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Research Article

Mitochondrial DNA cytochrome b diversity and phylogeny of the Eurasian coot (*Fulica atra*; Linnaeus, 1758) (Gruiformes: *Rallidae*)

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Abstract

The Eurasian coot (*Fulica atra*; Linnaeus, 1758) is a widely distributed waterbird consisting of four subspecies and found throughout northern Eurasia to the western Sahara, Australia, and New Zeeland that recently has experienced regional population declines. Population declines may impact genetic diversity and little information is available on genetic diversity in coots. We extracted genomic DNA from muscle tissues of coots collected from Fereidoonkenar International Wetland (FIW; n = 7) and Gorgan Bay (GB; n = 5) in Iran and sequenced 513-bp of mitochondrial DNA cytochrome b marker and published sequences from Genbank. Localities in Iran were characterized by two haplotypes high haplotype and low nucleotide diversities. The F_{st} value (0.177) indicated a high level of genetic differentiation between FIW and GB. The phylogenetic analysis that included published sequences, supported groupings that likely correspond to migration flyways of coots. Moreover, specimens from Australia were not associated with coots from other localities in our sample. Our results showed that coots number of cyt b haplotypes was low for the overall sample but have retained substantial levels of genetic diversity in our study sites.

Keywords: Biodiversity, Fereidoonkenar International Wetland, Genetic Conservation, Miankaleh International Wetland

Introduction

The Eurasian coot (*Fulica atra*; Linnaeus, 1758) is a widely distributed waterbird that consists of four subspecies; *F. atra atra*, *F. atra lugubris*, *F. atra novaeguaniae,* and *F. atra australis* (Cramp & Simmons, 1980) found throughout northern Eurasia to the western Sahara, Australia, and New Zeland and is currently listed globally as 'Least Concern' on the IUCN Red List of Threatened Species (IUCN 2019). Eurasian coots have three main populations all over Eurasia, including North-west Europe, the Black Sea, and West Siberia (CMS, 1994; Cramp & Simmons, 1980). Eurasian coot maintains small resident populations in the wetlands of Iran and populations seasonally grow as coots arrive to overwinter from other parts of the world (CMS, 2005). The Eurasia-East African Flyway is a 10,000 km system of largely overland corridors (Greenberg & Marra, 2005; Newton, 2008) that extends from northeastern Europe and western Siberia through the Middle East into southern Africa. Millions of ducks, geese, and coots in this flyway have long supported an annual harvest in the south Caspian region and are increasingly attracting the attention of sport hunters (CMS, 1994; Mansoori, 2009). Despite their global status, coots are undergoing a moderately rapid population decline in Europe from poisoning by ingestion of lead shot, oil and petroleum pollution, climate change and infectious disease (IUCN, 2019). Hunting and wetlands destruction have also caused fluctuations in populations of the species (Mondain-Monval, et al., 2002; Bartoszewicz & Zalewski, 2003; Aazami, et al., 2010; Bara & Luciano, 2019). Regionally, Eurasian coots are listed as 'Near Threatened' (BirdLife International, 2015; IUCN, 2015) and recent conservation measures have been taken to support them including listing in the EU Birds Directive Annex II and III (Directive, 2009). The Mediterranean and the Black Sea populations are listed on the CMS (Convention on Migratory Species) Appendix II and the species is legally protected in Britain (IUCN, 2019). It is also implied in the Conservation of African-Eurasian Migratory Waterbirds Agreement (AEWA, 2019) as a species with population decline that needs conservation and good quality habitat during their migrations. Population declines are often associated with the loss of genetic diversity (Hedrick 2011) and a recent paper suggests that low genetic diversity in birds is associated with an increased risk of extinction (Canteri et al., 2021). Human activities including overexploitation, unlimited poaching and recent unknown mortality of the Eurasian coot in the study areas may be responsible for the reported population declines especially in important breeding areas (Mondain-Monval, et al., 2002; Bartoszewicz & Zalewski, 2003; Grishanov, 2006; Lv, et al., 2017; Bara & Luciano, 2019) and

severe genetic bottleneck in Eurasian coots (CMS, 2005; IUCN, 2015; Nourani, et al., 2015; Parchizadeh & Williams, 2018). Despite reports of population declines of Eurasian coots and the importance of genetic studies to biological conservation, no studies have attempted to report on genetic variation of F. *atra* across their entire distribution including important wintering grounds like those found in Iran near the Caspian Sea. A limited number of studies were conducted for some areas occupied by F. *atra* (Alcaide, et al., 2014; Garcia, et al., 2014a, b; He, et al., 2015; Lv, et al., 2017; Tizard, et al., 2018); however, these did not include samples from Iran. In light of their recent regional population declines, we provide the first assessment of the genetic diversity of mitochondrial DNA (mtDNA) Cytochrome b (*cyt b*) from a small sample of Eurasian coots from the Fereydunkenar International wetland (FIW) and Miankaleh International wetland (MIW) near the southern Caspian Sea.

We combined our results with the limited data available for the species in other parts of the distribution range of the species (He et al., 2015; Lv et al., 2017) to; 1) establish a phylogenetic relationship among some populations of the Eurasian coot 2) assess levels of genetic diversity and differentiation of the Eurasian coot in Iran, and 3) compare the genetic variation of coots in Iran with published sequences from other parts of their distribution.

Material and methods

We sampled wintering populations of Eurasian coot (n=12) between 2017 and 2019 from Fereidoonkenar International Wetland (FIW; n=7) and Miankaleh International Wetland (MIW; n=5) located in the south and southeast coast of the Caspian Sea found at the coordinates of 36°40′N 52°33′E and 36°50′N 53°17′E, respectively (Ahmadpour et al. 2012; Ahmadpour et al. 2016; Birdlife International, 2020; Fig. 1a). The sampling localities were selected based on areas with suitable habitats that supported high densities of Eurasian coots (Yazdandad, 2007; Lantsheer, et al., 2008; Joolaee, et al., 2009). At each station, we collected muscle tissues taken from healthy carcasses that were confiscated from illegal hunters by rangers from the Department of Environment. Each sample was stored in a labeled plastic bag at -20°C for later use.

Genomic DNA was extracted using the WizBio Tissue DNA Extraction Kit (WizBio Co. Korea) according to the manufacturer's protocol. The quality and quantity of isolated DNA were analyzed following Green & Sambrook (2012). We amplified 513bp sequences amplified by polymerase chain reaction (PCR) using the protocol and primers L15413 (5′-GGGGGWTTYTCMGTNGAYAAYCC-3′) and H16064 (5′-

CTTCANTYTTTGGYTTACAAGRCC-3′) from MacCracken and Sorenson (2005). An mtDNA *cytb* gene is used in molecular evolution studies and has a high power to discriminate avian taxonomy (Seif et al., 2012; Prusak et al., 2004; Kuwayama and Ozawa, 2000).

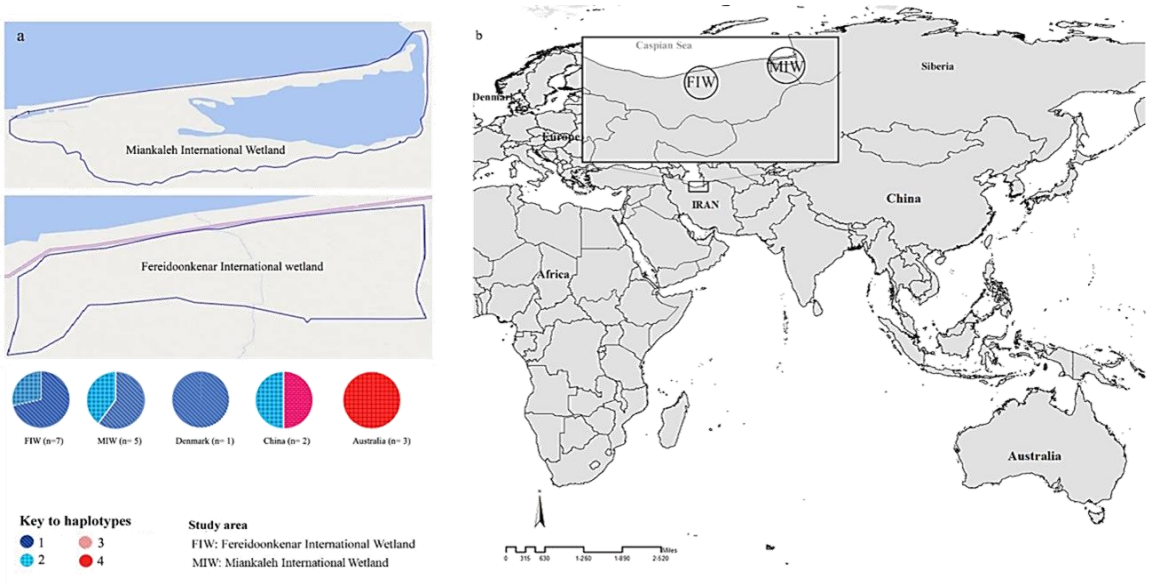

Fig. 1. The location of the study areas in Iran (insets a and b) and the distribution map of mtDNA haplotypes from Eurasian coots collected during this study combined with available sequences from GenBank. The exact locations of published sequences were

We amplified genomic DNA in reaction mixtures of 25 µl. Each reaction mixture contained 10 mM Tris-HCl (pH 8.5), 2 µ Taq polymerase, 200 µ dNTPs mix, 1.5 mM $MgCl_2$,and 50 mM KCl (WizBio Co. Korea), 1 µl each of forward and reverse primers, and 60 ng of template DNA. An initial denaturation step at 95˚C for 7 min was applied prior to 14 cycles of denaturation for 30 s at 95˚C, hybridization for 45s at 58˚C and extension for 1 min at 72˚C, and 36 cycles of denaturation for 30 s at 95˚C, hybridization for 45s at 51˚C and extension for 1 min at 72˚C followed by a final extension at 72˚C for 7 min. PCR products were electrophoresed in 2% agarose gel and DNA bands were then visualized using UV Trans-illuminator (ProteinSimple Co., California, USA; Fig. 2). All fragments were sequenced with the forward primer by Microsynth Co., Switzerland.

We used Seqscape v.2.6 (Applied Biosystems) to generate sequences of *cytb* using the complete genome of *Fulica atra* (accession number: KP313718) as a reference and after adjusting for gaps at the beginning of some of the sequences with MEGA7 (Tamura et al., 2019; Table 1), we based our analysis on a fragment of 513bp (position 588 and 1101). We performed a phylogenetic analysis of the mtDNA haplotypes using a Maximum Likelihood tree in MEGA v.7. We used

HKY+G as the best substitution model in MEGA to estimate evolutionary distance and used 1000 bootstrap replicates to assess nodal support for branches in the resulting tree. We also produced the ancestry of each individual (Ronquist, et al., 2019) using a Bayesian tree that was created with 1000000 repetitions in Mr.Bayes v.3.2.7 and used TreeView v.1.6.6 (Roderic, 2001) to observe the Bayesian phylogenetic tree.

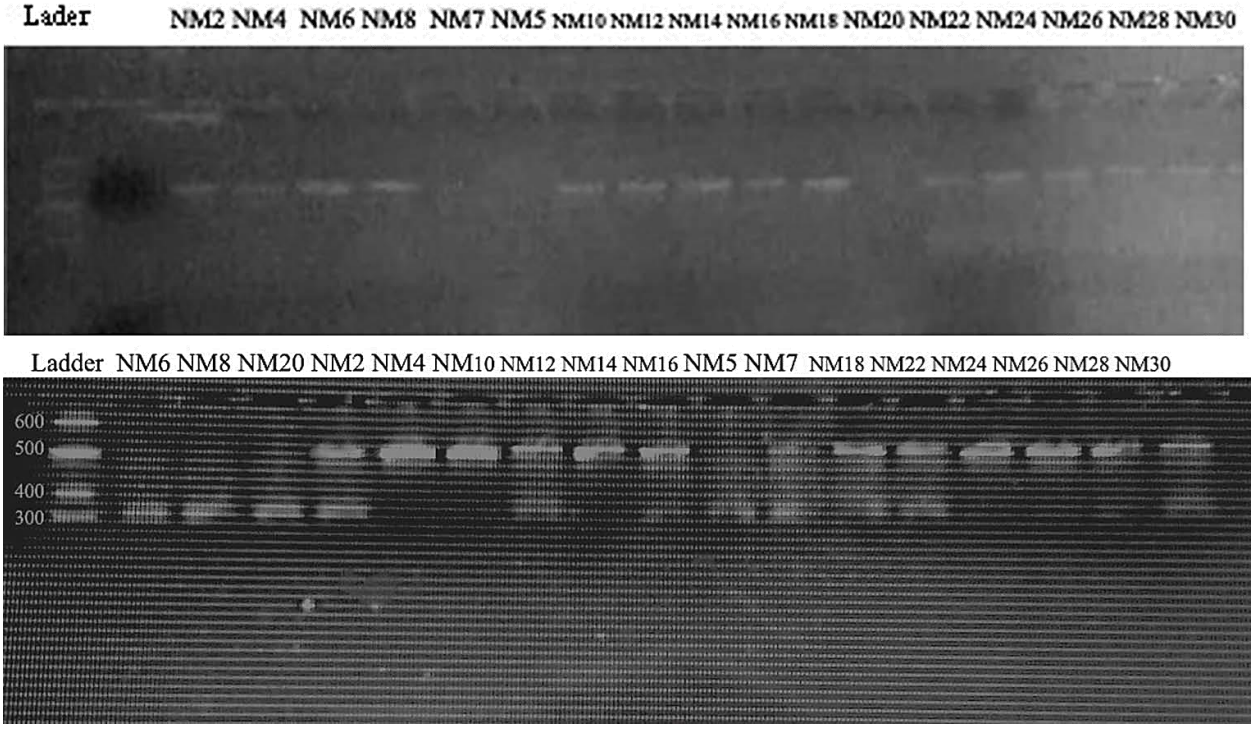

Fig. 2. Gel electrophoresis of PCR products. PCR products were loaded on 2% agarose gel containing ethidium bromide and visualized under UV light. Lanes show the 600bp DNA ladder.

We considered specimens from Iran to be from two populations based on previous findings of distinct morphometry and morphology (Yazdandad & Karami, 2009). We used the number of haplotypes (H) haplotype diversity (h), nucleotide diversity (π), and the number of polymorphic sites (P_S) to describe genetic diversity in each population using DnaSP v.6.12 (Rozas et al., 2018). We also calculated F_{st} in DnaSP to assess genetic differentiation between FIW and MIW. Finally, we combined our samples from Iran (n = 12) with all published sequences of the species (n=6) from the GenBank database (Table 1) to provide a broader understanding of the how genetic diversity of Eurasian Coots is spread across its distribution range. We visualized connections between haplotypes with a Median-joining haplotype network created in Popart v.1.7 (Bandelt et al., 1999).

Table 1: Details of sequence data from mtDNA *Cyt b* samples of *F. atra* obtained from this study. Sequences of the Ac.number= Accession number from Genbank

Ac. Number	Location	Reference
MT980781	FIW	This study
MW018479	FIW	This study
MW018480	FIW	This study
MW018481	FIW	This study
MW018482	FIW	This study
MW018483	FIW	This study
MW018484	FIW	This study
MW018485	MIW	This study
MW018486	MIW	This study
MW018487	MIW	This study
MW018488	MIW	This study
MW018489	MIW	This study
KM005730.1	China	(Ha et al., 2014)
KP313718.1	China	(He et al., 2016)
MN122918.1	Denmark	(Margaryan 2019)
KC614074.1	Australia	(Garcia et al., 2014a)
NC_025500.1	Australia	(Garcia et al., 2014b)
KF644582.1	Australia	(Garcia et al., 2014b)

Results

We detected four haplotypes from the combined sample of published sequences and samples from Iran. The first haplotype was found in Iran (Hap. 1) and overall, in 50% of the samples from the study. Second haplotype was also found in Iran (Hap. 2; 27%) while two other haplotypes were found from localities outside of Europe and the Middle East and associated with Australia and China (Hap. 3 & 4; Fig. 3, Table 1). The maximum likelihood tree of the sequences studied consisted of two main clades (A, B; Fig. 4a). Clade A contains two subclades (C and D) consisting of specimens from FIW, GB, China, and Denmark (Subclade C) with limited bootstrap support (22%) and specimens from Australia (Subclade D) with strong bootstrap support (85%). The

second clade (clade B) included specimens from FIW, GB, and China (Fig. 4a) separated with moderate bootstrap support (65%). Phylogenetic trees from maximum likelihood and Bayesian models produced similar results with two main clades (Fig. 4b).

o main clades (Fig. 4b).

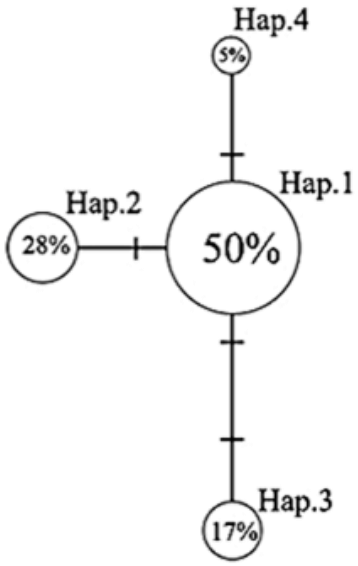

Fig. 3. Median-Joining network of Eurasian coot from Iran (FIW and MIW) and specimens from three other locations (China, Denmark, and Australia). Bars represent mutational differences and numbers inside circles represent the frequency of each haplotype in the sample.

The Median-Joining network showed a total of four mutational steps among all haplotypes. Two haplotypes were found at lower frequencies (Hap.2 and Hap.4) and were connected to the core-haplotype (Hap.1) by a single mutation. The final haplotype was separated by two mutations from the core haplotype and was found only in specimens from Australia (Hap.3; Fig. 3).

We detected two haplotypes among 12 individuals from FIW and MIW in Iran. Mean haplotype diversity in Iran was 0.562 ($h \geq 0.5$) and the average nucleotide diversity was 0.151 ($\pi < 0.5\%$). Haplotype diversity in MIW and FIW was 0.600 and 0.524 and nucleotide diversity was 0.135% and 0.168%, respectively Genetic differentiation (F_{st}) was high (0.177; P-value$_=$ 0.002) between FIW and MIW populations (Table 2).

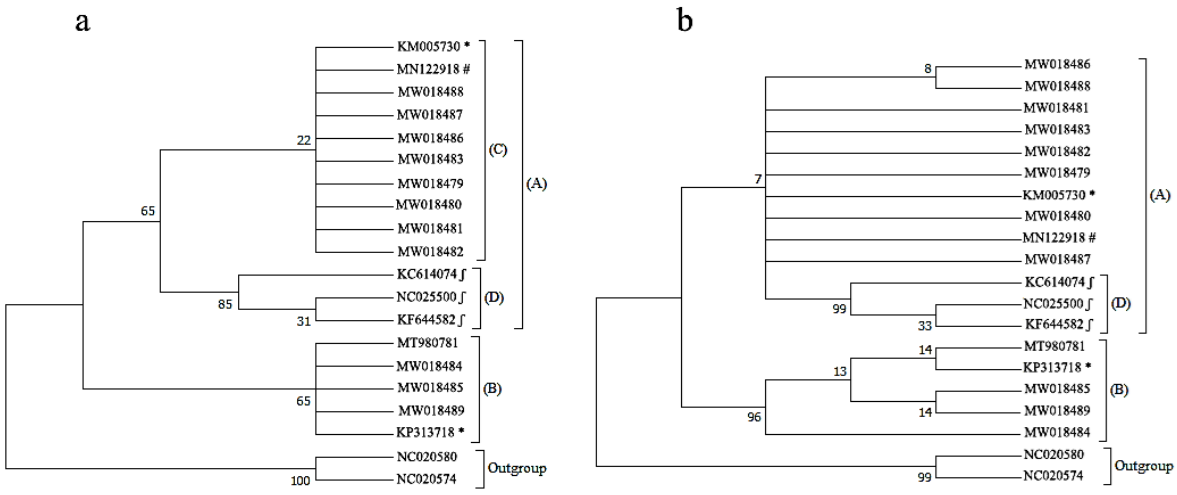

Fig. 4. a) Maximum likelihood tree and b) Bayesian tree of haplotypes (n = 18) of F. atra using 513bp of mtDNA cyt b Letters show clade or subclade membership and numbers at each haplotype represent bootstrap values. Two species of Gruidae: Grus rubicund

Table 2. Measures of genetic diversity for two populations of the *F. atra* from Iran. Abbreviations of populations as in Table 1.

	n	Hd±SD	π%±SD	PS	K	He		n	Hd±SD
FIW	7	0.524±0.209	0.168±0.072	1	2	0.055	FIW	7	0.524±0.209
MIW	5	0.600±0.175	0.135±0.039	1	3	0.070	MIW	5	0.600±0.175
Mean		0.562±0.038	0.151±0.016	1		0.062	Mean		0.562±0.038

n: sample size; Hd: haplotype diversity; π%: nucleotide diversity; PS: number of polymorphic sites; K: number of haplotypes; He: Expected heterozygosity.

Discussion

Our study is the first population-level assessment of mtDNA *cyt b* diversity of Eurasian coots in Iran. The number of *cyt b* haplotypes was low for the overall sample and similarly for FIW and MIW in Iran. Small resident populations of coot occur in wetlands of Iran and populations in the Caspian region seasonally grow as coots arrive from two main populations in northern Europe and western Siberia (Roomen et al., 2012) to overwinter. Cramp & Simmons (1980) proposed that coots from western Siberia migrate to Iran and China to overwinter. The geographic pattern of haplotypes from the overall sample provides some support for separate migratory pathways between populations in the north and wintering and breeding grounds further south in Europe and Australasia. Despite the low sample size in our study, the monophyletic group consisting of

specimens from Australia in our phylogenetic tree might suggest some degree of isolation from coots utilizing flyways further north.

Our sample of hunter-killed coots from FIW and MIW in Iran was likely a mixture of both resident and wintering birds, presumably representing different breeding populations. Despite our small sample, we expected to find a greater number of haplotypes. One possible explanation for the limited number of haplotypes is a past genetic bottleneck. Our sample size was not sufficient to conduct a formal test for a past bottleneck and a small sample size could explain the low number of haplotypes we found in our sample. However, the limited number of haplotypes, star-like pattern of haplotypes in the median joining network, and high level of *h* combined with the low level of π are consistent with a past genetic bottleneck (Lowe et al., 2004). It is plausible that the recent declines across their distribution have led to local reductions in genetic diversity of Eurasian coots. Additional samples would be needed to establish a potential timeline for the suspected bottleneck. Overharvesting of coots has been observed across their distribution (Grishanov, 2006; Lv, et al., 2017; Bara & Luciano, 2019; IUCN, 2019). For example, it is estimated that 3000 migratory birds are killed daily by local hunters in FIW and other protected wetlands in Iran and Eurasian coots comprise the largest percentage of the total kill (Aazami et al., 2012). Coots in Iran are now threatened by overexploitation along with the loss of critical wetland habitat and the absence of mitigating conservation measures.

Despite their close proximity and presence of similar haplotypes, we detected a high level of genetic differentiation between FIW and MIW (F_{st} = 0.171) supporting previous work that suggested coots from these two locations are distinct (Yazdandad & Karami 2009). Genetic differentiation between FIW and MIW may reflect differences in wintering coots that have arrived from separate breeding populations further north. However, before treating FIW and MIW as distinct populations for the purpose of conservation, those additional samples be collected to clarify the extent of genetic differentiation.

Conclusion

Our study provides the first range-wide examination of mtDNA *cyt b* diversity in Eurasian Coots in Iran. We detected low levels of genetic diversity in samples from Iran and in the overall sample. Unique haplotypes were found from several localities that are consistent with known migration flyways from northern Europe and Asia to southern wintering grounds. Genetic data from populations in Iran exhibit a pattern expected from experiencing a past population decline that

could potentially be associated with recent levels of hunter harvest. Additional samples would be important to verify this.

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