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

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**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*.**

## 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.

## 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.

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## 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 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).

**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.

## 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.

## 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 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.

**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 |

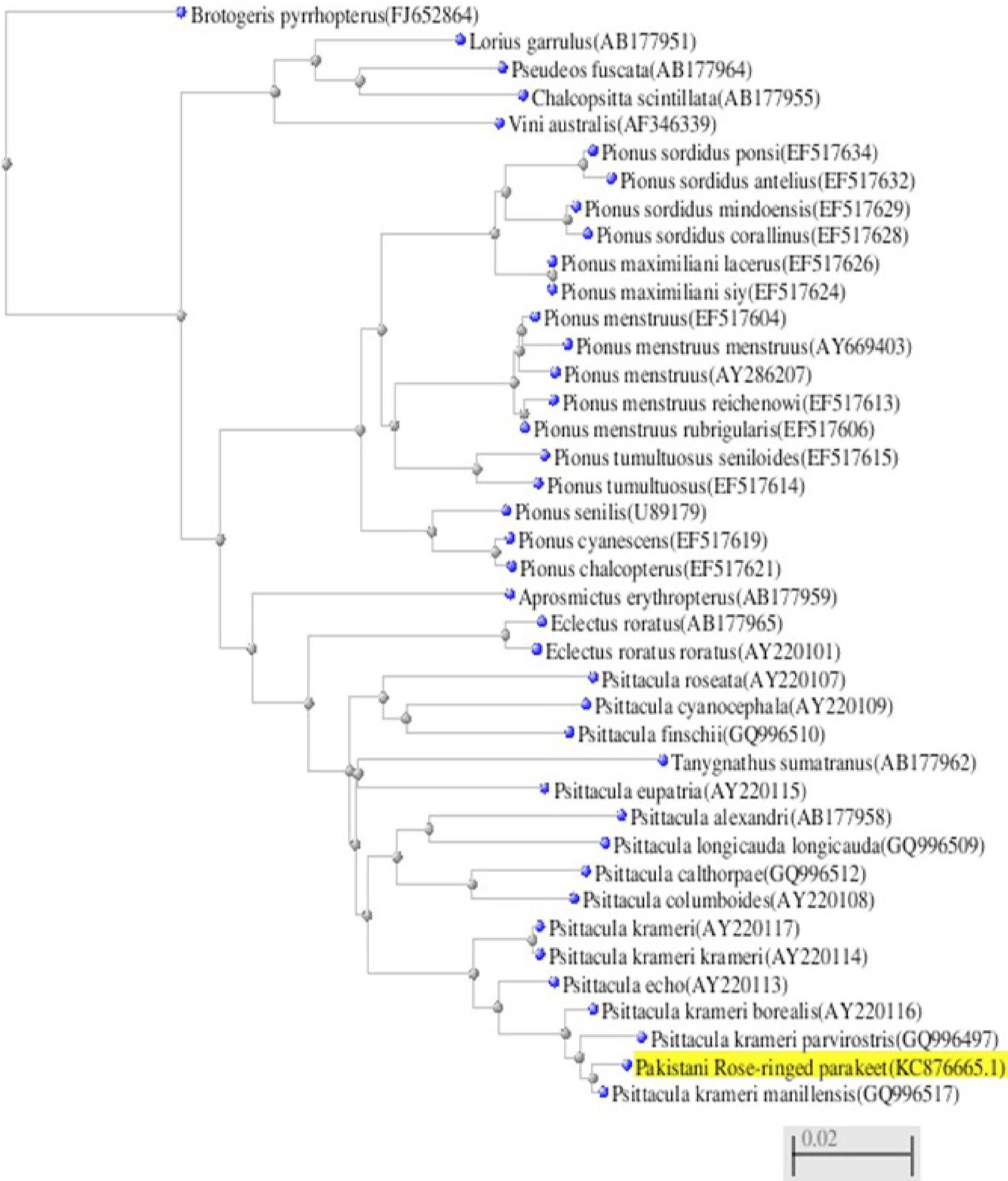


Fig. 2. The phylogeny based on the *Cytb* gene sequences of Pakistani wild rose-ringed Parakeet indicating the phylogenetic and molecular classification of the Pakistani wild rose-ringed parakeet.

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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