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BIOLOGY

Genetic variations and population differentiation in four species of shrikes based on mitochondrial (Mt) DNA cytochrome oxidase B gene

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ABSTRACT: Members of the genus *Lanius*, known as shrikes, are economically important song birds distributed widely in several Asian countries. DNA sequencing, based on a partial segment of the cytochrome oxidase b (Cyt b) gene, was used to determine genetic variations and population genetic structure in four populations of shrike samples from north, east, and west Pakistan. We observed nucleotide diversities in *L. isabellinus* (= 0.02), *L. schach* (= 0.005), *L. vittatus* (= 0.004) and *L. lahtora* (= 0.008) . FST values were significant (P < 0.05) in all species populations apart from *L. isabellinus*, which were non-significant. The negative values of Fu's Fs and Tajima's D showed significant population expansion in three species, *L. schach, L. vittatus* and *L. Lahtora,* whereas they were insignificant in populations of *L. isabellinus*. Fu's Fs and Tajima's D values explain that a strong genetic structure is present among members of *L. isabellinus.* On this basis, it is inferred that strong genetic structuring in *L. isabellinus* populations within this region was largely shaped by a common origin, with secondary influences from geographical factors and isolation.
Key words: *Lanius*, mtDNA, Cyt b, *L. lahtora*, shrikes, Pakistan.

Variações genéticas e diferenciação populacional em quatro espécies de picanços com base no dna mitocondrial (Mt) gene da citocromo oxidase

RESUMO: Membros do gênero *Lanius*, conhecidos como picanços, são pássaros canoros economicamente importantes, amplamente distribuídos em vários países asiáticos. O sequenciamento de DNA, baseado em um segmento parcial do gene citocromo oxidase b (Cyt b), foi usado para determinar as variações genéticas e a estrutura genética da população em quatro populações de amostras de picanço do norte, leste e oeste do Paquistão. As diversidades de nucleotídeos em *L. isabellinus* (= 0,02), *L. schach* (= 0,005), *L. vittatus* (= 0,004) e *L. lahtora* (= 0,008) foram observadas. Os valores de FST foram significativos (P < 0,05) em todas as populações de espécies, exceto L. isabellinus, que não foram significativos. Os valores negativos de Fs de Fu e D de Tajima mostraram expansão populacional significativa em três espécies, *L. schach, L. vittatus e L. Lahtora*, enquanto foram insignificantes em populações de *L. isabellinus*. Os valores Fs de Fu e D de Tajima explicam que uma forte estrutura genética está presente entre os membros de *L. isabellinus*. Com base nisso, infere-se que a forte estruturação genética nas populações de *L. isabellinus* nessa região foi amplamente moldada por uma origem comum, com influências secundárias de fatores geográficos e isolamento.
Palavras-chave: Lanius, mtDNA, Cyt b, *L. lahtora*, picanços, Paquistão.

INTRODUCTION

The presence of genetic diversity within and between natural populations is a basic element of evolutionary theory. Mitochondrial genes such as cytochrome oxidase b (Cyt b) help to demonstrate genetic variation and population differentiation in various species of birds. Cyt b genes have maternal inheritance and a high mutation rate due to base substitution when compared with nuclear genes (QIONGYING et al., 2006), and mitochondrial DNA has proved itself to be an efficient genetic marker in genetic differentiation studies. ERPENBECK et al. (2005) and ROCK et al. (2008) reported useful aspects of mtDNA that helped to investigate phylogeographic groups within a single species (YU et al., 2015). Members of the genus *Lanius* are passerine birds, commonly known as shrikes, that feed on arthropods, especially insects and sometimes vertebrates. They have been widely distributed in open habitats of savannah and steppe in Eurasian, African, and North American regions. Although, their distribution pattern varies from species to species, they also migrate from snowy to warmer climates in search of breeding grounds.

Various taxonomists have discussed the taxonomy of shrikes based on morphology, biology, and phylogeny. Previously, only 12 species of this genus were recognized as valid species (CHEN et al., 1998). To date, the genus *Lanius* consists of 27 species

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(PANOV, 1995), but their taxonomic status is still not clear, as various studies have reported that many species of this group have not yet established their taxonomic status and their status is still disputable (CHEN et al., 1998; HARRIS & FRANKLIN, 2000). MAYER & GREENWAY (1960) suggested that *L. isabellinus,* distributed in East Asia, originated from *L. cristatus,* whereas *L. isabellinus,* distributed in middle parts of Asia and Europe, was grouped with *L. collurio* and *L. fuscatus,* which previously belonged to a melanistic form *L. schach*. In Pakistan, four species of *Lanius*, *L. lahtora*, *L. isabellinus*, *L. schach*, and *L. vittatus,* have been widely distributed on the north and south sides of the Indus River regions. The Indus River acts as a small buffer zone to separate the Oriental and Palearctic biogeographical regions. Based on reports by GONZALEZ et al. (2008) and IMLAY et al. (2017), variable phenotypic traits, i.e. the body colour and plumage of a few species, suggested that there might be more than a single species within species distributed in Pakistan. Although, the status of these four species is least concerned in the Indian subcontinent (RASMUSSEN & ANDERTON, 2012), shrinking *Lanius* populations have also been reported in the Rajasthan and Gujrat areas of the Indian subcontinent due to a shortage of food availability and human interaction (GANPULE, 2016). In addition, as the species are widely distributed, the process of translocation is expected to occur, which may result in the disturbance of their natural genetic structures. In these scenarios, this study determined the genetic variations and population differentiations among four species of *Lanius, L. lahtora*, *L. isabellinus*, *L. schach,* and *L. vittatus,* distributed in Pakistan. This study will provide a preliminary assessment of population genetic diversity patterns of *L. lahtora*, *L. isabellinus*, *L. schach,* and *L. vittatus* in this region for conservation, management, and stock identification.

MATERIALS AND METHODS

Total 135 samples consisting 38 samples of *L. isabellinus* from five populations i.e. Kazakhstan, China, Pakistan, Mongolia and Sweden, 27 samples from six populations of *L. lahtora* i.e. Khantak Khor, Choil, Lodhri Mar, Rooh Wala of Pakistan, Kazakhstan and Mongolia, 44 samples from five populations of *L. schach* i.e. Bait Palowan and Hairo Gharbi, Hazrat Wala, Fazil Pur of Pakistan, India, China, Hong kong, Thailand and Java whereas 26 samples of *L. vittatus* from four populations of Pakistan i.e. Hairo Gharbi, Makwal Khurd, Jhoke Manjhotha and Bohar were randomly collected by using mist net method of birds catching (MACARTHUR & MACARTHUR, 1974) (Figure 1, Table 1) and were identified (GRIMMETT et al., 2008). One down feather was plucked from each

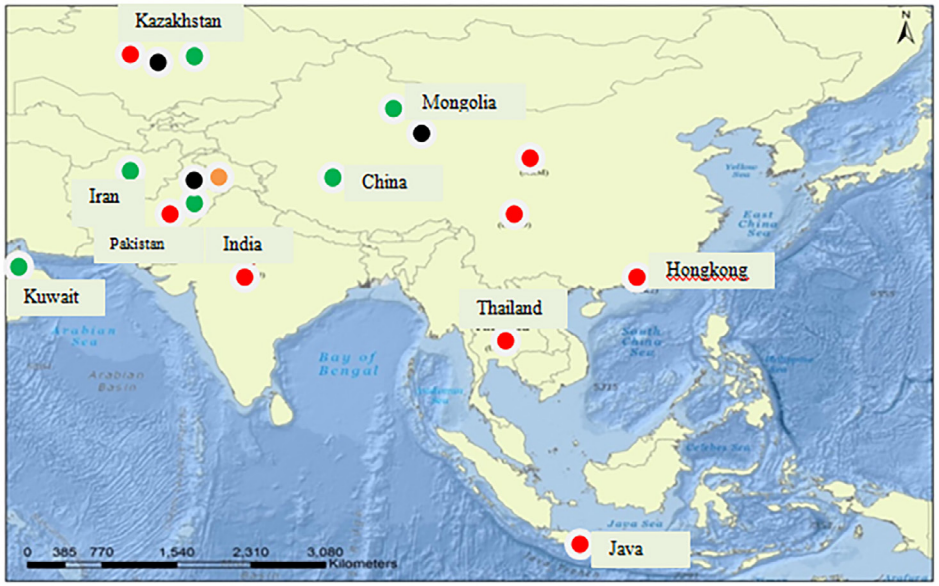

Figure 1 - Approximate sampling locations of *L. schach L. lahtora L. isabellinus and L. vittatus* collected from two different geographical locations of world i.e. palearctic (Kazakhstan, Mongolia, China, Iran, West side of Indus river in Pakistan, Kuwait) and Oriental region (Eastern side of Indus river in Pakistan, India, Thailand, Hongkong, Java).

Table 1 - The Species wise details of taxa (Shrikes, Genus Lanius) collected in this study along with sampling locations from different continents.

Species	----------Specimen No.----------	---N---	--Locality--	Sub area
L. isabellinus	L. isa114011	1	Pakistan	(HG)
L. isabellinus	L. isa107016	1	Pakistan	(LSNP)
L. isabellinus	L. isa U1723	1	Sweden	
L. isabellinus	L.isaU505,L.isaU124,L.isaU2149,L.isaU2151,L.isaU2154,L.isaU30612	6	China	
L. isabellinus	L. isa U369, L. isa U376, L. isa U377	3	China	(Qinghai)
L. isabellinus	L. isa U2115, L. isa U2143, L. isa U2147, L. isa U30627,	4	China	(Xinxiang)
L. isabellinus	L. isa U2140	1	China	(Turpan)
L. isabellinus	L. isa U2144, L. isa U2145, L. isa U2146	3	China	(Tarim)
L. isabellinus	L. isa U2955	1	China	Gobi
L. isabellinus	L. isa UL3, L. isa UL4	2	Kazakhstan	(Chokpak)
L. isabellinus	L.isakareliniU302,L.isakareliniU303,L.isaU305,L.isakareliniU309,	6	Kazakhstan	
	L.isakarelini U310, L. isa karelini U304			
L. isabellinus	L. isa U21, L. isa U38	2	Iran	-
L. isabellinus	L. isa U1005	1	Mongolia	Ulaangom
L. isabellinus	L. isa U2235, L. isa U2236, L. isa U2237	3	Mongolia	(OndorKhaan)
L. isabellinus	Lisa U5135, Lisa U5136, LisaU5137	3	Kuwait	
L. lahtora	Llahtora01, Llahtora02, Llahtora03, Llahtora04, Llahtora05, Llahtora06, Llahtora07, Llahtora12, Llahtora13, Llahtora14	10	Pak	LM
L. lahtora	Llahtora08, Llahtora09, Llahtora10, Llahtora11	4	Pak	CH
L. lahtora	Llahtora15, Llahtora16, Llahtora17, Llahtora18, Llahtora19,Llahtora20	6	Pak	KHK
L. lahtora	Llahtora21	1	Pak	RW
L. lahtora	Palli KZ U53, Palli KZ U300, Palli KZ U301	3	Kazakhstan	
L. lahtora	LpallMong U9523, LpallMong U9524, LpallMong U9787	3	Mongolia	-
L. schach	Lsh02,Lsh04,Lsh05,Lsh07,Lsh08,Lsh09,Lsh10,Lsh13,Lsh16,Lsh18,Lsh	28	Pak	HG
	19,Lsh20,Lsh26,Lsh27, Lsh28,Lsh29,Lsh30,Lsh31,Lsh32,Lsh33,Lsh34,			
	Lsh35,Lsh40,Lsh41,Lsh42,Lsh43,Lsh48,Lsh 49			
L. schach	L sh 03	1	Pak	HWFP
L. schach	L sh 22, L sh 23, L sh 24, L sh 45, L sh 50, L sh 51, L sh 52	7	Pak	BP
L. schach	L sh 36	1	Pak	HS
L. schach	L shach tricolor2660	1	India	-
L. schach	L shachU(KZ)	1	Kazakhstan	-
L. schach	L schach NRM	1	China	-
L. schach	L schach U (Java)	1	Java	-
L. schach	L schach U1263 Thai	1	Thailand	-
L. schach	L schach (U) HK2	1	Hongkong	-
L. schach	L tephronotus U4049	1	C.China	Sichuan
L. vittatus	L vi 01, L vi 02, L vi 03, L vi 04, L vi 05, L vi 06, L vi 07,	10	Pak	HG
	L vi 08, L vi 14, L vitt U3250			
L. vittatus	L vi 09, L vi 10, L vi 11, L vi 12, L vi 15, L vi 19, L vi 20, L vitt U3252	8	Pak	BH
L. vittatus	L vi 13	1	Pak	HS
L. vittatus	L vi 16, L vi 17, L vi 18	3	Pak	JM
L. vittatus	L vi 21, L vi 22, L vitt U3251	3	Pak	MK
L. vittatus	L vi 23	1	Pak	BD

Note: Localities (Pakistan): HG; Hairo Gharbi (30.6558517 o, 70.6863656 o), LSNP; Lal Sohanra National Park Bahawalpur (29.4426o,71.9852o), HS; Hairo Sharqi (30.6667 o, 70.7o), KHK; Khantak khor (30.4552280o,70.6863656o), RW; Rooh Wala (31.278046o,72.311760o), MK; Makwal Khurd (30.5387533o, 70.587533o), BH; Bohar (30.9841019o, 72.0403080o), CH; Choil (31.203965o, 66.8234593o), LM; Lodhri Mar Barthi (29.7638968o, 71.2733451o), JM; Jhoke Manjhotha (29.9638968o, 71.3733451o), BP; Bait Palowan (29.5158308o, 712224181o), BD; Basti Dajal (29.3334o, 70.223o), HWFP; Hazrat Wala Fazil Pur (29.293139o,70.452117o), Kazakhstan, Iran, Mongolia, China, Sweden, Kuwait, Hongkong, Thailand, Java, India).

bird and later the bird was set free in wild. The feathers were properly labelled, preserved in plastic bags and transported to the ornithology lab of the Institute of Pure and Applied Biology, BZU, Multan, Pakistan for further DNA extraction. The samples collected from outside Pakistan were provided and analyzed in Ornithology Lab University of Gothenburg, Sweden.

DNA was extracted by doing slight modification in the salt extraction method (Animal Genomics Laboratory, Liverpool University, UK, 2001). (2mm part of each feather was cut into very small pieces about the size of 1-2cm^3 and was put in prelabeled eppendorf tubes. 600 ul of TNES Urea lysis buffer with 10 ul of 30mg/ml of proteinase k was added in each eppendorf tube and was kept in incubator at 65 °C for 6 hours. The next day 390ul of 5M NaCl was added in each tube and was vortexed for 15 seconds. Each tube was centrifuged for 30 seconds at 13000 rpm and 400 ul of the supernatant was taken in a newly labeled 2ml eppendorf tube. Equal volumes (400 ul) of chilled ethanol was added in each tube and was inverted many times. Each tube was centrifuged for 15 minutes at 13000 rpm to get the pallet of DNA. Supernatant was pipette out in another eppendorf tube and 500 ul of 70% ethanol was added with a repeat of centrifugation step. The supernatant was discarded and each tube was kept slightly inverted on paper towel to dry for 20 minutes. Dry pallets in each tube were eluted with 50ul of sterile distilled water) and was stored at 4 °C for long term storage for further utilization. The forward and reverse primers were designed to amplify maximum 1100 base pairs of Cytochrome Oxidase b gene. PCR was conducted in the BioRad T100 thermal cycler, California, USA by preparing 50 ul of master mixture solution in an eppendorf Master Cycler i.e. 25 microlitres of 1x Red Taq ready mix, 1 µl of 0.5uM of two combinations of primers i.e. forward 5′- TCT TCG CTC TTC ACT TCC TC -3′, reverse 5′-GCT AGC TGG CCA ATG ATG AT -3′, forward 5′-TGG AGC CTC ATT CTT CTT C-3′ and reverse 5′-ATG CTC AGG GGA TTA GA-3′ 3 ul of DNA template, 20 microlitre of deionized H2O for each sample. All PCR tubes were vortexed for 5 seconds and placed in a PCR thermal cycler machine. The parameters for PCR cycles included: 10 minutes of denaturation at 94 °C, 2 minutes at an annealing temperature of 48 °C to 50 °C, 2 minutes at 72 °C, and termination temperature at 72 °C for 5 minutes. The amplification quality and quantity were checked by running a PCR product on Thermo Fisher scientific agarose gel electrophoresis and anew life scientific nanodrop ND-1000 UV/V spectrophotometer. Amplified PCR products were sent for sequencing to the Centre of Applied Molecular Biology Lab, Lahore, Pakistan.

Molecular genetic diversity and phylogeographic analysis

The sequences were trimmed and aligned using MEGA software version 7.0 (TAMURA et al., 2012). Inter-population genetic diversity was calculated by counting the number and diversity of haplotypes, available segregating sites as well as nucleotide diversity, mean pairwise distances, and the P-distances (NEI, 1987) of inter and intra populations were also calculated by using MEGA version 7.0 (TAMURA et al., 2012). We calculated population genetic differentiation and nucleotide variations in DnaSP software version 6.11 (ROZAS et al., 2017). Molecular diversity parameters: R2 test (Ramos-Onsins & Rozas, 2002), Tajima's *D* (TAJIMA, 1989), 'Fu's *FS* neutrality tests (FU, 1997), and mismatch distributions (HARPENDING, 1994) were calculated in DnaSP version 6.10 (ROZAS et al., 2017). The time of expansion ($\tau = 2\ \mu T$) was calculated by the 'mismatch calculator' (SCHENEKAR & WEISS, 2011) when "*u"*is the cumulative substitution rate/ no. of generation and "τ" is the unit of time mutation, whereas Fu's *FS*, Tajima's D, r, and R2 statistical tests were executed to determine the evidence for demographic equilibrium populations. Coalescent simulations, and the null hypothesis were calculated to test the statistical significance for all populations that were demographically at equilibrium.

Statistical Dispersal Vicariance Analysis (S-DIVA analysis version 1.9 Beta) (NYLANDER et al., 2008) was also performed to infer the bio-geographic histories of four populations of the genus *Lanius*. Molecular phylogenies were estimated by Bayesian inference using BEAST v. 1.8.4. The Cyt b Xml files for the BEAST analyses were generated in BEAUti v. 1.8.4. The choice of substitution model was determined based on the Akaike Information Criterion (AKAIKE, 1973) and a hierarchical likelihood ratio test (POSADA & CRANDALL, 1998), both of which were calculated in the J model test (NYLANDER, 2004). All loci were analysed separately (single-locus analyses, SLAs) and in a multi-locus analysis (MLA). The preferred model for single-locus analysis Cyt b was HKY+ I + G. An analysis was run using a fixed clock rate of 2.1% / mya (million years ago); a relaxed log normal clock was used for the clock model with a birth–death incomplete sampling prior. Other priors were used with default values. Four Markov Chain Monte Carlo (MCMC) chains were run for 5×108 generations, sampled every 10,000 generations. The

log file was analysed in Tracer version 1.6 to evaluate whether valid estimates of the posterior distribution of the parameters had been obtained.The first 10% to 25% of the generations were discarded as "burn-in", well after stationary chain likelihood values had been established. Trees were summarised using Tree Annotator version 1.8.4, choosing "Maximum clad credibility tree" (Maximum Likelihood , ML tree) and "Median heights", and displayed in Fig Tree v. 1.4.3.

Molecular phylogenies were estimated by Bayesian inference using Beast version 1.8.4. The Cyt b Xml files for the BEAST analyses were generated in BEAUti version 1.8.4. The choice of substitution model was determined based on the Akaiki Information Criterion (AKAIKE, 1973) and a hierarchical likelihood ratio test (Posada and Crandall, 1998) both were calculated in J Model test (NYLANDER, 2004). All loci were analysed separately (single-locus analyses, SLAs) and in a multi-locus analysis (MLA). The preferred model for single locus analysis Cyt b was HKY+ I +G. Analysis was run using a fixed clock rate of 2.1%/MY (million year) prior, a relaxed lognormal clock was used for the clock model with a birth-death incomplete sampling prior. Other priors were used with default values. Four Markov Chain Monte Carlo (MCMC) chains were run for 5 × 108 generations, sampled every 10000 generation. The log file was analysed in Tracer version 1.6 to evaluate whether valid estimates of the posterior distribution of the parameters had been obtained. The first 10-25% of the generations was discarded as "burn-in", well after stationary of chain likelihood values had been established. Trees were summarized using Tree Annotator version 1.8.4, choosing "Maximum clad credibility tree" (ML tree) and "Median heights" and displayed in Fig Tree version 1.4.3.

RESULTS

Among four *Lanius* species, *L. isabellinus, L. schach*, *L. lahtora* and *L. vittatus*, 55 haplotypes were distinguished, based on 155 variable sites and 77 parsimony informative sites (Table 2). P distance of 7.5% was observed between *L. isabellinus* and *L. vittatus*, 9.0% between *L. isabellinus and L. schach*, 9.4% between *L. lahtora* and *L. vittatus*, and 8.5% between *L. lahtora* and *L. schach* (Table 3).

The population genetics analyses of the Cyt *b* gene sequence data suggested that there is no significant departure of haplotypes from the null hypotheses. Populations of *L. isabellinus* are at demographic equilibrium as represented by Fu's *FS*, R2, Tajima's D and r values (Table 4). For *L. schach*, *L. lahtora,* and *L. vittatus* r value*,* Fu's *F*S, Tajima's D values are the most significant, rejecting the null hypothesis. The negative values of Fu's Fs, and Tajima's D show significant population expansion in three species, *L. schach*, *L. vittatus* and *L. lahtora,* whereas non-significant Fu's Fs and Tajima's D values explain that strong genetic structure is present among members of *L. isabellinus.* Moreover, low values of nucleotide diversity in *L. schach*, *L. vittatus,* and *L. lahtora* highlight the phenomenon of bottle necking in populations of these species (Table 4).

L. lahtora complex

OLSSON et al. (2010) reported that *L. lahtora* is a complex of five species: *L. lahtora, L. excubitor* (*elegans*, *koenigi*, *algeriensis*, *aucheri*), *L. borealis* (*invictus*, *mollis*), *L. meridionalis* (*pallidirostris*) and *L. ludovicianus*. The *L. vittatus* was chosen as the outgroup to construct the phylogenetic tree. A well-supported monophyletic clade topology in the *L. lahtora* complex was observed (Figure 2). Two distinct clades were identified: A1 and A2. Clade A1 consisted of *L. borealis, L. meridionalis, L. ludovicianus, and L. excubitor.* In Clade A1, *L. borealis, L. meridionalis, L. ludovicianus, and L. excubitor* settled in separate small clusters (Figure 2). Clade A2 consisted of all *L. lahtora*. *L. borealis* and *L. meridionalis* are the nearest neighbours with strong posterior probability support of 100%. Populations of *L. excubitor* settled in a separate cluster, with

Table 2 - Sigleton, Parsimony informative sites and variable sites in four *Lanius* species; *L. isabellinus, L. lahtora*, *L. schach* and *L. vittatus.*

Sr.	----------Sp. Names----------	----------Singleton----------	---Parsimony informative---	--------Variable sites--------
1	*L. vittatus*	20	7	27
2	*L. isabellinus*	4	36	40
3	*L. lahtora*	22	12	34
4	*L. schach*	32	22	54

Table 3 - Uncorrected mean P-distances between groups in four *Lanius* species; *L. isabellinus, L. lahtora*, *L. schach* and *L. vittatus.*

Sr.	------Sp. Names------	------*L. vittatus*------	-----*L. isabellinus*-----	-------*L. lahtora*-------	-------*L. schach*-------
1	*L. vittatus*				
2	*L. isabellinus*	0.0753			
3	*L. lahtora*	0.0944	0.0861		
4	*L. schach*	0.0831	0.0908	0.0851	

posterior probability support of 74%. This complex was separated by about 1.95MYA. The historical biogeography of *L. lahtora* was accessed by using likelihood-based statistical dispersal vacariance analysis (TAIKOVA & YU, 2016). About 1.8 mya, *L. lahtora* from Pakistan, L. *pallidirostris* from Mongolia and Kazakhstan (North Palearctic region) separated from the *L. excubitor* complex. Samples of *L. pallidirostris* from Mongolia and Kazakhstan (North Palearctic region) showed small mixing of genes with *L. lahtora* samples collected from the east bank of the Indus River in Pakistan (Oriental region) (red colour in figure 3).

L. schach complex

The *L. schach* complex consisted of the species *L. schach* and *L. tephronotus*. The maximum likelihood tree (Figure 4) of the *L. schach* complex clearly shows a monophyletic lineage with two major clades, Clade B1 and Clade B2. All the samples collected from Pakistan consisted of Clade B1, which was subdivided into two clusters, both settled at the upper half of the maximum likelihood (ML) tree representing the eastern populations. *L. vittatus* was used as the outgroup for this taxa. The third cluster consisted of two small subclusters: all the samples of *L. schach* collected from Java and Thailand settled in separate groups supported by strong posterior probability support of 0.93 (Figure 4). The second subcluster consisted of samples collected from China and Hong Kong with strong posterior probability support of 100%. These two subclusters show the populations of *L. schach* from the western side. The historical biogeography of *L. schach* was accessed using likelihood- based statistical dispersal vicariance analysis (TAIKOVA & YU, 2016). This group is separated by about 1.51 million years from the rest of the other species of the genus *Lanius*. *L. schach* showed a remarkable geographical structure with

Table 4 - Descriptive statistics on genetic variation in the four *Lanius* species, *L. isabellinus, L. lahtora*, *L. schach* and *L. vittatus* with significance determined using coalescent simulations; all calculated in DNASP 5.10 (LIBRADO & ROZAS, 2009).

Sr #	Species Names	*N*	Hd	π	θw	*S*	H	Π	FS	R2	Tajima *r* tau Tex 's D p
1	*L.*	3	0.9	0.021	0.018	4	6	16.6	1.997	0.2104	0.7373 0.1301 4.8 116
	isabelli	8	29	19	62	5		79			8 84 3
	nus										
2	*L.*	2	0.8	0.008	0.012	3	1	6.60	-1.843*	0.1169	- 0.0226 1.2 291
	lahtora	7	71	38	71	4	2	2			1.0127 ** 23
											1**
3	*L.*	4	0.8	0.005	0.013	4	2	4.20	-	0.0647	- 0.0103 0.0 11
	schach	4	94	47	90	6	3	9	12.113	**	2.1342 ** 45
									***		4**
4	*L.*	2	0.9	0.004	0.009	2	1	3.60	-5.931*	0.0690	- 0.0510 1.8 433
	vittatus	6	25	61	34	9	4	9		**	1.9127 ** 17 8**

*P < 0.05. **P < 0.01. ***P < 0.001. N – Number of sequences analyzed. Hd – Haplotype diversity (NEI, 1987). π-Nucleotide diversity. S – No. of segregating sites. h– Haplotype – Π Average number of nucleotide differences between sequence pairs (NEI, 1987). FS – Fu's neutrality test (FU, 1997). R2 – Ramos-Onsin & Roza's neutrality test (RAMOS-ONSINS & ROZAS, 2002). D – Tajima's neutrality test (TAJIMA, 1989). r– Raggedness statistic (HARPENDING, 1994) texp – Time since onset of population expansion (kilo years ago), in populations inferred to deviate from demographic equilibrium.

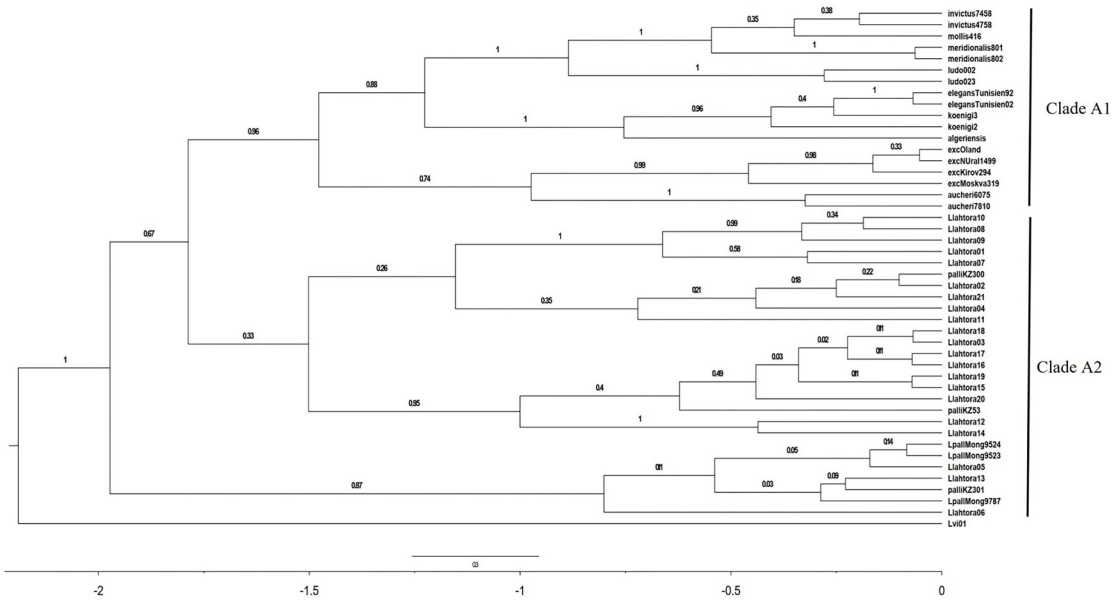

Figure 2 - Relationship among *L. lahtora* complex (OLSSON *et al*., 2010) (*L. excubitor* (*elegans*, *koenigi*, *algeriensis*, *aucheri*), *L. meridionalis* (*pallidirostris*), *L. borealis* (*invictus*, *mollis*), *L. ludovicianus* and its nearest relatives estimated by Bayesian Inference analysis using Cyt-*b* gene sequences.

good support 0.97 between the Clade B1 western population (Pakistan, India and Kazakhstan) (red colour) and the Clade B2 eastern population (China, Hong Kong, Thailand and Java) (Figure 5).

L.vittatus

The phylogenetic analysis of *L. vittatus* showed a well-supported monophyletic clade using the mitochondrial Cyt *b* gene shown in figure 6. *L. schach* was used as the outgroup. In Baysian tree the samples settled in two clads; A and B (Figure 6). The samples of *L. vittatus* were collected from six different geographical locations in Pakistan, but the clustering showed poor genetic structuring and random mixing of genes in all locations. The historical biogeography of *L. vittatus* was accessed by using likelihood-based statistical dispersal vicariance analysis (TAIKOVA & YU, 2016). The *L. vittatus* group is separated by about 0.71MYA. The populations of *L. vittatus* did not show any strongly supported genetic structuring (Figure 7).

L. isabellinus complex

In this *L. isabellinus* complex, samples were represented by *L. isabellinus*, *L. phoenicuroides, L. collurio,* and hybrid (*karelini* and *cobylini*). The samples of *L. isabellinus* were from China (Qinghai, Xinxiang, Tarim, Gobi), Kazakhstan, Western Mongolia (Ulaangom, Ondor Khaan), Bulgaria, South Africa (Kruger National Park), Kenya, and Kuwait. *L. isabellinus* samples from Pakistan were found to genetically overlap with those of samples procured from China (Tarim basin). *L. schach* was used as an outgroup to construct the cladogram, which represents four clades. Clade D1 shows the complex of *L. isabellinus* from north eastern Russia, while Clade D2 is represented by *L. collurio* from Western Europe with 0.99 and 1.00 nodal support, respectively. Clade D3 consists of *L. isabellinus* from the Tarim basin and Clade D4 from the Tarim basin and Pakistan (Figure 8). The historical biogeography of *L. isabellinus* complex was accessed by using likelihood-based statistical dispersal vicariance analysis (TAIKOVA & YU, 2016). The time scale analysis of the *L. isabellinus* complex shows that this species separated some 1.37 mya ago. The populations of *L. isabellinus* showed poor genetic structuring, but there was random mixing of genes. The populations distributed in Sweden showed genetic structuring, whereas there was some mixing of genes between populations from China, Pakistan, Iran, and Kazakhstan (Figure 9).

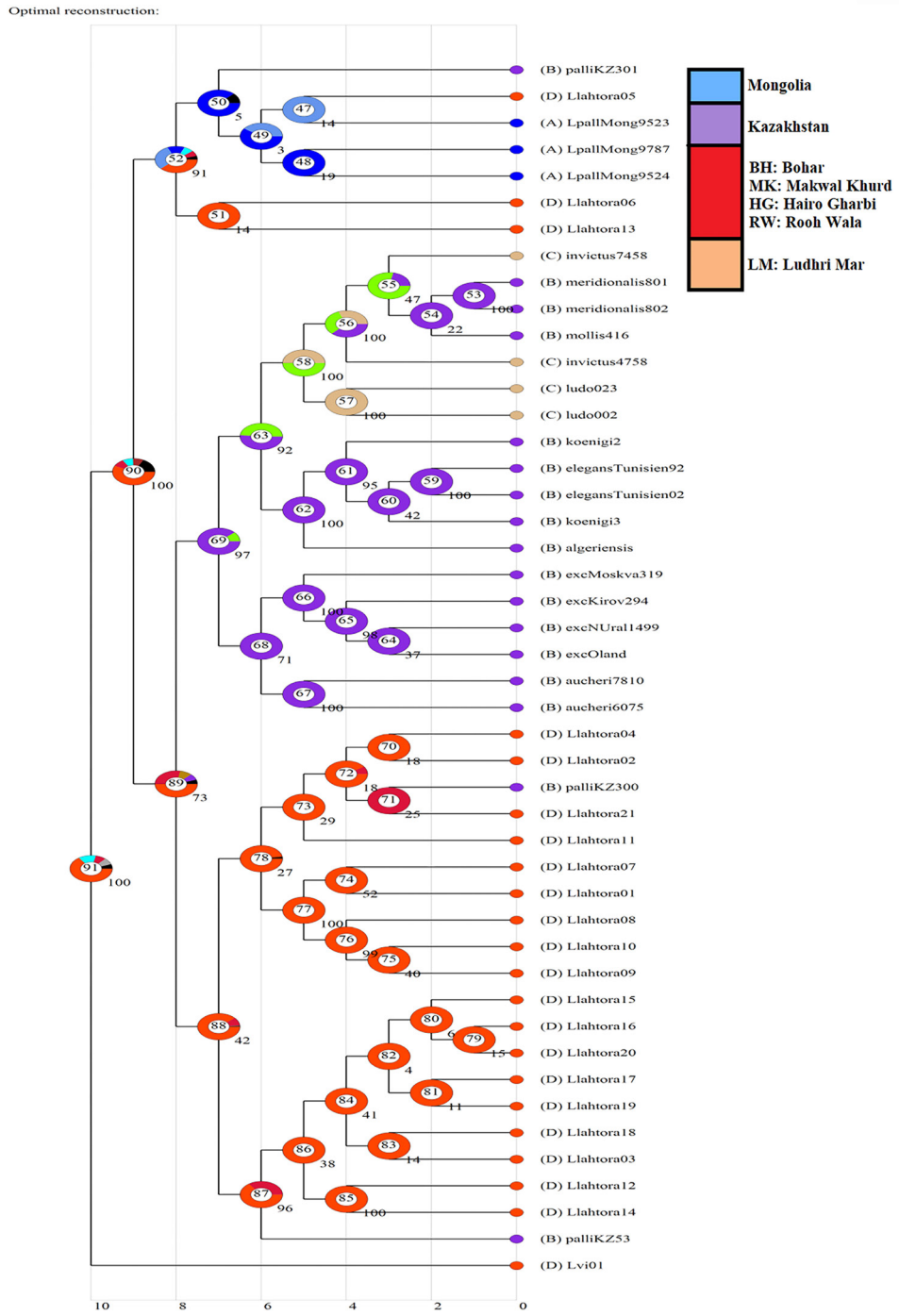

Figure 3 - Results from S-DIVA (Dispersal Vicarience analysis) performed in in RASP v-4.2. Tree topologies were from beast analysis of the *L. lahtora* complex (*L. excubitor* (*elegans*, *koenigi*, *algeriensis*, *aucheri*), *L. borealis* (*invictus*, *mollis*), *L. meridionalis* (*pallidirostris*) and *L. ludovicianus* using Cyt b gene dataset. Coloured circles at the node represents probabilities of the ancestral distributions, and the letters at the terminal branches denote which regions are coded by each colour in the circles. Letters in the parentheses following tip labels indicate the extant distribution of each tip. Annotations on branches refer to posterior probability support for each node (> 70%).

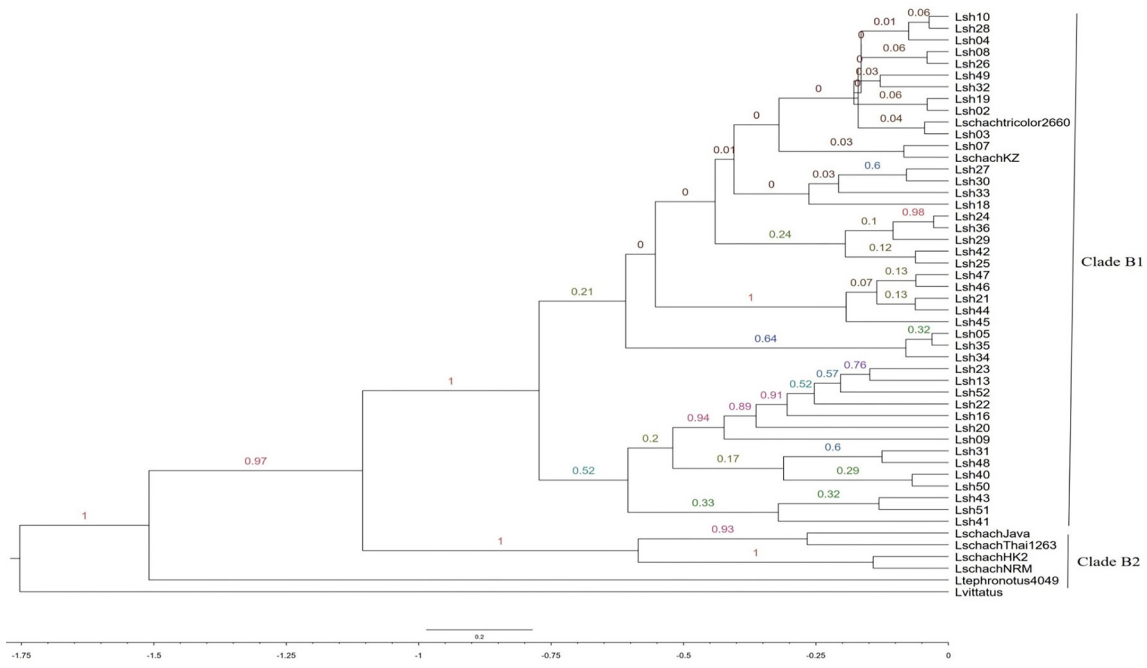

Figure 4 - Relationship among *L. schach* and its nearest relatives estimated by Bayesian analysis Cyt-*b* gene sequences.

We specified different areas for *L. lahtora*, *L. schach*, *L. vittatus,* and *L. isabellinus*, breeding and wintering areas were considered. For these analyses, the trees were generated in the BEAST program. Timescale data analysis revealed that *L. isabellinus,* Clade D, separated from other species of the genus *Lanius* nearly 2.37 mya. *L. vittatus* in the Clade co-originated around 1.9 mya, and both Clades C and D are sister clades to Clades A and B. Clade B, *L. schach,* is reciprocally a sister to Clade A *L. lahtora,* separated by some 1.70 million years. All Clades A, B, C, and D had relatively high posterior probability support of 0.96 and 1, respectively, with each other.

DISCUSSION

Shrikes belonging to the family *Laniidae* are a distinctive group that is insectivorous and consists of small to medium-sized colourful passerine birds. This family includes 31 species belonging to three genera that are widely distributed in Asia including Southeast Asia, Africa including North Africa, Europe, and North America. PANOV (1995) reported 27 species of the genus *Lanius* that are variable in size and morphology. Many species have not established their taxonomic status (HARRIS & FRANKLIN, 2000). The fragment of the Cyt b gene of mitochondrial DNA was amplified and sequenced in the current study to explore genetic diversity and phylogeographic structure in different populations of shrikes and, to some extent, the taxonomy of a few species.

The analysis of Cyt b showed that the P-distances of *L. isabellinus* and other members of the *Lanius* species was > 1% (XIANGYU et al*.,* 2000). Morphologically, most *L. isabellinus* have coloured patches on wings and long tail feathers; they live on dry and thin trees. CHEN et al*.* (1998) reported that members of *L. isabellinus* showed different colorations during moulting and breeding seasons. With respect to nomenclature, PEARSON (2000) proposed the Mongolian form as the nominate race of the isabelline shrike and the *L. speculigerus* its synonym that was proposed as *L. arenarius* for Tarim Basin birds. PANOV (2009) suggested that the type of specimen obtained by MLIKOVSKY & FRAHNERT (2009) belonged to the nominate race of *L. isabellinus,* which used to breed in the Tarim Basin. Nevertheless, WORFOLK (2000) named *arenarius* of the Tarim Basin as a subspecies. The *L. isabellline* complex is distinguished into two groups or forms: one is the '*phoenicuroides* group' (*phoenicuroides* and *speculigerus*) and

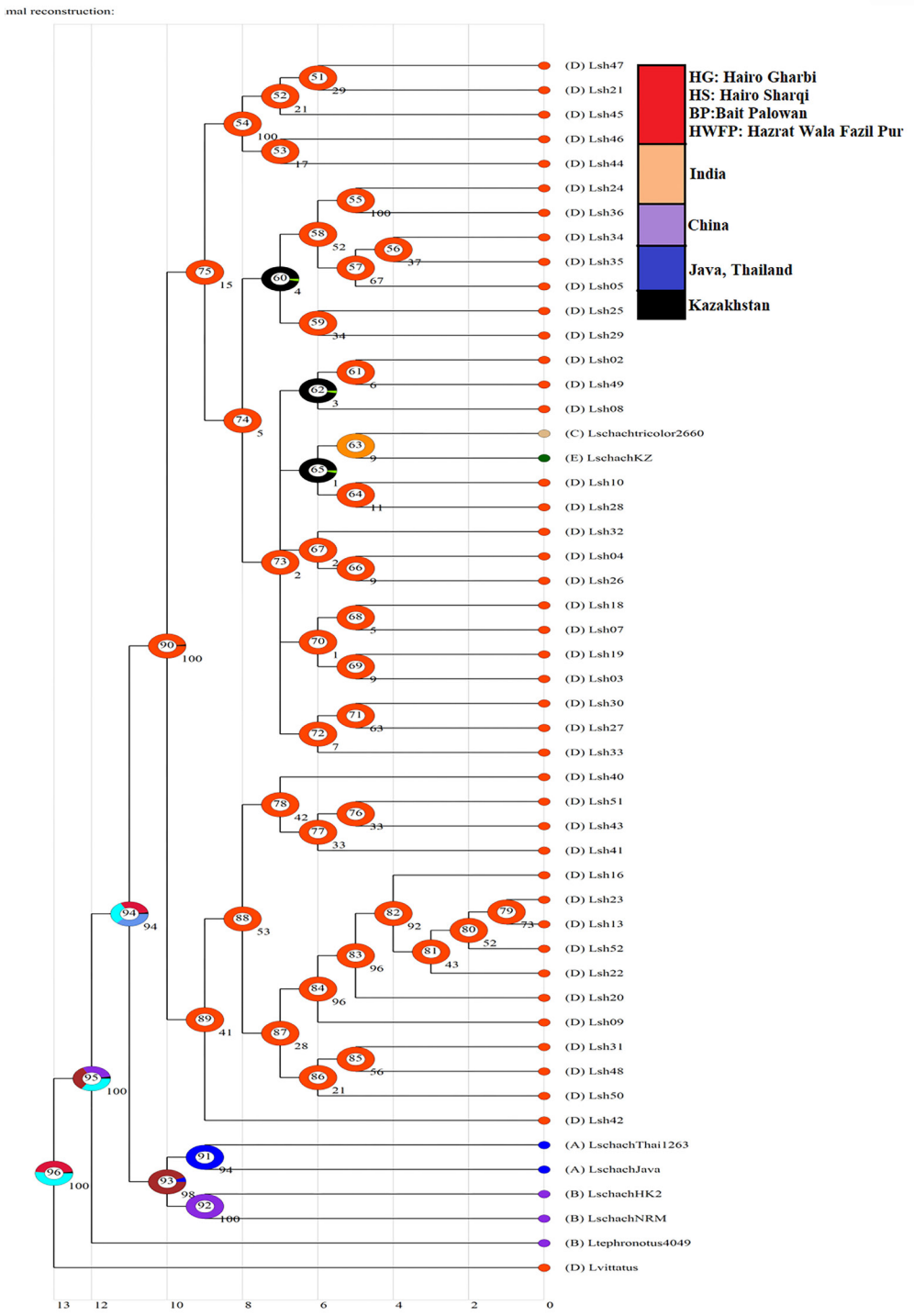

Figure 5 - Results from S-DIVA (Dispersal Vicarience analysis) performed in in RASP v-4.2. Tree topologies were from beast analysis of the *L. schach* using Cyt b gene dataset. Coloured circles at the node represent probabilities of the ancestral distributions, and the letters at the terminal branches denote which regions are coded by each colour in the circles. Letters in the parentheses following tip labels indicate the extant distribution of each tip. Annotations on branches refer to posterior probability support for each node (> 70%). As figure 1.

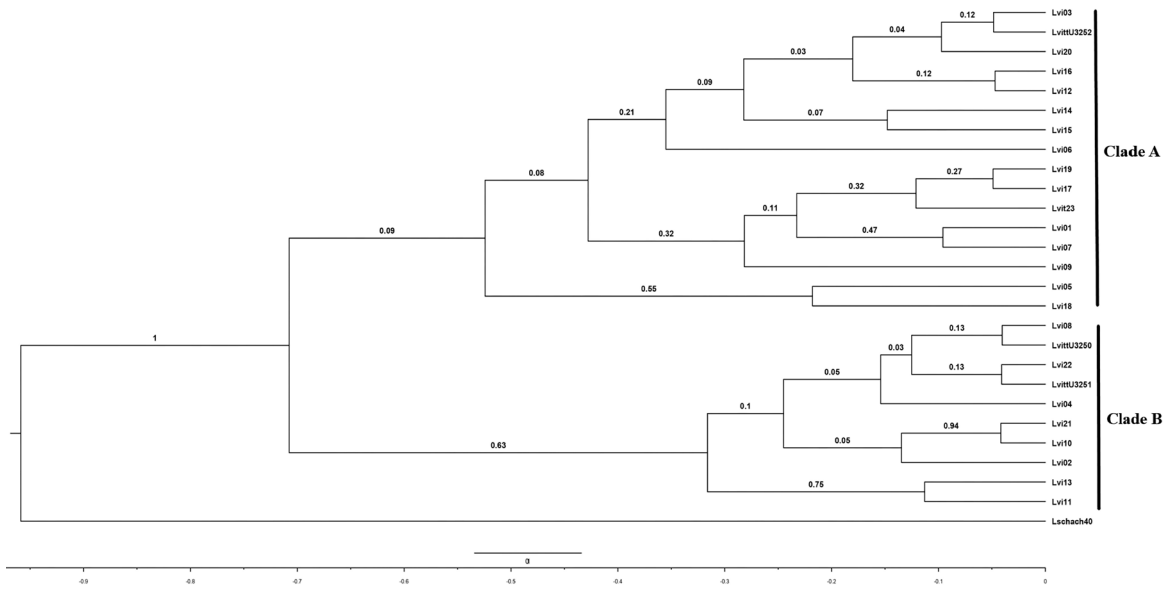

Figure 6 - Relationship among *L. vittatus* and its nearest relatives estimated by Bayesian Analysis Cyt-*b* gene sequences.

the other is the '*isabellinus* group' (*L. isabellinus tsaidamensis*). However, PANOV (2009) considered *L. phoenicuroides* as an independent species and suggested the old nomenclature for the polytypic *L. isabellinus*. There are four hybridization zones represented by WORFOLK (2000): northern Iran, western Kazakhstan, Tian Shan and Tarim Basin, and western Mongolia. Based on different genetic diversity tests, the populations of *L. Isabellinus* are at demographic equilibrium. *L. isabellinus* populations from the North Asian side that included China (Qinghai, Xinxiang, Tarim, Gobi), Kazakhstan, and Western Mongolia (Ulaangom, Ondor Khaan) were reported closer to the eastern side that included Pakistan. RASMUSSEN & ANDERTON (2005) reported that in the *L. isabellinus* species, spring and fall migration have been seen from north and central Afghanistan. During winters, they widely migrate through south Afghanistan, Pakistan, and the northwest of India. Sometimes a few individuals reach Nepal. This random migratory pattern can be the possible reason for low genetic diversity and low mixing of genes among populations of Pakistan and Afghanistan. *L. isabellinus* breeds in West and Central Asia to Mongolia, spending their winters from North East Africa to the Middle East RASMUSSEN (2012) findings supports the current study by settling Kuwait populations in a separate cluster. Moreover, STEPNIEWSKA et al. (2011) also reported similar findings that members of *L. isabellinus* migrate from North West Asia to Africa, which makes the African populations separate from the eastern populations.

The *L. schach* complex consists of the species *L. schach* and *L. tephronotus*. The maximum likelihood tree of the *L. schach* complex clearly shows monophyletic lineage. Based on various genetic diversity tests, the populations of *L. schach* are at demographic equilibrium. Previously, MAYR & GREENWAY (1960) reported that *L. tephronotus* consisted of a subspecies named *L. schach*, while even earlier, BISWAS (1950) reported that *L. schach* was a valid species. High P values of *L. schach* and high nodal support in the ML tree verify it as a separate species. All the samples collected from Pakistan settled in two clusters with high genetic diversity. RASMUSSEN & ANDERTON (2005) reported that in Pakistan, the hybrids *L. schach thronotus* are distributed towards the northeastern and northwestern parts of the country. They are distinguished as pale grey crowned races, whereas *L. schach,* also called rufous back shrikes,are distributed from Afghanistan and the northwestern Himalayas to Sri Lanka. Their colour pattern changes from rufous back to grey back. They are hybrids while migrating from north to south. The populations of Java and

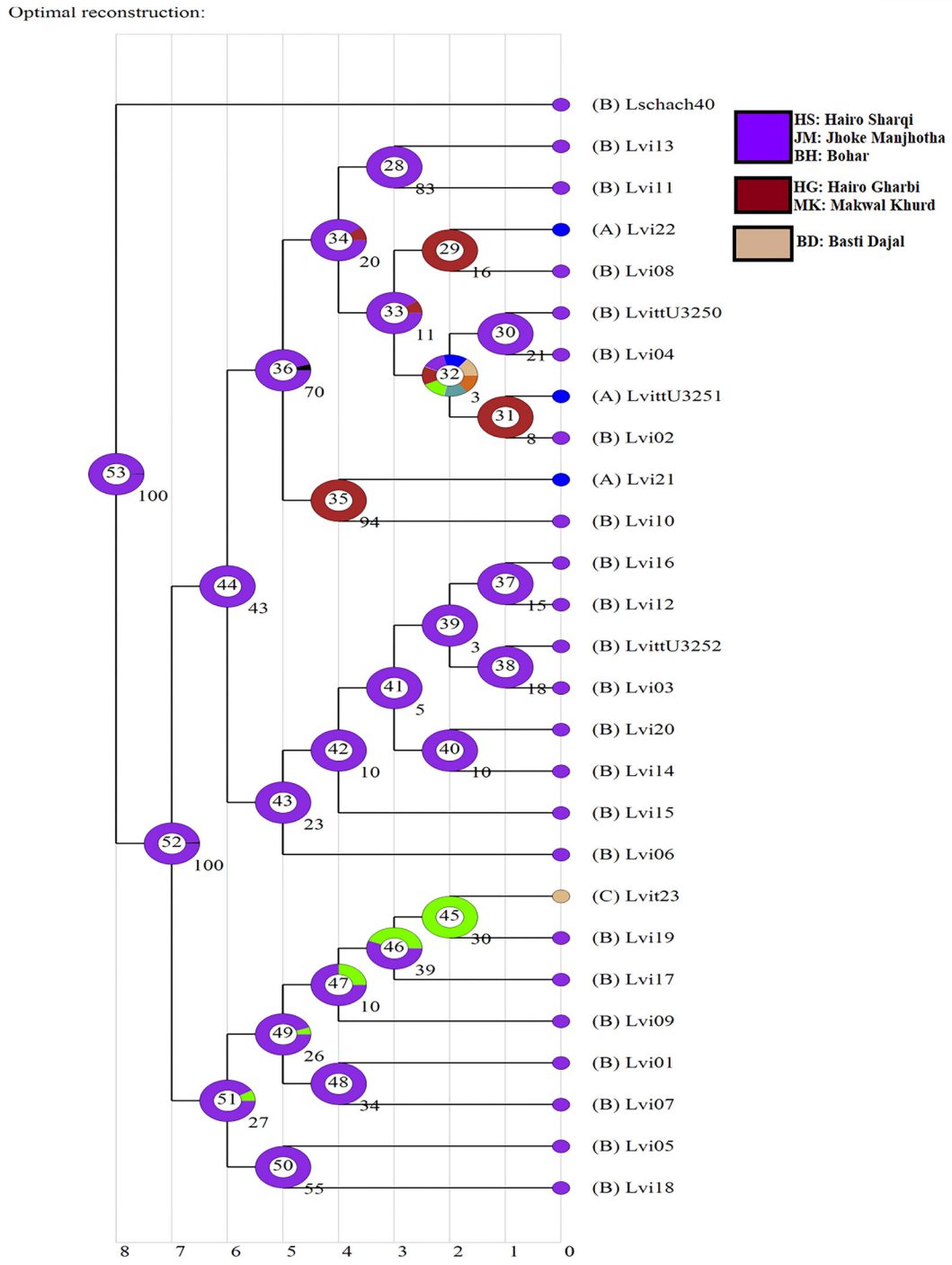

Figure 7 - Results from S-DIVA (Dispersal Vicarience analysis) performed in in RASP v-4.2. Tree topologies were from beast analysis of the *L. vittatus* using Cyt b gene dataset. Coloured circles at the node represent probabilities of the ancestral distributions, and the letters at the terminal branches denote which regions are coded by each colour in the circles. Letters in the parentheses following tip labels indicate the extant distribution of each tip. Annotations on branches refer to posterior probability support for each node (> 70%).

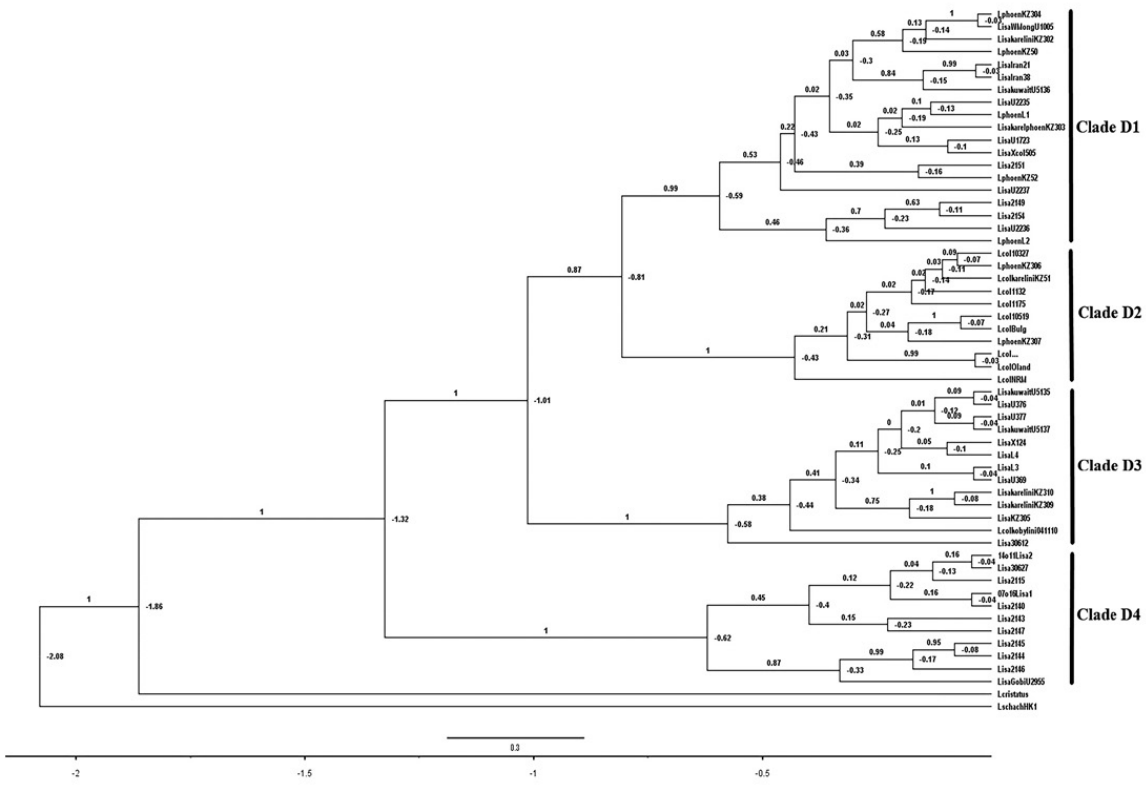

Figure 8 - Phylogenetic relationship among *L. isabellinus* complex and its nearest relatives estimated by Bayesian Analysis Cyt-*b* gene sequences.

Thailand have low genetic differences and represent a separate population group, whereas the populations of western China and Hong Kong are considered a separate group of *L. schach*. CHEN et al. (1998) reported overlapping breeding territories of *L. schach* and *L. tephronotus* from central and south western China, but they did not report any hybrids.

The current study suggested that the populations of Kazakhstan and India are similar to the Pakistan samples, which indicates there may be introgression, or no separation or divergence. Therefore, all samples belong to the same population, though *L. schach* colour looks different and in our analysis, it may be introgressive. Many years ago, *L. tephronotus* was considered a subspecies of *L. schach* (MAYR & GREENWAY, 1960). Our analysis distinguishes it, and *L. tephronotus* is sister to all the other samples of *L. schach*. The current study supports HARRIS & FRANKLIN (2000)'s view that *L. schach* and *L. tephronotus* are two separate species. A study by CHENG (2002) suggested that *L. schach* has colour morphs: a melanistic form (well developed) and not a distinct species, whereas CHEN et al. (1998) reported that the melanistic form of *L. schach* is a valid species and named it *L. fuscatus*. The current study reports that the intraspecific colour variation can be a plumage variation, but also indicate the presence of hybrids in Pakistan.

The phylogenetic analysis of *L. vittatus* showed that this group is monophyletic. The samples *L. vittatus* were from six different geographical locations in Pakistan, but the clustering showed poor genetic structuring and random mixing of genes in all locations. RASMUSSEN & ANDERSON (2012) reported that *L. vittatus* are summer visitors to Afghanistan and Balochistan but are residents of the subcontinent as far as western Bengal, Bhutan and Bangladesh, with the presence of hybrids in the north western zone of Pakistan. The hybrid *L. vittatus nargianus* is pale and large. All the populations from Pakistan had low genetic differentiations that showed low genetic structuring. In the phylogenetic tree, the settlement of all six populations into five small mixed population

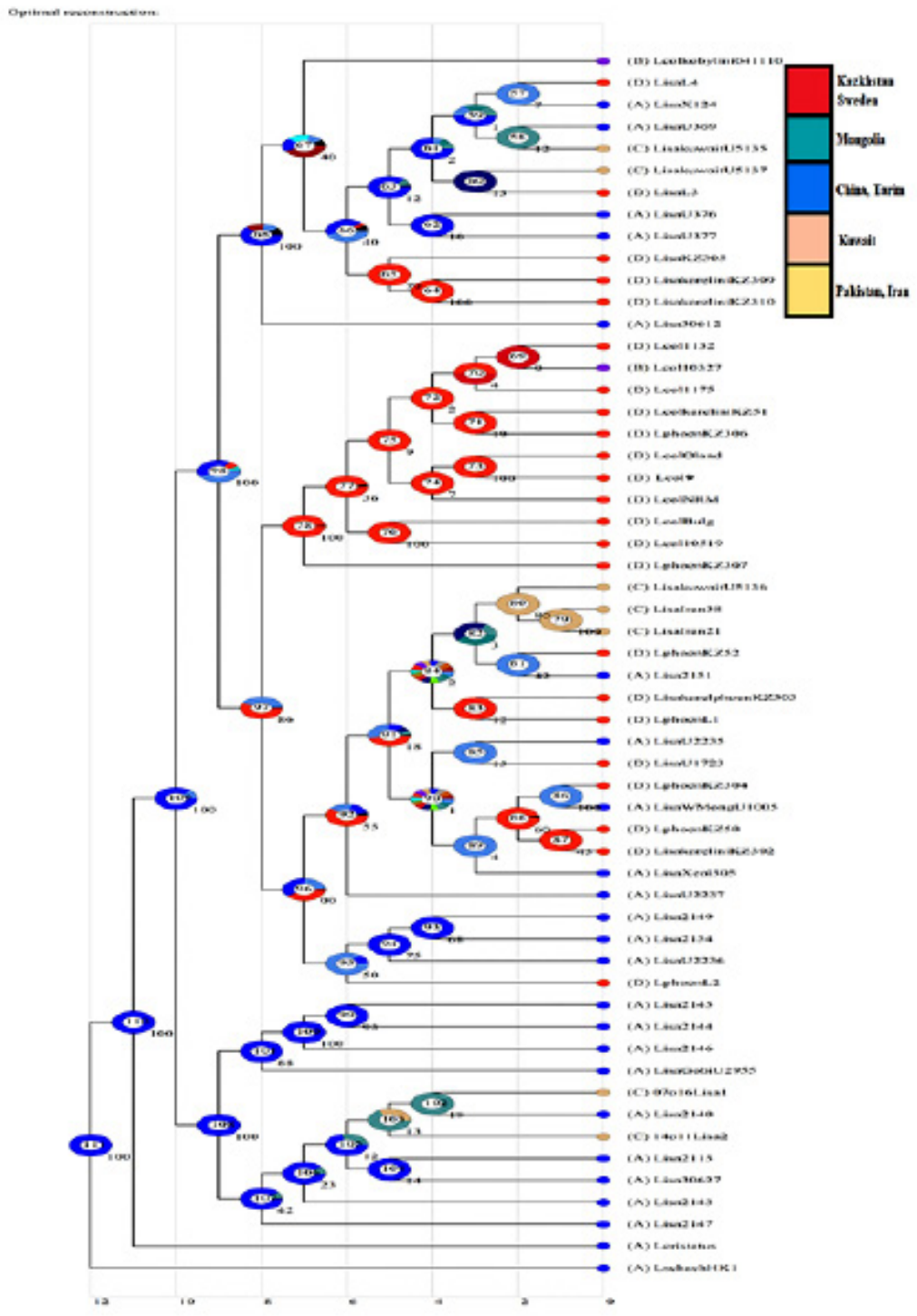

Figure 9 - Results from S-DIVA (Dispersal Vicarience analysis) performed in in RASP v-4.2. Tree topologies were from beast analysis of the *L. isabellinus* using Cyt b gene dataset. Coloured circles at the node represents probabilities of the ancestral distributions, and the letters at the terminal branches denote which regions are coded by each colour in the circles. Letters in the parentheses following tip labels indicate the extant distribution of each tip. Annotations on branches refer to posterior probability support for each node (> 70%).

clusters confirmed the intermixing of genes in all locations and supports the findings of FUCHS et al. (2019), who reported that *L. vittatus* is distributed in China and the Tibetan Plateau, and constituted one group. The population genetic analysis of the current study showed significant negative values that indicate rate of reduction in diversification with the passage of time. Moreover, the geographical distance between the sampling sites is not more than 200 km, which endorses the presence of low genetic diversity among all the populations present in the Punjab province of Pakistan.

L. lahtora complex, as reported by OLSSON et al. (2010), consists of *L. excubitor* (*elegans*, *koenigi*, *algeriensis*, *aucheri*), *L. meridionalis* (*pallidirostris*), *L. borealis* (*invictus*, *mollis*), and *L. ludovicianus*. Our study, based on population genetic analysis, confirmed that the populations of Mongolia and Kazakhstan represent a separate population that may be referred to as the north western population. RASMUSSEN & ANDERSON (2012) reported that in Pakistan, *L. lahtora* have coloured morphs named *pallidirostris* that are distinguished by dark grey, white rumps and whiter wings. Current research has reported strong genetic structuring among European populations that distinguishes them as separate populations, whereas populations from Pakistan are grouped into two small clusters showing two distinct populations and validating the presence of hybrids. This supports the findings of FUCHS et al. (2019), who also reported similar results. Our study revealed that *L. lahtora* and *L. pallidirostris* do not have many differences, at least at the molecular level (Cyt b) locus, but they are reported as coloured morphs of each other.

CONCLUSION

Based on this preliminary analysis, it can be inferred that the genetic structuring of the four species of the genus *Lanius* was largely shaped by a common origin, with secondary influences from geographical factors, food availability, migration, and isolation. Further studies with more efficient markers and larger populations are required to corroborate these findings.

RECOMMENDATIONS

Small sample size of populations is the limitation of this showing to avoid euthenization and scarcity of the birds. It is therefore recommended for further investigation on morphology, population diversity and molecular ecology of Lanius species with ample sample size to explore insight genetic capacity for adaptation, migration pattern and niche requirements of the species. However, current data is beneficial to the long-term conservation and management of this less studied avian group.

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BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

It is hereby clarified that above research work, relevant animal sampling and handling is done by following guidelines approved by the ethics committee, Institute of Zoology, Bahauddin Zakariya University Multan.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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