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Molecular Classification of Pakistani Wild Rose-Ringed Parakeet through Novel Cytochrome B Gene Polymorphism

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ABSTRACT

Rose-ringed Parakeet (*Psittacula krameri*) is sub-divided in 4 sub-species (*P. krameri krameri, P. krameri parvirostris, P. krameri manillensis* and *P. krameri borealis*). Identification of the sub-species is an intricate chore. This study aimed to genetically identify and classify the indigenous wild Rose-ringed Parakeet of Pakistan using *Cytochrome b* (*Cytb*) gene polymorphism. Mitochondrial DNA of 24 unrelated Pakistani wild Ring-rose Parakeets was isolated and utilized for amplification and DNA sequencing of *Cytb* gene. The Phylogenetic analysis of the *Cytb* gene indicated that the Pakistani wild Rose ringed Parakeet was mono-phyletically claded with *P. k. manillensis* with a sequence similarity of 99.37%. Comparative analysis indicated 4 Single Nucleotide Polymorphism (SNPs) sites in the Pakistani wild Rose-ringed parakeet's *Cytb* gene which tags the peculiarity to the Pakistani wild Rose-ringed parakeet. This is the first report that lays bare the molecular classification of Pakistani wild Rose-ringed Parakeet at sub-specie level using novel *Cytb* gene polymorphism.

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Authors' Contributions
ARA, SF conceived and designed the study, executed the experimental work, analyzed the data, and wrote the article. LR and MZH helped in sampling of birds. MT helped in data analysis. SS and MW helped in preparation of manuscript.

Key words
Parakeet, Parrot, Wildlife, Molecular classification, Cytochrome *b*.

INTRODUCTION

Pakistan is a habitat to hundreds of avian species with unique bio-diversity (Awan *et al*., 2013). Among the birds, parrots are considered unique cage-birds due to their bright colors, intelligence, social and affectionate nature and ability to imitate human voices. They attracted the focus of scientists due to their unique features *i.e*. vocal communication, brain evolution, nesting behavior, life-history trait evolution, global patterns of species richness, and evolution of mitochondrial control region duplications. Three hundred and seventy two species of parrots belong to order Psittaciformes and many of these species are the members of family Psittacoidae (Iwaniuk *et al*., 2004).

An indigenous parakeet species of Pakistan is phenotypically similitude to Rose-ringed Parakeet (*Psittacula krameri*). The local name of this parakeet is "Kathy" and this parakeet is least documented. The rose-ringed parakeets (*P. krameri*) are sub-divided into 4 sub-species; *P. k. krameri, P. k. parvirostris, P. k. manillensis* and *P. k. borealis* (Clements, 2007). Mead *et al*. (1993) differentiated the four sub-species on the basis of phenotypic characteristics including wing and tail length, body weight and differences in upper and lower mandible. Most phylogenetic studies (Astuti *et al*., 2006; Schweizer *et al*., 2009; Wright *et al*., 2008) have encompassed only the parrot genera giving a broad perspective of evolutionary trends (Kundu *et al*., 2012). Identification of wild rose-ringed parakeet sub-species is a complex chore because these sub-species share several common features and adaptive radiation wields strong influence on evolution of the parakeet species and sub-species along geographic coordinates. The taxonomic delimitation of taxa has to be carefully reviewed before any conclusion drawn with respect to bio-geographical history or population dynamics (Ribas *et al*., 2007). Dearth of dependable method for identification of wild rose-ringed parakeet at sub-species level led us to analyze Pakistani wild rose-ringed parakeet using *Cytb* gene polymorphism. *Cytb* gene analysis have become important tool in population genetics and in determination of phylogenetic relationship especially among the closely related avian species and sub-species (Allan and Max, 2010). In the present study, we have classified the indigenous rose-ringed parakeet of Pakistan at sub-species level using novel *Cytb* gene polymorphism.

MATERIALS AND METHODS

To explore the polymorphism in *Cytb* gene of Pakistani wild rose-ringed Parakeets; blood samples from 24 unrelated parakeets with typical phenotypic features were selected from different regions of Pakistan. The

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samples were named from PKPrC1 to PKPrC24. Standard organic method was used for isolation of the genomic DNA. Forward (5'-TCCTCCGCACTATCAATCCT-3') and reverse (5'-ATGCAAATAGGAAATACCATTC-3') primers were used for the amplification of *Cytb* genes of Pakistani wild rose-ringed parakeets using PCR. DNA sequencing of the *Cytb* genes was done using big dye terminator cycle sequencing kit and ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA). Sequence data was edited manually using Chromas Ver. 1.45, (http://www.technelysium.com.au/chromas.html) and BioEdit (Hall, 1999). Sequence homology analysis was performed using DNA sequences of *Cytb* gene of the Pakistani wild rose-ringed parakeets and the reported *Cytb* genes of *Psittacula* species available at NCBI Genbank; http://www.ncbi.nlm.nih. gov/genbank/ (Table I). Molecular Evolutionary Genetics Analysis (MEGA, V 5.0,) was used to estimate the evolutionary distances between sequences by computing the nucleotide differences between each pair of sequences. Phylogenetic tree was constructed using TreeView software. The rooted tree was formed by placing a root in the middle of the longest edge (Desper and Gascuel, 2004).

RESULTS

The comparative analysis of *Cytb* gene sequence of Pakistani wild rose-ringed parakeet with reported *Cytb* gene sequences indicated 4 novel SNPs sites in Pakistani wild rose-ringed parakeet (Fig. 1). The four SNPs were the substitutions of G to A at 270, T to C at 273, T to C at 276 and C to T at 663 positions in *Cytb* gene sequence of Pakistani wild rose-ringed parakeet (Fig. 1). All nucleotide substitutions were found at third base position of the codons and were not found changing any amino acids. The same pattern of same-sense substitutions were previously reported in Neotropical parrots (Tavares *et al*., 2006).

Psittacula krameri manillensis was found to be the closest homologue of Pakistani wild rose-ringed parakeet according to the sequence similarity analysis which is 99.37%.

Genetic distances were calculated for determination of relationship of Pakistani wild rose-ringed parakeet with other parrot species (Table II). Data demonstrated that, greater the genetic distance, more distinct will be the species. A value of 0.015 indicated closely related homologues *e.g*. Pakistani wild rose-ringed parakeet and *P. k. manillensis* share 99.37% identity. Similarly, genetic distance of 0.088 between Pakistani wild rose-ringed parakeet and *P. eupatria* corresponds to the sequence identity of 92.74% (Table II). Whereas a higher value (0.101) indicated that Pakistani wild rose-ringed parakeet was more genetically distinct from *P. alexandri* as these two share 91.1% sequence identity.

Table I.- The sequences utilized for the construction of phylogenetic tree.

Species	Accession No.	Species	Accession No.
Brotogeris pyrrhopterus	FJ652864	*Pionus sordidus ponsi*	EF517634
Chalcopsitta scintillate	AB177955	*Pionussordidus corallines*	EF517628
Eclectus roratus	AB177965	*Pionus sordidu smindoensis*	EF517629
Eclectus roratus roratus	AY220101	*Pionus tumultuo susseniloides*	EF517615
Lorius garrulous	AB177951	*Psittacula calthorpae*	GQ996512
Psittacula alexandri	AB177958	*Psittacula columboides*	AY220108
Psittacula cyanocephala	AY220109	*Psittacula echo*	AY220113
Pionus chalcopterus	EF517621	*Psittacula eupatria*	AY220115
Pionus cyanescens	EF517619	*Psittacula finschii*	GQ996510
Psittacula longicauda longicauda	GQ996509	*Pseudeos fuscata*	AB177964
Pionus maximiliani lacerus	EF517626	*Psittacula krameri*	AY220117
Pionus maximilianisiy	EF517624	*Psittacula krameri*	KC876659
Pionus menstruus	AY669403	*Psittacula krameri*	KC876665
Pionus menstruus	AY286207	*Psittacula krameri manillensis*	GQ996517
Pionus menstruus	EF517604	*Psittacula krameri parvirostris*	GQ996497
Pionus menstruus reichenowi	EF517613	*Psittacula roseate*	AY220107
Pionus menstruus rubrigularis	EF517606	*Pionus tumultuosus*	EF517614
Pionus sordidus antelius	EF517632	*Tanygnathussumatranus*	AB177962
Pionus senilis	U89179	*Vini australis*	AF346339

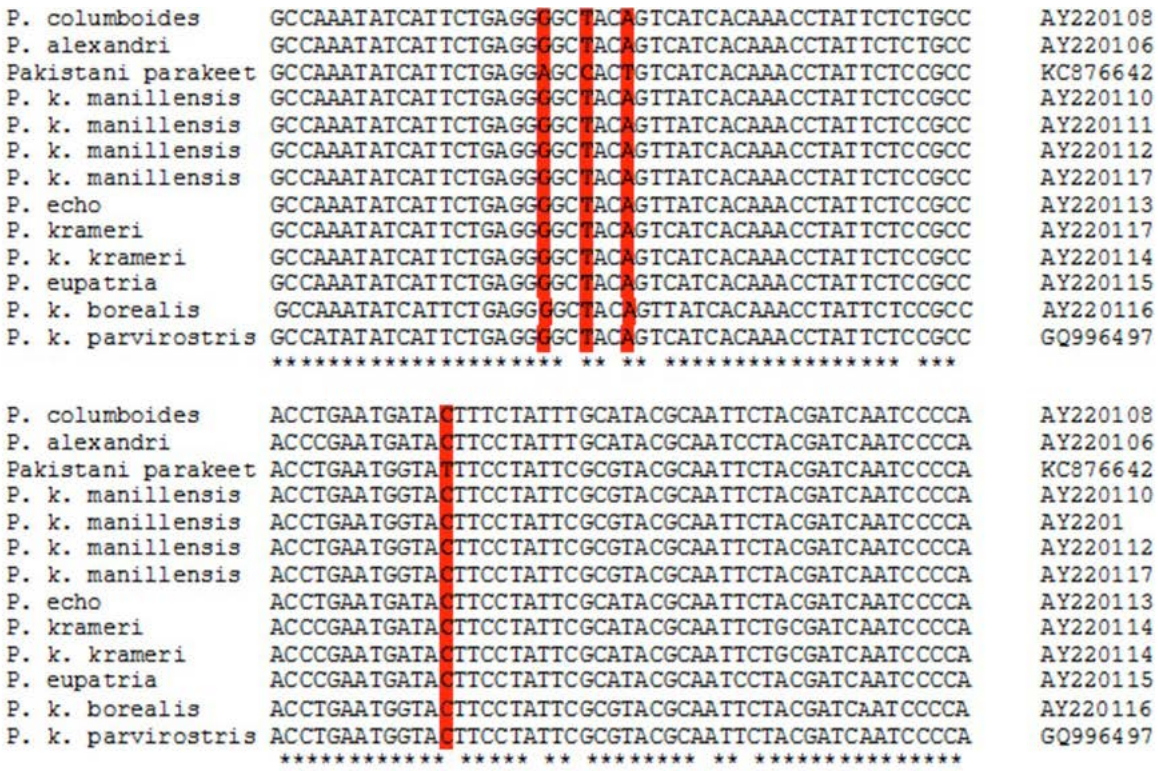

Fig. 1. *Cytb* gene homology sequence analysis of sequence of Pakistani wild rose-ringed parakeet and the reported *Psittacula* species.

Table II.- Genetic distances between the various parrot species.

Psittacula krameri parakeets (KC876642)												
Psittacula alexandri fasciata (GQ996507.1)	0.101											
Psittacula himalayana (AY220102.1)	0.097	0.096										
Psittacula alexandri (AY220105.1)	0.101	0.000	0.096									
Psittacula alexandri (AY220106.1)	0.101	0.000	0.096	0.000								
Psittacula cyanocephala (AY220109.1)	0.091	0.054	0.062	0.054	0.054							
Psittacula cyanocephala cyanocephala (GQ996508.1)	0.096	0.058	0.066	0.058	0.058	0.004						
Psittacula eupatria (AY220115.1)	0.087	0.104	0.101	0.104	0.104	0.099	0.095					
Psittacula eupatria magnirostris (GQ996496.1)	0.092	0.066	0.083	0.066	0.066	0.078	0.074	0.074				
Psittacula krameri manillensis (AY220110.1)	0.015	0.091	0.097	0.091	0.091	0.091	0.096	0.078	0.083			
Psittacula krameri manillensis (AY220111.1)	0.019	0.096	0.102	0.096	0.096	0.096	0.101	0.083	0.088	0.004		
Psittacula krameri borealis (AY220116.1)	0.019	0.096	0.101	0.096	0.096	0.096	0.100	0.074	0.087	0.004	0.007	
Psittacula krameri parvirostris (GQ996497.1)	0.015	0.092	0.097	0.092	0.092	0.092	0.096	0.079	0.083	0.007	0.011	0.011

DISCUSSION

This study describes the classification of indigenous Rose-ringed parakeet of Pakistan at sub-specie level using *Cytb* gene polymorphism. A wider relationship between evolution of morphology and distribution exists within *Psittacula* genus and make it highly speciose (Kundu *et al*., 2012). These spontaneous variations in *Psittacula*

morphology signify the identification of the *Psittaculla* species and sub-species through molecular tools. In the genomic era, phenotypic characters of species cannot represent the true depiction of the taxonomy as classical

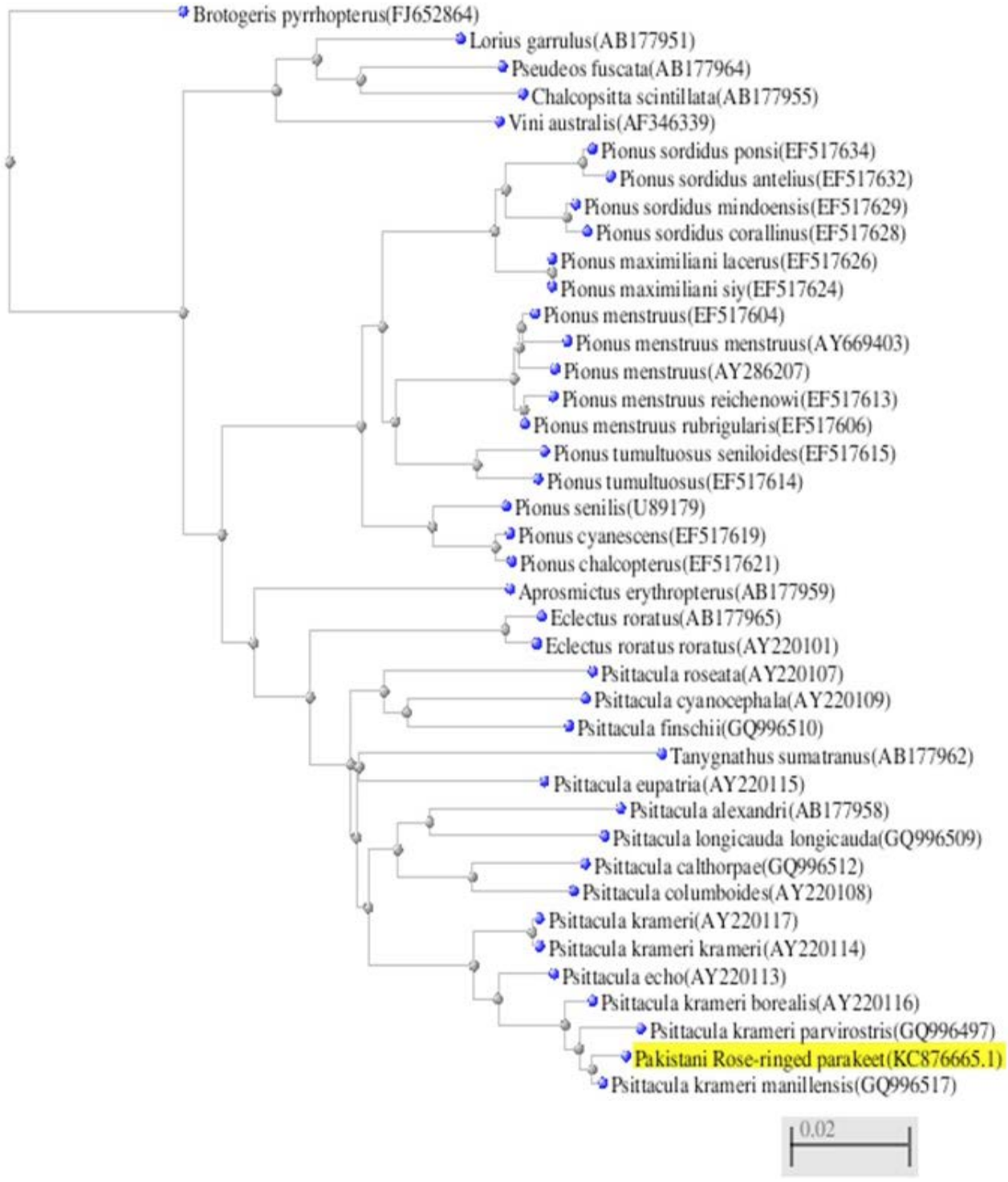

Fig. 2. The phylogeny based on the *Cytb* gene sequences of Pakistani wild rose-ringed Parakeet indicating the phylogenetic and molecular classification of the Pakistani wild rose-ringed parakeet.

identification approaches are not sufficient to elucidate the genetic bio-diversity and arrangement of hierarchical taxonomic and evolutionary tree (Ijaz *et al*., 2017). Mitochondrial genes are the molecular instruments used for genetic characterization and taxonomic investigations of avian families, species, sub-species and populations. Among the mitochondrial genes *Cytb* gene is extensively used for species and sub-species identification and has been considered one of the most useful genes for evolutionary studies. It has been extensively employed to address the diversity questions as it contains both rapidly and slowly evolving codon positions (Saif *et al*., 2012).

Cytb gene fragments of 799 base pairs (bp) from 24 unrelated Pakistani wild rose-ringed parakeet were amplified, sequenced and submitted to National Center of Biotechnology Information (NCBI) GenBank under the accession No. KC876642 to KC876665. The sequence similarity analysis shows the similarity of Pakistani Rose ringed parakeet with *P. k. parvirostris* (98.37%), *P. k. borealis* (98.2%), *P. echo* (96.37%), *P. k. krameri* (96.12%), *Psittacula columboides* and *Psittacula eupatria* (92.74%), *Psittacula alexandri* (91.99%), *Psittacula longicauda* (91.61%), *Psittacula calthorpae* (91.49%) and 90.99% with *Psittacula roseata.*

Further, phylogenetic analysis demonstrated that Indian *P. k. manillensis* was found to be the contiguous taxa of Pakistani wild rose-ringed parakeet, whereas Ethiopian *P. k. parvirostris,* Indo-Pak *P. k. borealis,* Mauritiusal *P. echo* and African *P. k. krameri* were found more distinct from the Pakistani wild rose-ringed parakeet (Fig. 2). *P. k. parvirostris* and *P. echo* were revealed polyphyletic with respect to other members of *P. krameri,* whereas, Kundu *et al*., (2012) reported the monophyletic existence of various *Psittacula* species.

On the basis of sequence homology of *Cytb* gene, the Pakistani wild rose-ringed parakeet can be classified as sub-species of *P. k. manillensis* but the deviation in *Cytb* gene sequence of Pakistani wild rose-ringed parakeets is pointing towards independent evolution of this species as an ecocline in Pakistan. The geographical heterogeneity exerts evolutionary pressure to a specie to adopt the status of an ecocline to that extent which evolves it into sub-species (Groombridge *et al*., 2004). For example mandible size in the African rose-ringed parakeets (*P. k. krameri*) increases westwards across its geographical range in Africa and mandible color of Asian rose-ringed parakeets changes from red (*P. k. borealis*) to black (*P. k. manillensis*) in longitudinal coordinates of South-Asia (Forshaw, 2006). The same pattern of adaptive radiation is observed in Pakistani wild rose-ringed parakeet which has been evolved independently in the indigenous environment.

CONCLUSION

This is the first report of classification of wild rose-ringed parakeet of Pakistan at sub-species level using novel *Cytb* gene polymorphism and this study indicated that the Pakistani wild rose ringed parakeet is mono-phyletically claded with *P. k. manillensis* having a sequence similarity of 99.37%.

Statement of conflict of interest

Authors have declared no conflict of interest.

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