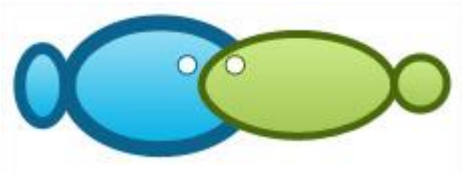

DNA barcoding analysis of fish species in the Marchica Lagoon, Morocco

[1]Aicha Hamid, [1]Imane Rahmouni, [1,2]Bazairi Hocein, [1]Halima Louizi, [1]Amal Lamkhalkhal, [3]Mohamed Selfati, [1]Oussama Bououarour, [1]Bouabid Badaoui

[1] Laboratory of Biodiversity Ecology and Genome, Faculty of Science, Mohammed V University in Rabat, Morocco; [2] Natural Sciences and Environment Research Hub, University of Gibraltar, Europa Point Campus, Gibraltar; [3] Laboratoire Santé Et Environnement, Faculté des Sciences Aïn Chock, Hassan II University of Casablanca, B.P 5366 Maarif, 20100 Casablanca, Morocco. Corresponding author: A. Hamid, aichahamid65@gmail.com

Abstract. This study conducted a DNA barcoding analysis of fish species from the Marchica lagoon, a Ramsar site in northeastern Morocco. The primary aim was to validate the efficacy of barcoding for species identification, genetic analysis, and phylogenetic studies. Tissue samples from various fish species in the lagoon were analyzed using mitochondrial DNA sequencing (COI). The resulting sequences were compared against genetic databases and evaluated with statistical methods including the fixation index (FST), discriminant analysis of principal components (DAPC), and haplotype comparison. The findings confirmed that DNA barcoding is a robust and reliable tool for these purposes. This initial investigation into fish barcoding in the Marchica lagoon offers critical insights into the genetic diversity of this Ramsar site and establishes a solid scientific foundation for conservation efforts to protect aquatic biodiversity.

Key Words: COI, DAPCs, FST, genetic diversity, haplotypes, Marchica lagoon, phylogeny, Ramsar site.

Introduction. Biodiversity conservation relies heavily on accurate species identification, yet a significant portion of global biodiversity remains undocumented or poorly understood (Faith 1992). Lagoons, as ecologically unique and dynamic coastal ecosystems, serve as biodiversity hotspots. These habitats, formed by natural barriers like sandbanks or coral reefs that partially enclose water from the open ocean, support an array of ecological processes and host diverse marine and terrestrial species. Lagoons provide critical functions as breeding grounds, nurseries, and feeding habitats, benefiting numerous organisms from fish to birds and invertebrates (Harley et al 2006; Waycott et al 2009; Camacho-Valdez et al 2013). Despite their ecological importance, lagoon biodiversity is often underexplored, leaving substantial knowledge gaps that hinder effective conservation (Kennish & Paerl 2010).

Current methods for describing species diversity rely predominantly on anatomical and morphological characteristics. However, these traditional approaches often underestimate species diversity due to challenges associated with identifying morphologically similar or cryptic species, juvenile specimens, and species with overlapping morphological traits. Furthermore, the limitations of morphology-based identification are compounded by the decreasing number of trained taxonomists and the high level of expertise required (Evans et al 2007). For instance, between 1950 and 2002, over a third of global fish catches lacked species-level identification, a critical shortfall in our understanding of marine biodiversity (Pauly et al 2005). These limitations underscore the urgent need for more accurate and efficient methods of species identification.

Molecular markers, including amplified fragment length polymorphisms (AFLP), simple sequence repeat (SSR), variable number tandem repeats (VNTR), mitochondrial DNA (mtDNA), relative afferent pupillary defect (RAPD), single nucleotide polymorphism (SNP), and systems security certified practitioner (SSCP), can aid in the identification of marine species. Hebert et al (2003) first recommended cytochrome c oxidase I (COI) DNA barcoding as a reliable method for animal species discrimination, validated at the 1st International Conference on DNA Barcode of Life. DNA barcoding leverages the high level of interspecific variation compared to intraspecific variation at specific genes, making it a superior method for species identification (Pegg et al 2006; Frantine Silva et al 2015; Trivedi et al 2016; Collet et al 2018). This technique excels beyond traditional identification methods and is effective in investigating marine biodiversity (Zhang & Hanner 2011), conducting biological monitoring, supporting conservation efforts, and detecting alien and cryptic species (Boissin et al 2017; Barman et al 2018).

Our study aims to identify fish species present in the Marchica lagoon, the only lagoon ecosystem on the Moroccan Mediterranean coast. This area is significant on multiple levels, including ecological, biological, and socio-economic aspects, due to increasing urbanization and population growth (AEFCS 1996). The lagoon is designated as a site of Biological and Ecological Interest and obtained RAMSAR site status in 2005, highlighting its crucial role in preserving globally important wetlands (Dakki et al 2011). However, rapid development has led to a deterioration of this ecosystem's natural capital, notably due to anthropogenic pressures related to urban activities in Morocco (Maanan et al 2015). The Marchica lagoon receives treated water from the Grand Nador and Beni Ensar treatment plants, contributing to environmental challenges (Bloundi 2005). As the lagoon is the site of significant fishing activity, identifying fish species in this environment could undoubtedly contribute to improving resource management and deepening our understanding of its ecology.

Material and Method

Description of the study sites. The Nador Lagoon, also known as "Marchica", stands out among Mediterranean lagoons for its imposing size, covering an area of 115 km², its unique geomorphological configuration, and its richness in biodiversity. Located in northeastern Morocco, between Cap des Trois Fourches and Cap-de-l'Eau (Figure 1), this lagoon holds importance both biologically, ecologically, and economically. Thus its existence represents a significant stake for regional development.

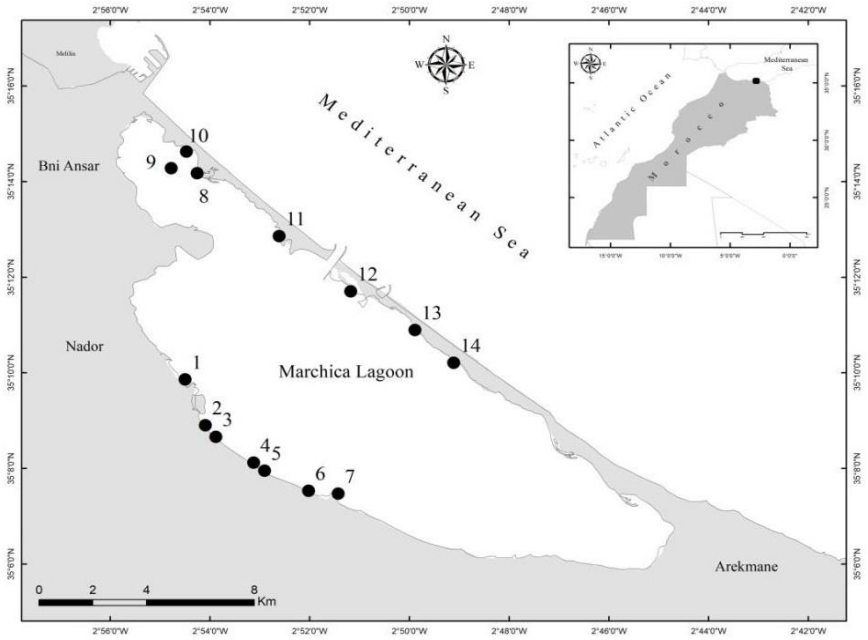

Figure 1. Geographical location of studied points in Marchica lagoon.

Sampling. Fish samples were caught at various points in the Marchica lagoon in January 2024 using a seine net, then brought back to the laboratory and frozen. After thawing the samples, each one was numbered, photographed, and tagged, and its size was measured using a graduated ruler. Subsequently, a piece of the right pectoral fin of each fish was preserved in an Eppendorf tube filled with ethanol for further genetic study.

Genetic study. DNA was extracted from the pectoral fin clips that were preserved in absolute ethanol using a rapid salt extraction protocol (Aljanabi & Martinez 1997). The extracted genetic material was then suspended in sterile distilled water and stored at -20°C until PCR amplification. The universal mitochondrial molecular barcode gene COI, already used to differentiate several fish species, was selected. For sample amplification, the FishF1 and FishF2 primers were used together as forward primers, and FishR1 as the reverse primer (Ward et al 2005). Each amplification followed the standard PCR protocol for Taq DNA polymerase, with standard Taq buffer (New England Biolabs) in a volume of 50 µL, including 5 µL of buffer (10× Standard Taq Reaction Buffer), 1 µL of 10 mMdNTPs, 1 µL of 10 µM forward primers, 1 µL of 10 µM reverse primer, 0.50 µL (2.5 units) of Taq polymerase, 1 µL of genomic DNA (0.1 to 0.5 ng), and 40.5 µL of nuclease-free water. The PCR reaction conditions were as follows: 94°C (3 min), 30 cycles of 94°C (30 s), 56°C (30 s), and 72°C (30 s), with a final step at 72°C for 10 min. PCR products were checked on 1% agarose gel, stained with ethidium bromide. Successful PCR products were sent to the National Center for Scientific and Technical Research (CNRST) for purification and sequencing. The sequencing was carried out by using the same primers as for PCR. The obtained sequences were cleaned manually with MEGA version X (Kumar et al 2018) and aligned using the computer program ClustalW, with multiple alignments running in MEGA version X. Identification was then performed by comparing the obtained sequences with those in the Basic Local Alignment Search Tool (BLAST) and the Barcode of life Data System (BOLD).

Our study based on the comparison of COI mitochondrial DNA sequences requires meticulous checks to ensure that the results obtained are reliable, so three summary statistical genetics were used to judge the results between groups of these identified species: the fixation index (FST), discriminant analysis of principal components (DAPC), and the number of haplotypes within a group.

Sequence data alignment, phylogenetic tree construction, and haplotypes identification COI genetic sequences were aligned using ClustalW software integrated within BioEdit. The aligned sequences were then saved in FASTA format for subsequent analyses in R software. Distance matrices were computed using the Kimura 80 (K80) model, and a neighbor-joining (NJ) tree was constructed. Unique sequences (haplotypes) were identified, and their frequencies were calculated.

Discriminant analysis of principal components (DAPCs). Discriminant analysis of principal components (DAPCs) was conducted on the COI dataset using the adegenet package for R. This method was employed to examine the genetic structure of species and assess genetic differentiation among the tested species through supervised clustering. The DAPC approach optimizes the separation of individuals into predefined groups (species) based on a discriminant function of principal components. This allows for individual assignment to species and the determination of membership probabilities, reflecting the overall genetic background of each individual.

Results and Discussion

Comparison of morphological and DNA barcode identifications. Initially, morphological identification of the fish sampled from the lagoon was conducted in the laboratory using the identification key established by Nelson et al (2016). The molecular characterization, which was consistent with the morphological identification, confirmed the presence of sixteen species (Table 1).

Table 1

Fish species identification in the GenBank database (BOLD and NCBI), number of representative samples, length of the Cytb gene and accession numbers

Scientific name	*Abbreviations*	*Identity (%)*	*No. of samples*	*Length of Cytb (bp)*	*Accession no.*	*BOLD (%)*
Salaria pavo	Sal_pvo	99.82	3	558	PQ057758	99.63
Diplodus sargus	Dipl_sar	98.47	3	526	PQ113380	98.66
Diplodus vulgaris	Dipl_vul	99.51	3	644	PQ121001	100
Symphodus cinereus	Sym_cin	99.49	2	590	PQ113336	100
Symphodus ocellatus	Sym_oce	99.34	3	606	PQ113342	99.67
Mullus barbatus	Mul_bar	99.67	3	609	PQ100676	100
Trachinotus ovatus	Trac_ova	96.88	3	484	PQ120678	99.14
Sardina pilchardus	Sar_pil	100	3	645	PQ057766	100
Trachurus trachurus	Trach_tra	99.69	3	671	PQ124105	100
Trigloporus lastoviza	Trig_las	99.85	3	702	PQ098926	100
Serranus cabrilla	Ser_cab	100	3	670	PQ098821	100
Conger conger	Cong_con	99.85	2	660	PQ057724	100
Engraulis encrasicolus	Engr_enc	100	3	689	PQ057231	100
Trachurus mediterraneus	Trach_med	100	3	684	PQ057234	100
Trachinus draco	Trach_dra	100	3	685	PQ057338	100
Scomber colias	Scom_col	99.71	3	689	PQ057543	100

The COI mitochondrial gene sequences of the samples exhibited a similarity of 98 to 99% compared to sequences recorded in the GenBank database using BLAST analysis and BOLD identification. Notably, samples 8, 11, 13, 14, and 15 were verified with a similarity score of 100%. According to Ward et al (2009), specimens with a similarity score between 98 and 100% with sequences in GenBank or BOLD can be confidently identified as the species recorded in these databases. This high similarity score serves as a reliable reference for species identification. Furthermore, the nucleotide sequences analyzed demonstrated that all individuals were polymorphic (Figure 2).

H1
CCGAGCAGAGCTGAGCCAGCCCGGAGCCCTTAGCCGCTATCTTATTCCAGTCTAAAGAACAGTGCCACTGACTACTCGAATTCCT
GCCTAGCCATCGACTCCCTCTCTCCCTCTGCCTGGTACTCGA-GTATCCTCCGGCGCCTACTCGCTGACCATTCCCTCGCGACCC--ACGT-
ATTCCACATTT-CGGCC-T-CAATCTCCAACACTCCG-CGTG-ATCAC-GATCC-CCCCT-AG-
CGACCTCCGCAGGCATCACAATGCTTCTAACAGACCGCAACC-TAAACACCACGTTCTTC-GACCCTG-CCGGAGG 3 1.81
H2
..... 6 3.61
H3
..... 1 0.6
H4
..... 1 0.6
H5
T.....A..A.....A..A.....A..CG..TAT..CAC.T.CA..ACTC.G..C.TCACTG.GT....AGT..T.CAG.AC.A.C...TGCT...C.T..T.....CTA.AA
..T..AG....A..A..CT.A.A.A.CC.C.AGA.GTT.AC.T.CT.A..T.A..G.A..C...TT...C..T.AA.....T..T.A....G.AT.A.TC....GT.T.A.CGA.A.TTA.TT.AA
CT.A.T..T..G..T..T.....T.....A.....T..T..T.....A...A..G.. 7 4.22
H6
T.....A..A.....A..A.....A..CG..TAT..CAC.T.CA..ACTC.G..C.TCACTG.GT....AGT..T.CAG.AC.A.C...TGCT...C.T..T.....CTA.AA
..T..AG....A..A..CT.A.A.A.CC.C.AGA.GTT.AC.T.CT.A..T.A..G.A..C...TT...C..T.AA.....T..T.A....G.AT.A.TC....GT.T.A.CGA.A.TTA.TT.AA
CT.A.T..T..A..T..T.....T.....A.....T..T..T.....A...A..G.. 1 0.6
H7
T.....A..A.....A..A.....A..CG..TAT..CAC.T.CA..ACTC.G..C.TCACTG.GT....AGT..T.CAG.AC.A.C...TGCT...C.T..T.....CTA.AA
..T..AG....A..A..CT.A.A.A.CC.C.AGA..TT.AC.T.CT.A..T.A..G.A..C...TT...C..T.AA.....T..T.A....G.AT.A.TC....GT.T.A.CGA.A.TTA.TT.AAC
T.A.T..T..G..T..T.....T.....A.....T..T..T.....A...A..G.. 1 0.6
H8
T.....A..A.....A..A.....A..CG..TAT..CAC.T.CA..ACTC.G..C.TCACTG.GT....AGT..T.CAG.AC.A.C...TGCT...C.T..T.....CTA.AA
..T..AG....A..A..CT.A.A.A.CC.C.AGA.GTT.AC.TTCT.A..T.A..G.A..C...TT...C..T.AA.....T..T.A....G.AT.A.TC....GT.T.A.CGA.A.TTA.TT.AA
CT.A.T..T..G..T..T.....T.....A.....T..T..T.....A...A..G.. 1 0.6
H9
T.....A..A.....A..A.....A..CG..TAT..CAC.T.CA..ACTC.G..C.TCACTG.GT....AGT..T.CAG.AC.A.C...TGCT...C.T..T.....CTA.AA
..T..AG....A..A..CT.A.A.A.CC.C.AGA.GTT.AC.T.CT.A..T.A..G.A..C...TT...C..T.AA.....T..T.A....G.AT.A.TC....GT.T.A.CGA.A.TTA.TT.A.C
T.A.T..T..G..T..T.....T.....A.....T..T..T.....A...A..G.. 1 0.6
H10
T..G.....A..A.....A..T..C.....C..T.ATC.CA..GCCT..A.....T...A.T.....G.TGTAG.CG.T.A.....T.GC....A..TC....TG..TA..TA.....G
.AT.T..A.TCA...T.G.CT.C.AGC..TT.CC..TC.AA.G..G...TAG.T..TTCT.....A-
TC....G...AAT.T.TCA.A.TT....GTCT.A..AT...T...TC.TTT..A.T..T.....T.....A..T.....T..TG.T.....T...T..A...A..... 8
4.82
H11
T..G.....A..A.....A..T..C.....C..T.ATC.CA..GCCT..A.....T...A.T.....G.TGTAG.CG.TCA.....T.GC....A..TC....TG..TA..TA.....
G.AT.T..A.TCA...T.G.CT.C.AGC..TT.CC..TC.AA.G..G...TAG.T..TTCT.....A-
TC....G...AAT.T.TCA.A.TT....GTCT.A..AT...T...TC.TTT..A.T..T.....T.....A..T.....T..TG.T.....T...T..A...A..... 1
0.6
H12
T..G.....A..A.....A..T..C.....C..T.ATC.CA..GCCT..A.....T...A.T.....G.TGTAG.CG.T.A.....T.GC....A..TC....TG..TA..TA.....G
.AT.T..ATTCA...T.G.CT.C.AGC..T..CC..TC.AA.G..G...TAG.T..TTCT.....A-
TC....G...AAT.T.TCA.A.TT....GTCT.A..AT...T...TC.TTT..A.T..T.....T.....A..T.....T..TG.T.....T...T..A...A..... 1
0.6
H13
T.....A..A.....A..T..CT.T..C.AT.A...CA.....A.....T...A...TG....TA.TA.A...AT.CAT..TATTGC....C.T.C...C.T.C.C..TA....AAT
AT.T..ATT...ATAATT....ATAC...T..CTTA..A.T.T.....T.G.TCCTT.....C.T.A-
TC...C..T.....A.....T...G.TT..A..AT.A.T.C.TT.TTT.....C..T..T.....A.....A.....T.....C.....G...A..... 2 1.2
H14
T.....A..A.....A..T..CT.T..C.AT.A...CA.....A.....T...A...TG....TA.TA.A...AT.CAT..TATTGC....C.T.C...C.T.C.C..TA....AAT
AT.T..ATT...ATAATT....ATAC...T..CTTA..A.T.T.....T.G.TCCTT.....C.T.A-
TC...C.....A.....T...G.TT..A..AT.A.T.C.TT.TTT.....C..T..T.....A.....A.....T.....C.....G...A..... 2 1.2
H15
T.....A..A.....A..T..CT.T..C.AT.A...CA.....A.....T...A...TG....TA.TA.A...AT.CAT..TATTGC....C.T.C...C.T.C.C..TA....AAT
AT.T..AT....ATAATT....ATAC...T..CTTA..A.T.T.....T.G.TCCTT.....C.T.A-
TC...C.....A.....T...G.TT..A..AT.A.T.C.TT.TTT.....C..T..T.....A.....A.....T.....C.....G...A..... 4 2.41
H16
T.....A..A.....A..T..CT.T..C.AT.A...CA.....A.....T...A...TG.....A.TA.A...AT.CAT..TATTGC....C.T.C...C.T.C.C..TA....AAT
AT.T..ATT...ATAATT....ATAC...T..CTTA..A.T.T.....T.G.TCCTT.....C.T.A-
TC...C..T.....A.....T...G.TT..A..AT.A.T.C.TT.TTT.....C..T..T.....A.....A.....T.....C.....G...A..... 1 0.6
H17
T.....A..A.....A..T..CT.T..C.AT.A...CA.....A.....T...A...TG....TA.TA.A...AT.CAT..TATTGC....C.T.C...C.T...C..TA....AAT
AT.T..ATT...ATAATT....ATAC...T..CTTA..A.T.T.....T.G.TCCTT.....C.T.A-
TC...C..T.....A.....T...G.TT..A..AT.A.T.C.TT.TTT.....C..T..T.....A.....A.....T.....C.....G...A..... 1 0.6

H18
T.....A..A.....A..T..CT.T..C.AT.A...CA.....A.....T...A...TG....TA.TA.A..CAT.CAT..TATTGC....C.T.C...C.T.C.C..TA....AA
TAT.T..ATT...ATAATT....ATAC...T..CTTA..A.T.T.....T.G.TCCTT.....C.T.A-
TC...C.....A.....T...G.TT..A..AT.A.T.C.TT.TTT.....C..T..T.....A.....A.....T.....C.....G...A..... 1 0.6
H19
.....T..A..A..T..A..A..GT....CCC..A.C.CA..G.C..TAC..T....GT...AG.....G..C.AG.CA..C.ATC.AT..C.....G.ATC.G.G.C.GTTTG
CA....TAT.C..ATT...C.T.G.CGAC..AA.TTT.CC.A.C.AATGT.....TG.C.C...A..C...AATGAG.-
TG.A.....CT....C....GAC..A..AT...T.C.TA.TA.T...T..C.....T.....C.....A..T.....T..T..C.....GG..... 7 4.22
H20
.....T..A..A..T..A..A..GT....CCC..A.C.CA..G.C..TAC..T....GT...AG.....G..C.AG.CA..C.A.C.AT..C.....G.ATC.G.G.C.GTTTG
CA....TAT.C..ATT...C.T.G.CGAC..AA.TTT.CC.A.C.AATGT.....TG.C.C...A..C...AATGAG.-
TG.A.....CT....C....GAC..A..AT...T.C.TA.TA.T...T..C.....T.....C.....A..T.....T..T..C.....GG..... 1 0.6
H21
.....T..A..A..T..A..A..GT....CCC..A.C.CA..G.C..TAC..T....GT...AG.....G..C.AG.CA..C.ATC.AT..C.....G.ATC.G.G.C.GTTTG
CA....TAT.C..ATT...C.T.G.CGAC..AA.TTT.CC.A.C.AATGT.....TG.C.C..CA..C...AATGAG.-
TG.A.....CT....C....GAC..A..AT...T.C.TA.TA.T...T..C.....T.....C.....A..T.....T..T..C.....GG..... 1 0.6
H22
.....T..A..A..T..A..A..GT....CCC..A.C.CA..G.C..TAC..T....GT...AG.....G..C.AG.CA..C.ATC.AT..C.....G.ATC.G.G.C.ATTTG
CA....TAT.C..ATT...C.T.G.CGAC..AA.TTT.CC.G.C.AATGT.....TG.C.C..CA..C...AATGAG.-
TG.A.....CT....C....GAC..A..AT...T.C.TA.TA.T...T..C.....T.....C.....A..T.....T..T..C.....GG..... 2 1.2
H23
T.....C.....A.....A..A..C..T.C..A...T...A...A..T.....T..CAG.....G.T..AG..ATT.AA.C....GC..G...T..T..G.G.C.C.T...TA.AG.C.
.T..A.T.TTA.T.A..GTCGAT...TT.ACTAT.T.ATGT.....A...C...A.....T.A.....T.....GT..AT...TT...GGTCT.A..TA.ATA...TA.TT.....T.....T..
T.....C.....A..T.....T..A..A.....T.....C...G..... 9 5.42
H24
T.....C.....A.....A..A..C..T.C..A...T...A...A..T.....T..CAA.....G.T..AG..ATT.AA.C....GC..G...T..T..G.G.C.C.T...TA.AG.C.
.T..A.T.TTA.T.A..GTCGAT...TT.ACTAT.T.ATGT.....A...C...A.....T.A.....T.....GT..AT...TT...GGTCT.A..TA.ATA...TA.TT.....T.....T..
T.....C.....A..T.....T..A..A.....T.....C...G..... 1 0.6
H25
T.....C.....A.....A..A..C..T.C..A...T...A...AC.T.....T..CAG.....G.T..AG..ATT.AA.C....GC..G...T..T..G.G.C.C.T...TA.AG.C
..T..A.T.TTA.T.A..GTCGAT...TT.ACTAT.T.ATGT.....A...C...A.....T.A.....T.....GT..AT...TT...GGTCT.A..TA.ATA...TA.TT.....T.....T.
.T.....C.....A..T.....T..A..A.....T.....C...G..... 1 0.6
H26
T.....T..A.....T..T..T.CCGA...T...A...A.AT.A.....A.....CA.T.....TA.TA.T.....CAA.C.AT.GC.....GA....T.A.A..TC.A..TG.C
T....ATTA....A.A.CT.CATAA.T...CCTTTC..TA.T.A..TTT..C..TTCA.....A...A..T....G...TG.A....TA....GTCT.A...T.T.A...T..TC.T...T..C..G..
.....C..T.....T..A.....T.....T.....G..--..G.. 1 0.6
H27
T.....T..A.....T..T..T.CCGA...T...A...A.AT.A.....A.....CA.T.....TA.TA.T.....CAA.C.AT.GC.....GA....T.A.A..TC.A..TG.C
T....ATTA....A.A.CT.CATAA.T...CCTTTC..TA.T.A..TTT..C..TTCA.....A...A..T....G...TG.A....TA....GTCT.A...T.T.A...T..TC.T...T..C..G..
.....C..T.....T..A.....T.....T.....G...G..G.. 10 6.02
H28
T.....T..AT.A..T..A..T.....T.CCTAT.....TCA.A..T.A..C...A.GA.A.AGTCT....AGGA.A...G.ACAATA.ATT.C...TATATAA.T.TA.ATA
ATA..A.G.GAA..A...TTCT...A.GTCG.CTAAA.T...ACTTAC.AAA.T.A..T.TA.T.CTTTT.....TAAA..A..T.C.A....T..CT.A.TA.....T..AGC...A.GT
A..C..C.....T..C..G..T.....T.....T..A..T...T..T..T..C.....T.....A...G..T.. 7 4.22
H29
T.....T..AT.A..T..A..T.....T.CCNAT.....TCA.A..T.A..C...A.GA.A.AGTCT....AGGA.A...G.ACAATA.ATT.C...TATATAA.T.TA.ATA
ATA..A.G.GAA..A...TTCT...A.GTCG.CTAAA.T...ACTTAC.AAA.T.A..T.TA.T.CTTTT.....TAAA..A..T.C.A....T..CT.A.TA.....T..AGC...A.GT
A..C..C.....T..C..G..T.....T.....T..A..T...T..T..T..C.....T.....A...G..T.. 1 0.6
H30
T.....T..AT.A..T..A..T.....T.CCTAT.....TCA.A..T.A..C...A.GA.A.AGTCT....AGAA.A...G.ACAATA.ATT.C...TATATAA.T.TA.ATA
ATA..A.G.GAA..A...TTCT...A.GTCG.CTAAA.T...ACTTAC.AAA.T.A..T.TA.T.CTTTT.....TAAA..A..T.C.A....T..CT.A.TA.....T..AGC...A.GT
A..C..C.....T..C..G..T.....T.....T..A..T...T..T..T..C.....T.....A...G..T.. 1 0.6
H31
T.....T..AT.A..T..A..T.....T.CCTAT.....T.A.A..T.A..C...A.GA.A.AGTCT....AGGA.A...G.ACAATA.ATT.C...TATATAA.T.TA.ATA
ATA..A.G.GAA..A...TTCT...A.GTCG.CTAAA.T...ACTTAC.AAA.T.A..T.TA.T.CTTTT.....TAAA..A..T.C.A....T..CT.A.TA.....T..AGC...A.GT
A..C..C.....T..C..G..T.....T.....T..A..T...T..T..T..C.....T.....A...G..T.. 1 0.6
H32
T.....T..AT.A..T..A..T.....T.CCTAT.....TCA.A..T.A..C...A.GA.A.AGTCTCT..AGGA.A...G.TCAATA.ATT.C.T.TATATAA.T.TA.AT
AATA..A.G.GAA..AG..TTCT...A.GTCG.CTAAA.T...ACTTAC.AAA.T.A..T.TA.T.CTTTT.....TAAA..A..T.C.A....TG.CT.A.TAGT....T..AGC...A
.GTA..C..C.....T..C..G..T.....T.....T..A..T...T..T..T..C.....T.....A...G..T.. 1 0.6
H33
T.....C..A..A..T..A..A.....T..CT.T.AT...AC.TCC.T.....A.....A...T....A.T..TG.CG.TCAA.C.ATTGCT...G..TCT....TTA.A...C.A...
G.C..A...TT.T..TA..TCG.CAA.A.TT..AC..TCTAGA.T.T...TCG.....T.....T.A...A..T....A.....AA.A.T....GGT...A..TA.A.....T..TCGT.C.A.....
A..T..C..A.....T.....T..A..T...T..T..A..C.....T.....C-.------- 1 0.6
H34
T.....C..A..A..T..A..A.....T..CT.T.AT...AC.TCC.T.....A.....A...T....A.T..TG.CG.TCAA.C.ATTGCT...G..TCT....TTA.A...C.A...
G.C..A...TT.T..TA..TCG.CAA.A.TT..AC..TCTAGA.T.T...TCG.....T.....T.A...A..T....A.....AA.A.T....GGT...A..TA.A.....T..TCGT...A.....A
..T..C..A.....T.....T..A..T...T..T..A..T------.-------.------- 1 0.6
H35
T.....C..A..A..T..A..A.....T..CTAT.AT...AC.TCC.T.....A.....T.....TA.TG.TG.CG.TCAA.C..TTGCT...G..TCT....TTA.A...C.A..
.G.C..A...T..T..TG.T.CCTCA..A.TT..AC.TTCTAGA.T.T...TTG.....T.....T.A...A..T..T.A.....AG...T....GGT...A..GA.A.T...TT.TCGT...A..C.
.A..T..T..A.....T.....A..T...G..T..A..T.....T.....T..G.. 1 0.6
H36
T.....C..A..A..T..A..A.....T..CTAT.AT...AC.TCC.T.....A.....T.....TA.TG.TG.CG.TCAA.C..TTGCT...G..TCT....TTA.A...C.A..
.G.C..A...T..T..TG.T.CCTCA..ATTT..AC.TTCTAGA.T.T...TTG.....T..A..T.A...A..T..T.A.....AG.T.T....GGT...A..GA.A.T...TT.TCGT...A..
C..A..T..T..A.....T.....A..T...G..T..A..T.....T.....T..G.. 1 0.6
H37
T.....C..A.....T..A..A.....T..CT.T.AT...AC.TCC.T.....A.....T.....TA.TG.TG.CG.TCAA.C..TTGCT...G..TCT....TTA.A...C.A...
G.C..A...T..T..TG.T.C.TCA..A.TT..AC.TTCTAGA.T.T...TTG.....T.....T.A...A..T..T.A.....AG...T....GGT...A..GA.A.T...TT.TCGT...A..C..
A..T..T..A.....T.....A..T...G..T..A..T------.-------.------- 3 1.81
H38
.....G..A..A.....CCT.T.A.C..TCA.A...T.....T...A.....G.C.TC.AG.CA.....TC....GC.....CA.....C....TC.CGGTGTTC

.C.GAG.ATTATT...CC.CAAAC.C.T.........G.........T.....TTCT..C..T........G..T.A..GT...T.A..C....G.TT.A...T.T.....T...TC....A..C.....T.....A
...........T..A......C........C..T..TT.......T.A..T.. 1 0.6
H39
.....G..A..A.................CCT.T.A.C..TCA.A...T..............A......G.C.TC.AG.CA.....TC....GC........CA......C....TC.CGGTGTTC.
C.GAG.ATTATT...CC.CAAAC.C.T.........G.........T.....TTCT..C..T........G..T.A..GT...T.A..C....G.TT.A...T.T.....T...TC....A..C.....T.....A.
...........T..A......C........C..T..T...........A..T.. 1 0.6
H40
.....G..A..A.................CCT.T.A.C..TCA.A...T..........T...A......G.C.TC.AG.CA.....TC....GC........CA......C....TC.CGGTG.TC.
C.GAG.ATTATT...CC.CAAAC.C.T.........G.........T.....TTCT..C..T........G..T.A..GT...T.A..C....G.TT.A...T.T.....T...TC....A..C.....T.....A.
...........T..A......C........C..T..T...........A..T.. 1 0.6
H41
.....G..A..A.................CCT.T.A.C..TCA.A...T..........T...A......G.C.TC.AG.CA.....TC....GC........CA......C....TC.CGGTGTTC
.C.GAG.ATTATT...CC.CAAAC.C.T.........G.........T.....TTCT..C..T........G..T.A..GT...T.A..C....G.TT.A...T.T.....T...TC....A..C.....T.....A
...........T..A......C........C..T..T...........A..T.. 3 1.81
H42
...G..G..A..A.................CCT...A.C.CTCGGA...C.......A.....C....T....C.TC.TG.CG.AC..TCG.T.GC....C..TCA..C...C....TA.CG.A
G.CC.C..AG.ATTA.T.TTCT.CAAAC.C.T......C..A..T..............C........A......G....A..GT..C..C..C...GGTTT.A...T.T.T.C.T..TCT....A..C.....
.....A.....T........A......T..T.....C..T..TT.........A..--- 1 0.6
H43
...G..G.....A.................CCT...A.C.CTCGGA...C.......A.....C....T....C.TC.TG..G.AC..TCG.T.GC....C..TCA..C...C....TA.CG.A
G.CC.C..AA.ATTA.T.TTCT.CAAAC.C.T......C..A..T..............C........A......G....A..GT..C..C..C...GGTTT.A...T.T.T.C.T..TCT....A..C.....
.....A.....T........A......C..T.....C..T..T...........A..C.. 1 0.6
H44
...G..G.....A.................CCT...A.C.CTCGGA...C.......A.....C....T....C.TC.TG.CG.AC..TCG.T.GC....C..TCA..C...C....TA.CG.A
G.CC.C..AA.ATTA.T.TTCT.CAAAC.C.T....T.C..A..T..............C........A......G....A..GT..C..C..C...GGTT..A...T.T...C.T..TCT....A..C.....
.....A.....T........A......C..T.....C..T..T...........A..C.. 1 0.6
H45
...G..G..A..A.................CCT...A.C.CTCGGA...C.......A.....C....T....C.TC.TG.CG.AC..TCG.T.GC....C..TCA..C...C....TA.CG.A
G.CC.C..AA.ATTA.T.TTCT.CAAAC.C.T......C..A..T..............C........A......G....A..GT..C..C..C...GGTTT.A...T.T.T.C.T..TCT....A..C.....
.....A.....T........A......C..T.....C..T..T...........A..C.. 5 3.01
H46
...G..G..A..A.................CCT...A.C.CTCGGA...C.......A.....C....T....C.TC.TG.CG.AC..TCG.T.GC....C..TCA..C...C....TA.CG.A
G.CC.C..AA.ATTA.T.TTCT.CAAAC.C.T......C..A..T..............C........A......G....A..GT..C..C..C...GGTT..A...T.T...C.T..TCT....A..C.....
....A.....T........A......C..T.....C..T..T...........A..C.. 1 0.6
H47
...G..G..A..A.................CCT...A.C.CTCGGA...C.......A.....C....T....C.TC.TG.CG.AC..TCG.T.GC....C..TCA..C...C....TA.CG.A
G.CC.C..AA.ATTA.T.TTCT.CAAAC.C.T......C..A..T..............C........A......G....A..GT..C..C..C...GGTTT.A...T.T...C.T..TCT....A..C.....
....A.....T........A......C..T.....C..T..T...........A..C.. 1 0.6
H48
...G..G..A..A.................CCT...A.C.CTCGGA...C.......A.....C....T....C.TC.TG.CG.AC..TCG.T.GC....C..TCA..C...C....TA.CG.A
G.CC.C..AA.ATTA.T.TTCT.CA.AC.C.T......C..A..T..............C..............G....A..GT..C..C..C...GGTTT.A...T.T.T.C.T..TCT....A..C.....
....A.....T........A......C..T.....C..T..T...........A..C.. 1 0.6
H49
T..T..C..A..C...........T..T.CC.AT.ATC.CAC.GC..T.A....G.A..T...AG.C.....A..ATAG..A....T.C..T.GC..G.C.TGCA....T.A.A..TA.C
..T.GCC.T.GAT.CTTA.A...C.ACT..C.T...C...T...AC.T.T..T.TG.T.C..CA.....T.A...A.....T.A..GTG.CA...T....GG.T..C..AT.T...C.T..GCCT...T
..T...........A.....T........A....C.......A........T...T..C...T..C.. 1 0.6
H50
T..T..C..A..C...........T..T.CC.AT.ATC.CAC.GC..T.A....G.A..T...AG.C.....A..ATAG..A....T.C..T.GC..G.C.TGCA....T.A.A..TA.C
..T.GCC.T.GAT.CTTA.A...C.ACT..C.T...C...T...AC.T.T..T.TG.T.C..CA.....T.A...A.....T.A..GTG.CA...T....GG.T..C..AT.T...C.T..GCCT...T
..T...........A.....T........A............A........T...T..C...T..C.. 10 6.02
H51 ------------------------
C..T.CCGA...T...A...A.GT.A......A......A.T......A..A.TG.CG.TCAA.C.AT.GC....G.A.GA..C...G.A..GC.A..TG.TT.A...TTG....A.T.CCTCATA
A.T.T..CTT.C.A.A.T.A..TTT..C....CA.......AT.CA..T....A...T..GATA.TA....G.CT.A.....A.T..TTA.TC.T...T..C..A..T........C........T..A.....
...T..T.CT.--.-------.------- 1 0.6
H52
T.....C..A..A........T..C..T.CCGA...T...A...A.GT.A......A......A.T......A..A.TG.C..TCAA.C.AT.GC.....A.GA..C...G.A..GC.A..TG
.TT.....T.G....A.T.CCTCATAA.T.T..CTT.C.A.A.T.A..TTT..C...TCA.......AT..A..T....A...TG.GATA.TA....G.CT.A.....A.T...TA.TC.T...T..C..
A..T........C........T..A............T..T.................A..G.. 8 4.82
H53
T.....T..A..A........T..C..T.CCGA...T...A...A.GT.A......A......A.T......A..A.TG.C..TCAA.C.AT.GC....C.A.GA..C...G.A..GC.A..T
G.TT.....T.G....A.T.CCTCATAA.T.T..CTT.C.A.A.T.A..TTT..C...TCA.......AT..A..T....A...TG.GATA.TA....G.CT.A.....A.T...TA.TC.T...T..C.
.A..T........C........T..A............T..T.................A..G.. 1 0.6
H54
T.....C..A..A........T..C..T.CCGA...T...A...A.GT.A......A......A.T......A..A.TG.C..TCAA.C.AT.GC....C.A.GA..C...G.A..GC.A..T
G.TT.....T.G....A.T.CCTCATAA.T.T..CTT.C.A.A.T.A..TTT..C...TCA.......AT..A..T....A...TG.GATA.TA....G.CT.A.....A.T...TA.TC.T...T..C.
.A..T........C........T..A............T..T.................A..G.. 1 0.6
H55 ----------------------------
CC.AT.AT...AC.GC.T.........A..T...A.T........T..AG.CA.T.AA.....TGC......A..T..A.T...C.T.A.AA.A..ACCA..A.....A.T.A.CTT.GAA..TTT.CC.
T.CTATA.TT.CT.TTGG..CTT.AGGA.T..A.TA.G.CG.T..ATG..T.A.AA.T..G.GT..TA..ATGT....TTCTATC..AA.C----------.-------.------- 1 0.6
H56
T...........A.....A.....C....CC.AT.AT...AC.GC.T.........A..T...A.T........T..AG.CA.T.AA.....TGC......A..T..A.T...C.T.A.AA.A..AC.
A..A.....A.T.A.CTT.GAA..TTT.CC.T.CTATA.T.....TTG...CTT.AG.A....A-
TA...CG.T..ATG..T.A.A..T....GT...A..AT.T.....TC.ATC..A.T........T........C..T..G.....G...T.....T...G.C.....T......A..... 3 1.81
H57
T...........T.....A.....C....CC.AT.AT...AC.GC.T.........A..T...A.T........T..AG.CA.T.AA.....TGC......A..T..A.T...C.T.A.AA.A..AC.
A..A.....A.T.A.CTT.GAA..TTT.CC.T.CTATA.T.....TTG...CTT.AG.A....A-
TA...CG.T..ATG..T.A.A..T....GT...A..AT.T.....TC.ATC..A.T........T........C..T..G.....G...T.....T...G.C.....T......A...T..... 1 0.6
H58
T...........T.....A.....C....CC.AT.AT...AC.GC.T.........A..T...A.T........T..AG.CA.T.AA.....TGC......A..T..A.T...C.T.A.AA.A..AC.
A..A.....A.T.A.CTT.GAA..TTT.CC.T.CTATA.T.....TTG...CTT.AG.A....A-

TA...CG.T..ATG..T.A.A..T....GT...A..AT.T.....TC.ATC..A.T.....T.....C.....C....------.----------.-------.------- 1
0.6
H59 -------
.....T.....A.....C....CC.AT.AT...AC.GC.T.....A..T...A.T.....T..AG.CA.T.AA.....TGC.....A..T..A.T...C.T.A.AA.A..AC....A.....A.T.A.CT
T.GAA..TTT.CC.T.CTATA.T.....TTG...CTT.AG.A....A-
TA...CG.T..ATG..T.A.A..T....GT...A..AT.T.....TC.ATC..A.T.....T.....C..T..G.....G...T.....T...---------.-------.------- 1
0.6
H60
T...........T.....A.....C....CC.AT.AT...AC.GC.T.....A..T...A.T.....T..AG.CA.T.AA.....TGC.....A..T..A.T...C.T.A.AA.A..AC.
A..A.....A.T.A.CTT.GAA..TTT.CC.T.CTATA.T.....TT....CTT.AG.A....A-
TA...CG.T..ATG..T.A.A..T....GT...A..AT.T.....TC.ATC..A.T.....T.....C..T..G.....G...T.....T...G.C.....T.....A...T..... 1 0.6
H61
T...........A.....A.....C....CC.AT.AT...AC.GC.....A..T...A.T.....T..AG.CA.T.AA.....TGC.....A..T..A.T...C.T.A.AA.A..AC.
A..A.....A.T.A.CTT.GAA..TTT.CC.T.CTATA.T.....TTG...CTT.AG.A....A-
TA...CG.T..ATG..T.A.A..T....GT...A..AT.T.....TC.ATC..A.T.....T.....C..T..G.....G...T.....T...G.C.....T.....A..... 2 1.2
H62
T...........A.....A.....C....CC.AT.AT...AC.GC.....A..T...A.T.....T..AG.CA.T.AA.....TGC.....A..T..A.T...C.T.A.AA.A..AC.
A..A.....A.T.A.CTT.GAA..TTT.CC.T.CTATA.T.....TTG...CTT.AG.A....A-
TA...CG.T..ATG..T.A.A..T....GT...A..AT.T.....TC.ATC..A.T.....T.....C..TT.C.....G...T.....T...G.C.....T.....A..... 1 0.6
H63
.....A..T.....A.....G..A..CCTTT.T..CAC.CGC.TG.CTC.GT....C..C.T.....AGT..TG.CAAT..A.A..T.GC..G.A.T.CA.....C....AA..
AGGGAAG...G...CT..TA..TCGA..AGA.TT.C...AAC.TTT.....TTG.TC.TTCA...C....T..G.....G.....A.....G..G...GGAT.T.T...TT.ACT.TG
.T..C..G.....C.....A..T...T.....A..C.....C...T..... 7 4.22
H64
.....T.....A.....G..A..CCTTT.T..CAC.CGC.TG.CTC.GT....C..C.T.....AGT..TG.CAAT..A.A..T.GC..G.A.T.CA.....C....AA..A
GGGAAG...G...CT..TA..TCGA..AGA.TT.C...AAC.TTT.....TTG.TC.TTCA...C....T..G.....G.....A.....G..G...GGAT.T.T...TT.ACT.TG.T
..C..G.....C.....A..T...T.....A..C.....C...T..... 1 0.6
H65
.....A..T.....A.....G..A..CCTTT.T..CAC.CGC.TG.CTC.GT....C..C.T.....AGT..TG.CAAT..A.A..T.GC..G.A.T.CA.....C....AA..
AGGGAAG...G...CT..TA..TCGA.TAGA.TT.C...AAC.TTT.....TTG.TC.TTCA...C....T..G.....G.....A.....G..G...GGAT.T.T...TT.ACT.TG
.T..C..G.....C.....A..T...T.....A..C.....C...T..... 1 0.6
H66
.....A..T.....A.....G..A..CCTTT.T..CAC.CGC.TG.CTC.GT....C..C.T.....AGT..TG.CAAT..A.A..T.GC..G.A.T.CA.....C....AA..
AGGGAAG...G...CT..TA..TCGA..AGA.TT.C...AA..TTT.....TTG.TC.TTCA...C....T..G.....G.....A.....G..G...GGAT.T.T...TT.ACT.TG.
T..C..G.....C.....A..T...T.....A..C.....C...T..... 1 0.6
H67
.....A..T.....A.....G..A..CCTTT.T..CAC.CGC.TG.CT..GT....C..C.T.....AGT..TG.CAAT..A.A..T.GC..G.A.T.CA.....C....AA..A
GGGAAG...G...CT..TA..TCGA..AGA.TT.C...AAC.TTT.....TTG.TC.TTCA...C....T..G.....G.....A.....G..G...GGAT.T.T...TT.ACT.TG.T
..C..G.....C.....A..T...T.....A..C.....C...T..... 1 0.6
H68
T..G.....A..A.....A..T..C.....C..T.ATCWCA..GCCT..A.....T...A.T.....G.TA.AG.CG.T.A.....T.GC....A..TC....TG..TA..TA.T...
G.AT.T..A.TCG...T.G.CT.C.AGC..TT.CC..TC.AA.G..G...TAG.T.CTTCT.....A-
TC....G...AAT.T.TCA.A.TT....GTCT.A..AT...T...TC.TTC..A.T..T.....T.....A.....A..T.....T..TG.T.....T...T..A...A..... 1
0.6
H69
T..G.....A..A.....A..T..C.....C..T.ATC.CA..GCCT..A.....T...A.T.....G.TA.AG.CG.T.A.....T.GC....A..TC....TG..TA..TA.T...G.
AT.T..A.TCG...T.G.CT.C.AGC..TT.CC..TC.AA.G..G...TAG.T.CTTCT.....A-
TC....G...AAT.T.TCG.A.TT....GTCT.A..AT...T...TC.TTC..A.T..T.....T.....A.....A..T.....T..TG.T.....T...T..A...A..... 6
3.61
H70
T..G.....A..A.....A..T..C.....C..T.ATC.CA..GCCT..A.....T...A.T.....G..A.AG.CG.T.A.....T.GC....A..TC....TG..TA..TA.T...G.
AT.T..A.TCG...T.G.CT.C.AGC..TT.CC..TC.AA.G..G...TAG.T.CTTCT.....A-
TC....G...AAT.T.TCG.A.TT....GTCT.A..AT...T...TC.TTC..A.T..T.....T.....A.....A..T.....T..TG.T.....T...T..A...A..... 2
1.2
H71
T..G.....A..A.....A..T..C.....C..T.ATC.CA..GCCT..A.....T...A.T.....G.TA.AG.CG.T.A.....T.GC....A..TC....TG..TA..TA.T...G.
AT.T..A.TCG...T.G.CT.C.AGC..TT.CC..TC.AA.G..G...TAG.T.CTTCT.....A-
TC....G...AAT.T.TCG.A.TT....GTCT.A..AT...T...CC.TTC..A.T..T.....T.....A.....A..T.....T..TG.T.....T...T..A...A..... 1
0.6
H72
T..G.....A..A.....A..T..C.....T.ATC.CA..GCCT..A.....T...A.T.....G.TA.AG.CG.T.A.....T.GC....A..TC....TG..TA..TA.T...G.
AT.T..A.TCG...T.G.CT.C.AGC..TT.CC..TC.AA.G..G...TAG.T.CTTCT.....A-
TC....G...AAT.T.TCG.A.TT....GTCT.A..AT...T...TC.TTC..A.T..T.....T.....A.....A..T.....T..TG.T.....T...T..A...A..... 1
0.6

Figure 2. Polymorphic nucleotide sequences of various fish species from Marchika Lagoon.

Genetic diversity, phylogenetic tree construction, and haplotypes identification. Comparisons using FST or the fixation index, as defined by Wright (1969), which evaluates pairwise differences in allelic frequencies between populations, revealed significant values in most cases. Specifically, FST values ranged from 0.17 between *Trachurus mediterraneus* and *Trachurus trachurus* to 0.235 between *Conger conger* and *Trigloporus lastoviza*, indicating considerable genetic differentiation among the species identified in the Marchica lagoon (Figure 3). This genetic differentiation aligns with broader research indicating that mitochondrial DNA, particularly the COI gene, offers effective resolution for species-level identification due to its high interspecific variability and moderate intraspecific consistency (Hebert et al 2003; Pegg et al 2006). The COI

gene is recognized as a reliable and widely accepted tool for species identification, as corroborated by multiple studies across diverse taxa (Hebert et al 2004; Mathur et al 2022). Further reinforcing this, studies have demonstrated its efficacy in distinguishing cryptic and closely related species within both animal and plant domains (Saddhe & Kumar 2018; Yang et al 2018).

Recent findings also underscore the application of COI barcoding in marine conservation, particularly for tracking biodiversity and detecting non-native species in sensitive ecosystems such as lagoons and estuaries (Boissin et al 2017). This utility is demonstrated in ecosystems with complex species assemblages, where genetic data significantly enhances taxonomic precision (Collet et al 2018). Therefore, our results not only confirm the COI gene's robustness in species identification but also highlight its critical role in monitoring biodiversity in ecologically vulnerable regions like the Marchica lagoon.

The phylogenetic tree in Figure 4 was constructed using ggtree and ggplot2, with estimates calculated using the neighbor-joining method supported by the ape package. Our molecular analyses, derived from DNA barcodes, allowed us to investigate the phylogenetic relationships among different species (Erickson & Driskell 2012). The phylogenetic tree clearly illustrates that the identified species form distinct clades with no overlap. For instance, *Trachinus draco* and *Sardina pilchardus* are represented by different colors with separate branches, indicating an early evolutionary divergence. Indeed barcoding has proven to be highly valuable for assessing species relatedness and providing a framework for investigating hypotheses regarding the evolution of traits and species distribution. Additionally, the COI gene has been instrumental in phylogenetic studies, offering insights into evolutionary lineages, as seen in studies on insect species (Pérez-Sayas et al 2022) and marine organisms (Hajibabaei et al 2007). According to Ptaszyńska (2012), Pérez-Sayas et al (2022) and Sajjad et al (2023), who used the COI gene to deduce the phylogeny of species, their results confirmed the usefulness of this gene in reconstructing evolutionary relationships, giving insight into genetic divergences and speciation events, the COI gene proved to be a solid tool for understanding the evolutionary history of various taxa.

A heatmap (Figure 5) with a dendrogram was used to visualize haplotype similarities and differences, effectively illustrating the genetic variation across species. Heatmaps, combined with dendrograms, are powerful for depicting clustering patterns among haplotypes, which has been supported in other biodiversity studies where mitochondrial DNA and SNPs were analyzed for genetic diversity (Labrador et al 2021; Fogliata et al 2022). The colors in the heatmap ranged from red to yellow, where red along the diagonal suggests high similarity among closely related haplotypes, such as H71 and H11 or H51 and H30, which share recent ancestry. Conversely, orange to yellow indicated lower similarity, highlighting distinct genetic lineages (e.g., H61 and H10 or H58 and H31). Dendrograms reveal the hierarchical relationships between haplotypes, where shorter branches reflect closer relationships, which can imply recent shared ancestry (Fogliata et al 2022). This approach is vital in studies of phylogenetic relationships and divergence levels, particularly among marine species in ecologically sensitive habitats, reinforcing similar results from other marine biodiversity studies (Boissin et al 2017; Collet et al 2018).

Analyzing the differences between haplotypes of various species aims to understand phylogenetic relationships, levels of divergence between groups, and genetic diversity within each species (Norman et al 1994; Branco et al 2000). SNP diversity was high, with a total of 72 haplotypes identified (Figure 6). Short connecting lines between similar haplotypes of different species suggest a recent common ancestor. For example, *Trachurus mediterraneus* and *Trachurus trachurus* share very similar dominant haplotypes, H69 and H10, respectively (Figure 6). This pattern is also reflected in studies where close genetic proximity and short phylogenetic distances in dendrograms have suggested recent divergence (Evans et al 2021; Han et al 2024).

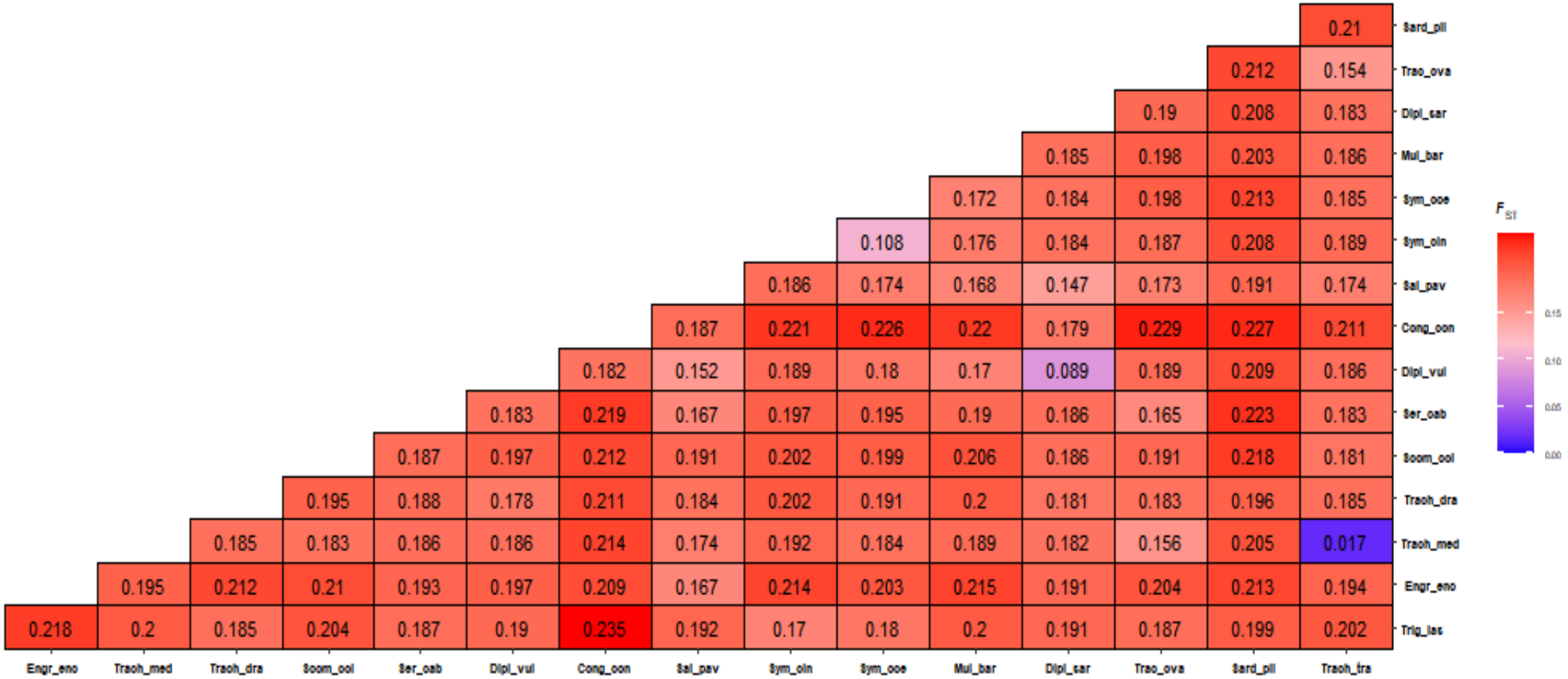

Figure 3. Analysis of species structure in Marchica Lagoon fish based on FST values.

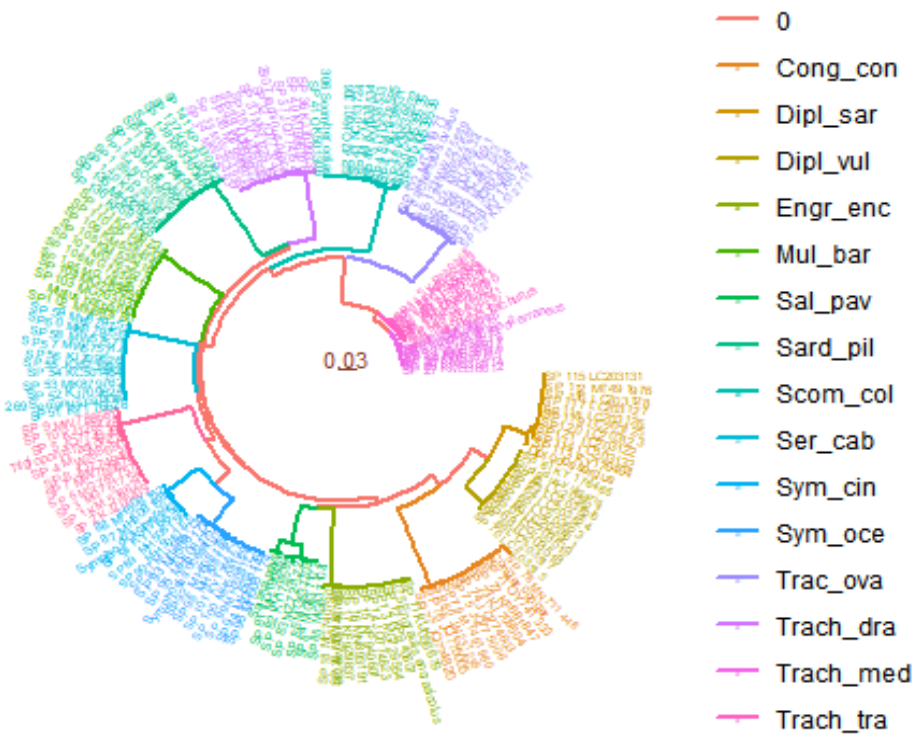

Figure 4. Colored phylogenetic tree representing fish species in Marchica Lagoon. The distance scale indicates a mean of 3% genetic variation due to nucleotide substitution.

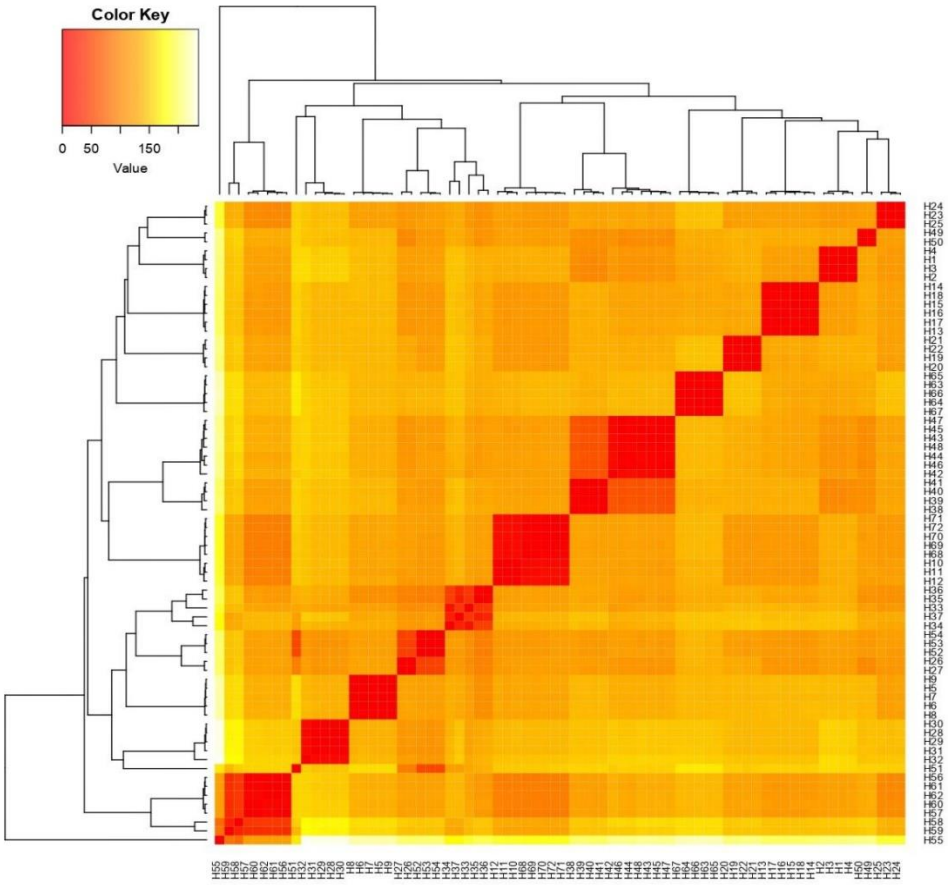

Figure 5. Heatmap illustrating the number of nucleotide differences between haplotypes. Each branch of the phylogenetic tree corresponds to a haplotype in the matrix. Close relationships are indicated by "dark red" color, while distant relationships are represented by "orange to yellow" color.

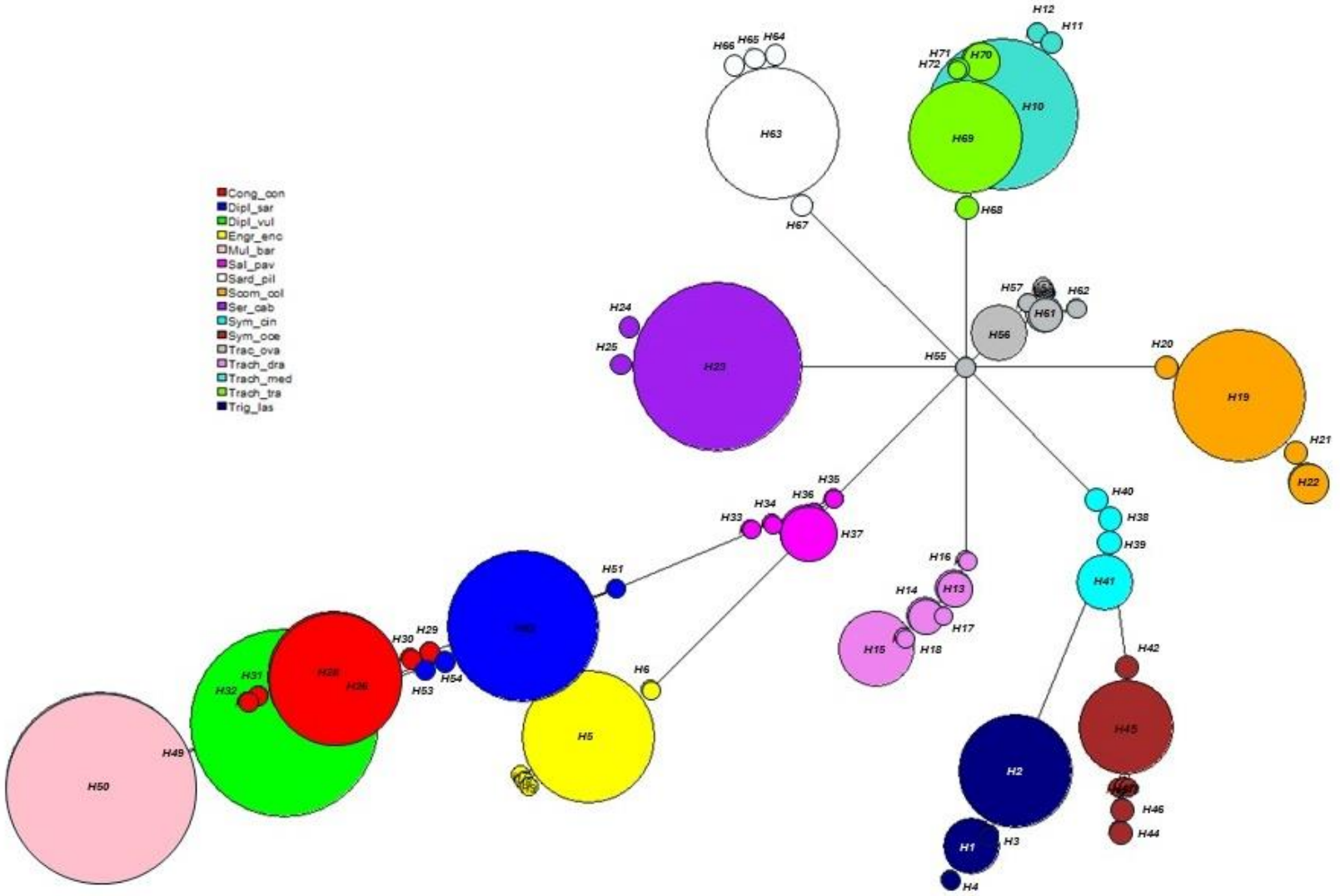

Figure 6. Haplotype networks of COI DNA sequences from fish in Marchica Lagoon. Circle size indicates haplotype frequency, and numbers mark the nodes.

In terms of intraspecific diversity, each species is represented by a distinct color. The dominant haplotype is depicted as a large circle, connected to several smaller circles representing less frequent haplotypes within that species. For instance, haplotype H23 of *Serranus cabrilla* is the most frequent, with smaller adjacent circles representing haplotypes 24 and 25. This visualization enables a more granular analysis of gene flow and genetic diversity within species (Wongfu et al 2022; Han et al 2024). Our findings align with Doorenweerd et al (2020) and Wei et al (2023), underscoring the COI gene's efficacy in evaluating intraspecific variability. These results collectively reinforce the robustness of the COI gene as a genetic marker for assessing genetic diversity and evolutionary relationships within and between species.

Principal component analysis (PCA), discriminant analysis of principal components (DAPC), and membership analyses. We analyzed the genomic variation of the COI gene among taxa using principal component analysis (PCA). The percentage of total data variance explained by PCA was low at 24.1% (Figure 7).

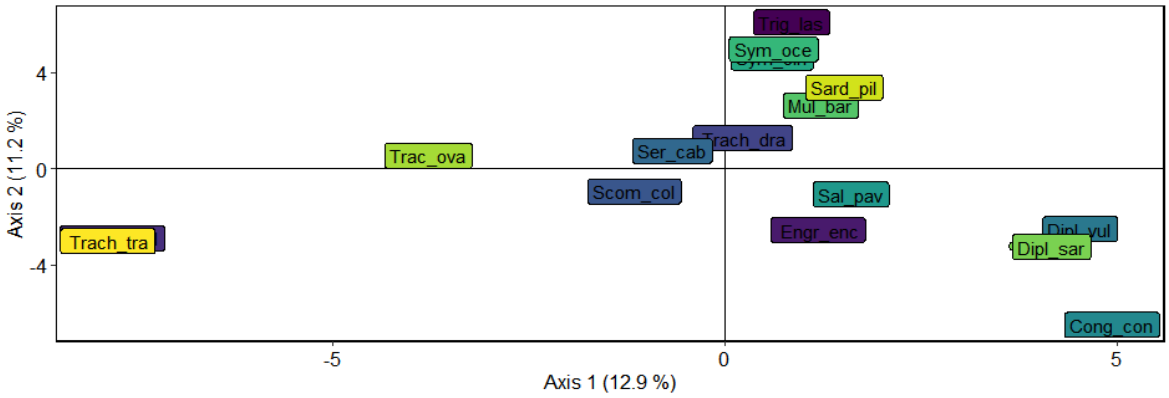

Figure 7. Visualization of genetic groups of Marchika Lagoon species by principal component analysis (PCA).

Consequently, we employed DAPC to better explore and visualize genetic structures within populations. DAPC relies on genetic variables such as SNPs to maximize the separation between identified clusters of species (Jombart et al 2010). The clusters in the DAPC were defined by a priori assumptions of species membership (K = 16). Using the α-score optimization method proposed by Jombart et al (2010), we retained 20 principal components for the discriminant analysis, which cumulatively explained about 57% of the total genetic variability among the studied species. The species structure revealed by the DAPC showed clear genetic differentiation between the tested species, with only a narrow overlap observed among a few of them (Figure 8).

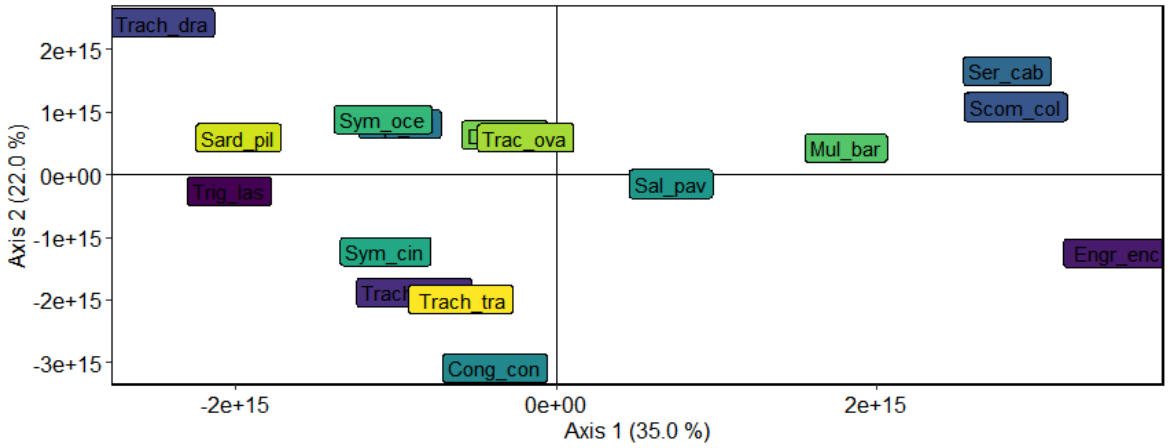

Figure 8. Visualization of genetic groups of Marchica Lagoon species using principal component discriminant analysis (DAPC).

Additionally, to validate our DAPC results, we used the membership relevance method to verify the probability of species membership. This analysis demonstrated that the 16 studied species are 100% distinct and well-differentiated from one another (Figure 9), echoing previous studies where DAPC and membership methods provided robust support for species delineation (Evans et al 2021; Labrador et al 2021). Our findings contribute to the growing body of research that leverages DAPC for evaluating genetic structures within biodiversity-rich and ecologically sensitive regions (Wongfu et al 2022).

Figure 9. Individual membership probabilities from discriminant analysis of principal components for 16 species in Marchica Lagoon.

Conclusions. Genetic identification using the mitochondrial COI gene as a DNA barcode facilitated the rapid and accurate identification of 46 samples from the Marchica Lagoon. The statistical analyses applied to these genetic sequences confirmed the robustness of barcoding as a tool for biodiversity studies and genetic and phylogenetic analyses of species. The results demonstrated significant genetic differentiation among the identified

species, revealing distinct clades and high SNP diversity with 72 haplotypes. The use of principal component analysis (PCA) and discriminant analysis of principal components (DAPC) further validated the genetic structure and differentiation among species. These findings underscore the effectiveness of genetic barcoding for identifying fish species and contribute to the conservation of vulnerable species, particularly in a Ramsar site. The insights gained provide a solid scientific foundation for understanding the genetic diversity and phylogenetic relationships of species within the Marchica Lagoon. It is crucial to disseminate these results to Ramsar site managers, researchers, policymakers, and the local community to highlight the importance of conserving vulnerable species. Promoting awareness and collaboration on conservation and sustainable management initiatives is essential for preserving the ecological integrity of this unique lagoon ecosystem.

Acknowledgements. The authors express their gratitude for the close collaboration between Mohammed V University of Rabat and the National Center for Scientific and Technical Research of Rabat (CNRST), whose support was invaluable in successfully conducting this study under optimal conditions.

Conflict of interest. The authors declare that there is no conflict of interest.

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Received: 21 July 2024. Accepted: 22 September 2024. Published online: 09 December 2024.
Authors:
Aicha Hamid, Mohammed V University in Rabat, Faculty of Sciences, Laboratory of Biodiversity, Ecology and Genome, 4 Avenue Ibn Battouta, B.P. 1014 RP, Rabat, Morocco, e-mail: aichahamid65@gmail.com
Imane Rahmouni, Mohammed V University in Rabat, Faculty of Sciences, Laboratory of Biodiversity, Ecology and Genome, 4 Avenue Ibn Battouta, B.P. 1014 RP, Rabat, Morocco, e-mail: i.rahmouni@um5r.ac.ma;
Bazairi Hocein, Mohammed V University in Rabat, Faculty of Sciences, Laboratory of Biodiversity, Ecology and Genome, 4 Avenue Ibn Battouta, B.P. 1014 RP, Rabat, Morocco, e-mail: hocein.bazairi@fsr.um5.ac.ma
Halima Louizi, Mohammed V University in Rabat, Faculty of Sciences, Laboratory of Biodiversity, Ecology and Genome, 4 Avenue Ibn Battouta, B.P. 1014 RP, Rabat, Morocco, e-mail: halima.louizi@um5s.net.ma
Amal Lamkhalkhal, Mohammed V University in Rabat, Faculty of Sciences, Laboratory of Biodiversity, Ecology and Genome, 4 Avenue Ibn Battouta, B.P. 1014 RP, Rabat, Morocco, e-mail: Amal.lamkhalkhal@um5r.ac.ma;
Mohamed Selfati, Laboratoire Santé et Environnement, Faculté des Sciences Aïn Chock, Hassan II University of Casablanca, B.P 5366 Maârif, 20100 Casablanca, Morocco, e-mail : selfatimohamed@gmail.com
Oussama Bououarour, Mohammed V University in Rabat, Faculty of Sciences, Laboratory of Biodiversity, Ecology and Genome, 4 Avenue Ibn Battouta, B.P. 1014 RP, Rabat, Morocco, e-mail: oussama.bouarour@gmail.com
Bouabid Badaoui, Mohammed V University in Rabat, Faculty of Sciences, Laboratory of Biodiversity, Ecology and Genome, 4 Avenue Ibn Battouta, B.P. 1014 RP, Rabat, Morocco, e-mail: bouabidbadaoui@gmail.com

How to cite this article:
Hamid A., Rahmouni I., Hocein B., Louizi H., Lamkhalkhal A., Selfati M., Bououarour O., Badaoui B., 2024 DNA barcoding analysis of fish species in the Marchica Lagoon, Morocco. AACL Bioflux 17(6):2775-2791.