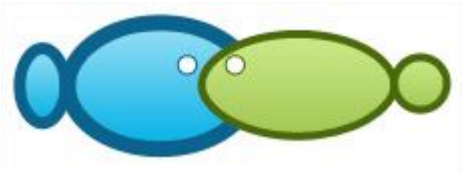

Assessment of genetic diversity in *Luciobarbus rifensis* Doadrio, Casal-Lopez & Yahyaoui, 2015 (Teleostei: Cyprinidae) using cytochrome b

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Abstract. Understanding the genetic diversity of native fishes distributed throughout Morocco, e.g., *Luciobarbus rifensis*, the target species of this study, is essential for the management of these species as a natural resource and for improving their conservation. In this study, total genomic DNA extracted from *L. rifensis* samples (n =128) belonging to four watersheds: Loukkos, Hachef, Laou and Hajera was analyzed to assess the genetic diversity of this freshwater fish. Sequencing of 1140 bp Cytochrome-b mtDNA fragment revealed the presence of 18 number of haplotypes (h) in the overall dataset with a high haplotype diversity value (Hd = 0.68) and a low nucleotide diversity value (π = 0.00090). Fixation index (Fst) values in the four populations ranged from 0.05326 to 0.53408. The pattern of haplotype network revealed no obvious genealogical structure. Our results showed a moderate to high value of Fst which means a moderate to high level of genetic divergence between the different localities. All results obtained in this study are the data that were extracted for *L. rifensis* species for the first time.

Key Words: *Luciobarbus rifensis*, genetic diversity, genetic differentiation, mitochondrial DNA, cytochrome b gene.

Introduction. The Rifian barbel (*Luciobarbus rifensis*) is a freshwater fish belonging to the Cyprinidae family and is endemic to northern Morocco, populating rivers from the Loukkos watershed on the eastern Atlantic slope to the Laou watershed on the south-western Mediterranean slope. In recent years, *L. rifensis* has faced the effects of several environmental and anthropogenic pressures such as pollution, damming, habitat degradation and proliferation of exotic species (Casal-Lopez et al 2015). In fact, those pressures can lead to reduced genetic diversity, modified population genetic structure and a decline of the total effective population size (Keyghobadi 2007; Radespiel & Bruford 2014). Thus, the conservation of genetic diversity is widely considered to be an essential basis of all conservation efforts because genetic diversity is thought to be mandatory to evolutionary adaptation, and such adaptation is the key to the long-term survival of any species (Schemske et al 1994).

To elucidate the genetic diversity of fish population, different molecular markers are used. The mitochondrial DNA is among the most frequent marker used. This marker is an excellent tool in conducting phylogenetic, phylogeographic, ecological and population genetics studies (Howell & Gilbert 1988; Galewski et al 2006; Galtier et al 2006). Likewise, it is recognized to have some advantages compared to nuclear DNA in the study of sequence divergence, due to its rapid evolution rate, low recombination rate, lack of introns and high copy number (Brown 1985; Luo et al 2011).

Until recently, no previous works have been conducted to assess the genetic diversity in *L. rifensis*. Therefore, the current study aims to determine the genetic diversity, the genetic differentiation and the demographical history of the endemic *L. rifensis* using the mitochondrial gene cytochrome b (Cytb).

Material and Method

Sampling. *Luciobarbus rifensis* individuals 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.

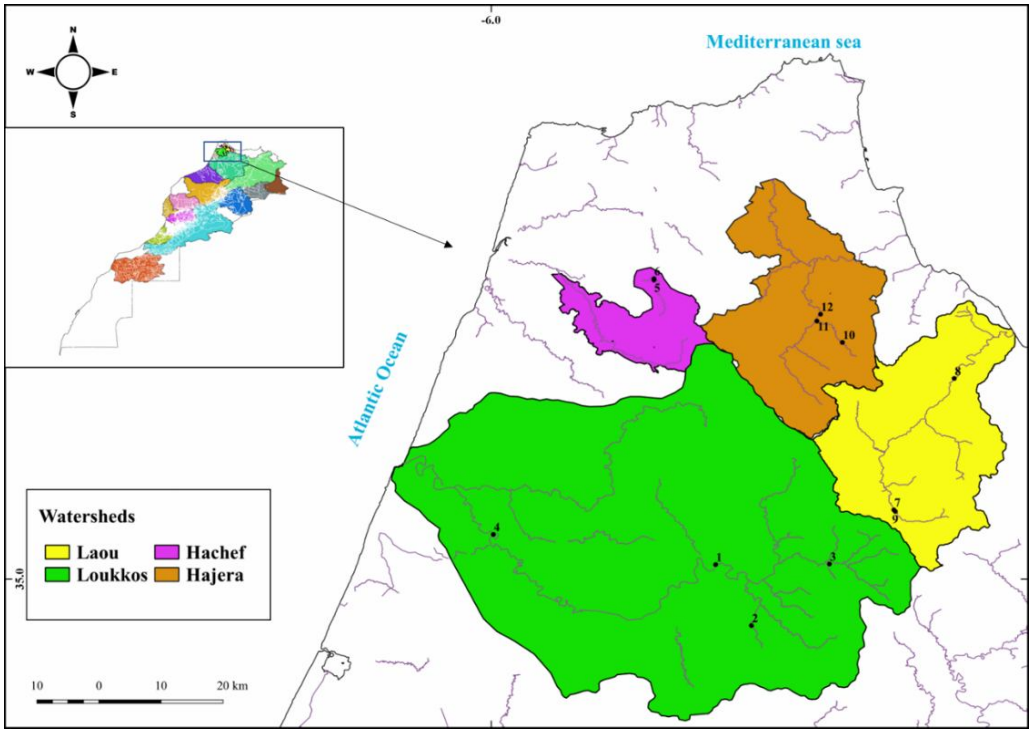

Figure 1. Sampling locations of *Luciobarbus rifensis* in four watersheds, Morocco.

Table 1

Details of *Luciobarbus rifensis* sample collection

	Sites of collection	*Coordinates*
1	Loukkos river, Loukkos watershed, Mouires	35°01'30.7"N 5°36'18.4"W
2	Zandoula river, Loukkos watershed, Douar Tiama	34°55'04.1"N 5°32'31.4"W
3	Loukkos river, Loukkos watershed, Laghdir	35°01'34.5"N 5°24'16.8"W
4	Sidi Amghar river, Loukkos watershed, Brikcha	35°04'41.9"N 5°59'47.4"W
5	Hachef river, Hachef watershed, Dar Chaoui	35°31'36.4"N 5°42'49.6"W
6	Hachef river, Hachef watershed, Dar Chaoui	35°31'44.3"N 5°42'49.1"W
7	Laou river, Laou watershed, Chefchaoun	35°07'18.1"N 5°17'28.9"W
8	Laou river, Laou watershed, Beni Ferten	35°21'11.7"N 5°11'05.4"W
9	Laou river, Laou watershed, Derdara	35°07'08.4"N 5°17'20.0"W
10	Hajera river, Hajera watershed, Zinate Tétouane	35°25'00.5"N 5°22'55.0"W
11	Hajera river, Hajera watershed, Zinate Tétouane	35°27'16.0"N 5°25'36.6"W
12	Hajera river, Hajera watershed, Zinate Tétouane	35°27'59.0"N 5°25'13.1"W

DNA extraction, PCR, and sequencing. In total, 128 sequences of Rifian barbels were studied, from which 25 were downloaded from GenBank Database (Table 2).

Whole genomic DNA was extracted from fin tissue of each specimen, using the commercial kit Biosprint15 for tissue and blood (Qiagen), according to the manufacturer's protocol. The entire mitochondrial cytochrome b gene (Cytb) (1140 pb) was amplified. Primers and protocols used for PCR for MT-CYB followed Palumbi et al (1991) (GluDGL:TGACTTGAAR AACCA YCGTGG) and Doadrio & Perdices (2005) (H16460:CGAYC TTCGG ATTAA CAAGA CCG). After checking PCR products on 1% agarose gels, they were purified by ExoSAPITTM (USB, Cleveland, USA) and were directly sequenced by

MACROGEN Inc (Amsterdam, the Netherlands; http://www.macrogen.org) using a 3730XL DNA Sequencer.

Table 2

GenBank accession numbers of downloaded sequences additional in this study

Species	*Locality*	*Code MNCN*	*GenBank acc. no.*
Luciobarbus rifensis	Loukkos river, Loukkos watershed, Tattofte	307M	JF798259.1
	Loukkos river, Loukkos watershed, Mouires	312M	KY457946.1
	Zandoula river, Loukkos watershed	AT22049	KY457919.1
	Zandoula river, Loukkos watershed	AT22051	KY457920.1
	Zandoula river, Loukkos watershed	AT22054	KY457921.1
	Zandoula river, Loukkos watershed	AT22055	KY457922.1
	Zandoula river, Loukkos watershed	AT22059	KY457923.1
	Zandoula river, Loukkos watershed	AT22065	KY457924.1
	Loukkos river, Loukkos watershed	MNCN:ADN:85616	KT003936.1
	Loukkos river, Loukkos watershed	MNCN:ADN:85617	KT003937.1
	Loukkos river, Loukkos watershed	MNCN:ADN:85618	KT003938.1
	Loukkos river, Loukkos watershed	MNCN:ADN:85619	KT003939.1
	Loukkos river, Loukkos watershed	MNCN:ADN:85620	KT003940.1
	Hachef river, Hachef watershed	MNCN:ADN:85613	KT003933.1
	Hachef river, Hachef watershed	MNCN:ADN:85614	KT003934.1
	Hachef river, Hachef watershed	MNCN:ADN:85615	KT003935.1
	Hachef river, Hachef watershed	MNCN:ADN:85611	KT003931.1
	Hachef river, Hachef watershed	MNCN:ADN:85612	KT003932.1
	Hachef river, Hachef watershed	210M	KY457925.1
	Hachef river, Hachef watershed	AT23602	KY457926.1
	Laou river, Laou watershed	MNCN:ADN:85636	KT003930.1
	Laou river, Laou watershed	MNCN:ADN:57479	KT003929.1
	Laou river, Laou watershed	MNCN:ADN:57478	KT003928.1
	Laou river, Laou watershed	MNCN:ADN:57477	KT003927.1
	Laou river, Laou watershed	MNCN:ADN:57476	KT003926.1

Data analysis. Cytochrome b sequences were preliminarily aligned using Clustal W (Thompson et al 1994) as implemented in the Mega software v 7.0.26 (Kumar et al 2015) and subsequently aligned manually.

To characterize genetic diversity for each population, haplotype diversity (Hd, Nei 1987), nucleotide diversity (π, Nei 1987) and the average number of pairwise nucleotide differences (K, Tajima 1983) were calculated with their standard deviation (SD) using DNAsp software package V.6.11.01 (Rozas et al 2017). The degree of genetic differentiation between populations was also tested using the fixation index Fst (Weir & Cockerham 1984).

For the genetic structure, the Network v.5.0.0.3 software (http://www.fluxus-engineering.com) was used to construct haplotype network from mtDNA data based on the Median-joining algorithm (Bandelt et al 1999).

In order to infer the population demographic history of *L. rifensis*, Tajima's D (Tajima 1989), Fu's (Fs) (Fu 1997) and mismatch distribution tests were performed with the program DNAsp. Harpending's raggedness index (r, Harpending 1994) and the R2 statistic of Ramos-Onsins and Rozas (Ramos-Onsins & Rozas 2002) also were determined.

Results. A total of 18 different haplotypes were identified within the 128 mtDNA analyzed sequences (1140 pb) of *L. rifensis*. The most haplotypes were detected in the Loukkos population. Overall genetic diversity showed a value of nucleotide diversity (π) of 0.0009 and haplotype diversity (Hd) of 0.68. For different populations, haplotype diversity ranged from 0.490 (Hachef) to 0.706 (Loukkos), and nucleotide diversity ranges between 0.00053 (Hajera) and 0.00134 (Laou). All population genetic statistics are listed in Table 3.

Table 3

Diversity indices for whole dataset and main watersheds

Population	*N*	*h*	*S*	*Hd (SD)*	*π (SD)*	*K*
Dataset	128	18	17	0.68 (0.029)	0.00090 (0.00009)	1.025
Loukkos	45	10	12	0.706 (0.058)	0.00103 (0.00018)	1.18
Laou	39	9	5	0.690 (0.066)	0.00134 (0.00016)	1.522
Hachef	21	5	5	0.490 (0.127)	0.00064 (0.00021)	0.733
Hajera	23	3	2	0.549 (0.060)	0.00053 (0.00008)	0.601

N = number of individuals analyzed; h = number of haplotypes; π = nucleotide diversity; Hd = haplotype diversity; S = number of polymorphic sites; SD = standard deviations; K = average number of nucleotide differences.

The result of pairwise population FST showed that genetic differentiation between the four populations ranged from 0.05326 to 0.53408 (Table 4). Overall, significant differentiation between populations was observed ($p < 0.05$). FST index is one of the most commonly used measures for assessing the genetic differentiation between/among populations (Holsinger & Weir 2009).

Table 4

Genetic differentiation (FST) based on cytochrome b DNA sequences of *Luciobarbus rifensis* in Morocco

Populations	*Loukkos*	*Hachef*	*Laou*	*Hajera*
Loukkos	–			
Hachef	0.53408	–		
Laou	0.31057	0.13702	–	
Hajera	0.19793	0.25759	0.05326	–

The genetic relationship was demonstrated by the haplotype distribution network (Figure 2), which revealed no clear geographic pattern among the 128 sequences included in this analysis. The haplotype network showed few mutational steps among the haplotypes found. The haplotype 3 (H_3) was the most common among the Loukkos population and the haplotype 5 (H_5) was the most common haplotype in the Hachef population.

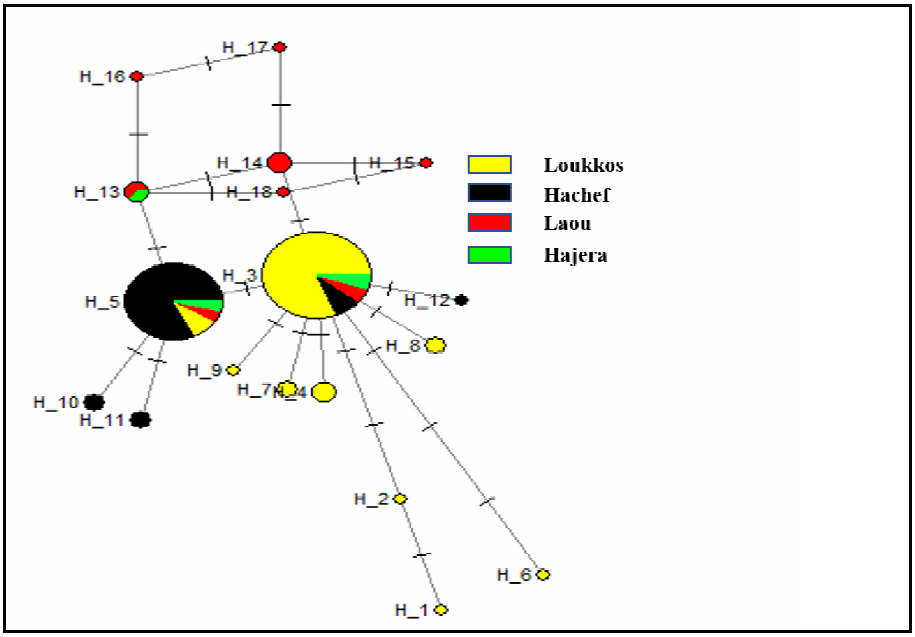

Figure 2. Haplotype network (based on Median-joining) of *L. rifensis* based on Cytb gene. Number of nodes between haplotypes are the mutational steps. Each circle represents a haplotype and their size is proportional to their frequencies. The color represents the geographic origin of haplotypes, as indicated in the legend.

The mismatch distribution shape including all populations was unimodal and perfectly adjusted to the distribution predicted by the growth-decline population model (Figure 3). Indeed, low values of the Raggedness index and R2 statistic, along with the negative values of Tajima's D and Fu's Fs neutrality tests (Table 5), further indicating that *L. rifensis* populations undergone a recent expansion.

Table 5

Demographic characteristics of each population and the entire dataset for mitochondrial cytochrome b gene

Population	*D (P)*	*Fs (P)*	*r (P)*	*R2 (P)*
Dataset	-1.84392 **(0.005)**	-14.392 **(0.001)**	0.1067 (0.23007)	0.0327 **(0.04)**
Loukkos	-1.71797 **(0.024)**	-4.739 **(0.042)**	0.0839 (0.19)	0.0564 **(0.03236)**
Hachef	-1.42367 (0.072)	-1.862 (0.147)	0.0822 (0.09434)	0.08 **(0.0)**
Laou	0.73507 (0.8)	-2.718 **(0.088)**	0.1230 (0.41625)	0.1522 (0.79477)
Hajera	0.24251 (0.634)	0.244 (0.722)	0.2044 (0.39769)	0.1640 (0.58304)

D = Tajima's D test; Fs = Fu's Fs test; r = raggedness index; R2 = Ramos-Osins and Rozas test. P-values in brackets, bold numbers correspond to significant values in neutrality tests.

Discussion. This is the first article discussing the genetic diversity of the freshwater fish *L. rifensis*. In the current study, the diversity analysis shows that the cytochrome b gene of *L. rifensis* has a moderately high haplotype diversity (0.490 to 0.706), considering that Hd ranges from 0.0 to near 1.0 (Avise 1994) and low nucleotide diversity (0.00053 to 0.00134), since that π ranges from 0.0005 to 0.2 (Stephan & Langley 1992). The low nucleotide diversity and the high haplotype diversity implied that the population of *L. rifensis* probably underwent expansion after a period of low effective population size.

The unimodal shape of mismatch distribution was reliable with the predicted distribution under a model of population expansion (Rogers & Harpending 1992). When a population is not under demographic equilibrium, it means that it is expected to be more sensible to diseases, parasites and environmental fluctuations (Atarhouch et al 2006). Tajima's D and Fu's FS values are sensitive to bottleneck effects and population expansion, which lead to the more negative values of Tajims's D and Fu's FS (Tajima 1993, 1996; Martel et al 2004). Furthermore, neither the Ramos-Osins nor the Rozasnor Harpending's raggedness index test reject the hypothesis of a sudden expansion model. Populations with a low nucleotide diversity value ($\pi < 0.005$) and high haplotype diversity ($Hd > 0.5$) show that they experienced a bottleneck effect (Grant & Bowen 1998). Likewise, low nucleotide diversity and high haplotype diversity values designate that the time after population expansion is long enough to observe the change in haplotypes that resulted from mutation, but it is not long enough to accumulate large differences among sequences (Avise 2000). Additionally, low nucleotide diversity values are revealing low population diversity; many factors namely habitation, bottleneck effects and anthropogenic activity perturb the genetic diversity (Fennando et al 2000; Ma et al 2010). Also, the structure of the median joining network indicated that there was no obvious genealogical structure among *L. rifensis* populations, and that a population expansion event may have occurred during evolution.

The overall FST value in the present study was found to be 0.27857 ($p < 0.05$) suggesting significant genetic differentiation among the sampled *L. rifensis* populations. Moderate to very great genetic differentiation was revealed among the four populations (0.053 to 0.534) and the highest FST value detected (0.53408) was between Loukkos and Hachef watersheds. As a general rule FST values of 0–0.05 characterize little differentiation, values of 0.05-0.25 designate moderate differentiation, and values higher than 0.25 indicate very great differentiation among populations (Wright 1978). The moderate to high level of population genetic differentiation suggest that the populations of *L. rifensis* have diverged for quite a long time and are characterized by a low connectivity and a low dispersion.

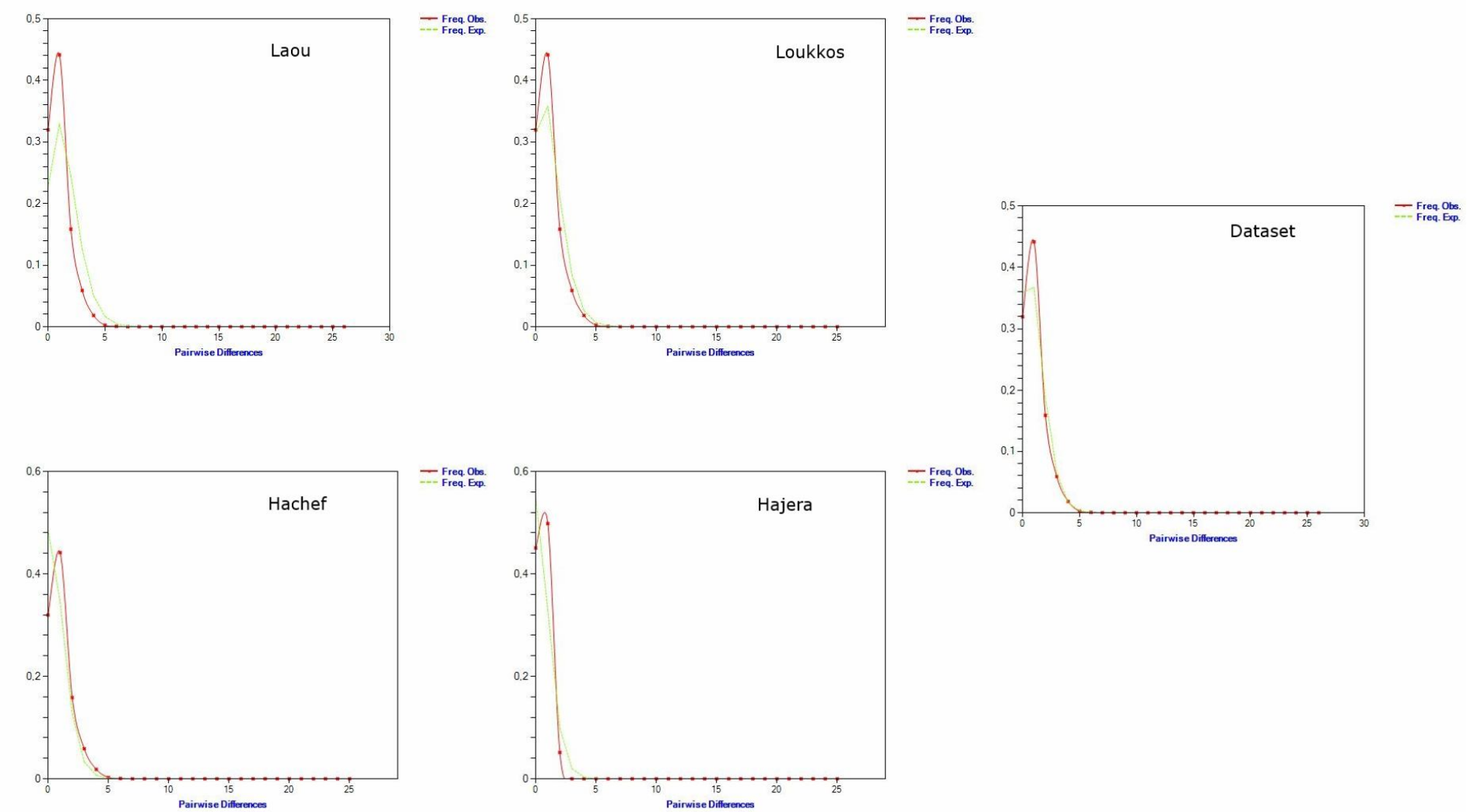

Figure 3. Mismatch distributions observed (green, dotted lines) and expected under an expansion model (red, continuous line) for *Luciobarbus rifensis* populations.

Conclusions. Our research proved the power of mtDNA fragments sequencing to give useful insights into the population genetic diversity and genetic structure of Rifian barbel populations.

Based on our results, the diversity analysis shows that the cytochrome b gene of *L. rifensis* has a moderately high haplotype diversity and low nucleotide diversity. The combination of low nucleotide diversity and high haplotype diversity implied that the population of *L. rifensis* probably underwent expansion after a period of low effective population size.

Our study provides a first description of the genetic diversity of *L. rifensis* in Morocco, information that could be useful in the implementation of conservation plans and fisheries management for this species aiming to maintain its genetic diversity. In addition, we have the prospect of expanding our study on other native species of freshwater fish of Morocco.

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