

Cytochrome b Mitochondrial Gene Revealed a Strong Genetic Differentiation between Two Populations of the Endemic Freshwater Fish Moroccan Loach *Cobitis maroccana* Pellegrin, 1929

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Abstract

Cobitis maroccana, also known as the Moroccan loach, is an endemic freshwater fish found in the rivers of the Loukkos and Sebou basins in Morocco. In order to gain a better understanding of the genetic differentiation within *C. maroccana* from the Loukkos and Sebou populations, a comprehensive study was conducted, focusing on the mitochondrial cytochrome b gene. A total of 30 sequences of the complete cytochrome b (1140 bp) were obtained from *C. maroccana* specimens. Through a combination of phylogenetic analysis, phylogeographic assessments, as well as the estimation of the F_{st} index, the research outcomes revealed significant genetic differentiation between the two juxtaposed basin populations. Notably, each of these basins displayed unique and distinct haplotype groups within the *C. maroccana* specimens. These intriguing findings strongly suppose that the populations in the Loukkos and Sebou basins have become reproductively isolated from each other over time, indicating limited or no interbreeding between them.

Introduction

Cobitids, commonly referred to as spined loaches, are a group of primary freshwater fish intolerant to water salinity (Myers, 1949; Nelson et al., 2016) distributed throughout Asia, Europe and North Africa (Nalbant et al., 2001; Kottelat & Freyhof, 2007). At present, the family of Cobitidae includes 232 valid species spread across 18 valid genera (Fricke et al., 2023). These species are known for their characteristic behavior of burrowing and dwelling at the bottom of water bodies (Slavik & Ráb, 1996).

In Morocco, *Cobitis maroccana* Pellegrin, 1929, which is commonly known as the Moroccan loach, is the only species identified within the Cobitidae family. Previous studies on the phylogeny of species in the

genus *Cobitis* showed that *C. maroccana* form a monophyletic Iberian-African group with the following Iberian species: *C. calderoni*, *C. paludica* and *C. vettonica* (Perdices et al., 1995; Perdices & Doadrio, 2001; Doadrio & Perdices, 2005; Doadrio et al., 2023). Based mainly on secondary sexual characteristics, these Ibero-African species have been divided into two subgenera: *Acanestrinia* (*C. calderoni*) and *Iberocobitis* (*C. maroccana*, *C. paludica* et *C. vettonica*) (Bacescu, 1961, 1962).

Cobitis Maroccana, like most *Luciobarbus* species found in Morocco, appears to be geographically isolated and endemic to specific river basins (Ouassal, 2022; Ouassal et al., 2022). This endemism may be attributed to the impact of various historical geological and climatic factors, including tectonic movements, climate

fluctuations throughout glacial-interglacial cycles, and successive marine regressions over time (Bianco, 1998; Hrbek & Meyer, 2003). These aforementioned factors may have led to geographical isolation and continental connections, favoring the processes of dispersion and vicariance of populations, the fauna migration, the confluence of alluvial plains, the capture of rivers, the connections of watersheds, and population isolation through geographical barriers (Tsigenopoulous et al., 2002; Aboim et al., 2013; Perea et al., 2015; Brahimi et al., 2016).

Cobitis maroccana is endemic to the northwestern region of Morocco and is found in the slow-flowing basins of rivers and in the backwaters of rivers, specifically in the Loukkos and Sebou basins. This particular species can typically be found in slow-moving streams close to the banks, hiding under rocks or in the mud. Additionally, it inhabits clear lakes with a sandy or sandy-muddy bottom (Yahyaoui et al., 2020). This species is diminishing as a result of habitat degradation, pumping, drainage, pesticides, and the introduction of invasive species. Consequently, *C. maroccana* has been classified as a vulnerable species (VU) by the International Union for Conservation of Nature (IUCN) (Crivelli, 2010).

Additionally, due to their bottom-dwelling behavior and lack of commercial value, cobitid fishes are often overlooked, leading to insufficient knowledge about their distribution and genetic structure in many regions throughout their habitat. The main objective of our study is to contribute to a better understanding of the phylogeography of Cobitidae species, with particular emphasis on *Cobitis maroccana* and its genetic differentiation using the cytochrome b mitochondrial gene as a molecular marker. The results obtained from

this research will contribute to the conservation and sustainable management of *Cobitis maroccana*.

Materials and Methods

Sampling

Cobitis maroccana specimens were collected throughout its distribution range in Morocco (Figure 1; Table 1) with standard electrofishing procedures. Fin clips were preserved in 96% ethanol (later kept at 4°C) and vouchers were deposited in DNA and Ichthyological Collection at the National Museum of Natural Sciences (MNCN-CSIC), in Madrid, Spain for subsequent DNA extraction, amplification and sequencing. The delineation of the river basins (Loukkos and Sebou) (Figure 1) was achieved using Qgis software (formerly known as Quantum GIS, (QGIS Development Team, 2021)). To facilitate the analyses, all samples collected within a single basin were pooled together. Thus, 12 sequences were used for Loukkos basin, while 18 sequences were included for the Sebou basin.

DNA Extraction, PCR and Sequencing

Genomic DNA was extracted from fin tissue samples using the Biosprint15 kit for tissue and blood (Qiagen), following the manufacturer's protocol. The amplification of the complete mitochondrial cytochrome b gene (1140 bp) was conducted. PCR primers and protocols for cytochrome b were adopted from Palumbi et al. (1991) (GluDGL: TGACTTGAAR AACCA YCGTGG) and Doadrio & Perdices (2005) (H16460:CGAYCTTCGG ATTAA CAAGA CCG). After checking PCR products on 1% agarose gels, they were

Table 1. Details of the target species: sampling localities, sample size, numbers in the map, coordinates and GenBank accession numbers

Species	River/Basin/Locality	N	Map	Latitude	Longitude	GenBank numbers	References
<i>C. maroccana</i>	Loukkos/Loukkos/Tattofte	2	1	35.034989	-5.767666	AY860123, 24	(Doadrio & Perdices, 2005)
	Sidi Amghar/Loukkos/Brikcha	2	2	34.973592	-5.9546273	OM386831, 32	This study
	Mlilah/Loukkos/Chefchaoun	4	3	34.885996	-5.4096	OM386833-36	This study
	Loukkos/Loukkos/Laghdir	3	4	35.026244	-5.404660	OM386837-39	This study
	Zandoula/Loukkos/Douar Tiama	1	5	34.55383	-5.32160	OM386840	This study
	Kherrouba/Sebou/Kherrouba	2	6	34.436755	-3.969711	AF263075,76	(Perdices & Doadrio, 2001)
		2				KP161091, 92	(Perdices et al., 2016)
	Oued rdat/Sebou/Ain ol fali	2	7	34.585318	-5.870019	OM386819, 20	This study
	Sebou/Sebou/Skhinate	10	8	34.0479	-4.85142	OM386817,OM386821-29	This study
	Sebou/Sebou/Zaouiat Sidi salem	2	9	33.99624	-4.78959	OM386818, 30	This study
<i>C. bilineata</i>	Croatia	1	-	-	-	MN995221	(Buj et al., 2020)
<i>C. brachysoma</i>	China	1	-	-	-	MF004256	(Chen et al., 2018)
<i>C. calderoni</i>	Spain	1	-	-	-	AY860122	(Doadrio & Perdices 2005)
<i>C. choui</i>	China	1	-	-	-	MN273780	(Zhang et al., 2020)
<i>C. dalmatina</i>	Croatia	1	-	-	-	EF605306	(Buj et al., 2008)
<i>C. elongatoides</i>	Black Sea	1	-	-	-	EF012715	(Papoušek et al., 2008)
<i>C. granoci</i>	China	1	-	-	-	DQ105242	(Tang et al., 2005)
<i>C. narentana</i>	Croatia	1	-	-	-	EF605316	(Buj et al., 2008)
<i>C. ohridana</i>	FYROM	1	-	-	-	EF597240	(Šanda et al., 2008)
<i>C. oxycephala</i>	China	1	-	-	-	MF004260	Unpublished
<i>C. paludica</i>	Portugal	1	-	-	-	MN583223	(Perdices & Coelho, 2020)
<i>C. takenoi</i>	Japan	1	-	-	-	LC744432	(Sado et al., 2022)
<i>C. vettonica</i>	Portugal	1	-	-	-	MN583211	(Perdices & Coelho, 2020)

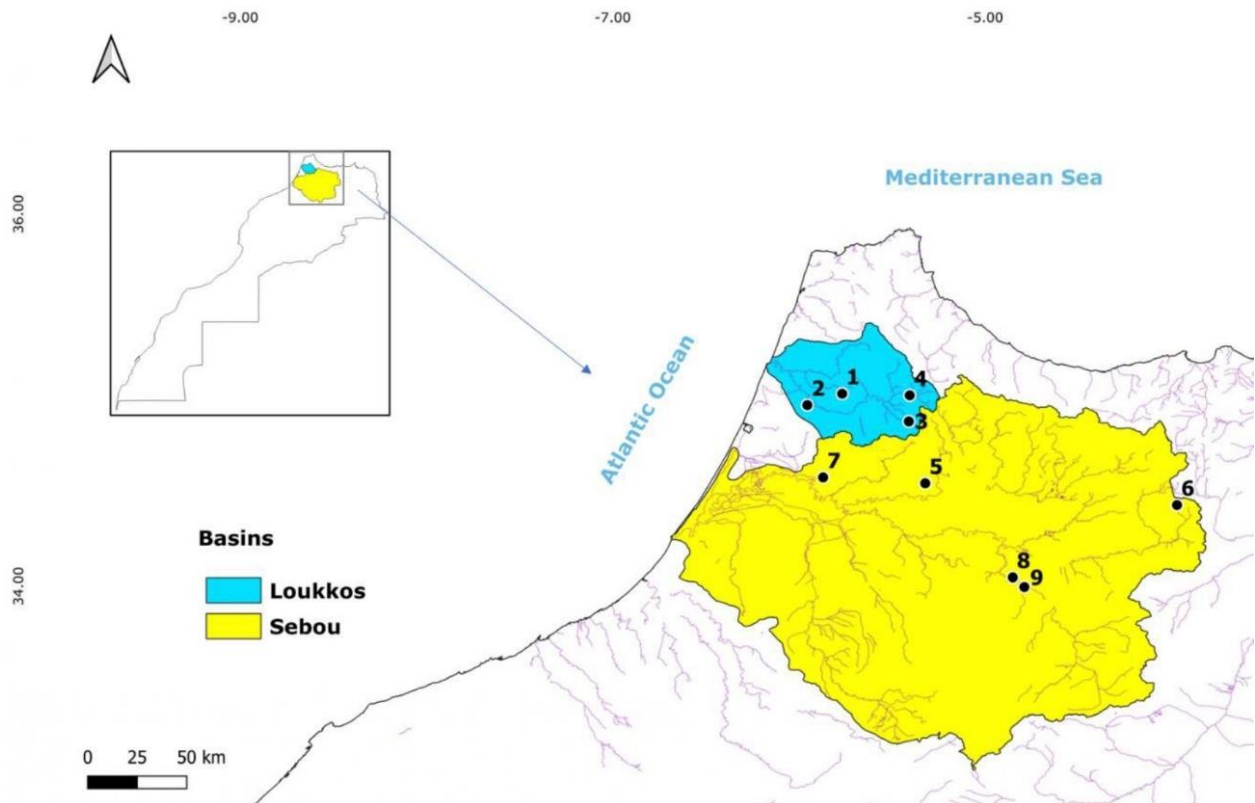


Figure 1. Sampling locations of *Cobitis maroccana*, Morocco. The numbers correspond to localities in Table 1.

purified by ExoSAPITM (USB, Cleveland, USA) and were directly sequenced using amplification primers by MACROGEN Inc (Amsterdam, the Netherlands; <http://www.macrogen.org>) using a 3730XL DNA Sequencer.

Analysis of DNA Sequences

Cytochrome b sequences were preliminarily aligned using Clustal W (Thompson et al., 1994) as implemented in the Mega software v7.0.26 (Kumar et al., 2015) and subsequently aligned manually. In total, 30 sequences of *C. maroccana* were studied, from which 6 were downloaded from GenBank Database. Sequences are submitted to GenBank and the accession numbers are listed in Table 1.

For phylogenetic analysis, identical sequences were collapsed using Alter software (Glez-Peña et al., 2010) in order to smooth computational processes. The best-fit model of sequence evolution for the cytochrome b under the Akaike Information Criterion (Akaike, 1973) were selected using jModeltest v2.1.10 (Guindon & Gascuel 2003; Durrin et al., 2012). In this case, GTR+I+G model was selected. The reference sequences of *Bibarba bibarba* Chen and Chen, 2007 and *Sabanejewia romanica* (Băcescu, 1943) (Genebank IDs: MG710539, AY059337) were used as outgroups. Likewise, additional sequences from different origins were included in the analysis and are listed in Table 1. Bayesian inference was performed using MrBayes v3.1.7 (Ronquist et al., 2012). Bayesian analyses were

performed using two independent runs of four Metropolis-coupled Markov Chain Monte Carlo (MCMC) of 10^6 generations each to estimate the posterior probability distribution. Topologies were sampled every 1000 generations, and majority-rule consensus tree was estimated after discarding the first 10% of generations. The phylogenetic tree was represented using Figtree v1.4.4 program (Rambaut, 2018). Haplotype networks for the cytochrome b gene was constructed using the Median-joining algorithm (Bandelt et al., 1999) as implemented in the program PopArt (Leigh and Bryant, 2015).

The fixation index (F_{st}) which is a measure of population genetic differentiation (Weir and Cockerham, 1984) was also estimated using DNAsp software v6.11.01 (Rozas et al., 2017). The haplotype diversity (H_d) and nucleotide diversity (π) (Nei, 1987) were calculated with their standard deviation (SD) using DNAsp software v6.11.01 (Rozas et al., 2017).

Isolation by distance (IBD) was tested by regressing the pairwise genetic distances (F_{st} in DNAsp) between the population river site samples and geographic distances between the sample locations (km), and using Mantel test (Pearson's correlation) with 10000 permutations of pairs of samples implemented in the XLSTAT trial version software (Addinsoft, Paris, France). The p-value was computed using 10000 Monte Carlo simulations. Geographic distances between sampling river sites were obtained using Google Earth based on longitude and latitude.

Results

Based on the findings of the BI phylogenetic tree (Figure 2), it is evident that the haplotypes of *C. maroccana* from Loukkos and Sebou belong to two distinct clades. The first clade consists of haplotypes from the Sebou basin, while the second clade comprises haplotypes from the Loukkos basin. Previous reports have noted that these two clades together form a well-supported monophyletic Iberian-African ($Pp=1$), along with the Iberian species *C. calderoni*, *C. paludica* and *C. vettonica*. The BI tree also indicates the presence of additional major clades in the phylogenetic analysis:

Asian Clade: This clade encompasses three species, namely *C. Takenoi* from Japan, *C. choii* from China, and *C. granoci* from China.

Balkan Clade: It includes several species, such as *C. ohridana* from FYROM and *C. bilineata* from Croatia.

Ibero-African Clade: This clade represents a distinct group consisting of *C. maroccana* from Morocco, *C. vettonica* from Portugal, *C. paludica* from Portugal, and *C. calderoni* from Spain. This clade is linked to a separate clade consisting of *C. elongatoides* from the Black Sea.

The haplotype network analysis (Figure 3) supported the phylogenetic analysis. The haplotype network illustrates the presence of two distinct mitochondrial lineages within the two juxtaposed basins. The first lineage gathers all the haplotypes of the Sebou basin, while the second lineage groups the haplotypes from the Loukkos basin. Among the three Loukkos haplotypes, they vary from one another by only a single mutation. The arrangement of these haplotypes suggests a star-like pattern, with three dominant haplotypes: HAP_9 shared by ten (33%) individuals from Loukkos, and HAP_1 and HAP_7 shared by five (17%) individuals from Sebou. A strong and significant level of genetic differentiation was identified between the two populations, as indicated by a high F_{st} value of 0.956 ($p < 0.001$) (Table 2).

This result is a very clear indication of low or no gene flow occurring between Loukkos and Sebou river basin populations. The overall haplotypic diversity H_d is 0.844 and the overall nucleotide diversity π is 0.02109. The calculations of genetic diversity for each population are outlined in Table 2. The highest number of haplotypes and the highest value of genetic diversity were identified in the Sebou population. In spite of high haplotype diversity, low nucleotide diversity values revealed that haplotypes of each population differed only slightly. This is also evident from the median-joining haplotype network, which displays few nucleotide differences between haplotypes of each population.

Furthermore, the Mantel test supports isolation by distance (IBD), showing a significant positive correlation between genetic differentiation and geographical distances ($r = 0.496$, $p < 0.05$) (Figure 4).

Discussion

Unlike the Cyprinidae, the Cobitidae family is poorly studied in biogeographical research, primarily because of significant taxonomic confusion among species, the presence of polyploid species and/or genera, and frequent instances of hybridization (Perdices et al., 2016).

Based on the analysis of the cytochrome b gene, the inferred phylogeny in our study (Figure 2) highlights the presence of two divergent clades within *C. maroccana* in Morocco. The phylogenetic clustering of these two clades with the Iberian species (*C. calderoni*, *C. paludica*, and *C. vettonica*) confirms the previously defined morphological lineages. *Cobitis calderoni* is included in the genus *Cobitis* as a sister species of the *Iberocobitis* lineage; this explains its differentiation from other Iberian species by constituting an isolated branch in our study based on the cytochrome b gene (Figure 2) and in the study by Perdices et al. (1995) based on allozymic markers.

The opening of the Strait of Gibraltar after the Messinian salinity crisis (5.5 Ma) (Krijgsman et al., 1999; Crespo-Blanc, 2016; Krijgsman et al., 2018), reflecting a scenario of dispersion by vicariance, led to the separation of the North African species *C. maroccana* from the Iberian species of subgenus *Iberocobitis* (Perdices & Doadrio 2001; Doadrio & Perdices, 2005) and favored its establishment in Moroccan rivers. Interestingly, a similar pattern emerges when examining the evolutionary history of *Luciobarbus guercifensis*. This species, located in the Moulouya basin of eastern Morocco, demonstrates a phylogenetic connection within the Iberian clade (Doadrio et al., 2016; Casal-López & Doadrio, 2018; Touil et al., 2019; Benovics et al., 2020; Ouassal, 2022; Ouassal et al., 2022). This suggests that the separation and colonization process observed in *Cobitis maroccana* could parallel the scenario involving *L. guercifensis*.

Patterns of genetic differentiation between populations were assessed by phylogeographic analyses, more precisely by graphical illustrations of haplotypes and the F_{st} differentiation index. In terms of population differentiation, obligate freshwater fish populations tend to display higher level of genetic differentiation than marine species due to the small effective population size and the isolating nature of riverine dendritic systems, which may act as a barrier to gene flow between populations (Gyllensten, 1985; Ward et al., 1994). In the present study based on the mitochondrial cytochrome b gene, results we obtained support this trend.

The phylogenetic distribution (Figure 2), the haplotype network indicating a very large number of mutations number of mutations (Figure 3) and the high and significant value of the differentiation index F_{st} (Table 2) revealed signs of very strong spatial structure, reflecting extreme allelic isolation between the Loukkos and Sebou.

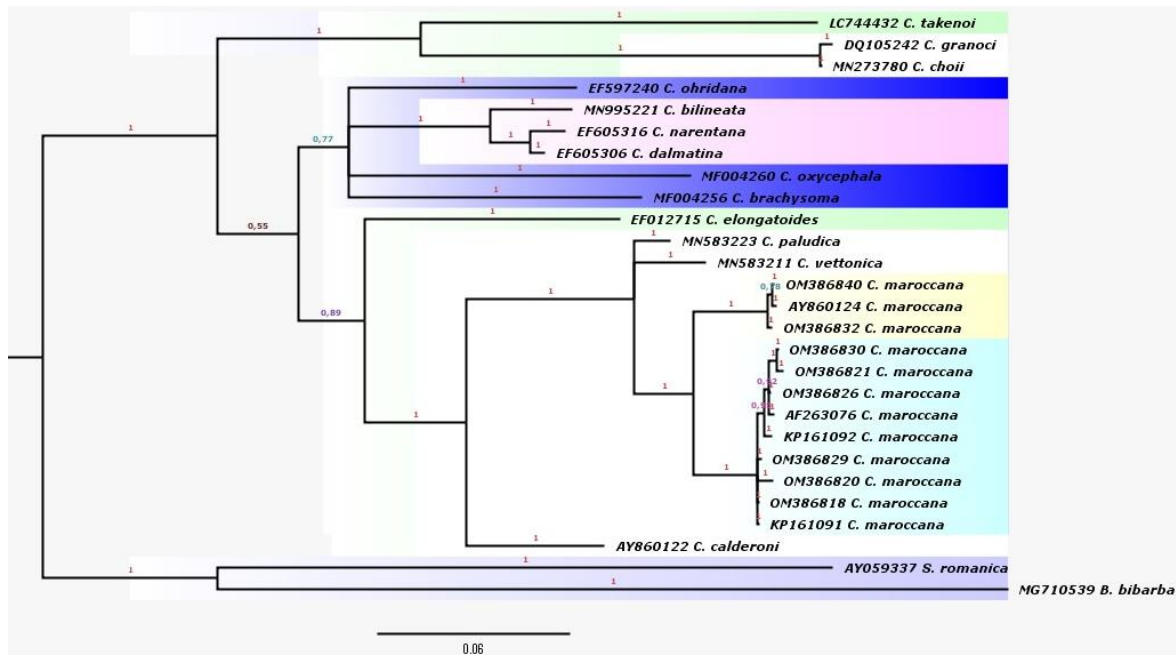


Figure 2. Phylogenetic tree of *Cobitis maroccana* obtained by Bayesian inference. Values indicated on the branches correspond to the posterior probability values (Pp).

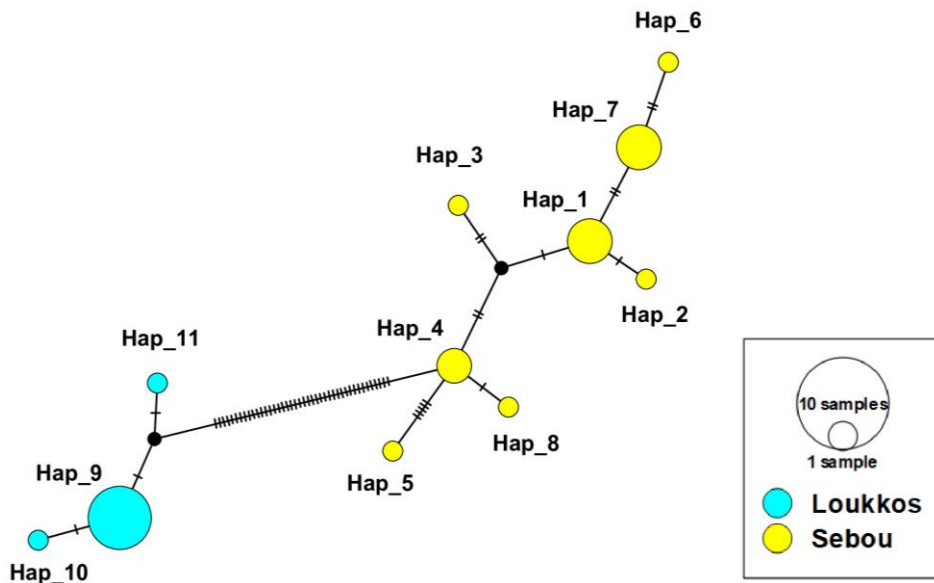


Figure 3. Haplotype network (based on Median-joining) of *Cobitis maroccana* based on cytochrome b gene. Bars reflect mutational steps between haplotypes. Each circle represents a haplotype, and its size is proportional to its frequency. The color denotes the geographic origin of haplotypes, as mentioned in the legend. The small black circles indicate median vectors which represent probable unsampled sequences and are created by the program while the network is being built.

Table 2. Number of haplotypes (H), number of polymorphic sites (S), Haplotype diversity (Hd) and nucleotide diversity (π), standard deviation (SD) and *Fst* values (underlined values)

	Loukkos	Sebou
N	12	18
H	3	8
S	3	15
Hd \pm SD	0.318 \pm 0.164	0.85 \pm 0.055
π \pm SD	0.00044 \pm 0.00025	0.00305 \pm 0.00056
K	0.5	3.471
Loukkos	-	-
Sebou	<u>0.956</u>	-

Three hypotheses can be put forward to explain this strong genetic differentiation. The first relates to natural selection. Faced with environmental pressures, the genotypes in a given population, and consequently the phenotypic traits, are favored by natural selection. The various interventions of this selection have the potential to cause differences between populations that indicate local adaptation and the emergence of the fittest phenotypes (Kawecki & Ebert, 2004). However, this hypothesis cannot be accepted for the simple reason that the two populations do not appear to be in geographical contact. It is in situations of sympatry that natural selection becomes involved (Coyne & Orr, 2004).

The second hypothesis involves demographic change, i.e., change in population size over time (reduction or expansion). This hypothesis has been reinforced by high values for haplotypic diversity H_d , low values for nucleotide diversity π (Table 1) and by the “star-like” pattern of the haplotype network (Figure 3), characteristic of unstable populations that have undergone rapid expansion after a period of low effective size (Slatkin & Hudson, 1991; Rogers & Harpending 1992; Grant & Bowen, 1998; Avise, 2004). This hypothesis is plausible. Thus, *C. maroccana* populations could be the result of a long-term history creating founder effects and/or a more recent history linked to habitat fragmentation due to increased human activities, which probably increases the effects of genetic drift.

The third hypothesis concerns allopatric divergence, which can occur when two initially similar and interfecund populations of the same species are isolated geographically. Over time, the isolated populations differentiate and establish reproductive barriers, eventually forming two distinct species. This is the most speciation mechanism for fish in inland waters (Jordan, 1908; Coyne & Orr, 2004). This hypothesis is also plausible. The significant isolation by distance (IBD) pattern ($r = 0.496$) supports this hypothesis. IBD indicates geographically closer populations share more genetic similarity, suggesting restricted gene flow. In our case study, the Rifian piedmont composed of a variety of reliefs between Loukkos and Sebou (Boudouhou, 2004) could have acted as a physical barrier, preventing gene flow from one basin to the other and thus divergence through allopatric speciation. We can therefore presume that the two populations, Loukkos and Sebou, belong to two sister species. According to Jordan's law (1908), the most closely related species, i.e., sister species, have a low probability of cohabiting in the same region, but rather a strong possibility of being located on either side of a dispersal barrier. This pattern is observed in several *Luciobarbus* species in Morocco (Ouassal, 2022; Ouassal et al., 2022) which are related and located in different but juxtaposed basins in Morocco.

However, this idea of allopatric speciation remains to be proven by morphological analyses, as well as by the use of other molecular markers (mitochondrial and

nuclear combined). The concordance between morphological divergence and molecular divergence could well reveal the speciation process within *C. maroccana*.

Additionally, one of the best ways of proving this allopatric speciation is conducting tests of reproductive isolation, such as examining the outcome of breeding between individuals from the Loukkos and Sebou populations when they meet in sympatry in a secondary contact zone (Coyne & Orr, 2004), which could provide valuable information about their status as separate species. If individuals from the two populations are unable to successfully interbreed or produce viable offspring, this would provide strong evidence in support of the hypothesis that the two populations represent separate species.

Conclusion

The current study emphasizes the usefulness of the cytochrome b mitochondrial gene as a potential marker to provide valuable insights into the population genetic differentiation of *C. maroccana* populations. Our finding exposed signs of very strong genetic differentiation between the Loukkos and Sebou populations, supporting the idea that the two populations are on different evolutionary trajectories and may be evolving into distinct species over time.

In summary, further research, combining genetic, morphological, ecological studies and reproductive isolation tests, would be needed to determine whether the two populations of *C. maroccana* should be considered separate species.

Ethical Statement

The authors claim that all procedures used in this study adhere to the ethical norms of the applicable national and institutional guidelines on the care and use of laboratory animals.

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Author Contribution

All authors contributed to manuscript preparation. First Author: conceiving the research, conducting laboratory experiments, data analysis, and interpretation; Second Author: supervision, fieldwork and collection of fish specimens; Third Author: supervision, fieldwork, collection of fish specimens and funding; Fourth Author: supervision, contributing to the discussion.

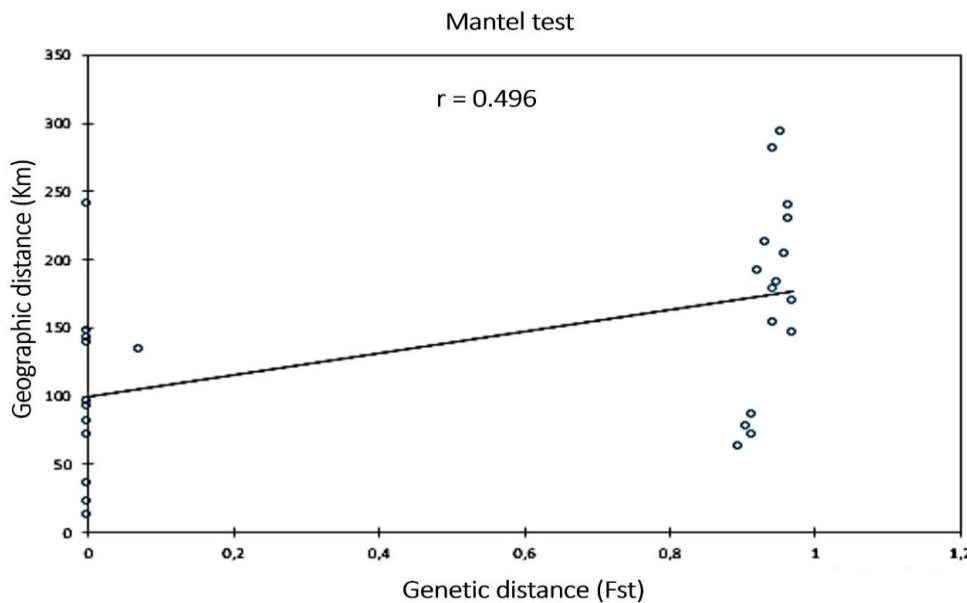


Figure 4. Mantel Test: Scatter plots of pairwise genetic distance (Fst) vs. geographic distance (km) between river site sample populations

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

- Aboim, M. A., Mesquita, N., Drago, M., Coelho, M. M., & Alves, M. J. (2013). Assessing interdrainage connections: patterns of genetic diversity in an Iberian cyprinid fish. *Biological Journal of the Linnean Society*, 109, 656–669.
- Akaike, H. (1973). Information theory and an extension of the maximum likelihood principle. In B. N. Petrov & F. Csaki (Eds.), *Proceedings of the Second International Symposium on Information Theory* (pp. 267–281).
- Avise, J. C. (2004). *Molecular markers, natural history, and evolution*. Sinauer Associates.
- Băcescu, M. (1961). Données nouvelles sur les loches (Cobitis) d'Europe avec des références sur les cagnettes de l'Italie du Nord. *Travaux du Museum National d'Histoire Naturelle Grigore Antipa*, 3, 281–301.
- Băcescu, M. (1962). Contribution à la systématique du genre *Cobitis* description d'une espèce nouvelle, *Cobitis calderoni*, provenant de l'Espagne. *Revue Roumaine de Biologie*, 4, 435–448.
- Bandelt, H. J., Forster, P., & Rohlf, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, 16, 37–48. <https://doi.org/10.1093/oxfordjournals.molbev.a026036>
- Benovics, M., Vukić, J., Šanda, R., Rahmouni, I., Šimková, A., (2020). Disentangling the evolutionary history of peri-Mediterranean cyprinids using host-specific gill monogeneans. *International Journal for Parasitology*, 12, 969–984. <https://doi.org/10.1016/j.ijpara.2020.05.007>
- Bianco, P. G. (1998). Diversity of Barbinae fishes in southern Europe with description of a new genus and a new species (Cyprinidae). *Italian Journal of Zoology*, 65(S1), 125–136. <https://doi.org/10.1080/11250009809386804>
- Boudouhou, N. (2004). La population de la région du piémont rifain entre le Loukkos et le Sebou (Maroc) de la période romaine à la période islamique. *Carocci L'Africa romana, Attide XV convegno di studio Tozeur*, 11–15.
- Brahimi, A., Tarai, N., Benhassane, A., Henrard, A., & Libois, R. (2016). Genetic and morphological consequences of Quaternary glaciations: a relic barbel lineage (*Luciobarbus pallaryi*) of the Guir basin (Algeria). *Comptes Rendus Biologies*, 339, 83–98. <https://doi.org/10.1016/j.crvi.2015.12.003>
- Casal-López, M., & Doadrio, I. (2018). The Messinian imprint on the evolution of freshwater fishes of the genus *Luciobarbus* Heckel, 1843 (Teleostei, Cyprinidae) in the western Mediterranean. *Journal of Biogeography*, 45, 15936–1603. <https://doi.org/10.1111/jbi.13244>
- Coyne, J. A., & Orr, H. A. (2004). *Speciation*. Sinauer Associates.
- Crespo-Blanc, A., Comas, M., & Balanyá, J. C. (2016). Clues for a Tortonian reconstruction of the Gibraltar Arc: Structural pattern, deformation diachronism and block rotations. *Tectonophysics*, 683, 308–324. <https://doi.org/10.1016/j.tecto.2016.05.045>
- Crivelli, A. J. (2010). *Cobitis maroccana*. The IUCN Red List of Threatened Species 2010: e.T40697A10351287.
- Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, 9, 772. <https://doi.org/10.1038/nmeth.2109>

- Doadrio I, & Perdices A. (2005). Phylogenetic relationships among the Ibero-African cobitids (*Cobitis*, cobitidae) based on cytochrome b sequence data. *Molecular Phylogenetics and Evolution*, 37(2), 484-493. <https://doi.org/10.1016/j.ympev.2005.07.009>
- Doadrio, I., Casal-López, M., Perea, S., & Yahyaoui, A. (2016). Taxonomy of rheophilic *Luciobarbus* Heckel, 1842 (Actinopterygii, Cyprinidae) from Morocco with the description of two new species. *Graellsia*, 72(1), e039. <https://doi.org/10.3989/graellsia.2016.v72.153>
- Doadrio, I., Sousa-Santos, C., Robalo, J., & Perea, S. (2023). Description of three new species of the genus *Cobitis* L., 1758 (Actinopterygii, Cobitidae) in the Iberian Peninsula. *Limnetica*, 43(2), 000-000.
- Fricke, R., Eschmeyer, W.N., & Van der Laan, R. (2021). *Eschmeyer's Catalog of Fishes*. <https://research.calacademy.org/research/ichthyology/catalog/fishcatmain.asp>
- Glez-Peña, D., Gómez-Blanco, D., Reboiro-Jato, M., Fdez-Riverola, F., & Posada D. (2010). ALTER : program-oriented format conversion of DNA and protein alignments. *Nucleic Acids Research*, 38(2), 14-18.
- Grant, W. S., & Bowen, B. W. (1998). Shallow population histories in deep evolutionary lineages of marine fishes: insights from sardines and anchovies and lessons for conservation. *Journal of Heredity*, 89(5), 415-426. <https://doi.org/10.1093/jhered/89.5.415>
- Guindon, S., & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, 52, 696-704. <https://doi.org/10.1080/10635150390235520>
- Gyllensten, Y. U. (1985). The genetic structure of fish: differences in the intraspecific distribution of biochemical genetic variation between marine, anadromous, and freshwater species. *Journal of Fish Biology*, 26, 691-699. <https://doi.org/10.1111/j.1095-8649.1985.tb04309.x>
- Hrbek, T., & Meyer, A. (2003). Closing of the Tethys Sea and the phylogeny of Eurasian killifishes (Cyprinodontiformes: Cyprinodontidae). *Journal of Evolutionary Biology*, 16, 17- 36. <https://doi.org/10.1046/j.1420-9101.2003.00475.x>
- Jordan, D. S. (1908). The law of germinate species. *American Naturalist*, 42, 73-80. <https://doi.org/10.1086/278905>
- Kawecki, T. J., & Ebert, D. (2004). Conceptual issues in local adaptation. *Ecology Letters*, 7, 1225-1241. <https://doi.org/10.1111/j.1461-0248.2004.00684.x>
- Kottelat, M., & Freyhof, J. (2007). *Handbook of European Fishes*. Publications Kottelat.
- Krijgsman, W., Hilgen, F. J., Raffi, I., Sierro, F. J., & Wilson, D.S. (1999). Chronology, causes and progression of the Messinian salinity crisis. *Nature*, 400, 652-655. <https://doi.org/10.1038/23231>
- Krijgsmana, W., Capellaa, W., Simona, D., Hilgena, F. J., Kouwenhovena, T. J., Meijera, P. T., Sierrob, F.J., Tulburea, M. A., van den Bergb, B. C. J., van der Scheeb, M., & Flecker, R. (2018). The Gibraltar Corridor: Watergate of the Messinian Salinity Crisis. *Marine Geology*, 403, 238-246. <https://doi.org/10.1016/j.margeo.2018.06.008>
- Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33(7), 1870-1874. <https://doi.org/10.1093/molbev/msw054>
- Leigh, J.W., & Bryant, D. (2015). Data from: PopART: full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, 6(9), 1110-1116. <https://doi.org/10.5061/dryad.4n4j1>
- Myers, G. S. (1949). Salt-tolerance of fresh-water fish groups in relation to zoogeographical problems. *Bijdragen tot de dierkunde*, 28(1), 315-322.
- Nalbant, T. T., Ráb, P., Böhlen, J., & Saitoh, K. (2001). Evolutionary success of the loaches of the genus *Cobitis* (Pisces: Ostariophysi: Cobitidae). *Travaux du Muséum National d'Histoire Naturelle Grigore Antipa*, 43, 277-289.
- Nelson, J. S., Grande, T.C., & Wilson, M. V. (2016). *Fishes of the world*. John Wiley and Sons.
- Ouassal, K. (2022). *Diversité génétique mitochondriale, phylogéographie et histoire démographique des poissons indigènes des eaux continentales du Maroc : Cas des genres *Luciobarbus*, *Cobitis* et *Coptodon**. Thèse de Doctorat, Université Mohammed V.
- Ouassal, K., Doadrio, I., Perea, S., Casal-López, M., Yahyaoui, A., & Jaziri, H. (2022). Mitochondrial genetic diversity, phylogeography and historical demography of Moroccan native freshwater fishes : a case study of the genus *Luciobarbus* Heckel, 1843. *Biologia*, 77(5). <https://doi.org/10.1007/s11756-022-01107-6>
- Palumbi, S. R., Martin, A. P., Romano, S., McMillan, W.O., Stice, L., & Grabowski, G. (1991). *The simple fool's guide to PCR Version 2.0*. Honolulu: Department of Zoology and Kewalo Marine Laboratory.
- Perdices, A., Machordom, A., & Doadrio, I. (1995). Allozyme variation of African and Iberian populations of the genus *Cobitis*. *Journal of Fish Biology*, 47(4), 707-718. <https://doi.org/10.1111/j.1095-8649.1995.tb01936.x>
- Perdices, A., & Doadrio, I. (2001). The molecular systematics and biogeography of the European cobitids based on mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, 19, 468-478. <https://doi.org/10.1006/mpev.2000.0900>
- Perdices, A., Bohlen, J., Šlechtotová, V., & Doadrio, I. (2016). Molecular evidence for multiple origins of the European spined loaches (Teleostei, Cobitidae). *PLoS ONE*, 11(1), e0144628. <https://doi.org/10.1371/journal.pone.0144628>
- QGIS Development Team, (2021). *QGIS Geographic Information System. Open-Source Geospatial Foundation Project*. <http://qgis.osgeo.org/en/site/>
- Rambaut, A. (2018). *FigTree v1.4.4*. <http://tree.bio.ed.ac.uk/software/figtree/>
- Rogers, A. R., & Harpending, H. (1992). Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution*, 9(3), 552-569. <https://doi.org/10.1093/oxfordjournals.molbev.a040727>
- Ronquist, F., Teslenko, M., Van der Mark, P., Ayres, D., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A., & Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, 61, 539-542. <https://doi.org/10.1093/sysbio/sys029>
- Rozas, J., Ferrer-Mata, A., Sanchez-Del Barrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., & Sanchez-Garcia, A. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution*, 34 (12), 3299-3302. <https://doi.org/10.1093/molbev/msx248>

- Slatkin, M., & Hudson, R. R. (1991). Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. *Genetics*, 129, 555-562. <https://doi.org/10.1093/genetics/129.2.555>
- Slavík, O., & Ráb, P. (1996). Life history of spined loach, *Cobitis taenia*, in an isolated site (Pšovka Creek, Bohemia). *Folia zoologica*, 45 (3), 247–252.
- Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22(22), 4673–4680. <https://doi.org/10.1093/nar/22.22.4673>
- Touil, A., Casal-López, M., Bouhadad, R., & Doadrio, I. (2019). Phylogeny and phylogeography of the genus *Luciobarbus* (Heckel, 1843) in Algeria inferred from mitochondrial DNA sequence variation. *Mitochondrial DNA Part A*, 30(2), 332-344. <https://doi.org/10.1080/24701394.2018.1526928>
- Tsigenopoulos, C. S., Kotlik, P., Berrebi, P. (2002). Biogeography and pattern of gene flow among *Barbus* species (Teleostei: Cyprinidae) inhabiting the Italian Peninsula and neighboring Adriatic drainages as revealed by allozyme and mitochondrial sequence data. *Biological Journal of the Linnean Society*, 75(1), 83-99. <https://doi.org/10.1046/j.1095-8312.2002.00007.x>
- Ward, R. D., Woodwark, M., & Skibinski, D. O. F. 1994. A comparison of genetic diversity levels in marine, freshwater and anadromous fishes. *Journal of Fish Biology*, 44, 213-232. <https://doi.org/10.1111/j.1095-8649.1994.tb01200.x>
- Weir, B. S., & Cockerham, C. C. (1984). Estimating *F*-statistics for the analysis of population structure. *Evolution*, 38(6), 1358-1370. <https://doi.org/10.1111/j.1558-5646.1984.tb05657.x>
- Yahyaoui, A., Perea, S., Garzon, P., & Doadrio, I. (2020). *Atlas des poisons des eaux continentales du Maroc*. Faculté des Sciences de Rabat, Maroc.