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Evidence for introgressive hybridization of wild black-necked pheasant with the exotic ring-necked pheasant during the past 50 years in the Hyrcanian zone, an integrative molecular and morphological approach

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Abstract

Hybridization with non-native-related taxa, especially with introgression, is a major conservation problem, as it may disturb local adaptations, resulting in population decline and biodiversity loss. It is important to identify hybrid individuals, which can be difficult to break up morphologically. Using morphometric and genetic approaches [multivariate analysis, microsatellites, and amplified fragment length polymorphism (AFLP)], this research attempts to classify the interaction and the prevalence of introgressive hybridization in four native pheasant subspecies of *Phasianus colchicus* with non-native *P. c. torquatus*. According to principal component analysis and canonical discriminant function results, hybrid individuals are placed between these two major groups of native and exotic birds. Also, we assessed the population structure, genetic variation, and gene flow between native subspecies and exotic pheasants using 16 microsatellite loci in 129 samples. Our findings revealed that hybridization occurred between two native subspecies, *P. c. talischensis and P. c. persicus*, and exotic subspecies (*P. c. torquatus*). Observation of hybrids in *P. c. persicus* were almost three times higher than *P. c. talischensis*. The levels of the genetic variation within and among the natural populations were investigated using the AFLP markers method to identify hybrids. A total of 202 distinct bands were amplified using four primer combinations, with 179 of them being polymorphic. AMOVA analysis revealed a low degree of genetic differentiation among the populations. The hybrid samples were closely related to the *P. c. talischensis* and *P. c. persicus* populations, according to the UPGMA clusters and discriminant analysis of principal components. It appears that disruption of gene flow between pheasant populations in the Hyrcanian zone is a serious issue. The data from molecular markers prompted us to raise a concern about the genetic integrity in some native subspecies in the Iranian plateau. As a result, a clear cut-level can now be used to differentiate the hybrids, which is also supported by morphological evidence. A direct relationship was found between farmed pheasants and hybrid frequency.

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Farmed individuals should be closely monitored, and non-native taxa should not be released into the wild.

KEYWORDS
amplified fragment length polymorphism, exotic pheasant, Hyrcanian zone, microsatellite, subspecies hybridization

1 | INTRODUCTION

The common pheasant (*Phasianus colchicus* Linnaeus, 1758) has a very large global distribution in the Palearctic and the edge of the oriental region, extending from eastern and southeastern Europe to the Caucasus in the West throughout a large part of China to Japan. It is widespread in China, except the Qiangtang Plateau in Tibet and Hainan Province. Because of the broad distribution, there is a lot of intra-species divergence. (Qu et al., 2009). Having a wide distribution, the common pheasant inhabits such a wide range of climatic and ecological conditions that its lineage diversification and intraspecific divergence can be affected (Liu et al., 2019).

There are 30 recognized subspecies classified into five groups based on morphological characteristics (Barilani et al., 2007; Gill et al., 2017). Based on phylogenetic data, the common pheasant could be divided into eight evolutionary lineages that only corresponds to four groups recognized in the traditional division. Eastern Palearctic groups included: *formosanus* group (four subspecies), *strauchi-vlangalii* group (six subspecies), *torquatus* group (five subspecies), *elegans* group (one subspecies), and *colchicus* group (four subspecies) *mongolicus* group (two subspecies), *principalis-chrysomelas* group (six subspecies), and *tarimensis* group (one subspecies) (Barnett & Larson, 2012).

The Qinghai-Tibetan plateau has been described as a common pheasant hotspot, with expansion to three areas from the originated region: (1) to the southeast, resulting in the *elegans* group that developed independently in the Hengduan Mountains. This is despite the fact that it was historically classified as a *torquatus* subspecies. (2) facing east and forming three distinct groups, namely the *torquatus* subtropics monsoon zone in the east, *strauchi-vlangalii* in arid and semi-arid climate in the west, and *formosanus* groups in Taiwan. (3) to the west, which leads to Central Asian groups (*tarimensis*, *mongolicus*, *principalis-chrysomelas*, and *colchicus* groups). Two colonization routes have been suggested from the Tarim Mountains, based on theoretical considerations. The *principalis-chrysomelas* group has colonized the valleys and foothills of the Kopet Dagh to the south of the Tien Shan highway. The *mongolicus* and *colchicus* groups are found to the north of the Tien Shan Mountains. The *principalis-chrysomela* route was shorter than the *colchicus* route. The discovery of genetically isolated populations of *P. c. persicus*, *P. c. talischensis*, and *P. c. colchicus* on the southern shore of the Caspian Sea resulted from a divergence in the *colchicus* tribe. The relationship between *P. c. persicus* and *P. c. talischensis* is likewise unclear and has not been studied by high-resolution methods (Kayvanfar et al., 2017).

Hybridization is an important general phenomenon in birds and it is on the rise all over the world (Champagnon et al., 2013; Chapuis & Estoup, 2007). Natural hybridization contributes significantly to adaptation and evolutionary diversification, but the first generation may experience infertility or viability issues especially in interspecific hybridization (Ottenburghs et al., 2016a, 2016b). Anthropogenic hybrids, in contrast to natural hybridization, will result in decreased local adaptation, population fitness, and, in the worst scenario, extinction (Nevard et al., 2020). Increased human activities such as habitat modification/fragmentation and bird introductions led to hybridization events with negative consequences. Human interventions become more evident when a rare species interacts with an abundant species (Allendorf et al., 2001). Under an uncontrolled hybridization process, genetic mixing between hybrids and their parents occurs, leading to the eventual transformation of the local population into a hybrid swarm (Todesco et al., 2016). The introduction of different subspecies of the common pheasant into the natural range of the native populations, followed by the loss of genetic purity, has been confirmed, which pose a serious threat to wild subspecies (Braasch et al., 2011).

Although the common pheasant is categorized as least concerned in the IUCN Red List, pheasant populations are decreasing (Ashoori et al., 2018), based on a variety of formal and informal sources, due to such factors as heavy hunting pressure/poaching, deforestation, loss or damage habitat, and the use of pesticides. In addition to the human threat to this species, there is a lack of data on some of its subspecies, especially the four subspecies that live around the Caspian Sea, which have been declared threatened. On the Talisch pheasant (*talischensis*) and the Persian pheasant (*persicus*), there is also no reliable information. The populations of these subspecies were reported to be 900 to 1000 birds in 1996 (Braasch et al., 2011).

In addition to a lack of knowledge on Iranian subspecies, there is a chance that hybridization will occur in some parts of the Hyrcanian ecosystem due to domesticating exotic subspecies, especially *torquatus*, which has become common in the last half-century and thousands of individuals are released. Human disturbance that causes range overlap of historically allopatric species is an increasing hybridization and conservation issue, and it is critical to conserve native biodiversity to maintain ecological balance (Rice, 2016). Anthropogenic hybridization may have benefits such as genetic or evolutionary rescue, but it also has drawbacks, such as wasted reproductive effort, which may lead to alleles being lost from the native populations or a taxon's extinction (Todesco et al., 2016). Despite the fact that pheasant classification and phylogeographic relationships have been resolved for the most members of Phasianidae (Gay et al., 2007), there is evidence of hybridization

that could drive wild subspecies to extinction. Our study has several goals and tries to fill the void information: (1) use microsatellite and AFLP markers, biometrics, and morphometric data to determine the existence of hybrids in nature, (2) collecting proof and hybrid specimens in nature between non-native and wild subspecies, (3) determining which subspecies is the most affected, and (4) identifying hybrids using a combination of molecular and morphometric methods.

2 | MATERIALS AND METHOD

2.1 | Study area

The study was performed in the northern strip of Iran from west to east, including the Arasbaran region, the Hyrcanian, and the Sarakhs-Dargaz zones:

2.2 | Arasbaran

The Aras River to the north, the Sarab and Tabriz Mountains to the south, the provinces of Ardabil and East Azerbaijan to the east, and the cities of Jolfa and Marand to the west define this area. It has a high biodiversity and an exclusive site where some uncommon species can be found.

2.3 | Hyrcanian zone

This zone is a green stretch of the northern slopes of the Alborz Mountain ranges to the Caspian Sea's southern coasts that extends from Astara in the northwest to the Gorgan area in the northeast of the Iranian plateau. The Alborz Mountains, which lie between the Caspian Sea and the Iranian plateau, have produced a climate that has resulted in a unique vegetation cover. The Hyrcanian evergreen strip stretches between East and West.

2.4 | Sarakhs-Dargaz

This area is located in the northeastern most point of Iran in the western and eastern Palearctic contact area and has a high richness of birds (43% of Iranian birds). The habitat is sparsely covered with Euphrates poplar and willow trees. There is a major distance gap among the pheasant subspecies in Sarakhs (*P. c. principalis*) and Hyrcanian zones (other subspecies), (Figure 1) (Sagheb-Talebi et al., 2014).

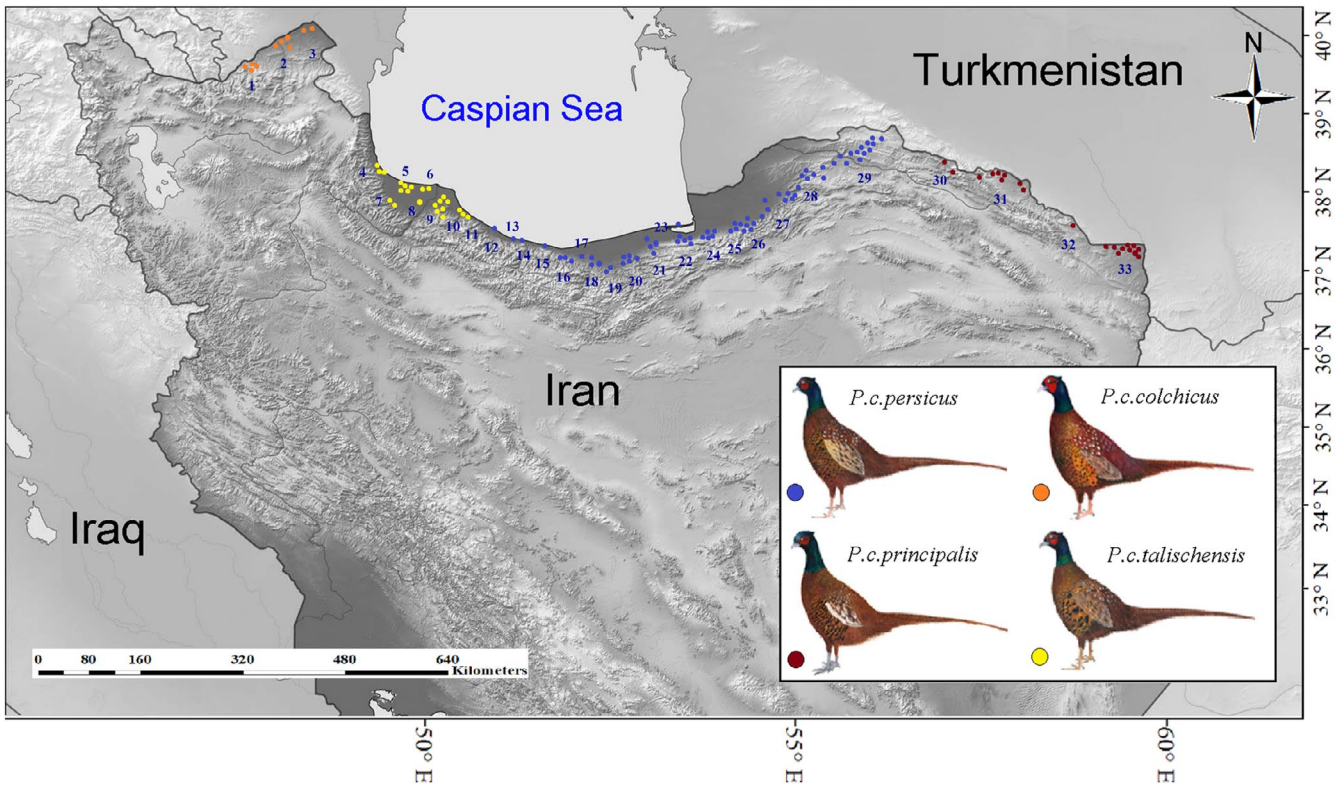

FIGURE 1 Sampling map of native pheasant subspecies in Iran, from West to East: 1—Khalafbaylou 2—Aslandouz 3—Parsabad (*P. c. colchicus*); 4—Talisch 5—Anzali 6—Anbarsar 7—Rezvanshahr 8—Lahijan 9—Siahkal 10—Oosa 11—Darvaz (*P. c. talischensis*); 12—Ramsar 13—Tonekabon 14—Chalus 15—Nowshahr 16—Nour 17—Amol 18—Babol 19—Band—e—pey 20—Savadkouh 21—Kiasar 22—Behshahr 23—Miankaleh 24—Galouhah 25—Kourdkoy 26—Gorgan 27—Minoodasht 28—Golestan National Park 29—Maravetapeh (*P. c. persicus*) 30—Raz—va—Jargalan 31—Dargaz 32—Lotfabad 33—Sarakhs (P. c. perincipalis)

2.5 | Sampling and DNA extraction

In total, 129 samples were collected, including tissue from taxidermized and dead individuals, and feather samples from illegal hunters under official correspondence licensed (Table S1). Genomic DNA was extracted using the standard phenol/chloroform protocol. A liquid-liquid extraction is a method that separates mixtures of molecules based on the differential solubilities of the individual molecules in two different immiscible liquids (Barnett & Larson, 2012). Further, to confirm the quality of the DNA, 0.8% agarose gel invitrogen was used. Gel pictures were taken in Gel Documentation CCD for quality checking of the DNA. After quantification with a Thermo Scientific NanoDrop™ 2000c spectrophotometers, T10 E1 buffer was used to dilute the DNA to a working concentration appropriate for AFLP and microsatellite analysis (Figure 2).

2.6 | Microsatellite and AFLP analysis

A total of 16 microsatellite primers were used, the accession numbers and sequences of which are given in Table 1. The polymerase chain reaction (PCR) amplification was carried out in a total volume of 15 μl using 1 μl DNA (approximately 50 ng), reagents 7.5 μl master mix (red2x, Takara), 1 μl of each primer, and 5 μl ddH2O. Temperature profiles for PCR (amplicon, Denmark) consisted of an initial denaturing of 5 min at 95°C. Then, the samples were cycled 35 times through the following steps: denaturing for 20 s at 95°C, annealing for 30 s in different temperature (Table 1), elongation for 20 s at 72°C, with a final extension of 5 min at 72°C. PCR products were denatured and visualized on an 8% denaturing polyacrylamide gel. Using FreeNA, the expectation-maximization algorithm was used to determine the frequency of null alleles per population and locus (Chapuis & Estoup, 2007). The procedures of the AFLP method were employed as described by Vos et al. (1995) with some modifications. The sequence of AFLP adapters and primers are listed in Table 2. Genomic DNA (500 ng) was digested with 1 μl of EcoRI (Invitrogen) at 37°C for 1 h, followed by second digestion with 1 μl of MseI at 60°C for 2 h. Double-stranded adapters were ligated to the restriction fragments, following the addition of a 5 pmol EcoRI adapter, a 50 pmol MseI adapter, 1 μl ATP and 1 μl of T4 DNA ligase at 22°C for 1 h. Pre-amplification was carried out in a PCR machine where adapters were used as primer. Only amplifying the DNA restriction fragments that had been ligated to adapters on both ends allowed for a first collection of fragments. Pre-amplified fragments were preselected using 0.5 μl each of EcoRI primer and MseI primer with a single selective nucleotide and then reaction mixtures were diluted 10-fold with 10 mM Tris–HCl (pH 8.0) and 0.1 mM EDTA and stored at −20°C. In order to restrict the level of polymorphism and to label the DNA, selective amplifications were performed using 1 μl of each of EcoRI primer and MseI primer with three selective nucleotides. Selective amplification was done using as template in reactions containing 10 μl PCR buffer, 0.5 μl Mse-+3 oligonucleotide, and 0.5 μl UFL in a total volume of 20 μl. Amplification was performed with an initial denaturation of 5 min at 95°C, then 30 cycles with the following cycle profile: 20 s at 94°C, 50 s at 58°C, and 2 min at 72°C, followed by 10 min at 72°C to avoid split peaks.

For capillary gel electrophoresis and data scoring, after selective amplification, 2 μl of PCR product was mixed with loading buffer (3 μl Hi-Di formamide and 1 μl GenScan 500 LIZ molecular weight marker). Samples were loaded on the automated DNA capillary sequencer ABI, 35000. The output file (peaks between 50 and 500 bp) was evaluated in fsa format in Geneious Prime and Peak Scanner™ Software v1.0 (Bin & Manli, 1996).

Population clustering and structure was analyzed with STRUCTURE 2.3.4 (Pritchard et al., 2000). For the whole dataset, the STRUCTURE runs utilized 1,000,000 (250,000 burn-in) MCMC, and simulations were run 10 times for each number of genetic clusters (*k*) ranging from 1 to 10, allowing admixture with five replicates of each run to reach convergence. We applied both likelihood of *K* and Evanno's Δ*K* (Evanno et al., 2005) of successive *K* values to determine the optimal number of clusters, using Structure Harvester (Earl, 2012). Gene flow, AMOVA, PIC, Shannon index, polymorphic percentage (%), and cluster analyses procedures were performed on the binary data (Table S2), using POPGENE (Gay et al., 2007).

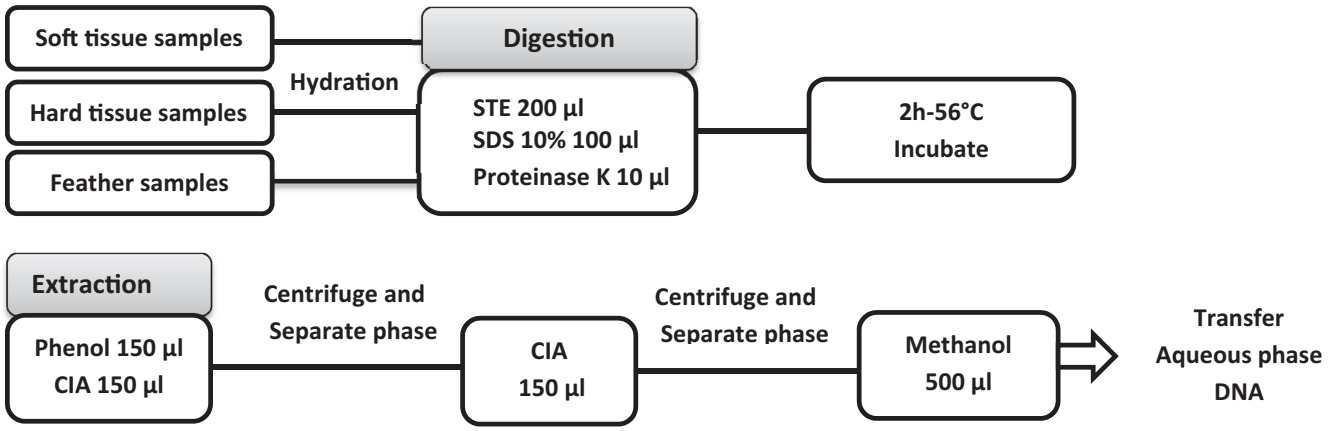

FIGURE 2 Flowchart of major steps for genomic DNA extraction method

TABLE 1 Characteristics of 16 primer pairs amplifying microsatellite DNA loci in common pheasant (*Phasianus colchicus*)

Primer name	Sequence (5′–3′)	Annealing temperature (°C)	Range band	Dye labelled
PC4-F[a]	F:**CAGTCGGGCGTCA** TTCCAAAAGCATATCCCAGAGC R:**GTTTCTT** TAAGATAGCCCATCCTTTGGGG	58	87–94	FAM
PC3[a]	F:**CAGTCGGGCGTCA**GAGGGTAGAGAGAAACAGGTGTTGA R:**GTTTCTT** GAGGTAATCTCTCACTGCTGATTGG	57	152–167	
PC8[a]	F:**CAGTCGGGCGTCA** GACCTCTGTCATTGGTTTTGGA R:**GTTTCTT** TATGATTGTGAACAGCTGCCAA	56	180–202	
PC2[a]	F:**CAGTCGGGCGTCA** AAAAAGCTCATTTGCTGTGGAA R:**GTTTCTT** TCTTTGTCTTCACCCTCATGGA	56	227–240	
MCW151[b]	F:**CAGTCGGGCGTCA** CATGCTGTGATACTACAATTCC R:**GTTTCTT** AACATCCTGGAGTTTGGGAAG	50	251–269	
MCW36[b]	F:**CAGTCGGGCGTCA** CCTCATGTGAAGCATCTTTTCATA R:**GTTTCTT** TGTCTTCAGTAGGACTGTGATAC	52	167–171	
PC1[a]	F:**CAGTCGGGCGTCA** AGCACATCACAGTGCTTTGAGC R:**GTTTCTT** TTTGCTCAGGAAAAGAAAATAAAGACA	58	201–205	HEX
PC10[a]	F:**CAGTCGGGCGTCA** GCTGCAAATCTCCTTAGCTCCA R:**GTTTCTT** GGAGCAACAGTGGGAGAAGAAA	58	207–242	
MCW97[b]	F:**CAGTCGGGCGTCA** GGAGAGCATCTGCCTTCCTAG R:**GTTTCTT** TGGTCTTCCAGTCTATGGTAG	50	266–292	
PC6[a]	F:**CAGTCGGGCGTCA** ACGGTCAGTAAGCATGTACCCC R:**GTTTCTT** AGCAGTCAATGGAGAGCAGGTT	57	84–101	
MCW55[b]	F:**CAGTCGGGCGTCA** TTTGTAGTTACCTGGTACTGA R:**GTTTCTT** GTTTGCATTGTCTACAGCTCCTTG	51	171–173	
MCW30[a]	F:**CAGTCGGGCGTCA** AGAGTG1TGTGTCAGTAAGAC R:**GTTTCTT**TTTGCTATCATAGCTGGAAGAGCT	50	194–198	
MCW127[a]	F:**CAGTCGGGCGTCA** TGCAATAAGAGAAGGTAAGGTC R:**GTTTCTT** GAGTTCAGCAGGAATGGGATG	50	209–240	TAMRA
PC5[a]	F:**CAGTCGGGCGTCA** TGACCACTACAGTTTCCCATTCTTC R:**GTTTCTT** AGATCTTCAGTAGCTCTTGGAACACA	57	284–286	
PC7[a]	F:**CAGTCGGGCGTCA** GGCTGTCCTTTTAGCTACAGCAG R:**GTTTCTT** CATCATCAAGAAGCATTGCAAAA	57	89–93	
MCW72[b]	F:**CAGTCGGGCGTCA** TAAACTGACTTCACTACTCAGCAT R:**GTTTCTT** AAAGGACATCTAACTTCAAAACAG	51	193–197	

[a]Ref: Wang et al 2017.

[b]Ref: Baraati et al 2001.

The bold text is CAG tail for forward and PIG tail for reverse primers.

TABLE 2 primer combination for AFLP marker

Primer combination
FAM- EcoRI + ACT/MseI + CAC
FAM- EcoRI + ACA/MseI + CAG
FAM- EcoRI + ACA/MseI + CCA
FAM- EcoRI + AAC/MseI + CAG

Abbreviation: AFLP, amplified fragment length polymorphism.

2.7 | Biometry and Statistical analysis

Based on Eck et al., (2012), we chose 13 characteristics to test our hybridization hypothesis: (1) BSK: bill to skull, (2) BF: bill to feathering, (3) BNdist: bill to nostril, (4) Bd: bill depth, at the distal edge of the nostrils, (5) Bp: bill depth, at the proximal edge of the nostrils, (6) Bwd: bill width, at the distal edge of the nostrils, (7) WL: wing length, (8) WtAl: distance between the tip of the longest alula feather and the tip of the wing, (9) dToec: central toe, (10) dClc: length of claws, (11) P9P6: tip of longest primary to sixth primary, (12) P9P5: tip of longest primary to fifth primary, (13) Tal: length of tarsus (Table S3).

2.7.1 | Principal component analysis

Principal component analysis (PCA) was used to look at the overall variance in the data without regard for any particular classification. To evaluate the dignity of three distinct classes, including native subspecies (including four subspecies with no major biometric differences), exotic, and hybrid samples. There were 13 morphological

variables in total. R packages were used to conduct all of the analyses from R core team. We used function "prcomp" from the package "stats" as a part of R (Venables & Ripley, 2013).

2.7.2 | Canonical discriminant function

Biometric variables are separately entered into the discriminant function in this method. The analysis of how the characters in the apparent hybrids differed from the parent species were conducted for each character separately and for a group of continuous characters using a canonical discriminant function (CDF) analysis. Wilks' lambda was used to evaluate which variables contributed more to the different discriminant functions. The smaller Wilks' lambda is (close to 0), the more, the variable contributes to the discriminant function. The corresponding function will describe the group membership well if the *p*-value is less than 0.05 (Queen et al., 2002). We used function "DiscriMiner" package in the R environment (Suggests, Sanchez, & Sanchez, 2012) for CDF.

3 | RESULTS

3.1 | Field observation

The majority of suspected hybrid specimens were found near the Caspian Sea, especially in the south, where there were various patterns of mixing between native black-necked and exotic ringed-necked pheasants. Ring-necked pheasant breeding farms have been practiced in Iran for at least 50 years and hybrid pheasants have also been reported in the wild. Hybrid males with different plumage have been encountered in central parts of the Hyrcanian green belt, but hybrid females are almost indistinguishable from the wild individuals. Hybrid pheasants, on the other hand, seemed to be easier to hunt for as these exhibit a different behavior. Figure 3 depicts the various patterns of male plumage. No specimens with mixed plumage patterns was found in the northeast and northwest, where there were no pheasant farms near the habitat of *principalis* and *colchicus* subspecies.

3.2 | Morphometric analyses

The coefficient of KMO = 0.8 was determined before evaluating the principal components, indicating that the sample size was sufficient, PCA analysis indicated that 55% of the total variance could be explained, of which WL and AtWt had the highest scores. The first three PCs referred to 82% of explained variation, mostly associated with WL, AtWt, and Tal. The native and hybrid samples overlapped in particular, while the exotic group was isolated from them (Figure 4).

Canonical discriminant function analysis, based on 13 morphometric characteristics of 45 individuals, show that DF1 had an eigenvalue of 6.421 (93.8% of variance explained) and DF2 had an eigenvalue of 0.422 (6.2% of variance explained) (Figure 5). Two characters, BF and BP showed the lowest scores for Wilks' lambda and thus contributed most to the discriminant function (Table 3).

According to predicted group membership, 16% of native specimens showed hybrid signs, thus posing a threat to the purity and genetic integrity of this subspecies. Assignment of individuals of the exotic to their original sample by the CDF analysis was 100%, which shows a clear-cut separation. Hybrids, according to discriminant function analyses are specifically placed between native and exotic individuals, though being more close to native (Table 4).

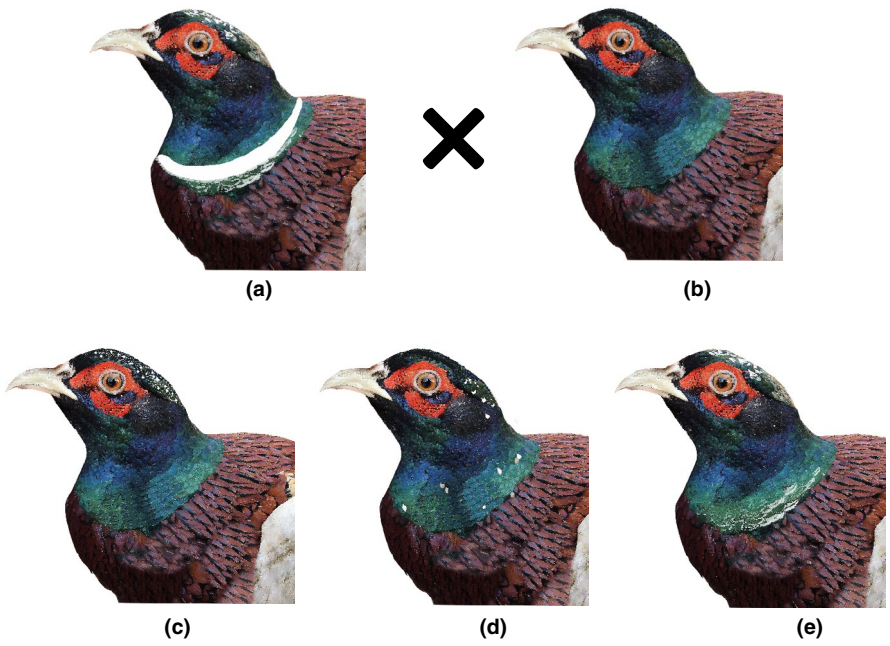

FIGURE 3 Various plumage patterns in male common pheasant (*Phasianus colchicus*). (a) Exotic. (b) Native. (c–e) The unusual pattern in suspected specimens which has no precedent in the past

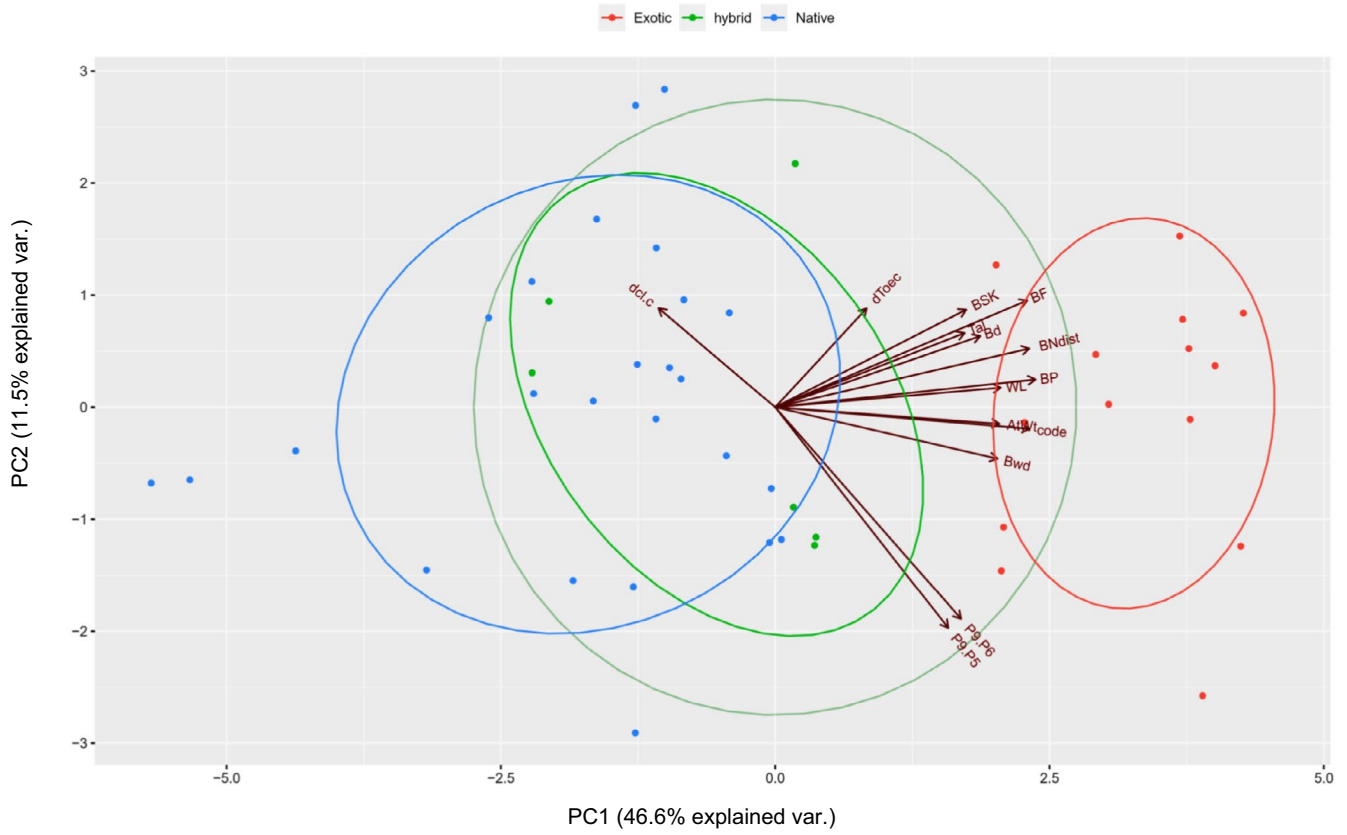

FIGURE 4 Principal component analysis (PCA) and biplots indicated that the first and second components (PC1 and PC2) justified 59% of variations among morphometrical quantitative traits the criteria for native ($n = 25$), exotic ($n = 15$), and hybrid ($n = 6$) specimens. Increasing body size in biometric traits means resembling exotic pheasants

3.3 | Molecular analysis

3.3.1 | Population structure

The study we carried out an analysis with STRUCTURE for various *K* values in order to assign individuals to certain subpopulations and detect the real number of subpopulations (*K*) using a Bayesian algorithm based on individual genotypes (Figure S1). Structure harvester suggested that the *P. colchicus* split into three distinct genetic clusters initially. At $K = 3$, all individuals of *P. c. colchicus* and *P. c. principalis-chrysomelas* are assigned to a common cluster. Three of the subpopulations assign to cluster II that included *P. c. talischensis*, *P. c. torquatus* locations and finally, the *P. c. persicus* subpopulation belonged to the third cluster. In this division, the Structure has not been able to distinguish within *P. c. colchicus* and *P. c. principalis-chrysomelas* and also in cluster II between native and exotic subspecies. At $K = 4$, *P. c. talischensis* and *P. c. torquatus* are separated from each other. In both clusters, individuals showed qi values (qi is threshold for discriminating pure from hybrid individuals, so that identified $0.20<q<0.8$ as the optimal threshold) supporting a possible mixing between clusters I and IV. At $K = 5$, *P. c. colchicus* and *P. c. principalis-chrysomelas* were further divided into two separate clusters (Figure 6). The two groups, eastern and western populations, which are geographically separated from central groups (Figure 1), show the complete assigned individuals percent in classification result (Table 5).

3.3.2 | AFLP profile and polymorphism

Two hundred and two peaks were generated with four FAM-EcoRI/MseI primer combinations carried out to assess 55 individuals; peak size ranged in size from 79 to 499 bp. A maximum of 179 polymorphic fragments was amplified and a minimum of 36 fragments, each fragment was obligated to represent a single locus, among these fragments and the average of PP value (percentage of polymorphism loci) for this species was 55.13% (Table 6). The genetic variance between pheasant groups was evaluated using adegenet's R Package via *k*-means clustering as a confirmation of population hybridization. A strong overlap is observed between the exotic and hybrid groups. Compared to central populations, the western and eastern groups show the least genetic influence. (Figure 7, Note S1) (Jombart, 2008). Electropherograms of AFLP peaks were evaluated with Geneious software (Figure S2).

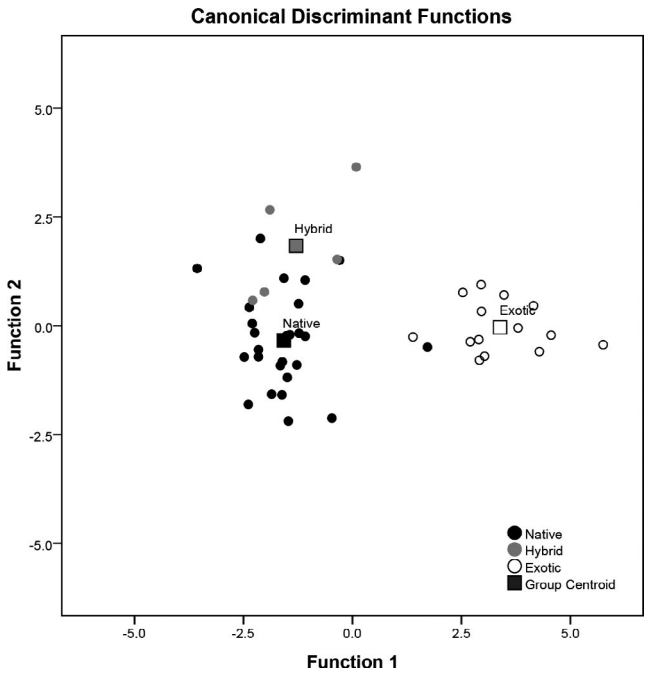

FIGURE 5 Canonical discriminant functions for native ($n = 25$), exotic ($n = 15$) and hybrid ($n = 6$) specimens, factor 1 and 2 variance were 93.8 and 6.2, respectively. In comparison with the exotic individuals, the centroid point of the hybrid pheasant, (number 2) is closer to the native centroid and individuals

TABLE 3 Discriminant test power which shows the Wilks' lambda values

No	Trait	Wilks' lambda	*F* values	*p*_values
1	BSK	0.751419	6.947135	$p < 0.01$
2	BF	0.483132	22.46636	$p < 0.001$
3	BNdist	0.607239	13.58274	$p < 0.001$
4	Bd	0.710640	8.550814	$p < 0.001$
5	BP	0.226470	71.72734	$p < 0.001$
6	Bwd	0.655862	11.01892	$p < 0.001$
7	WL	0.642453	11.68724	$p < 0.001$
8	AtWt	0.661529	10.74464	$p < 0.001$
9	dToec	0.866848	3.225705	$p < 0.01$
10	Tal	0.730486	7.747978	$p < 0.01$
11	dClc	0.722446	7.985521	$p < 0.01$
12	P9.P6	0.743350	7.250493	$p < 0.01$
13	P9.P5	0.782018	5.853600	$p < 0.01$

3.3.3 | Genetic diversity

Genetic diversity varied among populations with the PP values ranging from 17.72% (Pop 2) to 88.61% (Pop 3), with an average of 55.13%. With the assumption of Hardy–Weinberg equilibrium, the average Nei's (1973) gene diversity (*h*) was estimated to be 0.1153 within populations and 0.1451 at the species level. The observed number of alleles (N_a) and the effective number of alleles (N_e) showed differences in the numerical values, but with similar trend in Nei's gene diversity and Shannon's index. Among the five studied populations, population 2 (subspecies *P. c. colchicus*) exhibited the lowest levels of diversity (PP: 17.72%; h: 0.0419; I: 0.0701; Na: 1.1782; Ne: 1.0583), whereas among the native population, the population 3 (subspecies *P. c. persicus*) showed the highest variability (PP: 88.61%; h: 0.1531; I: 0.267; N_a: 1.8861; N_e: 1.2112) (Table 6a). In general, regardless of population 5 (subspecies *P. c. torquatus*, exotic population), the remaining populations of one (subspecies *P. c. principalis*) and four (subspecies *P. c. talischensis*) exhibited a range of diversity from low to high.

TABLE 4 Classified results of percentage allocation of species to homogeneous groups. The members of the exotic and then the native pheasants show the most allocation to their group respectively

Classification result[a]					
		Predicted group membership			
Factor		Native	Hybrid	Exotic	Total
%	Native	84.0	16.0	0.0	100.0
	Hybrid	33.3	50.0	16.7	100.0
	Exotic	0.0	0.0	100.0	100.0

[a]84.4% of original grouped cases correctly classified.

3.3.4 | Genetic structure

The coefficient of genetic differentiation among populations (G_{st}) was 0.1756, indicating only 17.56% genetic diversity among populations and 82.44% within populations. The level of the gene flow was 2.347 migrants per generation (Table 6b). AMOVA analysis further revealed a relatively low, but significant level of genetic differentiation ($p < 0.001$) among the five populations: 16% of the total variation was attributed to interpopulation variance and 84% to within populations (Tables 7 and 8).

3.3.5 | Cluster analysis

Nei's genetic distances (*D*) between population pairs ranged from 0.0023 (*principalis-colchicus*) to 0.0609 (*colchicus-torquatus*) ($p < 0.01$). Similarly, the genetic identity (IN) between population pairs varied from 0.9409 (*colchicus-torquatus*) to 0.9977 (*principalis-colchicus*) (Table 8, Figure 8). An UPGMA dendrogram based on Nei's genetic distance matrix revealed four distinct clusters. An UPGMA cluster analysis of 55 individuals indicated that the hybrids with exotic subspecies, were closely related to the *P. c. talischensis* and *P. c. persicus* populations. These two local populations are also geographically closest to the farm released exotic pheasant (Figure 9). This is one of the evidences that the exotic population is close to the two populations of *principalis* and *persicus*.

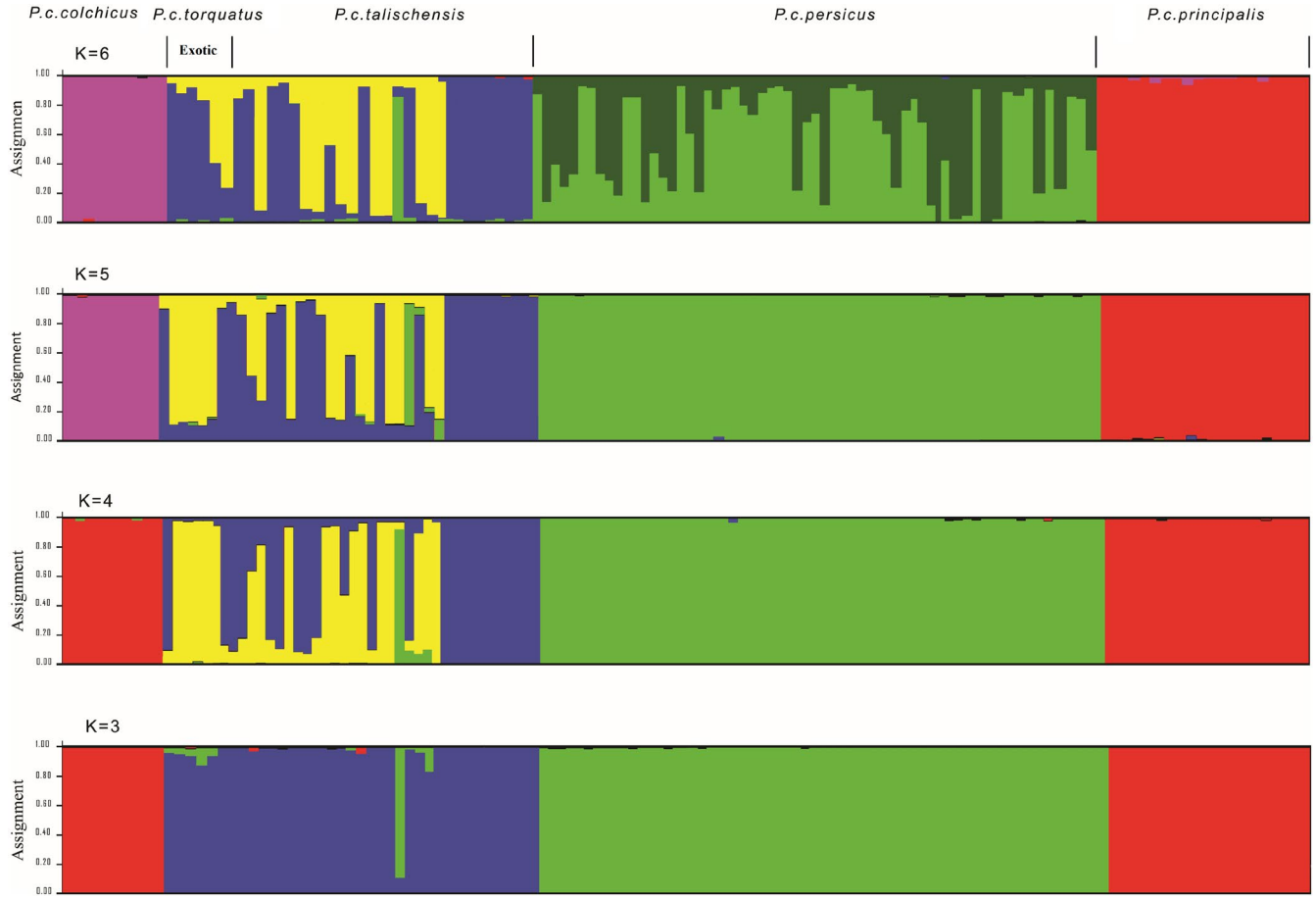

FIGURE 6 Microsatellite analysis for *Phasianus colchicus* subpopulations ($K = 3, 4, 5, 6$) using STRUCTURE program

TABLE 5 Proportion of membership (q) of each predefined group in each of two inferred clusters performed using STRUCTURE (admixture model without using the available previous population information)

Subspecies	*N*	Cluster					Unassigned individual%	Assigned individual %	Insider individual %
		I	II	III	IV	V			
P. c. colchicus	10	10	0	0	0	0	0	100	100
P. c. talischensis	23	0	10	1	0	10	0.09	91.30	43.48
P. c. persicus	70	0	1	63	0	0	0.04	94.29	90.00
P. c. principalis	20	0	0	0	20	0	0	100	100
P. c. torquatus	6	0	1	0	0	5	0	100	83.33

4 | DISCUSSION

Hybridization is a major conservation concern for many species. Genetic introgression between wild and domestic relatives is becoming more widely recognized as a major threat to biodiversity, as it can cause the loss of pure native genotypes and a reduction in global biodiversity (Steeves et al., 2010). Hybridization with domestic birds has already been reported for some birds, like native partridges, and wild ducks species (Chazara et al., 2010). Introduction of exotic species and/or artificial hybrids may lead to loss of local adaptations or fitness and decline in hybridizing native populations (Negri et al., 2013). There is considerable evidence that exotic pheasant subspecies hybridize with wild subspecies in the Hyrcanian central green belt of the southern Caspian basin. Almost all hybrid specimens were found in *persicus* or *talischensis* subspecies habitats, areas where there were several traditional and non-mechanized pheasant farms in recent years. More than a thousand ring-necked pheasant farms have been operating in Iran since the first exotic pheasants arrived at least 50 years ago that always release a number of pheasants into the wild accidentally.

TABLE 6 Genetic parameters for the five *Phasianus colchicus* subspecies based on AFLP data

(a)							
Population	**Subspecies**	**NPL**	**PP (%)**	N_a	N_e	*h*	*I*
Pop1	*P. c. principalis*	67	33.17	1.3317	1.062	0.0513	0.0943
Pop2	*P. c. colchicus*	36	17.72	1.1782	1.0583	0.0419	0.0701
Pop3	*P. c. persicus*	179	88.61	1.8861	1.2112	0.1531	0.2670
Pop4	*P. c. talischensis*	152	75.25	1.7525	1.1810	0.1340	0.2344
Pop5	*P. c. torquatus*	123	60.89	1.6089	1.3206	0.1966	0.3013
Average	—	111.4	55.128	1.5514	1.6666	0.1153	0.1934

(b)	
Statistic	**Calculated value**
H_t	0.1400
H_s	0.1154
G_{st}	0.1756
N_m	2.347

Abbreviation: NPL, number of polymorphic loci; PP, the percentage of polymorphic loci; N_a, Observed number of alleles; N_e, Effective number of alleles [Kimura and Crow (1964)]; h, Nei's (1973) gene diversity; I, Shannon's Information index [Lewontin (1972)]; H_t, Total genetic diversity; H_S, Genetic diversity within subpop; G_{st}, Gene differentiation coefficient; N_m, Gene flow.

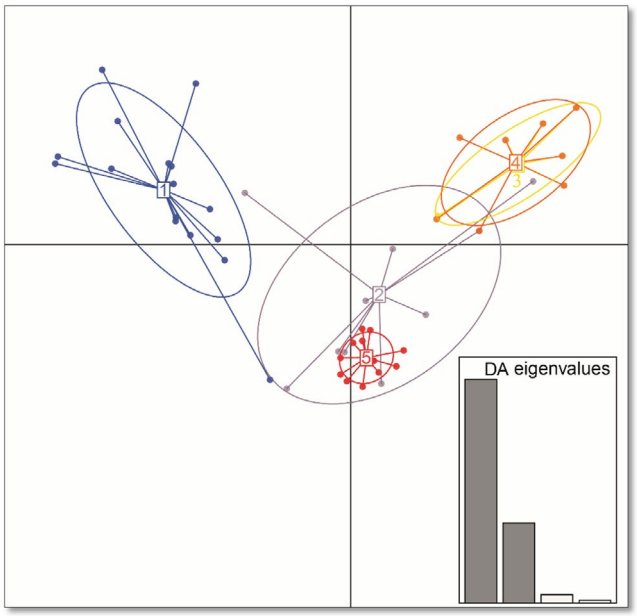

FIGURE 7 Genetic variation between pheasant groups was assessed using *k*-means clustering in r-cran-adegenet package. Each scatter plot depicts the DAPC's two initial principal component discriminant analysis. The following are the number of genetic clusters or geographical groups: (1) *P. c. persicus*, (2) *P. c. taleschensis*, (3) hybrid, (4) exotic, (5) *P. c. colchicus*, and *P. c. principalis* (Arasbaran and Sarakhs-Dargaz). Numbers 3 and 4 showed high overlap between *P. c. talischensis* and exotic or hybrid groups. In addition, similar to the structural results, AFLP evidence shows the influence of hybrid and exotic sign in the *P. c. taleschensis* group

4.1 | Morph evidence

At first glance, individuals hybridized with exotic subspecies, appear to be similar to the native in the field, because of the very narrow white ring and low difference in body size. Of the 13 characters checked, hybrids were significantly smaller than exotics in eight characters 1–10. The univariate statistics showed that these traits of hybrids are in the intermediate state. The remaining traits covered the diversity of individuals within each subspecies; hence, they played no role in segregation among the main groups. The acquired biplot diagram in multivariate analysis also shows that increasing the size of most of the major biometric characteristics, such as wing length, separates the two parental subspecies. The hybrid ellipse is very close to the native ellipse in the elliptical space between the three groups of native, exotic, and hybrid, though it tends toward the exotic ellipse. As a result, PCA confirms the hybrid group's intermediate state.

Canonical discriminant function analysis revealed a clear-cut separation between native and exotic groups, and hybrid was positioned intermediately between these two. In hybrids, the native value was 33.3% of the 50% allocation to the non-native group, which was twice the exotic value.

4.2 | Molecular evidence

We also used 16 microsatellite loci to estimate population genetic structure and see if any gene flow footprints could be found in exotic and native populations. Due to the distance and geographical

TABLE 7 Analysis of molecular variance (AMOVA) for AFLP data of 55 individuals in five populations of *Phasianus colchicus*

Source	df	SS	MS	Est. Var.	%	*p* value
Among pops	4	243.881	60.970	3.936	16	<.001
Within pops	50	1053.574	21.071	21.071	84	<.001
Total	54	1297.455		25.008	100	<.001

TABLE 8 Nei's genetic identity (IN) (above diagonal) and genetic distance (D) (below diagonal) among *Phasianus colchicus* populations

Pop ID	Subspecies	1	2	3	4	5
Pop1	*P. c. principalis*	****	0.9977	0.0609	0.9922	0.9421
Pop2	*P. c. colchicus*	0.0023	****	0.9861	0.9908	0.9409
Pop3	*P. c. persicus*	0.0145	0.0140	****	0.9876	0.9500
Pop4	*P. c. talischensis*	0.0078	0.0093	0.0125	****	0.9652
Pop5	*P. c. torquatus*	0.0597	0.0609	0.0513	0.0354	****

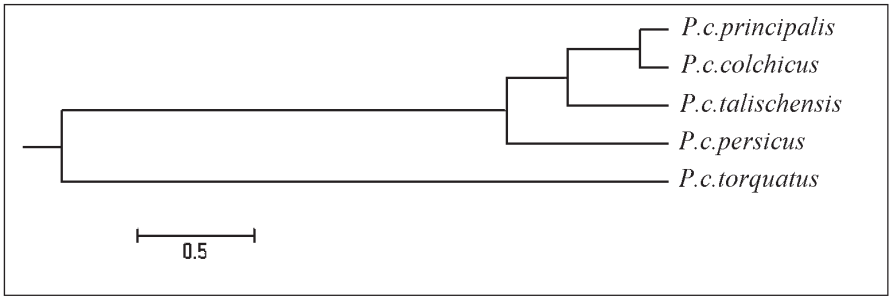

FIGURE 8 Dendrogram-based Nei's (1972) Genetic distance: Method = UPGMA

barriers between these regions and exotic pheasant farms, microsatellite markers agreed to exclude western and eastern subpopulations (*P. c. colchicus* and *P. c. principalis*) from hybridization with exotic (*P. c. torquatus*) pheasant. STRUCTURE analysis, on the other hand, detected hybridization signs in the west population of *persicus*, *talischensis*, and *torquatus* (exotic) populations (0.2 < qi <0.8). We found hybridization frequencies of 15% for microsatellite markers in these cases, which could be due to the deliberate or inadvertently release of exotic-farmed pheasants in nearby areas. In hybridization hotspots, however, only 4% of hybrids were visually identifiable based on field observations. The importance of using codominant markers in conjunction with morphometric traits in identifying interspecific hybridization events and gene flow between native and exotic birds is highlighted by our findings. However, the possibility of Bayesian statistical analyses of multilocus genotypes, particularly when a limited number of markers is used, is difficult to define, especially when hybrid samples or admixed populations are used (Chen et al., 2016). Although no evidence of two subspecies (*P. c. principalis* and *P. c. colchicus*) (Champagnon et al., 2013) with exotic pheasants was found between the eastern and western subspecies due to the lack of pheasant farms, it is reasonable to assume that such capability exists.

In applying AFLP marker, the mean final genetic diversity (Champagnon et al., 2013) was estimated to be 0.14, the mean index of gene diversity within subpopulations (Ottenburghs et al., 2016a, 2016b) was calculated to be 0.1154. The amount of gene diversity in the final population is the sum of gene diversity within subpopulations and gene diversity between subpopulations (Huang & He, 2010). The results showed that the amount of gene diversity in the final population is almost equal to the average gene diversity within the subpopulations and the amount of gene diversity between the subpopulations is close to zero. This shows that there are many similarities between the subpopulations of this study.

4.3 | Integrative approach

Due to the fact that in a part of the distribution area of native Iranian pheasants, the release of exotic subspecies has occurred and also the possibility of moving and breeding this non-native subspecies with native subspecies is obvious and expected, hybridization is an expected phenomenon. Besides, genetic similarity coefficients between the five subspecies ranged from 0.9409 to 0.9977 indicating a small genetic distance between these subspecies. According to the Jaccard similarity coefficients and both dendrograms, *colchicus* subspecies had a greater genetic similarity with *principalis* than other subspecies, indicating that genetic similarity does not have to be linked to geographical distance. On the other hand, *talischensis* subspecies had a higher genetic

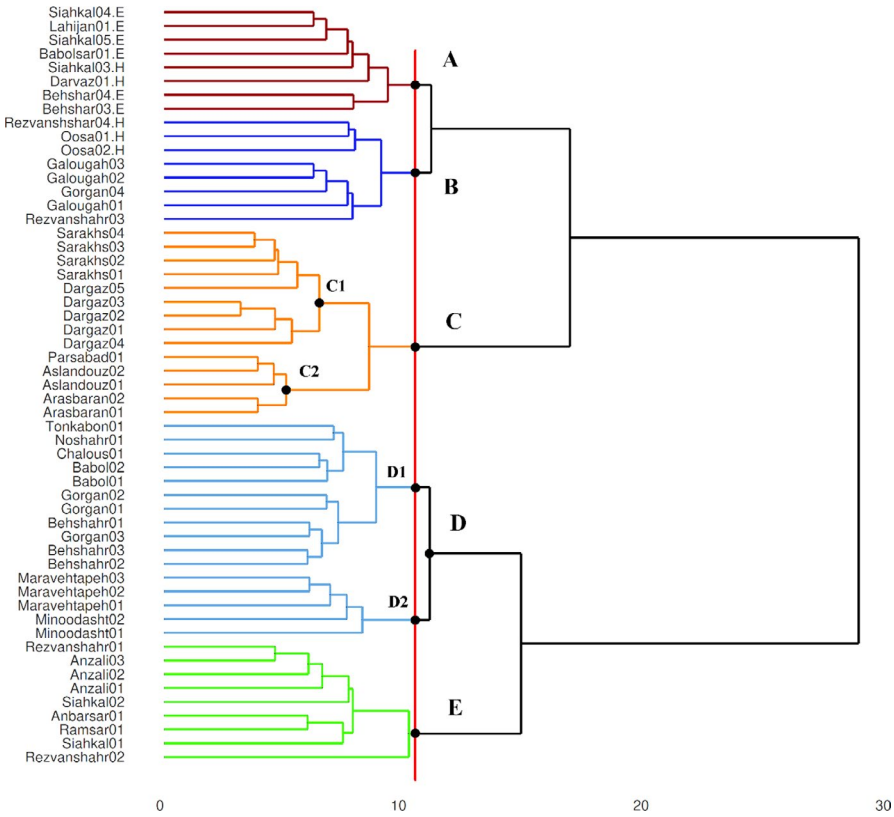

FIGURE 9 UPGMA tree for AFLP dataset based on between-group average linkage. Clades description is (a) all pure exotic "E" (*P. c. torquatus*) and two artificial hybrids "H", *torquatus* × *persicus*, (b) hybrids of reproduction in nature, (c) *P. c. principalis* (C1: Northern and Razavi Khorasan), and *P. c. colchicus* (C2: Azerbayjan), (d) *P. c. persicus* (D1: mostly Mazandaran and D2: Golestan), (e) *P. c. talischensis* Guilan province. Majority of hybrid specimens are found in *persicus* habitat and partially in *talischensis*. Just 3.6% incorrect placement due to pure subspecies

distance with exotic subspecies. The presence of hybrids in individuals with unusual signs, plumage and intermediate traits was confirmed in the *talischensis* and *persicus* subpopulations, as shown by the UPGMA dendrogram, PCA and CDF scatter plot. This is the first evidence of the existence of such hybrids of so closed pheasant subspecies in the north of Iran by morphometric, AFLP, and microsatellite marker techniques. Interestingly, the two eastern and western subspecies have a higher potential for hybridization because of genetic similarity, but they do not meet the exotic pheasants.

Many hybrid pheasants are also difficult to identify phenotypically. Almost all hybrid genotypes were discovered in high-stress exotic hybridization areas.

5 | CONCLUSION

The *persicus* subspecies with high mixing rate with exotics, which has a vast habitat range in Mazandaran province, is found near the majority of *torquatus* pheasant farms. In some areas, the hybridization phenotype rate was higher than 5%. In addition, the history of farms in this area is longer than other sites. On the other hand, hybrid specimens have not yet been recorded at both the eastern and western ends of the distribution range of native pheasant due to lack of such farms.

In this study, we demonstrate that pheasants with hybrid plumage are really hybrids, and that birds with mentioned plumage are most likely the result of backcrossing and/or hybrid mating. It is worth noting that the principal reasons for hybridization with domestic birds are: (1) Releasing domesticated animals in large numbers raises the likelihood of wild–domesticated hybridization and even backcrossing into their wild parental population, (2) Birds unintentionally fleeing from small and conventional farms established in large numbers near the natural habitats, (3) Pattern of introgression may be another result of artificial selection for domesticated populations, (4) Performing a voluntary release to preserve biodiversity or for humanity reasons, such as making a pledge to release an animal from captivity. Continuous and abundant release of domesticated animals raises the probability of hybridization, possibly resulting in wild population genetic swamping (Champagnon et al., 2013; Guo et al., 2020). This evidence enhances the need for a control plan for genetic purity of pheasant.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Table S1. List of all specimens in this article, including molecular and biometrics samples with voucher code.

Table S2. Input data for AFLP in binary format.

Table S3. (a) Biometric data for pheasants in 13 traits, (b) Mean and SD of each parameter for native, hybrid and toquatus.

Note S1. Web address contains three files for running "adegenet" R package for AFLP data.

Figure S1. ΔK graph based on STRUCTURE harvester regarding (a), mean of estimation Ln probability of data (b).

Figure S2. Geneious electropherograms of AFLP peaks.

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