Genetic evidence for the rediscovery in the wild of the critically endangered Sahara killifish *Apricaphanius saourensis* (Cyprinodontiformes: Aphaniidae)

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Abstract

Apricaphanius saourensis was described in 2006 from the Saoura River in western Algeria, and is currently listed as possibly extinct in the wild. We recently discovered an aphaniid population in a very isolated secondary wadi of the Guir River about 115 Km northwest of *A. saourensis'* type locality, which we hypothesized could belong to *A. saourensis* based on images taken from living individuals. We report here results of mitochondrial DNA analyses that suggest that this Guir River population indeed represents the rediscovery in the wild of *A. saourensis*

Keywords: *Aphanius saourensis*, aphaniid, possibly extinct in the wild, genetic species identification, endemic freshwater fish, conservation genetics

The Sahara killifish *Apricaphanius saourensis* (Blanco et *al*., 2006) is a cyprinidontiform freshwater fish of the Aphaniidae family that was described from the Saoura River in Mazzer, western Algeria. The species was originally assigned to the genus *Aphanius* (Blanco et *al*., 2006), but has been reclassified in *Apricaphanius* (Freyhof and Yoğurtçuoğlu, 2020). Blanco et *al*. (2006) showed that the species is distinct, both morphologically and genetically, from the other two known congeneric species: *iberus* (Valenciennes 1846) (Spanish killifish) and *baeticus* (Doadrio, Carmona and Fernández-Delgado 2002) (Baetican killifish). *Apricaphanius saourensis* still exists in captivity, in collections founded by individuals collected by Blanco et *al*. (2006) and transferred to European institutions, but it has not been observed in the wild since the time of its description, despite efforts to find it (Bacha et *al*., 2014; Freyhof and Ford, 2022). The apparent extinction of *A. saourensis* in the area where it was discovered is generally considered to be the result of a potential combination of several factors detrimental to the species: the presence and expansion of invasive species (Kara, 2012), such as the eastern mosquitofish *Gambusia holbrooki* Girard 1859 and the Nile tilapia *Oreochromis niloticus* (L. 1758), which compete with and prey on aphaniids; water consumption from the river for human use; water pollution; and increasingly frequent extreme drought events (Freyhof and Ford, 2022). Given the absence of records of *A. saourensis* in the wild since its description, the current conservation status of the species is critically endangered and possibly extinct in the wild (Freyhof and Ford, 2022), with prospecting for the species in the Saoura river valley and neighbouring wadis being considered a priority (Freyhof and Ford, 2022). One of us (Redouane Tahri) conducted such surveys for several years until, in late June 2024, he found an aphaniid population that may belong to *A. saourensis* in a very isolated secondary wadi of the Guir River, which flows into the Saoura (Tahri et *al*., 2025). The wadi is located in the Abadla district, at approximately 115 km northwest of *A. saourensis*' type locality. Comparisons of images of living individuals from said population with characteristics of the body pigmentation patterns mentioned as diagnostic for *A. saourensis* (Blanco et *al*., 2006) allowed the identification of colour morphotypes in both sexes that resemble *A. saourensis*, but also of phenotypes distinct from those presented in Blanco et *al*. (2006). It is however important to note that interpopulation morphological differentiation, including in body pigmentation patterns, has been observed within other aphaniid species (Bidaye et *al*., 2023; Teimori et *al*., 2018, 2021). Genetic analysis can be extremely useful and powerful in efficiently clarifying such uncertainties in taxonomic identification (e.g. Pereira et *al*., 2013; Melo et *al*., 2014; Jense et *al*., 2024). Here we present genetic analysis of specimens from the recently discovered aphaniid population in

the Guir River of uncertain species identity, and comparison with homologous DNA sequence data for the type population of *A. saourensis* and for the other two known *Apricaphanius* species. The results suggest that the study population belongs to *A. saourensis*.

Following the discovery of the aphaniid population, we made underwater recordings and captured images of live individuals to compare with *A. saourensis*, and reported the observations (Tahri et *al*., 2025). Subsequently, we decided to return to the wadi and tried to find and collect dead individuals, in recently dried pools or about to dry completely, for genetic analysis. We collected 20 specimens, which were placed in tubes filled with absolute ethanol and kept refrigerated until arrival at the laboratory, where they were stored in a freezer at -20°C until analysis. Genomic DNA was extracted from muscle samples using the EZNA Tissue DNA kit (Omega Bio-Tek). To polymerase chain reaction (PCR) amplify and Sanger sequence the entire mitochondrial cytochrome b (*Cyt b*) gene, we designed two primer pairs based on an alignment of mitochondrial DNA sequences available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) for the three *Apricaphanius* species. The primer pairs are Asaourensis Cytb F1 (5'-CCAGGACTAATGGCTTGA-3') and Asaourensis Cytb R1 (5'-TTATTTGAGCCTGATTCG-3'), and Asaourensis Cytb F2 (5'-AGGGGGCTTCTCAGTTGA-3') and Asaourensis Cytb R2 (5'-TTTAACCCTCGACATCCG-3'). For both primer pairs, PCRs were carried out in volumes of 15 µl with 0.3 µM of each primer, 1.5 U of Multiplex PCR NZYTaq 2x Colourless Master Mix (NZYTech), and 4 µl of DNA extract. Thermal cycling conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 45 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C, and a final extension of 7 min at 72 °C. The results of the PCR amplifications were visualized on 2% agarose gels to verify PCR quality, and the PCR products were purified with an Exo-SAP protocol (Hanke and Wink, 1994; Werle et *al*., 1994) and sequenced at Macrogen Inc.

Sequences were edited, assembled, aligned, and checked for the absence of indels and stop codons using SEQUENCHER 4.7 (Gene Codes Corporation). We downloaded *Cyt b* sequences among the longest available in GenBank for the three *Apricaphanius* species (for *A. saourensis* only one *Cyt b* sequence is available, published by Blanco et *al*. (2006)), and added them to the dataset generated by us (Table 1). In particular, we downloaded 20 sequences from each of the two congeneric species of *A. saourensis* (*A. iberu*s and *A. baeticus*), in both cases from across their geographic range (Perdices et *al*., 2001; Gonzalez et

al., 2014, 2018), to obtain a good representation of the mitochondrial genetic diversity in each species. Sequence alignments were analysed with FABOX 1.61 (https://users-birc.au.dk/~palle/php/fabox/index.php; Villesen, 2007) to identify samples with identical sequences. File format conversions of sequence alignments for use in different computer programs were done using ALTER (https://www.sing-group.org/ALTER/; Glez-Peña et *al*., 2010). To visualize the genealogical relationships among *A*. *saourensis* haplotypes, we built a haplotype network in POPART 1.7 (Leigh and Bryant, 2015). We also inferred a phylogenetic tree for the *Apricaphanius* sequences with the Bayesian method implemented in MRBAYES 3.2.6 (Ronquist et *al*., 2012). Based on the results of Esmaeili et *al*. (2020), which suggest a close phylogenetic relationship between the genera *Apricaphanius* and *Esmaeilius*, we used a *Cyt b* sequence from the Zagros toothcarp *Esmaeilius vladykovi* (Coad, 1988) (Genbank accession number DQ367526) as an outgroup for the *Apricaphanius* phylogenetic tree. Analyses in MRBAYES were conducted with two parallel Markov Chain Monte Carlo (MCMC) runs, each with four Markov chains (one cold and three heated), default heating parameter (t = 0.1), and 20 million generations. The first five million generations were discarded as burn-in and, thereafter, chains were sampled every 500 generations. The entire general time-reversible (GTR; Lanave et *al*., 1984) substitution model space was sampled within the analyses (Huelsenbeck et *al*., 2004), and the sequence alignment was partitioned by codon position, as this was the best-fit partitioning scheme according to the corrected Akaike information criterion (AICc) (Akaike, 1974) in PARTITIONFINDER 2.1.1 (Lanfear et *al*., 2017) using PHYML (Guindon et *al*., 2010). Convergence was indicated by an average standard deviation of split frequencies between parallel runs of less than 0.01. For all model parameters, the minimum and average effective sample sizes (ESS) among runs were respectively greater than 700 and 4850, and the potential scale reduction factor (PSRF) was 1.0. Support for tree nodes was determined according to the values of Bayesian posterior probability (BPP) obtained in a majority-rule consensus tree (Berry and Gascuel, 1996; Holder et *al*., 2008; Huggins et *al*., 2011). The majority-rule consensus tree was computed with SUMTREES 4.5.2 of the DENDROPY library version 4.5.2 (Sukumaran and Holder, 2010) and visualized and edited with FIGTREE 1.4.4 (available at https://github.com/rambaut/figtree).

We obtained complete *Cyt b* sequences for 19 of the 20 samples analysed in the laboratory (for sample 12 we obtained the first 1113 bp of the gene). The 19 sequences corresponded to two haplotypes, which we designated S1 and S2, with the second present only in sample 2

(for the 1113 bp of sample 12 the sequence was identical to that of haplotype S1). These S1 and S2 haplotypes were different from the only known *Cyt b* sequence of *A*. *saourensis* (from the Saoura River in Mazzer, Genbank accession number DQ367527, which we here designate as haplotype S3), and therefore we deposited their sequences in GenBank (accession numbers PV932924-PV932925). The genealogical relationships between haplotypes S1-S3 are depicted in the haplotype network in Figure 1a, with the genetic proximity between the three haplotypes being evident, with genetic distances of only one to two mutational steps between the haplotypes. This is the reason why we also used the initial S of *saourensis* for the two haplotypes found in the Guir River in Abadla. The close proximity between the Abadla haplotypes and the Mazzer haplotype is also evidenced in a phylogenetic tree for *Apricaphanius* (Figure 1d). The topology of this tree is similar to that inferred for the genus by Esmaeili et *al*. (2020) using the mitochondrial cytochrome oxidase I gene (COI).

Given the haplotypes found in 20 individuals from the wadi in Abadla and their great similarity with the known haplotype from the type population of *A*. *saourensis*, the most parsimonious interpretation of this result seems to be that the aphaniid population in the Abadla wadi also belongs to *A*. *saourensis*. It is true that the Mazzer haplotype was not found in Abadla and that therefore certain approaches to species delimitation (e.g. 'population aggregation analysis'; Davis and Nixon, 1992) would not be in agreement with this interpretation. It is also true that the result is based only on mitochondrial data, and that the divergence between the Abadla and Mazzer lineages should be evaluated in the future with nuclear genome data (e.g. Pedraza-Marrón et *al*., 2019; Campbell et *al*., 2022). But for now we believe that the most supported working hypothesis is to consider that the Abadla aphaniid represents the rediscovery of *A*. *saourensis* in the wild. Resolving this taxonomic issue is important, but equally crucial is ensuring the protection and conservation of the recently discovered population in the Abadla wadi. The observed results suggest low mitochondrial genetic variation, and it is necessary to confirm whether this is also the case for the nuclear genome, as the latter tends to be negatively associated with the ability to persist in the face of environmental adversities (which characterize the ecosystem in which the population lives) and for adaptive evolution. It is true that the included haplotypes of *A*. *baeticus* and *A*. *iberus* (Table 1) correspond to individuals from several populations of each species, but the combined results for Abadla and Mazzer suggest that genetic diversity may be much lower in *A*. *saourensis*. This would not be surprising since *A*. *saourensis* lives in an area where climatic conditions are more extreme in terms of the likelihood of watercourses drying up, and

therefore where drastic fluctuations and contractions in population size, with accelerated losses of genetic variation due to genetic drift, are probably much more recurrent and intense. It is therefore vital that protection and support be offered to the population in the Abadla wadi so that it can survive in the long term. In this context, it is a cause for hope and rejoicing that the Algerian government has decreed legal protection for the wadi area and the aphaniid population living within it, and established a captive breeding program for the population (Tahri et *al*., 2025).

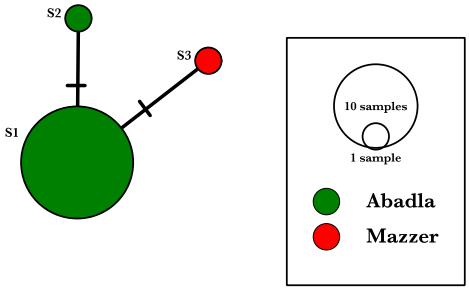
182 **Table 1.** Information on the analysed samples and previously published *Cyt b* sequences.

Population/species	**Country**	**Sample/sequence**	**Haplotype**	**Reference**
Newly discovered aphaniid population (Abadla)	Algeria	1, 3-20	S1	This study
		2	S2	This study
A. saourensis type population (Mazzer)	Algeria	DQ367527	S3	Blanco et *al*., 2006
A. iberus	Spain	AF299274	I1	Perdices et *al*., 2001
	Spain	AF299275	I2	Perdices et *al*., 2001
	Spain	AF299276, AF299277	I3	Perdices et *al*., 2001
	Spain	AF299278	I4	Perdices et *al*., 2001
	Spain	AF299279	I5	Perdices et *al*., 2001
	Spain	AF299286	I6	Perdices et *al*., 2001
	Spain	AF299287	I7	Perdices et *al*., 2001
	Spain	AF299288	I8	Perdices et *al*., 2001
	Spain	AF299289	I9	Perdices et *al*., 2001
	Spain	AF299290	I10	Perdices et *al*., 2001
	Spain	AY155569	I11	Doadrio and Dominguez, 2004
	Spain	DQ367528	I12	Blanco et *al*., 2006
	Spain	DQ367529	I13	Blanco et *al*., 2006
	Spain	KU174219	I14	Gonzalez et *al*., 2018
	Spain	KU174223	I15	Gonzalez et *al*., 2018
	Spain	KU174226	I16	Gonzalez et *al*., 2018
	Spain	KU174230	I17	Gonzalez et *al*., 2018
	Spain	KU174231	I18	Gonzalez et *al*., 2018
	Spain	NC072677	I19	López-Solano et *al*., 2023

Population/species	Country	Sample/sequence	Haplotype	Reference
A. baeticus	Spain	KF854343	B1	Gonzalez et *al*., 2014
	Spain	KF854345	B2	Gonzalez et *al*., 2014
	Spain	KF854395, KF854416	B3	Gonzalez et *al*., 2014
	Spain	KF854402	B4	Gonzalez et *al*., 2014
	Spain	KF854411	B5	Gonzalez et *al*., 2014
	Spain	KF854419, KF854420, KF854426, KF854427, KF854428	B6	Gonzalez et *al*., 2014
	Spain	KF854395, KF854416	B7	Gonzalez et *al*., 2014
	Spain	KF854442, KF854443, KF854449, KF854453, KF854454	B8	Gonzalez et *al*., 2014
	Spain	KF854476	B9	Gonzalez et *al*., 2014
	Spain	KF854477	B10	Gonzalez et *al*., 2014
E. vladykovi	Iran	DQ367526	E_vladykovi	Blanco et *al*., 2006

a) b)

c) d)



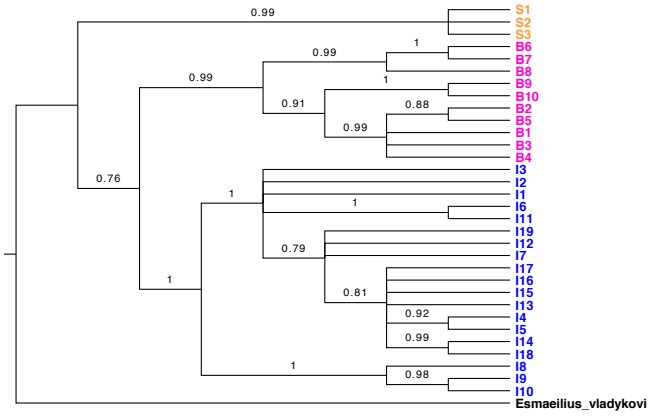


Figure 1 (a) Example of a male individual from the recently discovered aphaniid population in the Abadla district; (b) Example of a female individual from the same population; (c) Network of known *A. saourensis Cyt b* haplotypes. The circles represent haplotypes, with their size proportional to their frequency and coloured according to where they were found. Dashes on lines connecting haplotypes represent the number of nucleotide substitutions separating them. Haplotype designations and further information are given in Table 1; (d) Majority-rule consensus cladogram (cut-off 0.7) from the Bayesian inference analysis of the *Apricaphanus Cyt b* haplotypes dataset rooted with *Esmaeilius vladykovi*. Haplotypes are named and coloured according to species identity (initial 'S' and orange for *saourensis*, initial 'B' and magenta for *baeticus*, and initial 'I' and blue for *iberus*). See Table 1 for haplotype information. Numbers above branches are Bayesian posterior probabilities.

Author Contributions

L. D. and C. R. F. analysed and interpreted the data and wrote the manuscript. R. T. acquired funding, performed the fieldwork, made videos recordings and captured images of live individuals, and collected dead specimens for genetic analysis. Study conception and manuscript reviewing were done by all authors.

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