J. P., DAMIS, R., MORALES, J., LOPEZ-MARTINEZ, N., AUGUSTINI, J., ANADON, P., ARMENTEROS, I., CABRERA, L., CIVIS, J., CORROCHANO, A., DIAZ-MOLINA, M., MUÑOZ, A., PÉREZ-GARCÍA, A., PÉREZ-GONZÁLEZ, A., PORTERO, J. M., SANTISTEBAN, C., TORRES, T., VAN DER MEULEN, A. J., VERA, J. A., MEIN, P. (1993). Up-to-date Spanish continental Neogene synthesis and paleoclimatic interpretation. *Revista de la Sociedad Geológica de España* 6: 29-40.
- DOADRIO, I., CARMONA, J. A. (2006). Phylogenetic overview of the genus *Squalius* (Actinopterygii, Cyprinidae) in the Iberian Peninsula, with description of two new species. *Cybum* 30(3): 199-214.
- COELHO, M. M., BOGUTSKAYA, N. G., RODRIGUES, J. A. & COLLARES-PEREIRA, M. J. (1998). *Leuciscus torgalensis* and *Leuciscus aradensis*, two new cyprinids for Portuguese Freshwaters. *Journal of Fish Biology*, 52: 937-950.
- DOADRIO, I., KOTTELAT, Y., SOSTOA, J., PEREA, S., GARZÓN-HEVOT, P., GONZÁLEZ, J. L. (2011). *Isotofauna continental española. Bases para su seguimiento*. CSIC-Ministero de Medio Ambiente y Medio Rural y Marino. Madrid: 609 pp.
- DRUMMOND, A., RAMBAUT, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* 7: 214.
- GUINDON, S., GASCUEL, O. (2003). A simple, fast and accurate algorithm to estimate large phylogenies by Maximum Likelihood. *Systematic Biology* 52(5): 696-704.
- HAMMER, R., HARPER, D. A. T. & RYAN, P. D. (2001). PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Paleontologia Electronica* 4(1): 39p.
- LANFAR, R., CALCOTT, B., HO, S. Y. W., GUINDON, S. (2012). PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*, 29(6): 1695-1701.
- LOZANO-REY, L. (1935). Los peces fluviales de España. Memorias de la Academia de Ciencias Naturales, Tomo V, 367 pp.
- PHILLIPS, S. J., ANDERSON, R. P., SCHAPIRE, R. E. (2006). Maximum entropy modeling of species geographic distributions. *Ecol Model* 190: 231-259.
- RONQUIST, F., HUELSENBECK, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19(12): 1572-1574.

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.

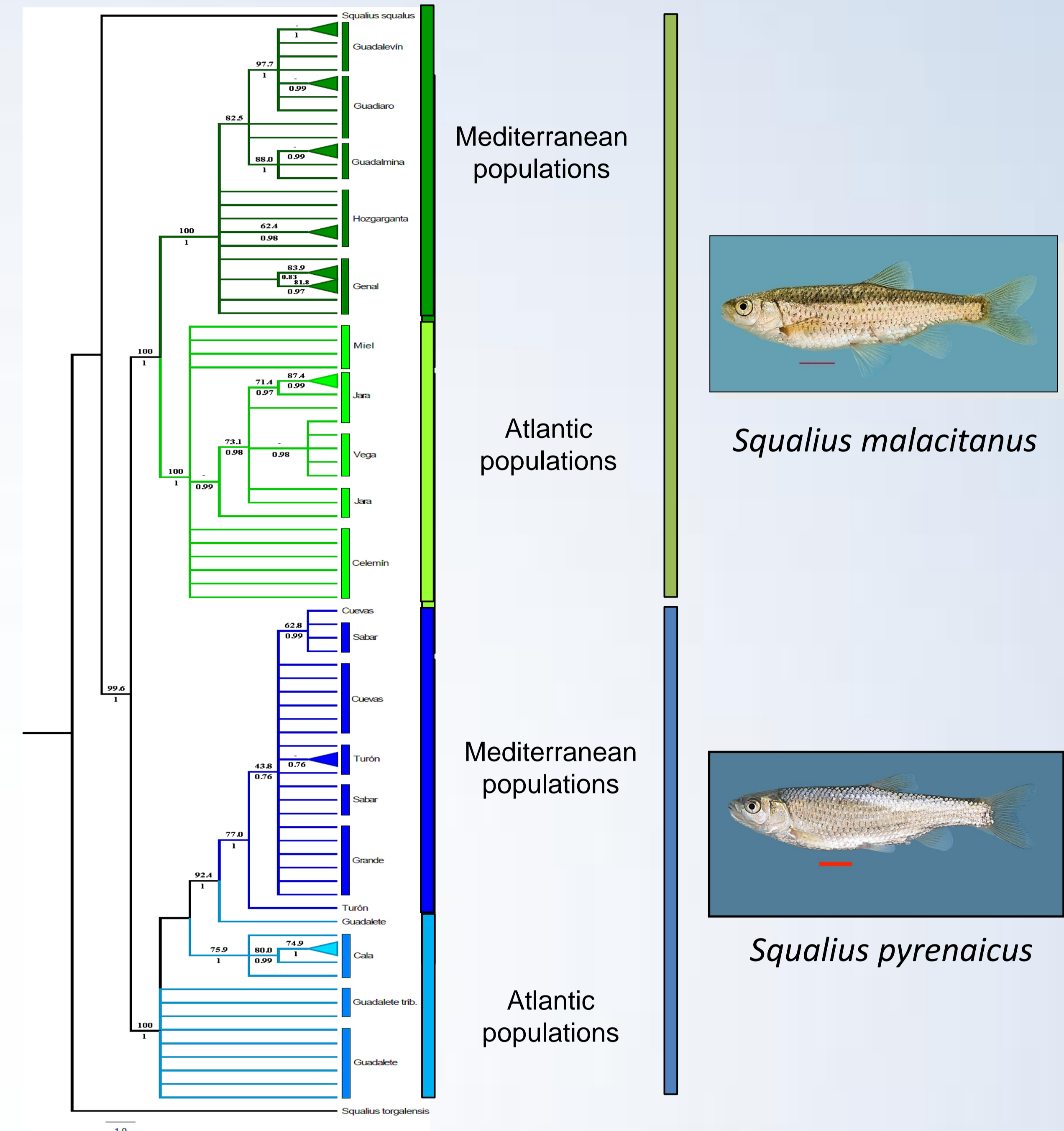


Figure 3. Phylogenetic reconstruction of *Squalius malacitanus* and *Squalius pyrenaicus* based on sequences of Cytochrome *b* and RAG-1 by Maximum Likelihood (bootstrap values above nodes) and Bayesian Inference (posterior probability values below nodes).

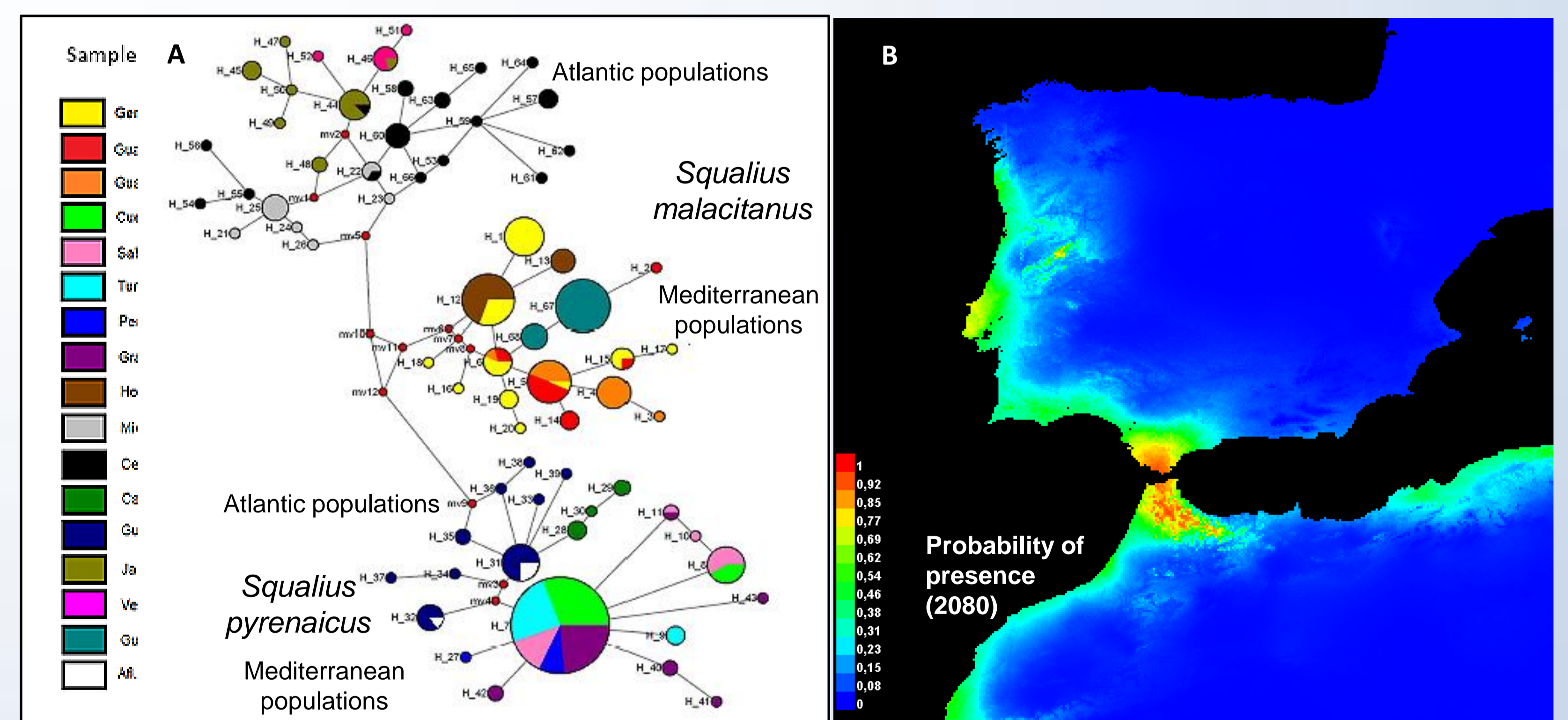


Figure 4. A. Haplotypes Network based on Cytochrome *b* gene of *Squalius malacitanus* and *Squalius pyrenaicus* samples obtained in this work. B. Potential distribution of *S. malacitanus* for future conditions (2080).

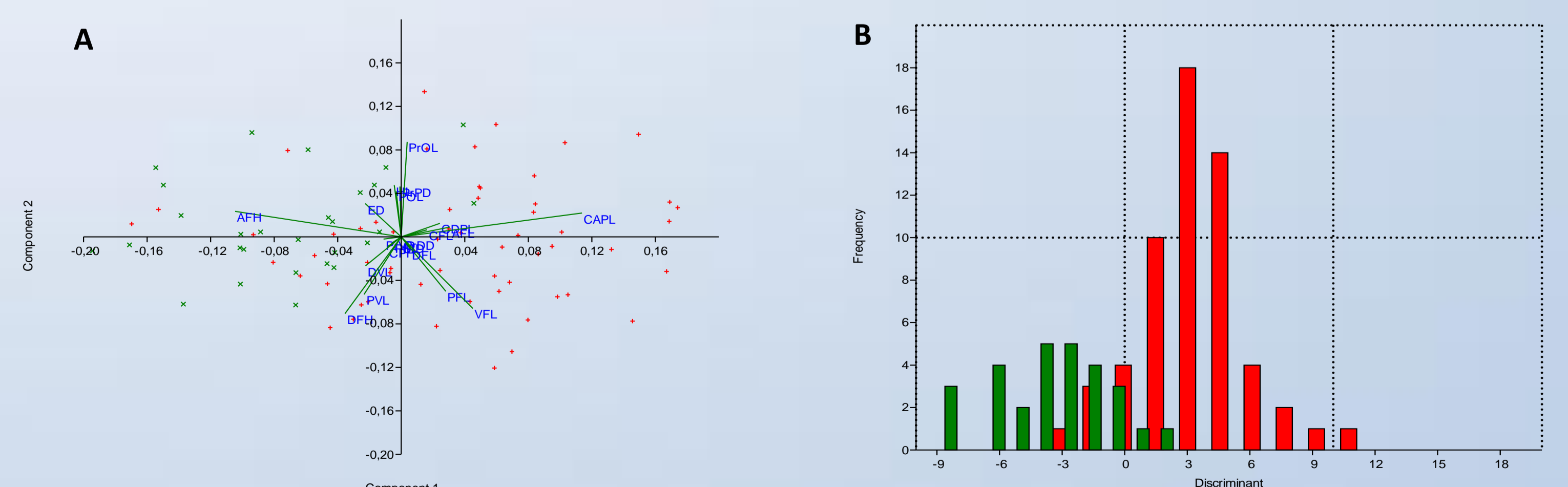


Figure 5. A. Variables that most contributed to the PCA analysis. Abbreviations are described in Figure 2. B. Two sample Hotelling's T* test from morphometric characters. Red, *Squalius malacitanus*. Green, *Squalius sp. nov.*