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Parallel Evolution in the βA Globin Gene of Black Kite, Bar-Headed Goose and Graylag Goose: Deciphering Hemoglobin Adaptation to High Altitude Hypoxia Using Birds as Models

Hylemariam Mihiretie Mengist[1,2,3], Ahmed Mohammed[2], Yuxi Wang[2],Tengchuan Jin[1,2], Lei Cao[4]

[1]Department of Biochemistry and molecular biology, Hefei National Laboratory for Physical Sciences at Microscale, CAS Key Laboratory of Innate Immunity and Chronic Diseases, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, Anhui, 230001, China
[2]Hefei National Laboratory for Physical Sciences at Microscale, CAS Key Laboratory of innate immunity and chronic disease, School of Basic Medical Sciences, Division of Life Sciences and Medicine, University of Science & Technology of China, Hefei, Anhui, 230027, China
[3]Department of Medical Laboratory Sciences, College of Health Sciences, Debre Markos University, Debre Markos, Ethiopia, P. O. Box 269
[4]State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

*Corresponding Author: Hylemariam Mihiretie Mengist

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Abstract

Parallel evolution involving historically independent amino acid substitutions in hemoglobin (Hb) is expected among closely related lineages; however, such studies in distantly related species are limited especially from the structural point of view. Here we reported structural and phylogenetic based parallel amino acid substitutions in the β^A globin gene of the Black kite (BK), Bar-headed goose (BHG), and graylag goose (GLG) to illustrate the adaptation of hemoglobin to high altitude hypoxia and high altitude sickness in humans and animals living at extreme altitudes. Based on our data, there are five to six parallel amino acid substitutions in the β^A globin gene of BK, BHG, and GLG. Four of the parallel amino acid substitutions (Asn-β^A83-Lys, Gln-β^A90-Lys, Asp-β^A125-Ala, and Ala-β^A128-Ser) are independently evolved only among BK, BHG, and GLG. The individual Ala-β^A128-Ser substitution resulted in a significant closeness of the α1 and β1 subunits which probably stabilizes the R state of oxy-Hb at high altitude. Our data contribute to evolutionary biology by providing pieces of evidence into the parallel evolution of BK, BHG, and GLG to high altitude hypoxia and laid a foundation to studying high altitude associated hemoglobinopathies in humans and other animals.

Keywords: Parallel evolution; β^A globin gene; Black kite; bar-headed goose; graylag goose

1.0 INTRODUCTION

The study of adaptive evolution is becoming ideal as different evolutionary lineages are confronted by similar environmental dissipations. In this regard, comparative phylogenetic study is a very important component of studying the adaptive evolution of different species sharing the same environment. Specifically, the combined study of phylogenetic analysis with structural and functional information is worse useful to identify significant changes and distinguish parallel evolution in different lineages from shared ancestral traits (1-4). According to evolutionary theory, parallel evolution could be common when the number of beneficial mutations is limited by selective constraints on the structure of the protein (5). A

study on the parallel evolution of Andean waterfowl suggested that adaptation to high altitude hypoxia resulted from unique but overlapping sets of a variable number of amino acid substitutions in each lineage (4).

Based on current satellite tracking, six different species of birds (Black kites, Bar-headed geese, Graylag geese, Ruddy shelducks, Demoiselle cranes, and Steppe carvings) are flying at high altitudes around the Himalayas. Although GLG is generally known fly at low altitudes (6), current satellite tracking showed that this bird is flying at high altitudes over a long period during wintering. Also, the black kites captured at different places showed different flying paths i.e. birds captured in western Mongolia cross the Himalayas to the Indian peninsula while those captured in the central part of Mongolia crosses the Hengduan Mountains. But black kites captured at the Eastern part of Russia did not cross the mountains.

Although studying parallel evolution is important to decipher evolutionary history, only a few studies have been conducted in birds. Of these, McCracken et al (4) reported parallel evolution in the major hemoglobin of Andean waterfowl. Besides, Zhu et al (7) also described the divergent and parallel evolution of high altitude passerine birds from Qinghai-Tibet plateau. Even though adaptation to high altitude hypoxia is due to a combination of unique, parallel, and collateral amino acid substitutions (8), convergence increase in Hb-O_2 affinity at high-altitude is reported so far to involve only limited parallelism at the molecular sequence level (4, 7, 8).

Humans are also living at extreme altitudes especially in Ethiopia (around mount Ras Dejen) and in China (around mount Tibet and Andean populations). Ethiopian highlanders are reported to have a comparatively high hemoglobin concentration (9) and hemoglobin from Chinese highlanders exhibited high oxygen affinity (10). It is reported that parallel evolution involving historically independent nucleotide and amino acid substitutions is expected among closely related lineages (11). But studies revealed that the parallel evolution of increased Hb-O_2 affinity in two distantly related highland waterfowl (Andean goose and BHG) involve similar basic mechanisms with different underlying sequence changes (4, 12-15). Therefore, the similarity of flying at high altitudes in Black kite, BHG, and GLG around the Himalayas could be due to parallel evolution in the globin genes. As hemoglobin is a well-studied protein coded by a small number of genes, a comparative analysis of molecular evolution in birds Hb provides insights into exploring the pattern and frequency of parallel adaptive evolution. Additionally, there is a paucity of published data illustrating parallel Hb evolution in birds and humans from the structural point of view. Here we report the structural and phylogenetic basis of parallel amino acid substitutions in the β^A globin gene of the Black kite, BHG, and GLG which provides insights into the parallel evolution of these birds to high altitude hypoxia. Based on the data from GLG, our findings also addressed an interesting question in evolution whether we can perceive evidence for convergent amino acid substitutions in the Hb of birds that spend significant time at high altitudes. Moreover this study laid a foundation to investigate the structural and molecular basis of high altitude hypoxia associated polycythemia and dyspnea in humans.

2.0 MATERIALS AND METHODS

Specimen collection, nucleic acid extraction, amplification and molecular cloning

Whole blood was collected from two black kites (BK), one bar-headed goose (BHG from Bayan Nuur, Mongolia), and one graylag goose (GLG from Yangtze River flying at high altitude for 16 hours, China). Genomic DNA was extracted from whole blood using DNeasy Tissue Kits (QIAGEN) according to the manufacturer's protocol. The quality and concentration of the extracted gDNA were measured and stored in -20 ºC for further experiments. Paralogspecific primers were designed using 5′ and 3′ sequences from annotated globin genes in the genome assembly of bald eagle, *Haliaeetus leucocephalus* isolate CR32 (16) for black kites and specific primers were designed from previously published sequences for amplifying BHG and GLG Hb genes (4, 17). The three globin genes (α^A, $\alpha^{D,}$ and β^A) of black kites, BHG and GLG were amplified from genomic DNA using Taq polymerase in standard reaction set-up. Following purification, the PCR products (α^A, $\alpha^{D,}$ and β^A adult globin genes) were cloned into pUCm-T (BBI Life Sciences, Hefei, China) TA cloning vector under a standard protocol. Sequencing was done by Sanger sequencing technique (Sigma Aldrich) at Sangon Biotech Ltd (Hefei, China) using M13F

(CTGGCCGTCGTTTTACAAC) and M13R (CAGGAAACAGCTATGACC) universal sequencing primers.

Phylogenetic analysis and model structure determination

Amino acid multiple sequence alignment and comparisons were done using DNA star version 7.1 software and phylogenetic trees were built using the Maximum likelihood method in MEGA-X software (18). Model homology structures were developed by the SWISS-MODEL server (https://swissmodel.expasy.org/). The quality of homology structural models was controlled by considering template structure with higher sequence similarity, using template structures solved only by X-ray crystallography, Global Quality Model estimation (GQME) scores, and QMEAN Z score (19-22). The major hemoglobin homology model structures were developed by using GLG hemoglobin (PDB: 1faw.1) (23) for BK-0 and GLG, and BHG hemoglobin (PDB: 1a4f.1) (24) for BHG as templates. The developed homology model structures have high sequence similarity with templates (90-96 %), high alignment quality with templates (GMQE ≥0.98), and excellent agreement with the predicted experimental model structures (QMEAN-Z score ≥-0.71). All the homology model structures were illustrated with Phyton Molecular Viewer (PyMOL) 2.3.4.

3.0 RESULTS

The hemoglobin amino acid sequences of BK-0, BK-1, BHG, and GLG were determined. The amino acid sequences of α^A and α^D globin chains of both black kites were identical while they have significant sequence differences at the β^A globin chain. Amino acid sequences of similar species show similarities and differences as compared to previously reported data in BHG and GLG (**Table 1**).

Phylogenetic patterns of parallelism

Comparison of Black kites, Bar-headed goose, and Graylag goose Hb amino acid sequences was done using multiple sequence alignment. The multiple sequence alignment results showed evolutionarily conserved sequences in α^A, $\beta^{A,}$ and α^D globin chains across birds' species. The presence of multiple amino acid substitutions occurred at different residues in the globin chains of birds Hb based on differences in altitude. Interestingly, based on the β^A phylogenetic pattern, the current black kite from Western Mongolia (BK-0) showed recent divergence from BK-1 (Chandmani, Mongolia) and bald eagle, and has, although not statistically significant, recent relationship with Geese species indicating parallel evolution to high altitude in a shared environment. Further, the current Geese (BHG and GLG), diverged from other Geese (BHG and GLG) reported previously (4, 17) indicating a difference in niche and altitude (**Fig. 1**). This shows that the β^A globin chain is becoming more evolutionary important to adapt high altitude hypoxia transiently and/or permanently.

Table 1: Summary of parallel and divergent amino acid substitutions among BK-0, BK-1, BHG, and GLG

Key: Red/green highlights = substituted amino acid, G= glycine, A= alanine, S= Serine, V= valine, P= proline, T= threonine, D= aspartic acid, E= glutamic acid, L= leucine, N= asparagine, C= cysteine, K= Lysine, M= Methionine, I= isoleucine= leucine

Bird	Location of specimen collection	Amino acid substitutions in the globin chains			Reference
		α^A	α^D	β^A	
BHG	Previous research	A12, S18, V63, A119	S3, V48	T4, N83, T84, E90, A116, A119, E121, D125, A128, L133	(4)
	Mongolia	A12, S18, V63, A119	S3, V48	T4, K83, C84, K90, S116, G119, D121, A125, S128, V133	Current
GLG	Previous research	G12, G18, A63, P119	T3, L48	S4, N83, T84, E90, A116, A119, E121, D125, A128, L133	(17)
	Yangtze	G12, G18, A63, P119	T3, L48	S4, K83, C84, K90, S116, G119, D121, A125, S128, M133	
Bald eagle	Previous research			I55, N83, T84, E90, S116, S123, D125, A128, L133	(16)
BK -0	West Mongolia	Identical		L55, K83, S84, K90, S116, T123, A125, S128, M133	Current
BK-1	Chandmani, Mongolia			I55, N83, T84, E90, S116, S123, D125, A128, L133	Current

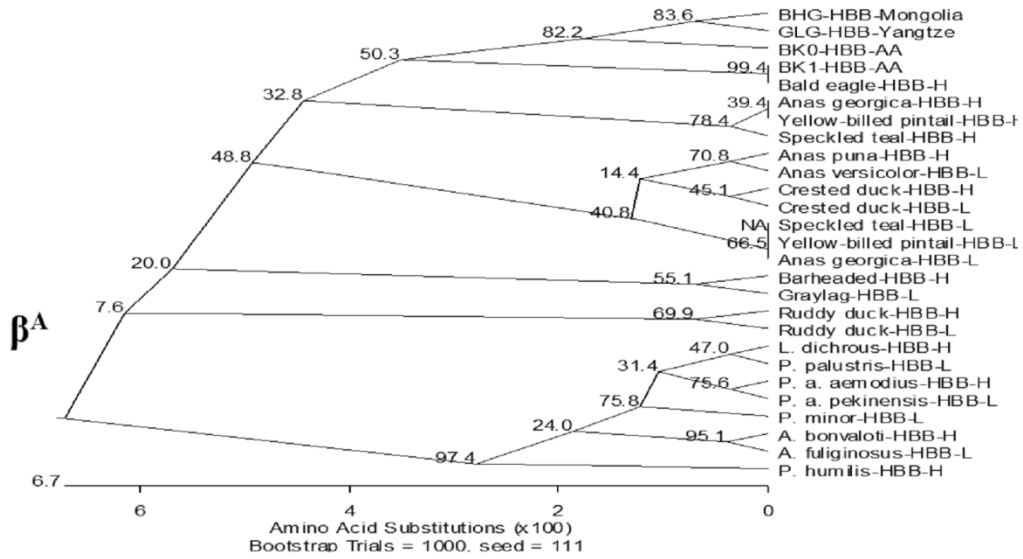

Figure 1. Phylogenetic tree analysis of the β^A globin chain of BK-0, BK-1, BHG, and GLG. The phylogenetic tree shows evolution and ancestral relationship among high altitude and low altitude birds. BK-0, BHG, and GLG show parallel evolution without a significant ancestral relationship

Structural and functional patterns of parallelism

We developed the homology model structure of the major hemoglobin (HBA) of BK-0, BHG, and GLG using the SWISS-MODEL server (19-22) to illustrate the nature, location, and function of the parallel amino acid replacements in the β^A globin chain. Here we reported the homology model structure of Black kite major Hb for the first time. The structural position of parallel and unique amino acid substitutions in BK-0, BHG, and GLG is described in **Fig. 2, Fig. 3 and Fig. 4.** Our data demonstrated unique and parallel amino acid replacements at adjacent sites within the same functional positions in the hemoglobin protein. BK-0, BK-1, BHG, and GLG shared an amino acid (α^A34-Thr) located at the α1β1 inter-subunit contact and another amino acid (α^A77-Ala) located in the 2nd IPP binding site of Hb. Whereas BK-0, BK-1, and BHG have α^A18-Ser located at the exterior solvent-accessible AB corner. But these amino acids are common for both low altitude and high-altitude flying birds. As reported previously (4, 8, 15, 25), all BHG isolates have a unique amino acid (α^A119-Ala) located at the α1β1 inter-subunit contact of Hb.

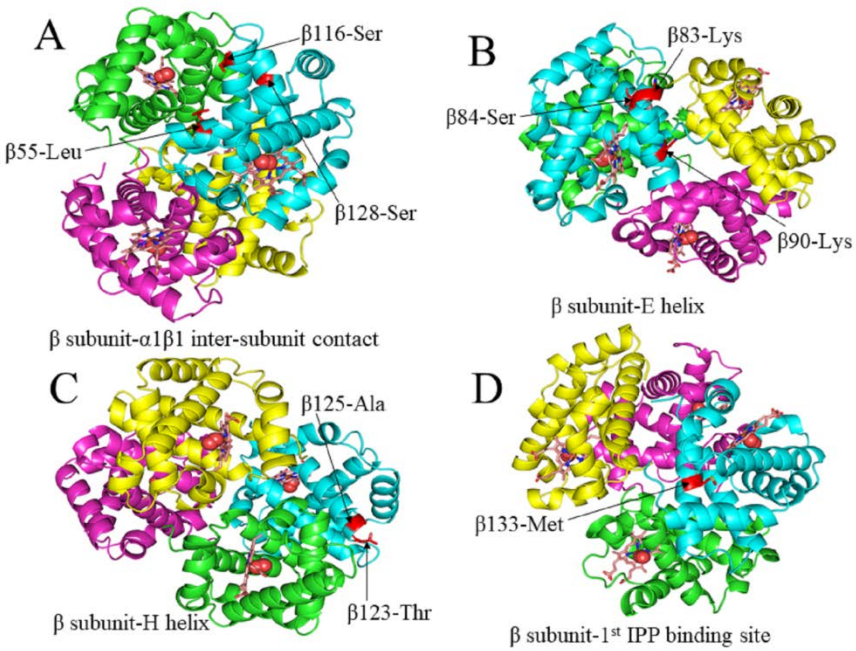

Figure 2. Structural positions of amino acid substitutions (except β116-Ser) in the β^A globin chain of the oxy (R-state) major hemoglobin of BK-0 compared to BK-1. (A) The β^A globin chain showing β55-Leu, β116-Ser and β128-Ser in the α1β1 inter-subunit contact (B) E helix of β^A globin showing β83-Lys, β84-Ser and β90-Lys (C) H helix of β^A globin showing β123-Thr and β125-Ala and (D) β^A globin chain showing β133-Met in the 1st IPP binding site.

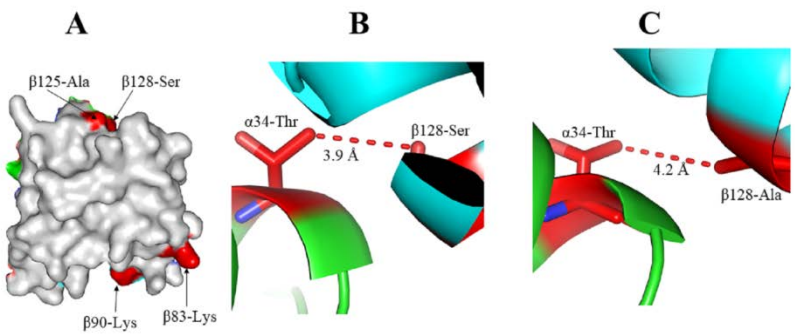

Figure 3. β^A globin chain of BK-0 oxy major hemoglobin. (A) A surface of BK-0 oxy major hemoglobin showing independent amino acid substitutions (β^A83-Lys, β^A90-Lys, β^A125-Ala, and β^A128-Ser) in the β^A globin chain shared with BHG and GLG (B) a 3.9 Å distance between α^A34-Thr and β^A128-Ser and (C) a 4.2 Å distance between α^A34-Thr and β^A128-Ala when serine was replaced with alanine in BK-0 oxy-Hb suggesting the replacement of alanine with serine in high altitude BK-0 result in the closeness of the α1β1 inter-subunit contact which probably causes the stability of the R state.

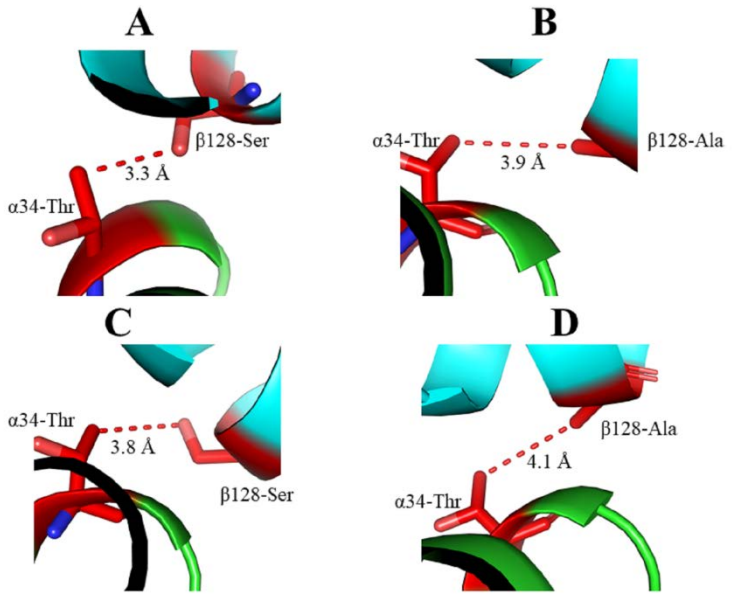

Figure 4. The effect of Ala-β^A128-Ser replacement on the structure and function of oxy major hemoglobin of BHG and GLG. (A) A 3.3 Å distance between α^A34-Thr and β^A128-Ser and (B) a 3.9 Å distance between α^A34-Thr and β^A128-Ala when serine was replaced with alanine in BHG oxy-Hb. (C) A 3.8 Å distance between α^A34-Thr and β^A128-Ser and (D) a 4.1 Å distance between α^A34-Thr and β^A128-Ala when serine was replaced with alanine in GLG oxy-Hb. This indicates that the individual Ala-β^A128-Ser substitution causes significant closeness of the α1β1 inter-subunit contact resulting in stability of the R state of BHG and GLG oxy-Hb at high altitude.

In the β^A globin chain of BK-0, GLG and BHG, three parallel amino acid substitutions (β^A55-Leu, β^A116-Ser, and β^A128-Ser) are located in the α1β1 inter-subunit contact, two parallel amino acid substitutions (β^A83-Lys and β^A90-Lys) are located at the E helix while other two parallel substitutions (β^A123-Thr and β^A125-Ala) are located at the H helix of Hb. All of the four studied birds shared the β^A116-Ser amino acid located at the α1β1 inter-subunit contact. BK-0, BK-1, and BHG shared a single amino acid (β^A4-Thr) located at the 1st IPP binding site. Similarly, BK-0 and GLG shared a single amino acid (β^A133-Met) located at the 1st IPP binding site of Hb (**Table 2**). From all parallel amino acid substitutions, Asn-β^A83-Lys, Gln-β^A90-Lys, Asp-β^A125-Ala, and Ala-β^A128-Ser are shared independently among BK-0, BHG, and GLG (**Fig. 3A**). Some of these replacements at specific locations are reported to contribute to the increased Hb-O_2 affinity at high altitude. Our data suggest structural and functional patterns of parallel evolution among the studied birds in the β^A globin chain. Further, parallel substitutions were found at adjacent amino acid positions on the same folded polypeptide and interacting positions in the same subunit coded by the β^A globin gene. The distance between the two atoms (α^A34-Thr and β^A128-Ser) linking the α1 and β1 subunits in BK-0 oxy-Hb is smaller than the distance between α^A34-Thr and β^A128-Ala in BK-1 oxy-Hb (3.9 Å versus 4.2 Å) (**Fig. 3B and C**). Similarly, the distance between α^A34-Thr and β^A128-Ser in BHG oxy-Hb is 3.3 Å which quite smaller than a 3.9 Å distance between α^A34-Thr and β^A128-Ala when serine was reverted into alanine (**Fig. 4A and B**). In addition, a 3.8 Å distance between α^A34-Thr and β^A128-Ser in GLG oxy-Hb is smaller than a 4.1 Å distance between α^A34-Thr and β^A128-Ala when serine was reverted into alanine (**Fig. 4C and D**).

4.0 DISCUSSION

Convergent changes in Hb–O_2 affinity at high-altitude in different species are seldom attributable to convergent or parallel changes at the amino acid sequence level. It is also reported that convergent increases in Hb-O_2 affinity were caused by parallel substitutions at key residues and were attributable to nonreplicated substitutions and/or parallel substitutions at sites that are not considered "key residues" (26). Here we made a comparison of amino acid substitution in the current birds with other phylogenetically diverse sets of birds reported before (4, 8, 15-17, 25, 27) and found a set of unique and parallel substitutions important for the unique and/or parallel evolution.

β^A83-Lys, β^A90-Lys, β^A125-Ala, and β^A128-Ser are the most prominent examples of parallel evolution among BK-0, BHG and GLG in our data sets, evolving independently only in the three birds. Most importantly, the replacement of alanine with serine at the β^A128 position might probably play a significant functional role at high altitude through stabilizing the R state due to the nature of serine to form additional hydrogen bonds between α1 and β1 Hb subunits. Additionally, although not an independent substitution in our birds, Ser-β^A116 and Met-β^A133 may result in a convergent increase in Hb-O_2 affinity in our birds at high altitudes as these mutations are reported to have this role in other birds (8). Especially when nonpolar residues are replaced with polar ones, they readily bind with a negatively charged ligand, inositol pentaphosphate (IPP), and produce hydrogen bonds resulting in a conformational change of the Hb heterotetramer. Moreover, the independently shared Asn-β^A83-Lys substitution could contribute to increasing Hb-O_2 affinity as lysine is a charged amino acid that can form salt bridges between the α1 and β1 subunits to stabilize the R state of oxy-Hb. Specific amino acid substitutions at this position (β^A83) are reported to enhance Hb-O_2 affinity at high altitudes in other birds (26, 28).

The shorter distance between α^A34-Thr and β^A128-Ser compared to α^A34-Thr and β^A128-Ala at the α1β1 inter-subunit in BK-0, BHG and GLG oxy-Hb indicates the role of the individual Ala-β^A128-Ser substitution in bringing about the closeness of the subunits where it stabilizes the R state of Hb which could result in increased Hb-O_2 affinity at high altitude. The distance between the two atoms (α^A34-Thr and β^A128-Ser) was the smallest in BHG oxy-Hb (3.3 Å) compared to BK-0 (3.9 Å) and GLG (3.8 Å) oxy-Hbs indicating synergy between the oppositely located β^A128-Ser and α^A119-Ala residues at the α1β1 dimer in stabilizing the R state and destabilizing the T conformations of the Hb tetramer, respectively. The distance between Alaα^A119 and Leuβ^A55 (4.5 Å), and Proα^A119 and Leuβ^A55 (3.8 Å) in the current BHG and GLG oxy-Hbs, respectively is similar with previous reports (23, 24) revealing the heightened role of amino acid substitutions at the β^A globin chain in stabilizing the R state than destabilizing the T state.

Studying the value of individual phenotypes is recommended to understand the evolutionary outcomes of group phenotypic composition (29). Our study has strengths in this regard presenting parallel evolution at the molecular sequence level supported with phylogenetic and structural data at individual bird level. But biochemical data is lacking which results in the scarcity of evidence to confirm whether the parallel substitutions, specifically those untested before, are uniquely associated with convergence in Hb function at high/low altitude. Despite these limitations, our study provides a glimpse into this interesting scenery of the structural and phylogenetic basis of parallel evolution in phylogenetically close and distant species of birds. Moreover, our findings evidenced the presence of convergence amino acid substitutions in the Hb of low altitude birds staying at high altitudes for a long time which could provide an important basis for future promising studies in both humans and other animals.

5.0 CONCLUSION

In conclusion, our data revealed that amino acid substitution in the β^A globin chain involved multiple parallelisms among the studied birds. Five to six parallel amino acid substitutions were observed in the β^A globin chain of BK-0, BHG, and GLG while four of them evolved independently in these birds. Our results suggest that parallel evolution in the β^A globin gene might be important to alleviate high altitude hypoxia in a shared environment among birds. More specifically, the Ala-β^A128-Ser parallel substitution provides structural evidence of stabilizing the R conformation of oxy-Hb at high altitude. Also, the nature and location of parallel amino acid substitutions in the Hb structure are determinants for the contribution to the increased convergence in Hb function at high altitude. Besides, our findings indicated that low-altitude birds spending longer at high altitudes involve convergent Hb amino acid

substitutions. Studying the parallel evolution of a combination of individual and group of birds' Hb supported with structural and biochemical data is highly recommended. This study deciphers an important evolutional scene which could be applied in humans.

6.0 ACKNOWLEDGMENTS

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