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Corvidae in Pakistan are Represented by Two Distinct Clades Revealed through Maternally Inherited Gene Region

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ABSTRACT

Corvidae is a species rich and morphologically diverse family of the order Passeriformes (Aves), generally well identified by barcodes globally. Species identification and phylogenetic analysis through DNA barcodes using mitochondrial *COI* gene (cytochrome c oxidase subunit I) was aimed for samples of birds collected from different regions of Pakistan. Mitochondrial DNA was successfully extracted from keel tissue and Folmer region of *COI* gene comprising ~650 bps was amplified using universal primers and PCR products were confirmed by 1% agarose gel electrophoresis. Sequencing was carried out by Sanger's method and BLAST analysis identified these samples as five species (*Urocissa flavirostris*, *Dendrocitta vagabunda, Corvus splendens, Corvus corax* and *Corvus macrorhynchos)* from three genera of family Corvidae. The nucleotide sequences were submitted to the Barcode of Life Data System (BOLD) and the eligible sequences were assigned the Barcode Index Numbers (BINs). The COI gene sequence data of 8 species of family Corvidae were retrieved from GenBank (NCBI) database and phylogenetic relationship was established in these 13 barcodes. K2-parameter distances were calculated on MEGA7 software with overall average distance of 0.141 calculated at 1000 bootstrap repetitions. Phylogenetic tree reconstructed by neighbour joining method discriminated all species into two distinct clades initially and then into subclades based on similarity and variations in their sequences. Species of the same genera were grouped into one clade as genus *Corvus*. DNA barcoding was proved to be an effective tool for species molecular identification and their phylogenetic analysis during this study which may help in identification and biogeographic studies of birds in future.

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Authors' Contribution
SF and FN conceived and designed the study; FN executed the experiment, analyzed the tissue samples, analyzed and interpreted the data. SF and FA critically reviewed and FN revised the manuscript for important intellectual contents and approved the final version.

Key words
DNA barcoding, *COI* gene, Phylogenetic analysis, Corvidae, mitochondrial DNA

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INTRODUCTION

The family Corvidae (crows and jays) is an important family of bird order Passeriformes with a worldwide distribution (Goodwin, 1976), comprising 21 genera and 131 species (Miller, 2017). The family is represented by 7 genera and 19 species in the fauna of Pakistan (Gill and Donsker, 2019).

Morphological characteristics provided the basis of taxonomy and phylogeny of species for a long time (Gibbs *et al*., 2001) but now genetic identification is considered as more reliable in species identification. Concept of DNA barcoding (Hebert *et al*., 2003a) has now been transformed into establishment of a public reference library for reliable species identification (Coissac, 2016). A 648 base pairs (bp) *COI* gene fragment (cytochrome c oxidase 1) from mitochondrial genome (mtDNA) a standard barcode (Hebert *et al*., 2003b; Cai *et al*., 2010; Breman *et al*., 2013) has been used for animal species identification, including birds (Hebert *et al*., 2004b; Kerr *et al*., 2007, 2009; Johnsen *et al*., 2010; Yang and Rannala, 2014). Recent mtDNA sequence based studies on phylogeny of Corvidae have changed the traditional relationships among its members (Ericson *et al*., 2005; Bonaccorso and Peterson, 2007).

Five species of family Corvidae were randomly collected from different regions of Pakistan based on their availability during the study period. Nucleotide sequences of other species of this family reported from Pakistan were retrieved from GenBank. The purpose of study was to identify samples genetically, find genetic distance among them and infer their phylogenetic relationship by reconstructing phylogenetic tree. The current research work on phylogenetic relationship among species of family Corvidae will help contribute in the construction of

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reference library of DNA barcodes of birds from different geographical areas of the world.

MATERIALS AND METHODS

Taxon sampling

Specimens of freshly killed birds, belonging to 5 species of family Corvidae; *Urocissa flavirostris* (yellow-billed blue-magpie), *Dendrocitta vagabunda* (Rufous treepie), *Corvus splendens* (house crow), *Corvus corax* (common raven), and *Corvus macrorhynchos* (large-billed crow), were provided by hunters. Samples were identified using morphological keys (Ali and Ripley, 1983; Roberts, 1991; Grzimek's Encycloclpaedia, 2002). Keel muscle tissues were taken and preserved separately in 70 % alcohol in plastic bottles (Kress and Erickson, 2012) and stored at room temperature at Centre for Bioresource Research (CBR) for molecular analysis. Voucher specimens were deposited at Museum of CBR.

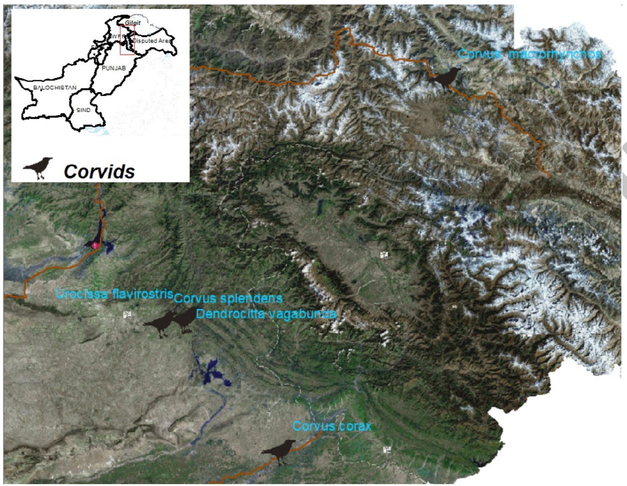

Fig. 1. Map of study area with geographical locations indicating collection sites of samples from Pakistan (Google Earth).

PCR amplification of mitochondrial COI *gene*

Genomic DNA from each tissue sample was extracted using phenol-chloroform extraction protocol with some modifications (Sambrook *et al*., 1989; Zou *et al*., 2005). Folmer region of *CO1* gene (~650 bp) of mtDNA was amplified (Folmer *et al*., 1994; Hebert *et al*., 2004; Dawnay *et al*., 2007) by PCR sprint thermo cycler (Thermoelectron Corporation, USA) using universal primers (Ivanova *et al*., 2006) supplied by Oligo, MACRO GEN (Seoul, Republic of Korea) (Table I).

PCR reaction was carried out by denaturation of DNA for 5 minutes at 94 ℃; followed by 35 cycles for 1 min at 94 ℃, primers annealing for 1 min at 48 ℃ and the process of elongation for 2 min at 72 ℃, and the extension of the target region for 5 min at 72 ℃. Amplified PCR product confirmed using 1 % agarose gel by gel electrophoresis.

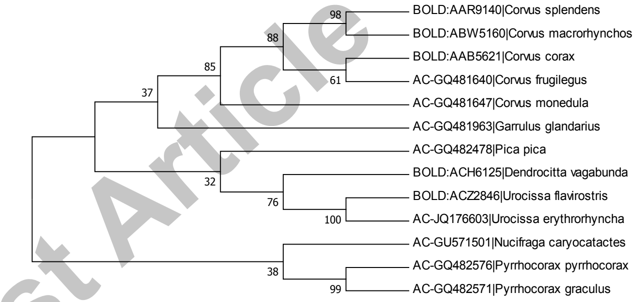

Fig. 2. The bootstrap consensus tree of thirteen nucleotide sequences of taxa of family Corvidae reported from Pakistan inferred by the Neighbor- Joining method using 1000 replicates to show their evolutionary relationships with final dataset of total 563 positions by MEGA7. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

Nucleotide sequencing of mitochondrial COI PCR product

Sequencing was carried out by Sanger's method (Sanger *et al*., 1977), edited using Bio Edit software Version 7.0.2 (https://bioedit.software.informer.com/7.0/), converted into FASTA format and after proof reading, BLAST (Basic Local Alignment Search Tool) was applied at NCBI (National Centre for Biotechnology Information) GenBank portal (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for species genetic identification (Table II). Nucleotide sequences were then submitted to BOLD

Table I. Primers and amplicon size (bp) used for amplification of *COI* gene.

Primer	Sequence (5ˊ to 3ˊ)	Target gene	Amplicon size (bp)	Reference
Bird F1	TTCTCCAACCACAAAGACATTGGCAC	CO1	˜650	Ivanova *et al.*, 2006
R1	ACGTGGGAGATAATTCCAAATCCTG			
Falco F	ATCAACAAACCACAAAGACATCGGCAC			
Bird R2	ACTACATGTGAGATGATTCCGAATCCAG			

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(www.boldsystem.org) database and Barcode Index Numbers (BINs) System assigned BINs to them (Ratnasingham and Hebert, 2013) and confirmed their molecular classification by comparing with current zoological nomenclature (Birky Jr, 2013; Fujisawa and Barraclough, 2013; Edwards *et al*., 2016).

Out of 19 species of family Corvidae reported from Pakistan (https://avibase.bsc-eoc.org), COI gene sequence data of 14 species was searched from GenBank at NCBI database; sequences of only 8 species were available and retrieved (Table III). *COI* gene region sequences data of six species of family Corvidae (*Garrulus lanceolatus, Dendrocitta formosae, Nucifraga multipunctata, Corvus corone, Corvus cornix, Corvus ruficollis*) was not available on NCBI database during study time therefore, was not included in this phylogenetic analysis of family Corvidae.

Table II. Accession numbers of *COI* gene sequences of species of family Corvidae with similarity percentage after BLAST analysis of five samples with data available at NCBI.

Species	Similarity %	Accession number
Urocissa flavirostris	96%	JQ176603.1
Dendrocitta vagabunda	99%	KT240059.1
Corvus macrorhynchos	97.98%	AB842677.1
Corvus splendens	99.56%	GU326327.1
Corvus corax	100%	GQ481624.1

Table III. Name of species of family Corvidae with accession numbers retrieved from NCBI database used for phylogenetic analysis.

Common name	Species	Accession number
Eurasian Jay	*Garrulus glandarius*	GQ481963
Red-billed Blue-Magpie	*Urocissa erythrorhyncha*	JQ176603
Eurasian Magpie	*Pica pica*	GQ482478
Eurasian Nutcracker	*Nucifraga caryocatactes*	GU571501
Red-billed Chough	*Pyrrhocorax pyrrhocorax*	GQ482576
Yellow-billed Chough	*Pyrrhocorax graculus*	GQ482571
Eurasian Jackdaw	*Corvus monedula*	GQ481647
Rook	*Corvus frugilegus*	GQ481640

Phylogenetic analysis

Multiple sequence alignment of nucleotide sequences of 13 species, reported from Pakistan, was performed by ClustalW software (Thompson *et al*., 2003). Pair wise distance of evolutionary divergence was calculated by K2 parameter method (Kimura, 1980), and 1000 bootstrap (Felsenstein, 1985) repetitions were used to find the variance in genetic distances between taxon pairs. Phylogenetic tree by the Neighbor-Joining method (Saitou and Nei, 1987) was reconstructed for final dataset of total 563 positions, including some positions (1st, 2nd, 3rd and noncoding codon) while excluding other positions (with gaps and missing data) in this analysis (Wei *et al*., 2014) by MEGA7 (Kumar *et al*., 2016).

RESULTS

MtDNA was successfully extracted from keel muscle tissues of 5 samples and *COI* gene amplified and nucleotide sequenced. BLAST analysis of nucleotide sequences confirmed the taxonomically determined species status of these specimen showing high similarity percentages with the respective sequences retrieved from NCBI database (Table II). The Accession numbers of 8 other species, retrieved from NCBI appear in Table III.

Multiple sequence alignment (MSA) of nucleotide sequences of 13 species of the family Corvidae, followed by estimates on evolutionary divergence, suggested overall average distance 0.141 at 1000 bootstrap repetitions, as calculated by K2 parameter method (Kimura, 1980) on MEGA7 (Kumar *et al*., 2016) for 563 positions in the final dataset. The distances between different species ranged between 0.195±0.026 (SE) between *D. vagabunda* and *P. pyrrhocorax* and 0.044±0.009 (SE) between *C. splendens* and *C. macrorhynchos* (Table IV). The values of genetic distances between different species were supported by phylogenetic tree also.

A bootstrap consensus tree of 13 nucleotide sequences of taxa of family Corvidae reported from Pakistan was inferred by the Neighbor Joining method using 1000 replicates to show their evolutionary relationships with final dataset of total 563 positions by MEGA7. This tree showed a very clear and close relationship among different species of same genus and distinct relationship among species of the different genera of family Corvidae as indicated in Figure 2.

COI gene region sequences of 13 species of family Corvidae were distinctly divided into two main clades. One was comprised of Pyrrhocorax genus with two species *P. pyrrhocorax, P. graculus* and other Nucifraga genus with one species *N. caryocatactes* while the rest of ten species were falling under the second main clade. This second main clade was further divided into two subclades one consisting of Corvus genus with all five available species *C. monedula, C. splendens, C. frugilegus, C. macrorhynchos,* and *C. corax* while, Garrulus genus with one species *G. glandarius*. The second subclade was comprised of three

Table IV. Estimates of evolutionary divergence between sequences of family Corvidae.

	1	2	3	4	5	6	7	8	9	10	11	12	13
Dendrocitta vagabunda		0.025	0.024	0.024	0.020	0.024	0.019	0.022	0.023	0.025	0.022	0.021	0.022
Corvus splendens	**0.188**		0.014	0.009	0.022	0.020	0.021	0.020	0.021	0.021	0.019	0.016	0.014
Corvus corax	**0.174**	**0.086**		0.013	0.017	0.019	0.016	0.019	0.017	0.021	0.018	0.014	0.013
Corvus macrorhynchos	**0.182**	**0.044**	**0.079**		0.019	0.019	0.018	0.018	0.017	0.020	0.019	0.014	0.014
Urocissa flavirostris	**0.146**	**0.162**	**0.134**	**0.132**		0.021	0.009	0.018	0.021	0.023	0.022	0.018	0.019
Garrulus glandarius	**0.188**	**0.151**	**0.135**	**0.132**	**0.151**		0.020	0.020	0.021	0.021	0.018	0.018	0.019
Urocissa erythrorhyncha	**0.143**	**0.157**	**0.122**	**0.127**	**0.046**	**0.142**		0.018	0.020	0.022	0.021	0.018	0.018
Pica pica	**0.173**	**0.155**	**0.139**	**0.128**	**0.132**	**0.145**	**0.140**		0.019	0.021	0.020	0.019	0.021
Nucifraga aryocatact	**0.180**	**0.157**	**0.127**	**0.124**	**0.154**	**0.145**	**0.138**	**0.133**		0.021	0.019	0.018	0.019
Pyrrhocorax pyrrhocorax	**0.195**	**0.171**	**0.178**	**0.163**	**0.193**	**0.172**	**0.183**	**0.173**	**0.156**		0.016	0.020	0.023
Pyrrhocorax graculus	**0.172**	**0.141**	**0.133**	**0.138**	**0.168**	**0.143**	**0.159**	**0.156**	**0.139**	**0.107**		0.019	0.018
Corvus monedula	**0.161**	**0.108**	**0.089**	**0.094**	**0.131**	**0.122**	**0.124**	**0.139**	**0.131**	**0.174**	**0.153**		0.016
Corvus frugilegus	**0.165**	**0.084**	**0.071**	**0.092**	**0.135**	**0.135**	**0.124**	**0.162**	**0.138**	**0.189**	**0.136**	**0.099**	

genera Pica with one species *P. pica*, Urocissa genus, having *U. erythroryncha* and *U. flavirostris* two species and genus Dendrocitta having one species *D. vagabunda* (Fig. 2).

DISCUSSION

According to avibase database retrieved (2019) (https://avibase.bsc-eoc.org), 770 species of birds are reported from Pakistan out of which 45 are globally threatened species. The family Corvidae (crows and jays) belongs to order Passeriformes and suborder Passeri. This family of birds is comprised of 21 genera, with 131 species globally (Miller, 2017), and locally 7 genera with 19 species reported from Pakistan retrieved (2019) from (https://avibase.bsc-eoc.org). Out of these 131 species of Corvidae family, 14 species are near threatened (NT), 8 are vulnerable (VU), 3 are critical (CR), 4 are endangered (EN) and 1 is (EW) globally (Del Hoyo and Collar, 2016). Therefore, it is the need of time to study the geographical status of these species of family Corvidae to help take measure to conserve them.

These 13 species fall in least concern (LC) in the red list category of IUCN (2018). The justification for this is the reason that they show attributes which fall in the criteria of range size (the extent of occurrence is <20,000 km^2), Population trends (>30 % decline over ten years or three generations) and population size (<10,000 mature individuals with a continuing decline estimated to be >10 % in ten years or three generations. All these qualities of the species under study help avoid approaching the thresholds for Vulnerability according to Bird Life International (2021), http://www.birdlife.org).

The morphological characteristics of an organism help in determining the taxonomy and phylogeny of a particular species (Gibbs and Matzkin, 2001). Collected samples were successfully identified on the bases of their morphological features and identified as species of family Corvidae, order Passeriformes (Manegold, 2008). In this study the COI region of mitochondrial genome was proved as rapid and accurate genetic marker capable of identifying unknown sample of birds to species level (Herbert *et al*., 2004) and same was proficient in reconstructing the phylogenetic relationship among bird species (Hebert *et al*., 2004; Hajibabaei *et al*., 2007; Kerr *et al*., 2007, 2009; Borisenko *et al*., 2008; Johnsen *et al*., 2010; Yang and Rannala, 2014).

MEGA7 software successfully determined the evolutionary history and reconstructed phylogenetic tree (Kumar *et al*., 2016) of 13 nucleotide sequences of species of family Corvidae reported from Pakistan. Genetic distances were compared between pairs of species resulted as follows: the pairwise distance between *P. pyrrhocorax* and *D. vagabunda* was observed 0.195 the highest and second highest was 0.193 again between *P. pyrrhocorax* and *U. flavirostris*. On the other hand, the species of genus *Corvus* showed least sequence divergence as highlighted in Table IV, and all five species of this genus showed least pairwise distances. For example, *C. splendens* shows 0.044 pairwise distance with *C. macrorhynchos* and *C. corax* has 0.079 pairwise distances from *C. macrorhynchos*. In the same way *C. monedula* and *C. frugilegus* also showed less pairwise distances in comparison with species of other genera (Ericson, 2005).

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It was expected that the MEGA should keep all species of family Corvidae in the corresponding taxonomic clusters if the genetic alignments and the taxonomy were concordant. In this study most species were discriminated into their respective distinct clusters and all species of one genus were clustered together in one specific clade or sub clade (Fig. 2). Phylogenetic tree is an efficient representation of the genetic distance between different individuals under study. Phylogeny was constructed at 1000 bootstrap replications and p distance was found. The nearest-neighbour distance is the smallest distance to another species within a defined group (Kerr *et al*., 2007). A tree was reconstructed for 13 nucleotide sequences of family Corvidae. According to the phylogenetic analysis of mitochondrial *COI* gene by neibour joining method all species under study were segregated into two main clades. One main clade was comprised of genus *Corvus* whose all five species were closely related and clustered in one large sub clade (Huang *et al*., 2016) along with *Garrulus* genus with one species *G. glandarius* which is treated as conspecific with *G. bispecularis* and *G. leucotis* (http://www.birdlife.org).

The species of genus *Dendrocitta, Urocissa* and *Pica* were part of one main clade but were distinctly related to *Corvus* genus therefore were latter split into another subclade due to large clade distance. These three species belong to different genera but have more association with each other as compared to genus *Corvus* (Ericson *et al*., 2005; Ekman and Ericson, 2006). The *U. flavirostris* belongs to race cucullata which intergrades with nominate in Central Nepal (Madge and Burn, 1994; http://www.birdlife.org). The *D. vagabunda* species also occur in Indomalayan, Palearctic Realms with population decreasing slowly due to habitat loss and hunting (Tracewski *et al*., 2016; http://www.birdlife.org). *Pica pica* Eurasian Magpie species relationships are confused, differ widely or sometimes contradictory to limit species and subspecies concluded by phylogenetic analyses in various studies and advocate numerous splits, while others suggested to treat all taxa in this genus into single species; therefore, further research needed in this regard.

Members of genus *Corvus* were closely related to each other as considered genetically associated and were further segregated into smaller clades based on the similarity in their sequences *C. macrorhynchos* was separated as individual clade and *C. splendens* and *C. corax* were further grouped together as a clade on the basis of similarity in their sequences (Cibois and Pasquet, 1999). The *C. splendens* is suggested based on head and bill shape as an early offshoot and close to the “C. macrorhynchos complex”, and overlaps with the members of this group, but somewhat separated ecologically (http://www.birdlife.org). This analysis shows that *C. splendens* and *C. corax* have more association as compared to *C. macrorhynchos* irrespective of the fact that all belong to the same genus (Li *et al*., 2016; Huang *et al*., 2016; Johnsen, 2017; Iqbal *et al*., 2020).

The *C. macrorhynchos* in older literature was included in *C. coronoides*, and listed as *C. levaillantii*. Variations in vocal and morphometric characters have elevated many taxa to species level recently and there is a strong need for some integrative comprehensive review to fully establish an evidence-based species limits (http://www.birdlife.org). Recent genetic investigations on common raven *C. corax* (Haring *et al*., 2012) suggested three evolutionary paths occupied by this species therefore, any little genetic exchange between new world and old world populations, will allow these two groups to maintain slight, but distinct genetic signatures. A variety of morphological forms may have been especially distinct in old world, 1 million years ago, but today they do not show unique mitochrondrial signatures as intergrade extensively. Genetic diversity is reducing in the New World through similar process, in which mtDNA samples were used to recognize two clades currently (http://www.birdlife.org). Increasing populations of this species led to targeted killing campaigns to benefit crops in western U.S.A. (Marzluff *et al*., 2010) and currently, the human disturbance, removal of woodlands, and intensive farming, may affect the species (Hagemeijer and Blair, 1997). Molecular study on *C. frugilegus* Rook, has indicated that its subspecies form a clade with *C. hawaiiensis*; (Jonsson *et al*., 2012) but further investigation is required (http://www.birdlife.org). *C. monedula* Eurasian Jackdaw showed geographical variation complex, with clinal and individual variations. *C. dauuricus* along with *C. monedula* sometimes separated in Coloeus, but recent studies (Haring *et al*., 2012; Jonsson *et al*., 2012) found them to form a basal clade within present genus. The two species geographically replace each other in L Baikal and N Mongolia but sometimes considered conspecific, with few reports of mixed pairings.

In this study the species *C. macrorhynchos* and *C. splendens* had shown close relationship with each other therefore were grouped in one subclade while *C. frugilegus,* and *C. corax* were grouped in second subclade showing close relationship while C. *monedula* was separately assigned to third subclade indicating distinct relationship with other species of genus Corvus (Haring *et al*., 2012; Li *et al*., 2016).

Second main clade was comprised of Nucifraga and Pyrrhocorax genera in this phylogenetic analysis. The species of *Pyrrhocorax* genus (*P. graculus, P. pyrrhocorax*) were grouped in one clade showing close association among them than Nucifraga which was further

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split into another branch showing distinct relationship with them (Haring *et al*., 2012; Huang *et al*., 2016; Johnsen *et al*., 2017; Iqbal *et al*., 2020). *N. caryocatactes* (Northern nutcracker) is treated as conspecific with *N. hemispila*, and *N. multipunctata* and in Urals, its subspecies intergrades with C. *macrorhynchos*. On the basis of some minor differences its several populations have been separated racially and are best treated as synonyms (http://www.birdlife.org). *P. pyrrhocorax* (red-billed Chough) hybridizes with *P. graculus* (yellow-billed chough) but very rarely reported showing slight geographical variation.

Relationship between different species of family Corvidae resulted in our study were supported by the work of other researchers as citied above but the genetic markers and genetic distance estimation methods were varying some time.

In conclusion, DNA barcoding has been proved an effective tool for identification and inference of evolutionary relationship among species of family Corvidae. MEGA software was equally proved good to confirm the evolutionary relationship among different species. However, multiple genetic markers or whole genome sequences should be used in future studies to effectively infer the phylogenetic relationships among different species of Corvidae.

Statement of conflict of interest

The authors have declared no conflict of interest.

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