Pakistan J. Zool., vol. 51(4), pp 1299-1307, 2019. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2019.51.4.1299.1307

Analysis of the Phylogeny and Evolutionary Selection Pressure of the *Mx* Gene in 10 Wild Birds

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ABSTRACT

The Myxovirus-resistance (Mx) gene has broad-spectrum antiviral effects. This gene is particularly important for the prevention and treatment of avian influenza. Currently, this gene is more studied in poultry, and studies in wild birds are rare. To understand the antiviral site of the Myxovirus-resistance (Mx) protein in the evolution of different wild birds, 10 wild bird species, including *Anas formosa*, *Anas crecca*, *Anas strepera*, *Mergus squamatus*, *Accipiter nisus*, *Buteo hemilasius*, *Buteo lagopus*, *Passer montanus*, *Psittacula roseata* and *Emberiza elegans*, were selected. The sequences of the GTPase effector domain (GED) of the *Mx* gene were determined by PCR sequencing. The haplotypes were analysed by DNA SP software. The Datamonkey Adaptive Evolution Server was used to detect the selection pressure. Phylogenetic analyses of the reported sequences of jungle fowl (*Gallus gallus*) and the 10 tested birds were performed. A total of 10 nucleotide sequences of the GED region of the *Mx* gene in wild birds were obtained, with a length of 231 bp, encoding 77 amino acids. The third locus in all tested amino acid sequences contains a Ser residue that corresponds to amino acid position 631, the virus resistance site, in the chicken *Mx* gene. The third locus in all tested amino acid sequences are Ser and corresponds to the chicken 631 amino acid position, the virus resistance site. Ten haplotypes were found with 60 nucleotide mutations. Phylogenetic analysis by MEGA 7.0 revealed that the evolution of the tested genes is consistent with the evolution of the tested birds. The results of the selection stress test show that only a few sites in the GED region of the *Mx* gene are positively selected during evolution, and the majority of the amino acid sites are constrained by the strong structure and function of the protein, indicating a high risk of avian influenza infection in the tested wild birds.

Article Information
Received 13 March 2018
Revised 11 May 2018
Accepted 13 June 2018
Available online 01 May 2019

Authors' Contribution
XLW conceived the study. SRW and SYB designed the experiments. SRW and Joka conducted the experiments in the field. SRW and SYB analysed the results. SRW and Joka drafted the first manuscript. XLW and SYB revised the draft.

Key words
GTP activity region, Mx gene, Phylogenetic analysis, Selection pressure.

INTRODUCTION

Myxovirus-resistance (Mx) protein belongs to the dynamin-like GTPase family with a broad anti-virus spectrum. This protein confers resistance to RNA viruses, such as *Orthomyxoviridae, Rhabdoviridae*, *Paramyxoviridae*, *Bunyaviridae*, and *Togaviridae*, as well as some DNA viruses, including *Hepadnaviridae* (Frese *et al*., 1996; Gordien *et al*., 2001; Haller and Kochs, 2002; Sasaki *et al*., 2013).

The structures of Mx proteins in different species are basically the same, with three domains: a GTP-binding domain (GD), comprising 3 regions near the N-terminus, which exhibits an important antiviral function (Pitossi *et al*., 1993), and the central interactive domain (CID) and the GTPase effector region (GED) in the C-terminus. The evolution of the leucine zipper at C-terminus is highly conserved, suggesting that this domain plays a vital role in the Mx protein function (Tao *et al*., 2016).

The *Mx* gene has been found in higher vertebrates, including humans, livestock and poultry (Lindenmann, 1962). Subsequently, the *Mx* gene was also found in invertebrates, such as abalone (de Zoysa *et al*., 2007), indicating that the *Mx* gene exists in a variety of animals,

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0030-9923/2019/0004-1299 $ 9.00/0

Abbreviations
Mx gene, Myxo-virus resistance gene; GED, GTPase effector domain; GD, GTP-binding domain; CID, central interactive domain; AIV, avian influenza virus; d_S, synonymous replacement rate; d_N, non-synonymous replacement rate; ω, non-synonymous/synonymous replacement rate ratio; BIC, Bayesian information criterion; IFEL, internal fixed effects likelihood method; FEL, fixed effects likelihood method; SALC, single-likelihood ancestor counting method; FUBAR, fast unconstrained Bayesian AppRoximation for rehabilitation method; Post Pr, post processing.

primarily in the form of a recessive allele. Tumpey *et al.* (2007) found that the mouse Mx protein confers resistance to the avian influenza virus (AIV). Ko *et al.* (2002) found that the specific antiviral site of the chicken *Mx* gene is located at the 631st amino acid position encoded by exon 14, which determines whether the Mx protein is resistant to avian influenza and herpes viruses, indicating that a Ser-to-Asn substitution at this position is the source of this antiviral ability (Ko *et al.*, 2004; Sironi *et al.*, 2008). Most studies have focused on the relationship between amino acid positions and disease resistance, among which most of studies have focused on the *Mx* gene in poultry (Li *et al.*, 2009; Yin *et al.*, 2010; Zhang *et al.*, 2013; Niu *et al.*, 2014). Recent studies have demonstrated that the specific variant in the *Mx* gene encoded by exon 14 is responsible for the antiviral activity of the protein (Fulton *et al.*, 2014). The non-synonymous G/A polymorphism at position 2032 of chicken Mx cDNA results in a change at the 631st amino acid position of the Mx protein. The *Mx* gene has become an effective candidate for disease resistance in some poultry breeds. Studies have shown that the antiviral function and intracellular localization of the *Mx* gene are both dependent on the change at amino acid position 631. The Mx protein with an Asn at the 631 aa distributes in a granular-like pattern in the cytoplasm and shows the capacity for inhibiting viral growth. However, Mx protein with a Ser at the 631 aa did not inhibit viral growth and homogenous spread throughout the cytoplasm (Sasaki *et al.*, 2013). The replacement of Glu→Arg near the carboxyl terminus of human *Mx* gene encoded by exon 14 can inhibit the proliferation of influenza virus, but it has no resistance to (Vesicular Stomatitis Virus, VSV) infection (Zurcher *et al.*, 1992).

As a spreader and reservoir of avian influenza virus (Zhang *et al.*, 2012; Piaggio *et al.*, 2012), wild birds are an increasing concern of many researchers. Avian influenza virus has been found in multiple species of wild birds, such as Anseriformes, the largest population, followed by Passeriformes (Zhao, 2008). Most aquatic migratory birds that have the capabilities of cross-boundary and long-distance migration are the main hosts of avian influenza (Olsen *et al.*, 2006).

The *Mx* gene is the only gene identified as resistant to avian influenza virus. However, there are few studies confirming whether this speculation is true in wild birds. The important variation and potential virus resistance of the *Mx* gene have intrigued researchers. In the present study, samples from 10 common wild birds from the East Asian migration route were used to amplify the nucleotide sequences of the GED coding region of the *Mx* gene and analyse their evolution; additionally, amino acid analysis of the S631N site was conducted to report the susceptibility of hosts to avian influenza. The present study provided a theoretical basis for studying the anti-influenza virus activity of the *Mx* gene in broader hosts.

MATERIALS AND METHODS

The samples were obtained from individuals that died during the rescue process in recent years and stored in our laboratory (Table I).

Table I.- Information of samples.

Family / Scientific name	Common name	Code	Year
Anatidae			
Anas formosa	Baikal Teal	N1-2	2016
Anas crecca	Green-winged Teal	N2-1	2016
Anas strepera	Gadwall	N4-1	2016
Mergus squamatus	Chinese Merganser	N5-2	2012
Accipitridae			
Accipiter nisus	Sparrowhawk	N6	2010
Buteo hemilasius	Upland Buzzard	N7	2010
Buteo lagopus	Rough-legged Buzzard	N8	2010
Passeridae			
Passer montanus	Eurasian Tree Sparrow	N10	2010
Psittacula roseata	Blossom-headed Parakeet	N11	2010
Emberizidae			
Emberiza elegans	Yellow-throated Bunting	N13	2010

Table II.- The sequences of primers for PCR.

Primers	Samples	Sequence (5'~3')	Product size (bp)	Tm value (°C)
CMX1 CMX2	N6, N7, N8	AGTTCCTAGAAGCACTCACTTT GATTAACTCGGCCACTGAGGT	356	49
DMX1 DMX2	N11, N13	GCATGAGAGAGACTAACAGGAAAC ACTCGGCCACTGAGGTAATTC	382	51
EMX1 EMX2	N1, N2, N4, N5, N10	GCATGAGAGAGACTAACAGGAAAC ACTGGCAGTAAAGGTCAGCG	535	51

PCR for Mx *genes samples*

Total DNA was extracted from the chest muscle by using the Animal DNAout Kit (Beijing TIANDZ Gene Technology Co., Ltd.). Three pairs of primers for *Mx* gene exon 14 were designed according to the mallard genomic sequence in GenBank (NW004677804) by Primer Premier 5.0 and NCBI online (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The primers were synthesized by Invitrogen Trading (Shanghai) Co., Ltd. The primer information is shown in Table II.

Exon 14 of the *Mx* gene was specifically amplified with the primers described above. The PCR reaction mixture contained two 20 µM primers at 0.8 µl each, Taq PCR master mix 10 µl, template 1.6 µl, and ddH_2O 6.8 µl to obtain a final volume of 20 µl. The following programme was used for the *Mx* gene PCR amplification: 95°C for 5 min, followed by 30 cycles at 94°C for 30 s, Tm values of the different primers in accordance with the primers used (Table II) for 30 s, and 72°C for 45 s, with a final extension at 72°C for 5 min. The PCR product was detected by 1% agarose gel electrophoresis. The PCR products were recovered by the TIANGEN Universal DNA Purification Kit (TIANGEN Biotech (Beijing) Co., Ltd.). Each sample was sequenced three times by Invitrogen Co., Ltd. (Shanghai) for bidirectional sequencing.

Bioinformatics analysis

The PCR products were confirmed as *Mx* gene sequences by alignment in the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The haplotype analysis was performed by DNA SP 5.10.1 software (Librado and Rozas, 2009) (http://www.ub.edu/dnasp/).

The evolution mode test was performed to determine the haplotype of the 10 GEDs in the *Mx* gene by the modified Nei-Gojobori method in Jukes-Cantor of MEGA7.0 software and the Datamonkey Adaptive Evolution Server (http://www.datamonkey.org/) (Delport *et al*., 2010).

Prior to the phylogenetic analysis, the 10 obtained nucleotide sequences were tested for substitution saturation by DAMBE6.4 software (Xia *et al*., 2003) to determine their suitability for further phylogenetic tree construction.

The phylogenetic tree was constructed by ML methods, in which the Bayesian information criterion (BIC) standard offered by MEGA7.0 software was used to select the most suitable model, and the lowest BIC value was adopted to represent the best model selection. A topological structure with highest value of maximum-likelihood was selected as the final phylogenetic tree, and the result was verified by the MP method. The *Mx* exon 14 sequence of *Gallus gallus* was selected as an outgroup to assist in locating the evolutionary tree roots. The confidence of the branches of phylogenetic tree was tested by bootstrap analysis for 1000 replicates. The sequences used are shown in Table III.

Table III.- Accession numbers of sequences used for phylogenetic analysis.

Name	Accession number
Anas platyrhynchos	XM013105472
Gallus gallus S	Z23618
Gallus gallus RIR	EF575619
Gallus gallus TB	EF575627
Gallus gallus SK	EF575630
Gallus gallus	DQ788616
Gallus gallus Rhode Island Red	DQ788613
Gallus gallus Silkie	DQ788614
Gallus gallus BY1	EF575623
Gallus gallus WL-N	EF575630
Gallus gallus N	EU348752
Meleagris gallopavo	XM003202961
Caprimulgus carolinensis	XM010173565
Coturnix japonica	XM015882889

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                                        111 1111111111 1111111111 1111111222
          11122334 4445566677 7788889011 1122222344 4455556667 7788889223
         5612947031 2590306923 8912596601 4724579504 8927893592 4923894260
H1       CAAATTGTTT CAGCTTCCCA GCGTTTTGGG ATAAGTAAGT GTTATGATCA TATTGGGCCT
H2       .......... .......... .......... .A........ .CC.....TG .G.....TT.
H3       .......... .......... .......... .A........ .CC....... .G.....TTA
H4       ......A... .......... ..AA...... .A.T....A. .CC..T.... .G.....TT.
H5       .......A.. AGTTCCTGA. A..AA...AA CGGCACCGAC .CCCAT.C.. .G........
H6       ....A..A.. .GT....GTC ...AA.C.AA CG.CACCGA. .CCCATGG.. .G........
H7       TCTGG..A.. .GT....GTT ...AA...AA CG.CACCGA. .CCCATGG.. .G..AA....
H8       .......... .......... .......... .A.......G CCC....... GG....CTTA
H9       .......... .......... .......... .A........ .CC....... GG.A......
H10      .....CA.CC .......... .G.C.G.A.. .A........ .CC....... G.AA......
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Fig. 1. The nucleotide variation sites of *Mx* gene exon 14 in 10 birds.

RESULTS

Mx *gene amplification*

The obtained *Mx* gene exon 14 was identified as 231 bp, encoding a total of 77 amino acids.

Sequence variation analysis

The complete coding regions of *Mx* gene exon 14 were obtained from 10 species of birds: *Anas formosa*, *Anas crecca*, *Anas strepera*, *Mergus squamatus*, *Accipiter nisus*, *Buteo hemilasius*, *Buteo lagopus*, *Passer montanus*, *Psittacula roseata* and *Emberiza elegans*.

Ten haplotypes of *Mx* gene exon 14 were confirmed in the present study. No cross-species-shared haplotypes were found. The coding region length was 231 bp (excluding the stop codon) with 60 nucleotide mutation sites, 28 parsimony-informative sites (*i.e.,* the variation contains at least two types of nucleotide or amino acid) and 33 single nucleotide polymorphism sites (Fig. 1). The average contents of A, T, G and C were 29.4%, 25.5%, 21.2% and 23.9%, respectively, among which the A + T content was higher than the G+C content. Our finding also indicates that 32 amino acid mutation sites were found in the 78 amino acids encoded by *Mx* gene exon 14 (Fig. 2).

Test of selection pressure for the GED region

The test of selection pressure for the GED region indicates that the average non-synonymous nucleotide substitution rate in the GED region of the *Mx* gene (exon 14 coding) was $d_N = 0.117$, whereas the average synonymous nucleotide substitution rate was $d_S = 0.26165$, giving $d_N/d_S = 0.447$ (<1). The model selection result shows that the most suitable nucleotide substitution model for the detected sequence was HKY85. In the present study, the results of different methods were not the same. Three positive selection sites and 2 negative selection sites were detected by the Internal Fixed Effects Likelihood (IFEL) Method. No positive selection site was detected by other methods. Seven negative selection sites were found by the Fixed Effects Likelihood (FEL) Method. Each positive selection site was detected by the Single-Likelihood Ancestor Counting (SALC) Method and the Fast, Unconstrained Bayesian AppRoximation for Rehabilitation (FUBAR) Method (Table IV).

Phylogenetic analysis for Mx *gene*

The test of substitution saturation satisfied the conditions P = 0.0000 and ISS <ISS.C (Table V); thus, the sequences were unsaturated and suitable for establishing the phylogenetic tree. In the present study, the maximum likelihood and K2 model were adopted to establish the phylogenetic tree. The topological structures were identical, except for differences in confidence between the phylogenetic trees constructed by the ML and MP methods (Fig. 3). The sequences of the species in the phylogenetic tree were clustered together according to their respective classifications. *Galliformes* differentiated into two large groups. In another large group, two subgroups were observed: the first group consisted of *Passeriformes*, *Anseriformes* and *Psittaciformes*, and the second group comprised *Falconiformes* and *Caprimulgiformes*.

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                1112222 2333334444 5555556666 77
           2474784578 9067891278 0135780135 67
H1         AKSIAVDNQS SMERENNSGS VVMNLSQVRS RL
H2         .......... .....K.... AA..FGR... C.
H3         .......... .....K.... AA....R... CQ
H4         .........T .....K.CD. AAI...R... C.
H5         ....FAE..T T..KDKSHD. AAH...R... C.
H6         ..T.S..H.T T..KDK.HD. AAHE..R... C.
H7         VMA.S..Y.T T..KDK.HD. AAHE..R.Q. C.
H8         .......... .....K...R PA...RR..T CQ
H9         .......... .....K.... AA...RR... C.
H10        ...T....EP .RK..K.... AA...R.EG. C.
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Fig. 2. The amino acid variation sites of Mx protein in 10 birds.

Table IV.- Selection pressure analysis of *Mx* gene exon14.

Codon	FUBAR		SLAC		IFEL		FEL	
	d_N/d_S	Post. Pr	d_N/d_S	P-value	d_N/d_S	P-value	d_N/d_S	P-value
8								0.0868
9	0.0244	0.9832	0.7437	0.0687		0.0234		0.0051
10								0.0810
20								0.0792
22								0.0675
32						0.0921		0.0433
42					>100	0.0594		
45								0.0810
53					>100	0.0883		
74								0.0794
77					>100	0.0958		

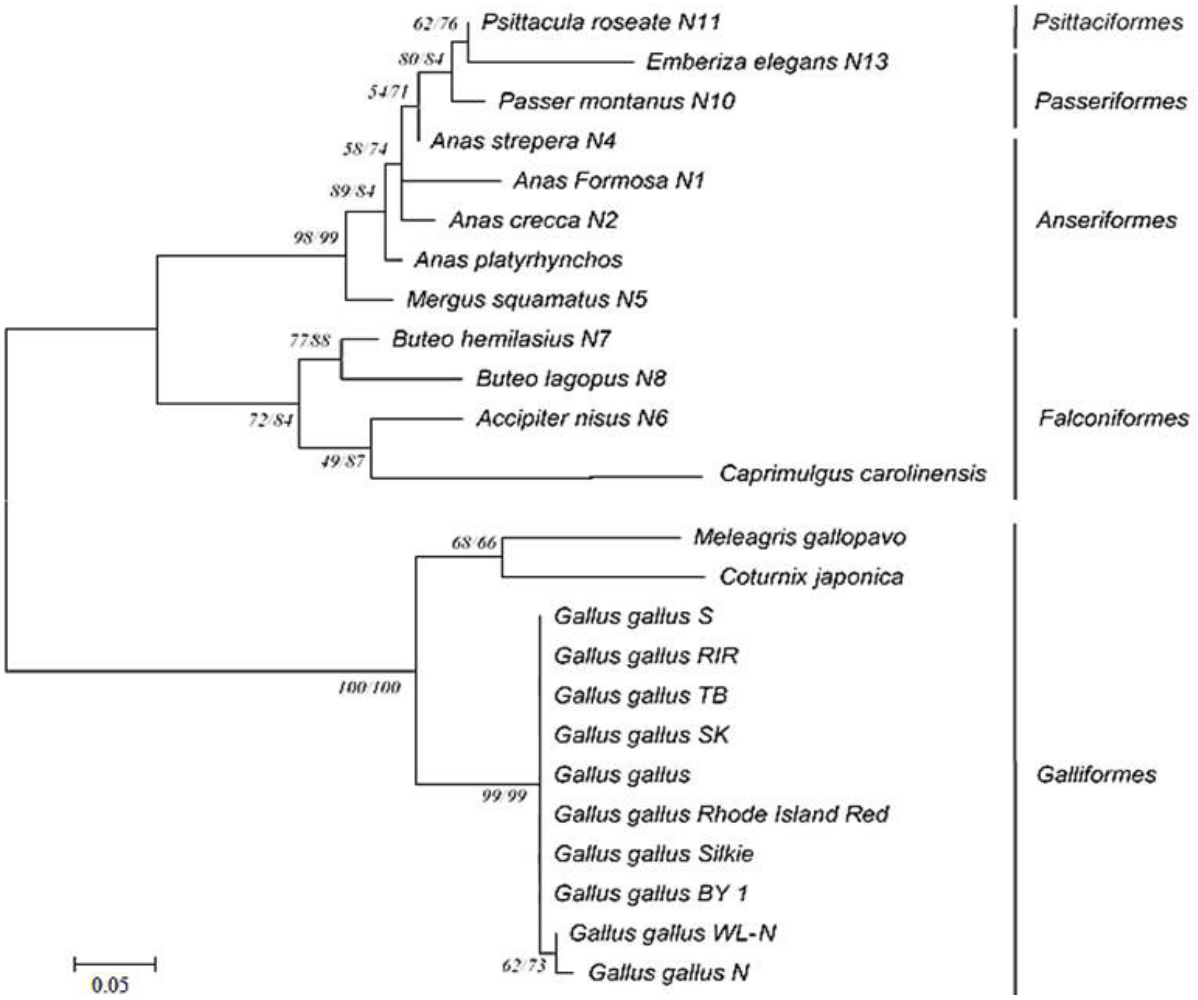

Fig. 3. The phylogenetic trees of Mx exon 14 plotted by Maximum Likelihood and Maximum parsimony methods. The confidence of the branches of phylogenetic tree of Maximum Likelihood and Maximum parsimony were symbol in the figure and separated by'/'. GenBank accession numbers for: N1, MF667526; N2, MF667527; N4, MF667528; N5, MF667529; N6, MF667530; N7, MF667531; N8, MF667532; N10, MF667533; N11, MF667534; N13, MF667535.

Table V.- The test of substitution saturation for nucleotide sequences.

Gene	ISS	ISS.C		P value (Double tail)	Significance level
		Symmetric tree	Unbalanced tree		
Mx	0.3477	0.7034	0.5710	0.0000	Extremely significant

DISCUSSION

Mx *gene antiviral sites*

In poultry, the susceptibility and resistance to a virus are determined by the amino acid at the 631st site in the Mx protein. When this site is occupied by Asn, the Mx protein shows virus resistance, whereas when a substitution of Ser occurs, the birds are susceptible to a virus (Ko *et al*., 2002). All amplified complete sequences from the coding region of exon 14 were compared with those of the complete coding region of the jungle fowl gene (EF575689). The results showed that the target 631st amino acid in the entire Mx protein sequence was located at the 3rd amino acid site of the amplified complete coding region of exon 14 in all 10 birds. All 3rd amino acid sites detected in the present test samples were Ser residues, demonstrating that these 10 wild birds were susceptible to avian influenza virus, and the functions of their Mx proteins were lost during the course of the early replication of influenza virus. As all subtypes of avian influenza viruses have been detected in wild birds (Zhao, 2008), a large group of carriers and

spreaders of avian influenza viruses are under great threat. Further understanding of the role of the Mx protein may contribute a new strategy in the prevention of the global spread of avian influenza and other viruses.

Test of selection pressure for the GED region in the Mx *gene*

The role of natural selection in species differentiation has become a topic of renewed interest in the past few decades (Schluter and Conte, 2009; Arnegard *et al*., 2014). Selection pressure is the main driving force for the genetic evolution of biological groups. Different selection pressures may lead to different evolutionary directions. With the synonymous mutation rate as a criterion, it can be concluded that the retention of the non-synonymous mutation is supported by natural selection or obstruction. Non-synonymous substitutes can directly affect the function of the protein and are therefore more likely to alter biological adaptability than synonymous substitutions. A significantly higher mutation rate of the non-synonymous mutation than that of the synonymous mutation is evidence of the adaptive evolution of the protein (Yang, 2006). The maximum likelihood method for the selection pressure has been widely accepted, in which the selection coefficient ω (non-synonymous/synonymous replacement rate ratio, d_N/d_S) intuitively reflects the evolutionary trend of the organism at the codon level. These parameters can be used to measure the selection pressure at the protein level and are important measures based on the codon level analysis of the genetic evolution of the coding gene. Additionally, $\omega > 1$, $\omega = 1$ and $\omega < 1$ represent positive selection, neutral selection and purification selection (negative selection), respectively, during evolution (Yang and Bielawski, 2000; Choisy *et al*., 2004). If natural selection has no effect on the fitness of the gene, then the non-synonymous mutation will be retained at the same rate as the synonymous mutation, *i.e.,* $d_N = d_S$, $\omega = 1$. If the non-synonymous mutation is adverse, then the purifying selection will reduce its retention rate, resulting in $d_N < d_S$, $\omega < 1$. If the non-synonymous mutations are favoured by Darwinian selection, then these polymorphisms will be preserved at a greater rate than the synonym mutations, resulting in $d_N > d_S$, $\omega > 1$. This method has been widely used for the analysis of gene adaptive evolution related to reproductive performance and disease resistance (Yang, 2000; Sainudiin *et al*., 2005).

The three methods used for pressure selection detection on the genetic locus are maximum likelihood, distance and parsimony. Among these methods, the maximum likelihood method is more accurate for the study of species fecundity and the adaptive evolution of antiviral and other related genes (Koch *et al*., 2007). Therefore, in the present study, the maximum likelihood method based on the online software was selected for testing. With a selection coefficient of 0.447 ($d_N / d_S = 0.447$) <1, the select pressure can be considered as purely selective. Further, 5 detection methods of pressure selection, provided by Datamonkey were used. Among these methods, three positive selection sites were detected by the IFEL method. To a certain extent, these sites were affected by the positive selection pressure at the population level. In the REL method, $d_N > d_S$ was not detected; that is, the codon was subject to positive selection pressure, and the results showed that the GED region was under pure selection. This result is consistent with the average stress level of protein in the GED region. The results also showed that the antiviral site S631N was under neutral evolution, and compared with other loci in the GED coding region, this codon was under weaker mutation restriction of the protein structure. In the present study, the 631st sites of different bird families were under neutral selection pressure. Notably, the 9th codon was under purifying selection by the other four methods, except for the REL method. The mutation of the *Mx* gene may occur in all hosts; thus, the discovery of new antiviral sites can be expected. It is necessary to carefully analyse the variation caused by each mutation.

In summary, most of the amino acid sites in the GED region of the *Mx* genes from 10 species of birds were under strong limitation of protein structure and function, and only a small number of sites were under the pressure of positive selection during the evolutionary process. The results revealed that the Mx proteins in the tested birds were not prone to mutation.

Evolution of Mx *gene*

Mitochondrial DNA(mtDNA) is widely used in the classification of birds for its unique advantages (Awan *et al*., 2017). The species classification of birds in the present study indicates that different species are roughly clustered together as a group on the taxonomic category of Order, including the Galliformes group with related species, the Caprimulgiformes and Falconiformes group, and the Anseriformes and Passeriformes group. There are related records in GenBank showing higher sequence homology for the Mx protein in Galliformes and the 631st amino acid site in most species is occupied by Serine, which implies a great potential for susceptibility to virus. Asn has only been observed in a few Galliformes species, implying antiviral resistance. This finding confirms the Jungle fowl as the wild ancestor of the domestic chicken, and to obtain good breeds in the poultry industry, chickens have undertaken great anthropocratic selection pressure, during which the effectiveness of artificial selection overwhelms that of natural selection. Thus, the S631N sites of a few Galliformes

incline to express as environmental adaptability and evolve in the direction of disease resistance. Wild birds are different from poultry, primarily undertaking natural selection rather than aggressive anthropocratic selection for chickens.

The evolution of the GED sequence of the *Mx* gene is roughly consistent with the sequence of bird evolution (Hackett *et al*., 2008; Jarvis *et al*., 2014). The 10 species of birds are among the list of animal species infected with avian influenza published by the US Geological Survey (USGS National Wildlife Health Centre, 2016), which suggests that wild birds are highly susceptible and show great potential as hosts for the spread of bird flu on the basis of the defence mechanism of the Mx protein.

CONCLUSIONS

Studies of the *Mx* gene in wild birds are rare. The present study is the first to report the sequences of the *Mx* genes of 10 species of birds and characterize their relationships among other birds. The relationship between the classification status of the 10 tested bird species in the evolutionary category and the potential resistance to virus of the *Mx* gene still require much experimental verification in more avian hosts.

ACKNOWLEDGEMENTS

This work was financially supported through a grant from "the Fundamental Research Funds for the Central Universities" (2572016AA44).

Statement of conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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