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Identification of intra- and inter-specific variations in Passiformes of Kabul province using *COX1* mitochondrial gene

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Abstract
The objective of this study was to assess interspecific and intraspecific variation of Afghan passiformes birds by extracting DNA from the *COX1* mitochondrial gene. In the case of this family of birds, it has been thought that is a single group of mono-descendent origin. However, new studies have shown that many passiformes families are not common in traditional classifications, and a more complete understanding of the phylogeny of passiformes can only be achieved by extensive molecular studies. Sampling were done from ten (stations during three periods in 2015 and 2016 in Kabul province included Deh sabz, Bagrami, Sarobi, khak-i-Jabbar, Shakar-daraeh, Gol Dareh, Paghman, Char- asiabe, Road Andarabi and Kafroshi). 190 samples were collected using Miss net and 148 samples were transferred to the laboratory. Of these, 110 specimens of passiformes and 38 non- passiformes samples are in the sample. The hunted birds were related to 13 families, 23 genera and 35 species. The family of Emberizdae with 7 species the most diverse and the family of Passeridae with the highest number of 45 individduals. After morphometry, the specimens were transferred to the laboratory for molecular studies, and DNA extraction and sequencing of the COX1 gene were performed to identify the species and to study the molecular distances. From 7704 pairs of intra-species genetic variations were, variations ranged from zero to six, while from 814700 pairs of inter-species genetic variations, the variation was greater than zero to 25.

Keywords: transferred, variations, greater, genetic

Introduction
Birds with more than 10,000 species form a diverse range of vertebrates in dry and wet lands of the world (Britannica ISLS, 2008). This category of vertebrates with their own flying ability, which made them unique among other vertebrates, are scattered annually in various habitats of Afghanistan. Kabul Province is one of the most important provinces in the country in terms of protecting seasonal migratory birds. The presence of Hindu Kush Pamir Mountains in the province represents great habitat diversity for birds (Arinml, 2014). The province of Kabul has an area of 3128 square kilometers, equivalent to a half percent of the total area of the country (Noristani, 1971)
Kabul province has a dry climate with an average temperature of 40 ° C above zero and 17 ° C below zero and an average rainfall of 100-150 mm per year. Afghanistan's underground water resources are relatively rich (Arianmal, 2014) [2] Kabul weather is a function of latitude and longitude, elevation and mountain range, and ahstance from the sea. With respect to above issues, Kabul has 4 kinds of climates e.g. Mediterranean, temperate-cold, temperate-warm in east, west, north and author of the province, respect whey In Afghanistan, barcoding DNA has not yet been used to identify birds. In the only report that has been made by the researchers on birds, 483 species have been listed (Avibase, 2012) [2] of which 140 species of Sparrow sprouts belong to the province of Kabul. As a result, due to the abundance and diversity of sparrows in Afghanistan, the province of Kabul is a good place to begin the identification of birds based on morphometric and molecular methods.

One of the best ways in which it has been possible to identify and categorize animals, especially birds, quickly and reliably over the past years, is to detect a DN marker (DNA barcoding) using molecular markers of DNA. In this method, the sequences of small patron of the mitochondrial genome (mt DNA) are used as a precise and rapid tool for the identification and classification of species. Based on this method, changes, sequencing, number and sequence of 650 nucleotides of the standard gene are defined. Cytochrome oxidase 1 can indicate unique variations for any species. For this reason, this is called the DNA molecule barcode or identifier. The gene encoding the COXI protein is generally more distinct than other mitochondrial ribosomal genes ate species level and is more suitable for the detection of very close species (Aliabadian *et al*., 2009) [1].
The objective of this study was to investigate in the province with regard to the ability to separate and differentiate the species based on the percentage of intra interspecific and interspecific changes.

Materials and methods
Computation of the molecular distance of intra-specific and inter-specific variations
The genetic distance reflects the level of nucleotide differentiation between the two sequences. The degree of genetic divergence between different species is far greater than the intra-species genetic divergence, but is also called the so-called barcoding chat. An interdisciplinary variation of 10 species is used to identify species at the species level (Hebert *et al*., 2004) [18]. The greater the difference between

the nucleotide difference between the taxon and the anus is that it takes longer to split the taxon (Hall, 2008) [17]. The Kimura's Two Parameters (K2P) model is used by MEGA 4.1 to measure the divergence between individuals, or the same genetic distance. When the genetic distance is small, the best model is to calculate the K2P distance. The average K2P intervals are based on the number of pairings of comparisons for intra-and inter-species variation for all genes. The interspecific and inter-species variation pairs are calculated using the output matrix of the MEGA 4.1 program and compiled with the Convertor program SPD 1.1 converter program written in C (Aliabadian *et al.*, 2009) [1].

Sampled areas

Sampling was carried out in the Kabul province (the capital of Afghanistan) during two consecutive years (2015, and 2016) in seven sampling sites as Shawn in fig 1.6 and seven cities in the province of Kabul, including Baghrami, Sorobi, khaki Jabbar, Shakardareh, Guldareh and Paghman. This sampling included the collection of full and muscle for molecular studies, from the sample The species of this order and a number of other types of orders, a total of about 190 samples. Sampling stations are shown on the map of the province of Kabul with a red mark (Fig. 1).

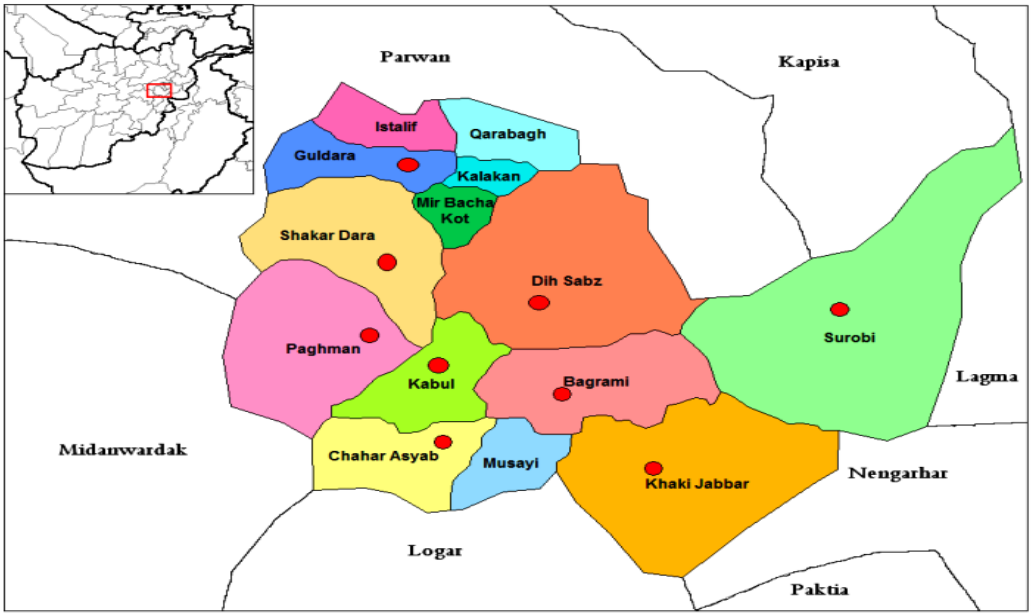

Fig 1: Map of the Kabul Province. The Kabul Central Border, the areas sampled from it them, are marked with red color. During sampling, a number of specimens of different species were found that were not trapped in the sampling net, but their names were given in table (1).

Table 1: Sampling areas with sampling dates and geographical location

Altitude	Latitude	longitude	Sampling date	Sampling number	Sampling area
1917m	-	-	2016	4	Andrabi
1940m	-	-	2016 2016 2016 2015	3 2 15 14	Kafroshi
1748m 1748m 1748m 1748m 1749m	69°14'54.44"E 69°14'54.44"E 69°14'54.44"E 69°14'40.61"E 69°14'40.61"E	34°38'54.06"N 34°38'54.06"N 34°38'54.06"N 34°83'44.72"N 34°83'44.72"N	2015 2015 2016 2016	5 6 35 22 15	Deh sabz
1791m 1791m	69°16'31.61"E 69°16'31.61"E	34°28'63.32"N 34°28'63.32"N	2015 2016	4 1	Bagrami
1001m 1001m 1001m	69°44'48.94"E 69°44'50.53"E 69°44'48.94"E	34°35'18.09"N 34°35'19.42"N 34°35'18.09"N	2015 2016 2016	5 5 5	Serobi
2390m	69°26'00.09 "E	34°20'00.02"N	2016	3	Khakie Jabar
2337m	69°66'51.91"E	34°24'57.55"N	2016	5	Chaar Asiab
2208m	68°58'41.62"E	34°35'05.24"N	2016	20	Paghman
1991m 1991m	69°02'24.70"E 69°02'24.70"E	34°44'41.94"N 34°44'41.94"N	2016 2016	9 4	Guldareh
1994m	69°04'18.87"E	34°41'51.09"N	2015	4	Shakardara

Sampling method

In different habitats, the Misnet was used for catching samples, and by singing the sound of the expected species in each habitat,. After catching the bird (Fig. 2.1.2), it was removed from the net, and photographs were taken from the back, abdomen and lateral surfaces before changing the

feathers. Then separate a few samples (such as those from the primary and secondary master bird) and put in a zipper bag. The sample number, the specifications of the sampling site (name of the location, the geographical location) and the sex of the bird were recorded on the envelope. Then, from the chest and muscle of the bird (Fig. 2.1), the tissue samples were taken into vials containing alcohol, and the vials were numbered.

Sampling equipment

The equipment used in this study were

Misnet (for catching), GPS for geographical location, binocular, camera, alcohol, containing, containers, vials, enveloper, digital caliper, plaster bags, paper, permanent marker

Invisible net (Misnet) for tracking GPS bird to measure longitude and latitude of area 3 Map of the area, binoculars, cameras, containers containing alcohol to store dead specimens, vials containing alcohol, to fix muscle tissue, envelope, to hold the full Digital caliper for measuring morphological traits, anatomical instruments for removing tissue, plastic box or paper for storing vials, padding for measuring wings, markers

Molecular studies

For molecular studies, tissues and bird feathers were used.

(a). Separating and storing tissues for molecular studies

The tissues were kept in alcohol. The tissue size was not too large because the water in the tissue dilutes the alcohols were replaced after a few hours. Different tissues such as muscle, liver and kidney can be used to extract DNA but the molecular tissues were used in this study. The tissue parts were separated from the tissue samples in a completely sterile condition and placed in another vial and the sample number was recorded. The parts are better in size and small in size.

DNA extraction

Necessary materiel for DNA Extraction are:

1. Extraction buffer (µL140)
2. Proteinase k (µL10)
3. Sterile water (µL50)
4. NaCl (µL180)
5. 10% SDS (µL80)
6. Isopropanol (µL420)
7. Ethanol 80% (2 × 250 µl)

DNA extraction steps

10 µl Proteinase K, 80 µl SDS 10%, and 410 µl Extraction Buffer were added to the sample containing vial,and were placed in 37c temperature for 12_244 in a____. After that, the sample were centrifuged at about 13000 rpm for 5 minutes and the supernatant was transferred to a new sterile vial. After adding 180 µl of NaCl to vial, it was again centrifuged for 5 minutes at 13,000 rpm. The supernatant was poured into a new sterile vial and added 420 microliters of cold isopropanol to each vial. After mixing the contents of the vial, they were centrifuged for 5 minutes at 13000 rpm and the supernatant was discarded. The contents of the vial were washed with alcohol in two steps and, at each washing step, 250 µl of 80% ethanol was added to each vial and centrifuged for 5 minutes at about 13000 rpm. In the final wash step, in order to completely eliminate the effect of alcohol, they can be placed in a vacuum or in the absence of a vacuum, the vials were left open and placed in the hood for several hours to evaporate the alcohol. After assuring evaporation of alcohol, 50 µl of distilled water were added and mixed. After that, the extracted DNA can be stored for a long time at -20 ° C.

Quantifying the quality and quantity of DNA

The quantity and quality of extracted DNA are measured using a Nanodrope device or electrophoresis gel. In the measurement with a Nano drop device, the device is calibrated with the solution used for diluting the DNA (here were distilled water), and then the amount of 1 microliter of extracted DNA wase placed on the sensitive surface of the device and the genome was studied quantitvely and quantitvely (Fig. 2)

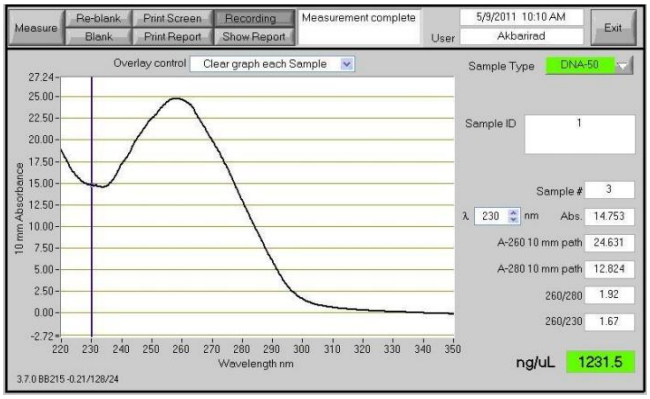

Fig 2: Diagram of DNA Acquiring in Nano drop System.

By Using nanodrop, the amount of DNA present in the cell is expressed in ng / µl, as shown in Figure 2-2, the DNA value in the sample is equal to 2,123 ng / µl. The quality of the extracted DNA is calculated based on the wavelength ratio A-260 / A-280, and it appronate whene the ratio is between. 1 and 2

Agarose gel electrophoresis

An electrophoretic gel can also be used to determine the quantity and quality of DNA. The brightness and thickness of the desired bands show good information about the DNA at the extraction stages as well as in the PCR stage. Electrophoresis at the extraction stage can reflect the state of DNA in terms of degeneration and in the PCR stage it can indicate the proliferation of the desired bond or the formation of dimer dimer on the gel.

Preparation of the gel and its buffer

To make the gel, the following procedure was performed: 0.4 g of agarose was dissolved in 50 ml of TBX buffer and placed on microwave to clarify the gel solution.

TBX Buffer Material

1. TRIS 2. Boric acid 3. EDTA (ethylene diamine tetraacetic acid)

The buffer wase prepared initially 5X and converted to 0.5X when used.

To prepare a TBX 5X buffer, 3.44 g boric acid, 6.75 g Tris and 2.5 ml EDTA were mixed and by adding distilled water was reached to a volume of 125 ml. At the time of use, it was diluted 10 times with distilled water and prepared to 0.5X.

Preparing the gel to put the samples

After the mixture of agarose and TBX buffer was cooled slightly, 5 µl of Green Viewers was added to it, and was poured into a gel plates whose tow ends were previously sealed with gel. After the gel was formed, the shoulders were removed and the gel was placed in an electrophoresis tank containing 0.5 X TBX solutions.

Transfer of sink sump well

After adding 4 µl of leading solution to 2 µl of PCR product or extraction of DNA, the sample was placed inside the gel well, and the DNA ladder was put in the first well. Then, due to the negativity of the DNA charge, the product was placed in the negative pole to the positive pole on a gel at a voltage of 90 volts. The time required for electrophoresis is 20-15 minutes.

See the gel in the gel documenting machine

The documenting is used to see bends on the gel and the gel is examined under UV light. With this device you can take a gel image.

Results

In this chapter, results from the molecular approach were obtained using the gene sequences found in the NCBI and BOLD gene search engines. In this study, 1240 sequences of the gene bank and 86 sequences from 190 samples of birds captured from Kabul province were investigated in a molecular analysis. Finally, the distinction between interspecific and interspecific distances in the study of the phylogenetic relationships of the aves in the province of Kabul was shown in two diagrams. (fig 1 fig 2)

Identification of samples of passeriformes in Kabul province

A total of 190 specimens of passeriformes and non-sparse forests were captured during the study in Kabul province in 2015 and 2016. Based on morphological traits and using valid identification keys from existing samples, 148 samples were selected for morphological and molecular study.

Among 148 samples, 110 specimens were related to other with 13 families, 23 genera and 35 species. Their scientific names based on family presented in table 1.

Table 2: Birds Species Caught in Kabul Province by Separation of Number, Scientific and local Names and Family.

S. No	Scientific name	Family name	Number of Sp.
1	*Lanius vittatus*	Laniidae	1
1	*Lanius schach*	Laniidae	2
1	*Golden oriol*	Oriolidae	3
1	*Corvus monedula*	Corvidae	4
1	*Alauda arvensis*	Alaudidae	5
1	*Eremophila alpestris*	Alaudidae	6
1	*Alauda gulgula*	Alaudidae	7
3	*Calandrella brachydactyla*	Alaudidae	8
2	*Hirundo rustica*	Hirundinidae	9
2	*Phylloscopus inornatus*	Phylloscopidae	10
12	*Sylvia curruca*	Sylviidae	11
1	*Sitta tephronota*	Sittidae	12
1	*Acridotheres tristis*	Sturnidae	13
1	*Temenuchus pagodarum*	Sturnidae	14
2	*Luscinia megarhynchos*	Muscicapidae	15
2	*Luscinia svecica*	Muscicapidae	16
1	*Muscicapa striata*	Muscicapidae	17
12	*Passer domesticus*	Passeridae	18
8	*Passer montanus*	Passeridae	19
3	*Passer hispaniolelsis*	Passeridae	20
2	*Motacilla flava*	Motacillidae	21
2	*Anthus hodgsoni*	Motacillidae	22
2	*Anthus pratensis*	Motacillidae	23
1	*Calandrella brachydactyla*	Alaudidae	24
1	*acutirostris*	Alaudidae	25
2	*Carduelis carduelis*	Fringillidae	26
3	*Pyrrhula pyrrhula*	Fringillidae	27
1	*Serinus pusillus*	Fringillidae	28
12	*Emberiza bruniceps*	Emberizidae	29
2	*Emberiza hortulana*	Emberizidae	30
8	*Emberiza buchanani*	Emberizidae	31
1	*Emberiza leucocephalos*	Emberizidae	32
1	*Emberiza cia*	Emberizidae	33
5	*Emberiza melanocephala*	Emberizidae	34
9	*Emberiza stewarti*	Emberizidae	35

Molecular results of Passeriformes in Kabul province

A total of 1414 sequences were examined. The 148 collected specimens of birds from the province of Kabul, of which 38 were belonging to different non-asbestos orders, 110 belonged to the passeriformes, and other sequences belonging to the passeriformes in Kabul province were taken from the Gene Bank.

Intra-species and interspecific distances of samples from Kabul province

Of the 1281 joint sequences of species downloaded from the gene bank and caught specimens from Kabul province, 22 species were impossible to become red on. Only one sample was a violable but in case of one sample the diagram of intraspecific distance was located ()and diagram of all available inter species distance was also plotted (FIG) As a result, 693 nucleotide pairs were belonging to COX1 gene and 1281 sequences, 7704 intra-species pairs and 814700 inter species pairs of comparisons were. The genetic distance for intra-species variation was from zero to 2.49, and for inter-species variation above 2.49. Finally 7704 pairs of genetic variations within a species from zero to 6, with an average of 1, a maximum of 6, and a Minimum of zero. For six samples, interspecific variations were higher than 2.49 interspecific. caught variations have shown that interspecific for Troglodytes *troglodytes*, *rupestris*, *Sitta neumayer*, *Phylloscopus Schwarz*, *Cinclus pallasii*, *Saxicola torquata* were higher than the threshold, so variations these taxon may give us new information. Of the 814,700 pairs of interspecific variations, the interspecific were 25 to zero, with an average of 16, a maximum of 25, and at Minimam zero. After studying the genetic distances, the species remind between 2 and zero were 26 species, among which 6 species were placed oppositely due to spelling errors and 20 species were my birds as showed in table with opposite spelling errors, and 20 species were hybrid with each other (see Table 3) is shown.

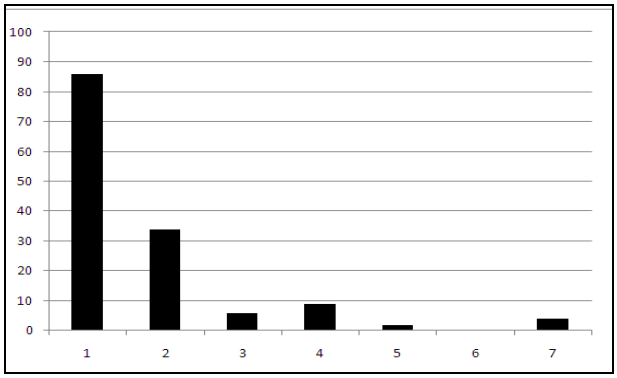

Fig 3: Interspecific genetics distance as diagram of Afghan species in Kabul province; Axis X: Intera genetic distance, Y axis: Intra-species comparison pairs.

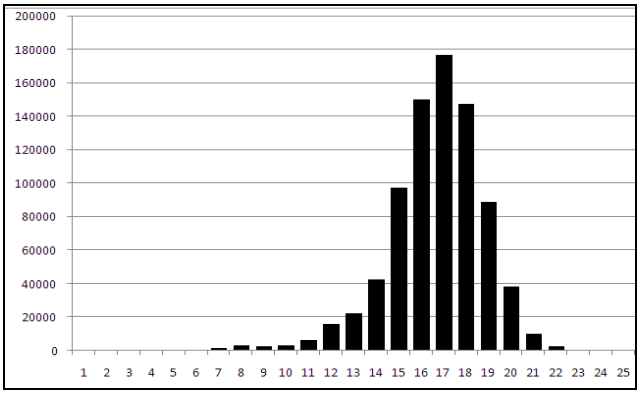

Fig 4: Diagram of interspecific genetic distance of Passeriformes spiny species in Kabul province; Axis X: Interspecific genetic distances, Y axis: Number of interspecific genetic interbreeding of Passeriformes sprouts in Kabul province (Afghanistan).

Discussion and Conclusion

The Taxonomic Situation of the Passeriformes

Passeriformes includes a mono-descendant species that seems to be a successful group of bird and rapidly separated in the late Tertiary period According to the transitional classification based on the recognition birds are divided into the subclasses (Tyranny) and Oscines (*Passer*), they were introduced with *Acanthisittidae's* recent molecular studies as the third chord and the sister group for the two previous clusters. and its molecular traits (Johansson *et al*., 2001).

On the basic of resent molecule studies a canthi's ttidae was introduced was introduced as Third substance and sisten group joheroon at, 2001).

The Oscines group, with more than 4500 species, is the largest group with highest global distribution and probably originated from the Australian Region (Johansson, 2008) [22]. The division of Oscines into tow sisten taxon Sorta by Sibley and Ahlquist in 1990, which was rejected in the recent years, and *Corvida* was introduced as a *paraphilithic* group (Barker *et al*., 2002; Ericson *et al*. 2002).

Sibley and Ahlquist (1990) defined the cluster for *Passerida*, including *Passerioidea* and Sylvioidea, and established Muscicapoidea as a regular group for two other clouds.

In addition to above contradictory phylogenetic hyphothesis has been reported for phylogenetices, sub groups espeacilly with passerida with including *muscicapoidea sylvioidea* and passerida families (Ericoson and johansson, 2003) [15].

Monodescenant (Sibley and Monroe, 1990) [30]. For example, the sequences of the RAGI and RAGII markers showed that *Paramythiamontium*, *Toxorhamphus*, *Oedistoma* and *Melanocharis*, belonging to the Corrvida nucleus in Sibley and Monroe (1990) [30] in the Passeria group (Barker *et al*., 2004) [10]. Also, molecular studies have shown that taxa that are not in the Passerida group may be part of the split. For example, *Culicicapa*, *Chloropsis*, Irena, and some other species that were basically in the scavenger group are in the Passerida group (Barker *et al*., 2004) [10]. The *Pseudopodoces humilis* species previously categorized in Corvini (crows) is associated with recent studies in the Passeria family (James *et al*., 2003) [20]. So far, many molecular studies have been carried out on passeriformes, but the largest number of taxon studied from passeriformes has been studied in research is 173 taxa of passeriformes (Beresford *et al*., 2005) [11]. Typically, most studies have been based on one or more nuclear genes as phylogenetic markers, and the use of several molecular markers has been proven, but so far few studies have used the combination of more than two molecular markers (Moore, 1999) [26].

Intra-spelling variation of COX1 mitochondrial gene in Kabul province's Passeriformes

In this study, 693 nucleotide pairs of COX1 gene were studied. The number of pairs of intra-species comparisons was 7704 and the number of interspecific pair compression was 814700. The result of our study was 7704 pairs of genetic variations within a species from zero to six, with an average of 1, a maximum of 6 and a minimum of zero. Based on studies, done by Ali Abadian *et al.* In 2009 and Koor *et al*. (2012), the maximum variation in intraspecific variation was 2.47. In this study, 6 samples of the distance above the 47.2 were given in the table below.

Table 3: The number of species whose interspecific distance is 4 and 6.

Species with means above 3	Mean	Mean × 100
Troglodytes troglodytes	0.035283	4
Ptyonoprogne rupestris	0.038667	4
Sitta neumayer	0.0622	6
Phylloscopus schwarzi	0.0642	6
Cinclus pallasii	0.064433	6
Saxicola torquata	0.064769	6

These results indicate that taxonomic classification of these species should be given more attention because, due to morphological changes and severe molecular divergence with other subspecies in these species, further studies are necessary. Therefore, more comprehensive studies and Is more complete for the species mentioned, so that in many taxonomic books, so far, the separation of these species in Afghanistan with other subspecies has been discussed and the studies confirm the divergence of these subspecies, but the final decision It will require further and broader further studies.

Cross-Intraspecific variation of COX1 mitochondria gene in Kabul province's Passeriformes

On the other hand, from 814700 pairs of genetic variations of interspecific, values range from zero to 25% with an average of 16. Among the variations between 26 pairs of species, intraspecific in intrinsic and intermediate variations ranged from 2.4 to zero percent, as shown in Table (2). Different groups at the gene bank (NCBI), such *as Delichon urbicum, Phylloscopus trochiloides, Muscicapasibirica, Sitta tephronota, Galerida cristata and Muscicapa striata*, were used. Sixteen pairs of species have intraspesific or interspecific variations that are based on a hybrid source of birds (Mccazhy, 2006) of 16 pairs of species, sister species, and two hybrids in their contact area. These 16 pairs of species are listed in Table (2). Most of these species with their sister species taken from genbanke have, hybrids in Afghanistan or neighboring countries, such as *Motacilla flava with Motacilla cinerea* in Europe, Asia and Russia (Hopkinson, 1926; Jotgensen, 1996) [19]; *Emberiza citricella* hybrid with *Emberiza leucocephalos* found in Asian regions (Jihuetm 2003; Johansen, 1944). *Corvus corone* hybrids with *Corvus cornix*, which have contact areas in Scotland, Ireland and Germany (Figure 5) (Parkin *et al*., 2003) [28].

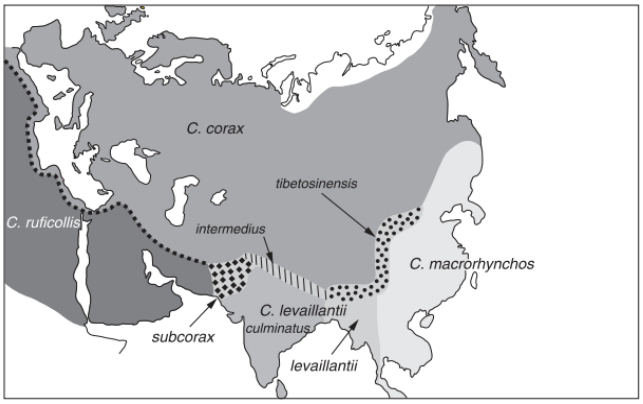

Fig 5: Map the contact areas between *Corvus corone* and *Corvus cornix* species.

Also, hybrids of *Lanius cristatus* with *Lanius excubi* in Russia; *Sitta neumayer* dcotuibuted in European region hybrid with *Sitta tephronota* in Central Asia; *Anthus spinoletta* hybrid with *Anthu spratensis* (Bures *et al*., 2002); Hybrid *Lanius isabellinus* with *Lanius collurio*, whose hybrid is seen in TianShan of Russia is shown in Figure 3 (Panov, 1993) [27]. Hybrid *Lanius bucephalus* with *Lanius cristatus* in the S-region of japan (Takagi, 2000) [32], and hybrid *Lanius bucephalus* with *Lanius excubitor*.

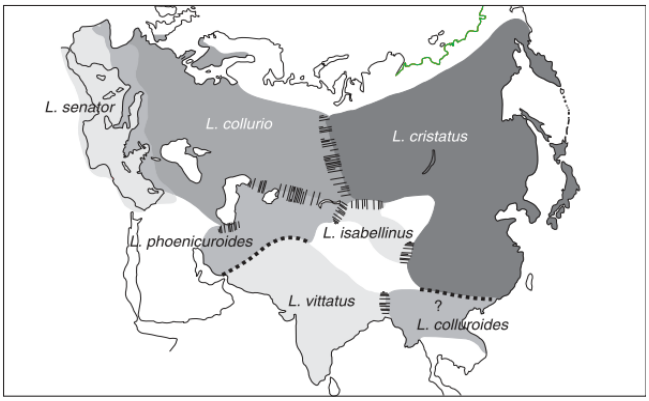

Fig 6: Hybrid in genes the map to lanius.

Of *Motacilla flava* hybrid with *Motacilla citreola*, which has been distributed in Asia and Eastern Europe (Mayud, 1994; Panov, 1993) [27]? Hybrid *Sylvia mystacea* has not been seen with *Sylvia communis, but hybrid Sylvia communis* has been seen with other species in Europe and Asia.

Turdus ruficollis hybrid with Turdus naumanni in Turkey is seen as an area near the Balcal Lake (Clement, 1999) [14].

The *species Sylvia curruca has been hybridized* with other species of the species found in Asia in Kazakhstan. Hybrid *Sylvia curruca with Sylvia communis* is found in Russia, *hybrid Sylvia hortensis with Sylvia crassirostris* seen in Siberia, Russia (Figure 7) (Johnsen, 1999; Shihai *et al*., 2001).

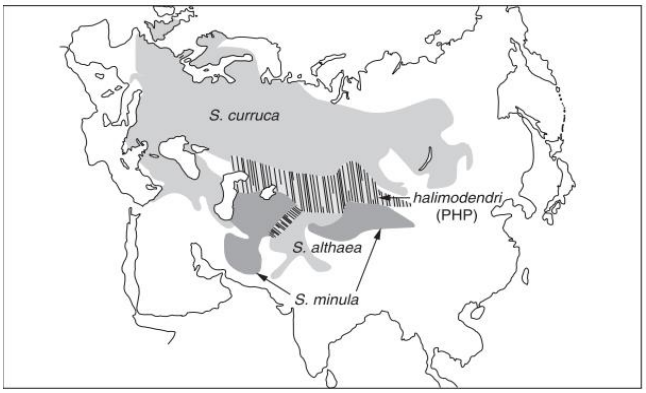

Fig 7: The map of hybridization of sites of *Sylvia* species.

Passer hispaniolensis hybrid with Passer domesticus was found in West Eurasia and North Africa (Jiguet, 2003; Alanso, 1984) [23]. Hybrids of other species such *as Saxicola torquata with Saxicola maura* were found in different hybrid regions of different species (Urquhart, 2002). Hybrid of *Acridotherestristis* has not been reported *with Acridotheres ginginianus* species, but these two species are *individually* hybridized with other species of this genus (Ali and Ripley, 1973) [7].

Since the precision and accuracy of DNA barcoding using molecular distance methods depends on the existence of the

difference between interspecific and intraspecific distances, the best time is that there is no any overlap between intraspecific and interspecific variations and as the amount of this gap is larger, the marker is more powerful in differentiating intraspecific variations.

Previous studies using intra and make species variation to *COX1* gene have showed that a threshold of mean intraspecific variation (10x) variation of individually of new species by using interspecific and inter-species genetic variations for the COX1 gene. In comparison the sites species of 10 orders of 6 birds, in each pair, each person in the neighboring tree was single-stranded, and each had a constant mutational difference that caused the diagnosis and separation of its sister species (Tavares *et al*., 2008) [31].

Table 4: Genetic distance of 1-2-0 species and species on which comparison is based

No	Species with below 3	Species compared to	0.4	1.4	2.4	Total
1	*Galerida crisiata*	*Galerida cristata*	66			66
2	*Galerida cristata*	*Lanius isabellinus*	18			18
3	*Saxicola torquata*	*Saxicola Maura*	10	3		13
4	*Motacilla flava*	*Motacilla cinerea*	4			4
5	*Emberiza leucocephalos*	*Emberiza citronella*	20			20
6	*Corvus corone*	*Corvus cornix*	17	2	3	22
7	*Delichon urbica*	*Delichon urbicum*	22	1		23
8	*Muscicapa sibiric*	*Muscicapa sibirica*	37			37
9	*Muscicapa striat*	*Muscicapa striata*	109	2		111
10	*Sylvia curruca*	*Sylvia communis*	12			12
11	*Sitta tephronota*	*Sitta tephronota*	9	3		12
12	*Sylvia hortensis*	*Sylvia crassirostris*	1			1
13	*Lanius cristatus*	*Lanius excubito*	2			2
14	*Sitta neumayer*	*Sitta tephronota*	2	4		6
15	*Anthus spinoletta*	*Anthus pratensis*	2		4	6
16	*Lanius isabellinus*	*Lanius collurio*	2	1	47	50
17	*Motacilla flava*	*Motacilla citreola*		16	8	24
18	*Sylvia mystacea*	*Sylvia communis*		12		12
19	*Turdus ruficollis*	*Turdus naumanni*		95	9	104
20	*Sylvia curruca*	*Sylvia mystacea*		1		1
21	*Acridotheres tristis*	*Acridotheres ginginianus*			6	6
22	*Sylvia curruca*	*Muscicapa striata*			9	9
23	*Phylloscpus trochilo*	*Phylloscopus trochiloides*			7	7
24	*Lanius bucephalus*	*Lanius cristatus*			16	16

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