*J Genet Resour2019;5(1): 51-58* *Homepage: http://sc.journals.umz.ac.ir/*

**RESEARCH ARTICLE** *DOI: 10.22080/jgr.2019.16484.1134*

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# A Comparative Analysis of Genetic Diversity and Structure of Whooper Swan (*Cygnus cygnus)*: A New Considerable Established Population in Iran

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**ARTICLEINFO**

***Article history:***
Received 23 February 2019
Accepted 02 April 2019
Available online 15 April 2019

***Keywords:***
Population structure
Genetic differentiation
SSR markers
New wintering population

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***Print & Online ISSN:***
p-ISSN 2423-4257
e-ISSN 2588-2589

**ABSTRACT**

New wintering populations of Whooper Swan have been recently reported from west Asia, a lack of information about the population and its origin. The understanding the genetic structure and connectivity are crucial for determining strategies of management for its conservation programs. The samples were collected from two populations in northern Iran, Finland, Sweden, and Iceland, where with large breeding populations and Poland, where it has started nesting recently. Total genomic DNA was isolated from fresh blood or feather samples and six microsatellites markers were chosen based on their level of polymorphism. The results indicated that the maximum and minimum of Allele richness were observed for Iranian (5.6) and Polish (3.44) populations respectively. The analysis of molecular variance revealed that 77 % and 23% of the total diversity belong to within and between populations, respectively. The 114 Whooper Swans from six populations were categorized into four gene pools. Structure analysis suggested that the Scandinavian populations (Finland, Sweden, and Iceland) were differentiated from the Poland population and from both the old and new Iranian populations. Generally, both populations from Iran have comprised maximum variety in terms of having different gene pool and it is likely that the new population of Iran (Feredonkenar), is composed of individuals that migrated from an old Iranian population (Guilan) to this area as well as some individuals from Scandinavian.



***Please cite this paper as:*** Delpassand A, Ghasempouri M, Yousefzadeh H. 2019. A Comparative Analysis of Genetic Diversity and Structure of Whooper Swan (*Cygnus cygnus)*: A New Considerable Established Population in Iran. *J Genet Resour* 5(1): 51-58. doi: 10.22080/jgr.2019.16484.1134

## Introduction

Climate change would be caused by shifting the bird migrations routes and changing their habitats conditions. Parmesan (2006) compiled studies on many species, including alpine herbs, birds, and butterflies, and found an average poleward shift of 6.1 km per decade. outstanding influences on species range expansion and contraction due to climate change and global warming has been confirmed by fossil records (Woodward, 1987; Davis and Shaw, 2001) and reported trends (Hughes, 2000; McCarty, 2001; Walther *et al.*, 2001). Birds that migrate long distances usually show less resistance to temperature increases are more likely to be victims than all other species. Therefore, moving to a new habitat is just one response to climate change. The Whooper Swan (*Cygnus cygnus*) has a wide range in contrast to all swans and is frequently distributed in latitudes between 45° to 70°N. Main breeding populations of this species are present in Iceland, Sweden, Finland and Russia (Brazil 2003; del Hoyo and Collar, 2017). There are five populations of Whooper Swan: the Iceland population, winters primarily in Britain and Ireland, with smaller numbers remaining in Iceland and some also migrating as far as the near European continent.; ii) The Scandinavia and North European Russia winters in northwest and central Europe; iii) The northern Europe and western Siberia population, winters in the Black Sea and eastern Mediterranean; iv) The central and east Siberia

population to northeast China winters in east Asia; and v) population from West and central Siberia winters in the Caspian and Central Asia (Mathiasson 1991). The entire population of West and Central Asia may be present in the South Caspian region (Amini and Sehhatisabet, 2007). The Whooper Swan population are increasing across the world (Nilsson 1997, Brazil 2003). The maximum number is estimated that 150 000 to 200 000 individuals (Brazil, 2003). The Iranian Population of this species has significantly increased during the years 2014 and 2015 (more than 5000 individuals) and a new population also have recently been established in Fereydunkenar International wetlands. This wetland includes three small sites (Sorkhroud, Azbaran, and Fereydonkenar) which recorded as a twenty-second international wetland of Iran.

The wetland complex also has man-made wetlands (farmland) that are prepared for rice cultivation in late March. This artificial wetland is cultivated in the spring and summer season and is flooded in autumn and winter to prepare habitat for overwintering large numbers of migratory birds. Although the Whooper Swan is classified as least concern in the IUCN's Red List categories, information about its genetic diversity and connectivity among populations is rare.

Understanding population dynamics of species, genetic structure and connectivity are crucial for determining units of management for wildlife conservation programs (Schtickzelle *et al.*, 2005; Palsbøll et al., 2006; Anderson *et al.*, 2009). Utilizing DNA-based markers is an efficient and reliable tool for evaluating the genetic diversity of birds. A few molecular studies were performed on different species of the genus *Cygnus*, especially the Whooper Swan. Ransler *et al.* (2011) assessed the consequences of reintroduction program on genetic diversity of new populations of the Trumpeter Swan *Cygnus buccinator* using comparing patterns of genetic variation at 17 microsatellite loci across four restoration flocks (three wild-released, one captive) and their source populations. They found that a wild-released population established from a single source displayed a trend toward reduced genetic diversity relative to and significant genetic differentiation from its source population, though small founder population effects may also explain this pattern.

Among different molecular markers, Simple sequence repeats (SSRs) are frequently used for evaluating the genetic diversity of various birds with low taxonomic levels (Wilson, 2013; Ahmadi *et al.*, 2007; Arruga *et al.*, 2007; Roshier *et al.*, 2012), because they are codominant and many loci are available in comparison with dominant markers.

The objective of this study was to establish management strategies for the conservation of a new population of *Cygnus cygnus* in the north of Iran by evaluating the genetic diversity of this new site, comparing the levels of genetic variability within this population and its genetic differentiation with other populations of this species.

## Materials and Methods

### DNA extraction, SSR amplification, and PCR

Tissue samples include feathers were collected from two small ponds in Northern Iran (42 individuals from old and 27 from new population: Iran 2) and four countries across the world with corresponding including Finland (10 samples), Sweden (9 samples), Iceland (8 samples) and Poland (10 samples). The locations of the sampled sites, as well as pictures of new habitat of Whooper Swans in northern Iran, are presented in figure 1. Total genomic DNA was isolated from fresh blood using the DNeasy Blood and tissue kit (Qiagen, Hilden, Germany). Six microsatellites markers were chosen based on their level of polymorphism reported in John *et al.* (2006). PCR amplifications were accomplished in 20 µl reactions includes DNA, primers, Master Mix with the Accu Power HotStart PCR Premix kit (Bioneer, Korea). Microsatellite primer sequence and locus information are presented in Table 1. Amplification of the DNA was performed using a Biorad (ICycler) thermocycler with the following parameters: (a) initial denaturation at 95 ºC for 11 min; (b) 38 cycles of denaturation at 95 ºC for 40 sec, primer annealing at the adequate temperature following to in St. *John et al.* (2006 ) for each primer pair for 60 second at 54 ºC; and extension at 72 ºC for 2 min; (c) final

extension at 72 ºC for 10 min. PCR products were electrophoresed on 8% polyacrylamide gels and detected by silver staining.

**Genetic diversity and population structure**

The Micro-checker software was used to detect null alleles at the loci used in this analysis, also GenAlEx 6.501 (Peakall and Smouse 2012) to calculate various measures of genetic diversity including observed and expected heterozygosities (Ho, He, respectively) (Nei 1973), number of alleles (Na), and Nm (the product of the effective population number and rate of migration among populations). Analyses of molecular variance (AMOVA) were carried out in GenAlEx based on $F_{ST}$ model (Peakall and Smouse 2012). Genetic distance based on $F_{ST}$ was calculated between all pairs of populations. In addition, we examined genetic structure using STRUCTURE version 2.3.1 software (Pritchard *et al.*, 2000) with the admixture model, correlated allele frequencies, and a burn-in of 100000 followed by ten trials of $20^5$ Monte Carlo Markov Chain (MCMC) replications. The software Structure Harvester was used to calculate determine the optimal K value (Earl and von Holdt 2011). We computed allelic richness (AR) and private allelic richness (Ap) per population using ADZE (Szpiech *et al.*, 2008).

**Table 1.** Microsatellite primer sequence and locus information.

| Locus | GenBank AC. | Primer sequence (5′→3′) | *T* a (°C) | Allele size range |
|---|---|---|---|---|
| TSP.1.20.2B | DQ453778 | F: TCTCCACTGCCCTGTTCTC<br>R: GTAAGGTCTGCAGTGGCAC | 59 | 228-230 |
| TSP.1.20.5 | DQ453779 | F:GGTTCTTATCTTTTGGTTCTTCC<br>R: CCTGCCTCAATTTCAAGGTT | 59 | 178-191 |
| TSP.1.20.9 | DQ453780 | F:ACCATTCCCCAGCTGAATTT<br>R: CTTCCTGAGTCTGGCTCTGC | 58 | 180-194 |
| TSP.1.20.16 | DQ453782 | F:TCTGCCAAGCATGTTCAAAG<br>R: GGTGAGCGAATTACAAATCCA | 58 | 155-168 |
| TSP.1.20.43 | DQ453787 | F:CAGGAGCTCGAGGCTACAGT<br>R: TGCAGGGAACACTTCTTGTG | 58 | 223-229 |
| TSP.1.20.46 | DQ453788 | F: TGCACTAGCAATAGCATCACG<br>R: TGCGAATTTCAAAAGCTGTG | 58 | 161-173 |

**Results**

**Genetic diversity**

The genetic diversity parameters are shown in Table 2. TSP1202B and TSP12043 primers with 30 and 20 alleles, respectively had the highest and lowest number of the allele. The highest and lowest effective number of alleles was observed in Iran population (11.01) and Poland population (3.52), respectively. Furthermore, the maximum and minimum of Allele richness (an average for all population= 4.21) were observed for Iran (5.6) and Poland (3.44) populations.

**Genetic differentiation**

The results of the analysis of molecular variance (AMOVA) showed that 77 % and 23% of the total diversity belong to within and between populations, respectively (Table 3). The average gene flow (Nm) and F*st* were 0.23 and 0.82, respectively. Maximum genetic distance was observed between Iceland and Poland populations (Table 4). The 114 Whooper Swans from six areas were further studied for population classification using the STRUCTURE program.

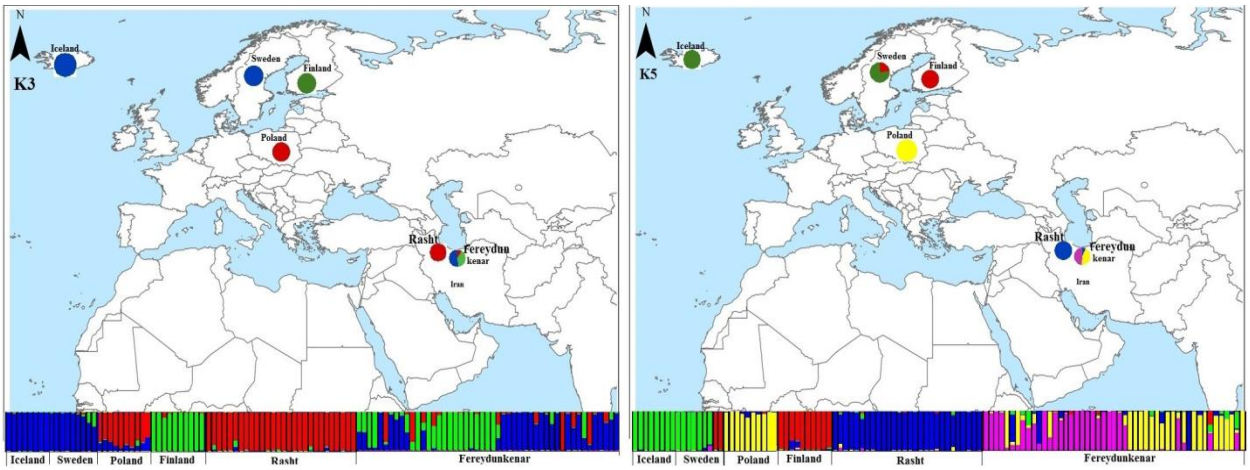


**Fig. 1.** Estimated population genetic structure based on allele frequency variation from 6 microsatellite loci as calculated in STRUCTURE. Each vertical bar represents an individual Whooper Swan partitioned into K genetic clusters (K= 3 and K=5), represented by unique colors. Population names are given below the figure.

A sharp signal was found at K=4, thus we categorized six populations of Whooper Swan into four gene pools (Fig.1). Structure analysis suggested that the Scandinavian populations (Finland, Sweden, and Iceland) were differentiated from the Poland population from both the old and new Iranian populations. Within the Old Iranian (Guilan), two subgroups were present with one showing similarities with the population from Poland. It is likely that the new population of Iran (Feredonkenar) is originated of individuals that migrated from an old Iranian population (Guilan) to this area as well as some individuals from Scandinavia.

The assignment of an individual to a specific genetic cluster was provided by a membership probability of qi (the mean proportion of ancestry). Genotypes with a membership probability lower than 55 % were considered to belong to more than one genetic cluster. All individuals from Scandinavian populations (Finland, Sweden, and Iceland) were comprised of the genetic cluster represented in red. The cluster in green was comprised of individuals from Poland (90%) and more than 39 % of individuals from the new Iranian population. Generally, both populations from Iran have comprised a maximum variety in terms of having different gene pool (Table 5). More precisely, the assignment proportions of each individual to population are reported in fig. 2.

**Table 2.** Genetic variability within six Whooper Swan populations based on SSR markers.

| Pop | Na | Ne | I | Ho | He | uHe | Ar | Apr | FIS | Sample size |
|---|---|---|---|---|---|---|---|---|---|---|
| Iran | 18.833 | 11.014 | 2.615 | 0.560 | 0.904 | 0.918 | 5.6 | 2.8 | 0.380 | 42 |
| Finland | 5.000 | 3.738 | 1.386 | 0.950 | 0.708 | 0.748 | 3.67 | 1.42 | -0.361 | 10 |
| Sweden | 7.833 | 5.870 | 1.819 | 0.919 | 0.794 | 0.842 | 4.74 | 2.18 | -0.170 | 09 |
| Iceland | 4.833 | 4.084 | 1.304 | 1.000 | 0.672 | 0.718 | 3.52 | 1.37 | -0.545 | 08 |
| Poland | 4.333 | 3.527 | 1.245 | 0.872 | 0.661 | 0.710 | 3.44 | 1.27 | -0.384 | 10 |
| Iran2 | 8.167 | 5.585 | 1.716 | 0.749 | 0.756 | 0.797 | 4.33 | 1.28 | -0.025 | 27 |
| Average | 8.167 | 5.637 | 1.681 | 0.842 | 0.749 | 0.789 | 4.21 | 1.72 | -0.184 | |

Na: mean number of observed allele per locus, Ne: effective number of alleles, I: Shannon index, Ho: observed heterozygosity, He: expected heterozygosity, uHe: mean unbiased estimate of expected heterozygosity, Ar: Allelic richness, Apr: private allelic richness, FIS: Fixation index.

**Table 3.** Analyses of molecular variance (AMOVA) for six populations of *Cygnus cignus*). Statistics include sums of squared deviations (SS); mean squared deviations (MS), variance component estimates (Est. Var.), the percentage of the total variance contributed by each component.

| S.O.V. | D.f | SS | MS | Est. Var. | %of Variance | Fst | Nm |
|---|---|---|---|---|---|---|---|
| Between groups | 5 | 185.008 | 37.002 | 1.859 | 23% | | |
| Within groups | 108 | 661.413 | 6.124 | 6.124 | 77% | 0.233* | 0.823 |
| Total Sum | 113 | 846.421 | | 7.983 | 100% | | |

* P-value< 0.05

**Table 4.** Pairwise estimated genetic distance based on F*st* value (Below) and gene flow (Above).

| | Iran | Finland | Sweden | Iceland | Poland | Iran2 |
|---|---|---|---|---|---|---|
| Iran | 0 | 1.238 | 1.487 | 0.959 | 1.596 | 1.103 |
| Finland | 0.087 | 0 | 0.586 | 0.303 | 0.455 | 0.414 |
| Sweden | 0.066 | 0.12 | 0 | 0.469 | 0.581 | 0.508 |
| Iceland | 0.1 | 0.17 | 0.135 | 0 | 0.341 | 0.359 |
| Poland | 0.107 | 0.174 | 0.151 | 0.193 | 0 | 0.657 |
| Iran2 | 0.073 | 0.144 | 0.11 | 0.152 | 0.146 | 0 |

**Table 5.** The proportion membership of each individual of the six populations in each of the 4 gene pools identified by Structure analysis.

| | Red | Green | Yellow | Blue | Admixture | Total |
|---|---|---|---|---|---|---|
| Iran (new) | 6.1 | 2 | 40.8 | 40.8 | 10.2 | 42 |
| Finland | 100 | 0 | 0 | 0 | 0 | 10 |
| Sweden | 100 | 0 | 0 | 0 | 0 | 9 |
| Iceland | 100 | 0 | 0 | 0 | 0 | 8 |
| Poland | 10 | 90 | 0 | 0 | 0 | 10 |
| Iran-(old) | 0 | 39.2 | 35.7 | 25 | 0 | 27 |

## Discussion

This study was performed to assess population genetic structure within and among the often-large populations of Whooper Swan across the world and to clarify the origin of a newly formed wintering population of this species in the North of Iran. One the outstanding result of this research was the higher amount of Ho from He in all populations except two Iranian populations. If heterozygosity is higher than expected, we might suspect an isolate-breaking effect (the mixing of two previously isolated populations). This is strongly confirmed that the population of Iceland, Finland, and Sweden have the same origin. On the other hand, there is a significant difference between observed heterozygosity and expected heterozygosity in the Iranian population where it shows a high rate of inbreeding.

Swans distinct sexual behavior (monogamy) in the population with low gene flow increases population differentiation over time (Oyler-McCance *et al.*, 2007). In our study, the average value of *Fst* was 0.16, which mentioned for a relatively high moderate level of population differentiation. The AMOVA analysis also confirmed this result, which indicated that only 23% of the variation was attributable to differences among populations. High levels of total genetic differentiation were also reported at the population level in Trumpeter Swan using SSR markers. 24.11% of the genetic variation was found within populations, and 75.89% was found among populations (Oyler-McCance *et al.*, 2007).

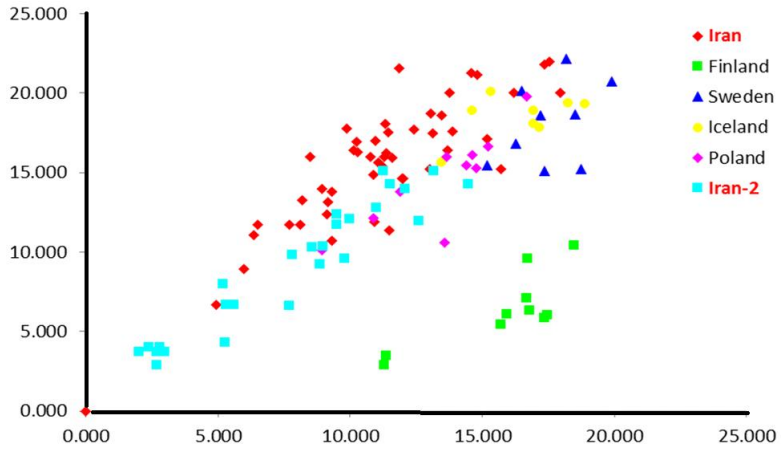


**Fig. 2.** Assignment percentage of individuals based on structure analysis per each population.

Low gene flow could be the cause of relatively high genetic differentiation and may have resulted from a lack of genetic exchange of populations from each other. We suggest that different migration routes and low gene flow may be the foremost evolutionary force that influences the genetic structure in swans, and this is in line with a previous study on Trumpeter swans of the same genus (Oyler-McCance *et al.*, 2007).

The results of the genetic data show the presence of four Whooper Swan groups in the study area. The Finland and Sweden population of Whooper Swan are distinct genetically and show differences from the other populations. The Iceland populations comprised an integrative structure originated from Finland and Sweden populations. The Iceland population of Whooper Swan breeds exclusively in Iceland and winters primarily in Britain and Ireland, with smaller numbers remaining in Iceland and some also migrating as far as the near European continent (Oyler-McCance *et al.*, 2007). The first group comprises the Scandinavian populations (Iceland, Sweden, and Finland) and two populations from Iran as well as the populations from Poland were located in group 2. In the Scandinavian group including population from Sweden and Finland are close together, but the Iceland swans spend their breeding and overwintering in this region. This pattern is in accordance with Dudzik *et al.*, (2015) expressed that Whooper Swan travels a short distance due to the mild climate. Hence, it is more likely that more geographically close populations have more close genetic relationships.

It seems that the Iranian Whooper Swan population (especially new population) revealed a common genetic structure. but the clear differentiation of Iranian populations from the Scandinavian populations is expectable because of their origin from the West and center of Siberia. In addition, the assignment analysis revealed that the relatively high dedicated genetic structure (89%) in Whooper Swan may also be due to very low gene flow.

## Conclusion

The medium to relatively high and significant F*st* values among Whooper swan population revealed that gene flow and connectivity among whopper population especially among Scandinavian groups and Iranian populations is limited. Of course, a short-distance pattern of dispersal (mean value 19.9 km, range 0-46 km) were shown for Whopper population from south of Poland (Dudzik *et al.*, 2012). Individual-based Bayesian clustering approaches also detect highly genetic structure among the study populations. Since Whooper Swan is a migratory bird and has extensive dispersal; this rate of differentiation is surprising for a wetland species that breed in patchy habitats like a swan.

## Acknowledgments

The authors wish to thank Tarbiat Modares University, Ministry of Science and National Science Foundation for their financial support. We also thanks to Kane Brides of the Wildfowl & Wetlands Trust (WWT) who collected

samples dropped by moulting birds at various sites in northern Iceland. We wish to thank Krzysztof Dudzik from Swan Specialist Group (SSG, IUCN) for constructive and useful comments on the manuscript and kindly checked the English editing.

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