EVOLUTIONARY RELATIONSHIPS AMONG THE REGIONAL POPULATIONS OF *TURDOIDES AFFINIS TAPROBANUS* IN SRI LANKA, REVEALED THROUGH MORPHOLOGY AND GENETIC CHARACTERISTICS

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Turdoides affinis (Yellow-billed babbler) is a member of the family Leiothrichidae, which is endemic to Southern India and Sri Lanka. The objective of this project was to study the morphological and genetic characteristics of *Turdoides affinis taprobanus*, the Sri Lankan subspecies and hence to evaluate the intraspecific relationships between the regional populations in Sri Lanka. Field sampling was carried out in Jaffna, Pooneryn, Mannar, Hiyare Sanctuary and Colombo city areas. Each captured bird using mist netting method was sampled for 10 morphometric parameters and plumage colourations focusing on 21 identified areas of the body *in situ*. DNA was isolated from a total of 15 blood samples (3 from each site) drawn from a wing vein of the captured birds (40µl) using QIAGEN DNeasy blood and tissue kit, a 653 bp region of CO1 subunit was PCR amplified using primer combinations BirdF1 (TTCTCCAACCACAAAGACATTGGCAC) and BirdR1 (ACGTGGGAGATAATTCCAAATCCTG) and visualized through agarose gel electrophoresis. The PCR products were sequenced and were aligned using the BioEdit software. Sequences for the Indian species were downloaded from NCBI GenBank and all the sequences were aligned with clustalW in Mega 5.5. Phylogenetic trees were generated using RaxML using rapid bootstrap, for 1000 replicates with the GTR+G model using different outgroup species. Morphologically and plumage-wise, a significant difference exists between the Northern population and the Southern population. Body size, beak and tail lengths were significantly higher in Jaffna population than that of Galle ($P < 0.001$). Even though there's a considerable genetic difference between the Indian subspecies and Sri Lankan species, the molecular phylogenetic analysis implies that the Northern and Southern populations have no significant genetic divergence in mitochondrial CO1 gene, because all the Sri Lankan populations are placed in two clades, each with > 98% bootstrap support, though they showed a significant morphological and plumage difference. In conclusion, this study demonstrates a clear illustration of local adaptation of regional populations of *Turdoides affinis taprobanus* in Sri Lanka leading to isolation by distance and resulting in phenotypic variation.

Key words: Genetic characteristics, morphology, Yellow-billed babbler *

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Introduction

The process of evolution in which a single population separates into two or more distinct, reproductively isolated species due to the impediment of gene flow is known as speciation. Sri Lanka is a continental island on the Indian Plate with a diverse geological and climatic environment. Periodic changes in climate and the sea level changes may have made the geographic barrier between Sri Lanka and the mainland throughout geologic time, allowing local endemics to interact with their mainland relatives.

The ultimate objective of this research project was to study the taxonomic status of *Turdoides affinis taprobanus* (Yellow billed babblers) in Sri Lanka based on the morphological and molecular characteristics. The specific objectives are to characterize the variation of morphology and plumage between regional populations of Yellow-billed babblers, to investigate the phylogenetic affinities among them using nuclear mitochondrial markers.

Methodology

Field sampling

Field sampling was carried out during February to March, 2021 in Jaffna Peninsula, Pooneryn, Mannar, Hiyare Sanctuary at Galle and Colombo city areas under the permit obtained from the Department of Wildlife Conservation (WL/3/2/40/21). Birds were located using visuals, acoustic cues and playback calls and they were captured using mist netting method from 06.00 to 11.00 hrs and 16.00 to 18.00 hrs.

Collection of morphological data

Morphometric variables:

For each captured bird, their culmen length, bill height, bill width, head length, head width, flattened wing length, tail length, eye ring width, first claw length and tarsus length were measured. (Baldwin *et al*. 1931, Seneviratne *et al*. 2012).

Plumage data

The plumage colours of captured birds were evaluated according to Munsell (1976) colour charts, focusing on 21 identified areas of the body; the fore head, crown, nape, mustache (malar) band, face region, supercilium, iris, chin, neck (throat), breast, abdomen, scapulas, back (mental), rump, upper tail covers, tail, belly, primaries, secondaries, middle coverts and upper and lower wing coverts.

Collection of blood samples

A wing vein was pricked using a 25- gauge needle and a sample of 40 µl blood was drawn into a heparinized hematocrit tube attached to a dropper. The collected blood was then transferred to a tube containing 500 µl of SET buffer (EDTA) and the pricked site was cleaned with cotton wool and an antibiotic cream was applied.

DNA extraction

DNA was isolated from a total of 15 collected blood samples (three samples from each site) using QIAGEN DNeasy blood and tissue kit according to the manufacturer's instructions. 5 µl of anticoagulated blood sample was digested at 56°C for 10 minutes in 20 µl of Proteinase k and 220 µl of PBS inside a microcentrifuge tube. Digested samples treated with Buffer AL, were transferred to Deneasy mini spin columns placed in 2 ml collection tubes were subjected to several centrifugation steps. The latter steps of extraction include thorough washing with buffers (AW1 and AW2) and finally eluted in buffer AE.

DNA amplification

The PCR master mixture was prepared according to the procedure given by Seneviratne *et al*., 2012. A 653 bp region of CO1 subunit was amplified using the primer combination BirdF1 and BirdR1. The PCR reaction procedure was as follows: 1X PCR buffer (Invitrogen, Life Technologies, USA) 2.5 µl, 1.5 mM $MgCl_2$ (Invitrogen, Life Technologies, USA), 0.2 mM dNTP mix (Promega, USA) 1 µl, 0.5 mM BirdF1 (TTCTCCAACCACAAAGACATTGGCAC) 1 µl and BirdR1 (ACGTGGGAGATAATTCCAAATCCTG) primers 1 µl, 0.04 units/µl Taq DNA polymerase (Invitrogen, Life Technologies, USA) 0.5 µl and template DNA 3 µl, in a total reaction volume of 25 µl. The thermal cycling profile was 3 min at 94°C, 30 s at 94°C, 30 s at 53°C, 30 s at 72°C and ending with 10 min at 72°C followed by 34 cycles.

DNA visualization

PCR amplified DNA strands were visualized by subjecting to gel electrophoresis under a voltage of 70 V for 1 hour in a 2% agarose gel, which was prepared by adding Ethidium bromide, a light sensitive dye. 2.5 µl of each post pcr product was loaded into a well after mixing with 3 µl of gel loading dye. The loaded samples were subjected to run in 0.5X TAE as running buffer along with 0.5 µl of "1Kb Plus DNA Ladder" mixed with 3 µl of the dye in the first well.

DNA sequencing and sequence analysis

PCR products were sequenced commercially using Sanger sequencing (Macrogen Inc., Seoul, Republic of Korea). Obtained sequences were trimmed and aligned using BioEdit software (Ibis Theraputics, CA). Sequences for Indian species were downloaded from NCBI GenBank and all the sequences were multiple aligned with clustalW (MBL-EBI, Welcome Trust Genome Campus, Cambridge shire) in Mega 5.5. Phylogenetic trees were generated using RaxML (Exelixis Lab, Scientific Computing Group, Heidelberg Institute for Theoretical Studies, Heidelberg), using rapid bootstrap, for 1000 replicates with the GTR+G model using different out group species.

RESULTS AND DISCUSSION

Morphological data analysis

According to the Principal Component Analysis (PCA) for morphological traits, PC1 explained 64.45% of the variation and described size differences between the regional populations (Figure 1). Many traits contributed heavily to PC1 and there was a clear separation of Jaffna population (Northern) and Galle population (Southern) in PC1 ($P < 0.001$). The variation captured by the morphometric PC2 was noticeably lesser than PC1 (15.3%), describing differences in shape. The length of the flat wing and tail of Jaffna regional population are significantly higher than that of Galle ($P < 0.05$), indicating that Jaffna regional population is larger in size.

Plumage data analysis

In the plumage PCA, PC1 captured 56.5% of the variation, correlating well with all characters that tend to differ between regional populations ($P < 0.001$; (Figu.re 2). There is a slight difference in belly, rump and secondary's colour between Galle and Jaffna/Mannar regional populations.

Phylogenetic analysis

Phylogenetic reconstruction generated two major clades each with more than 98% bootstrap support revealing an interesting pattern. The first clade is represented by the Sri Lankan Colombo and Jaffna regional populations. This major clade is comprised of two sub-clades. According to this analysis, there is no diverse difference between the *Turdoides affinis taprobanus* regional populations in Sri Lanka; but, there is a considerable genetic difference between the Indian subspecies and Sri Lankan species since they are placed in 2 different major clades in the phylogenetic tree. (Figure 3).

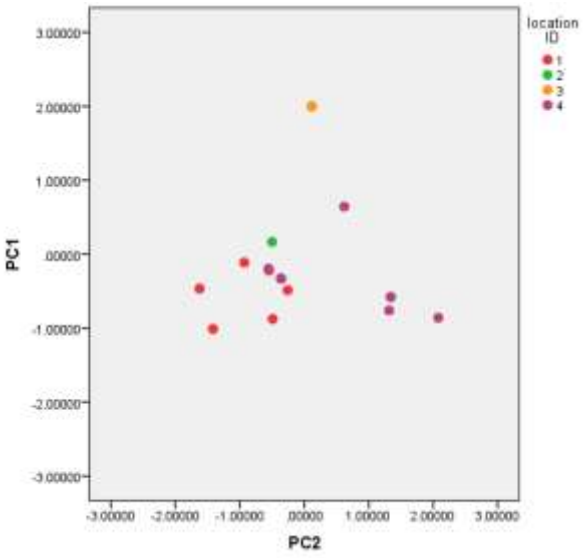

Figure 1: PCA for morphological traits

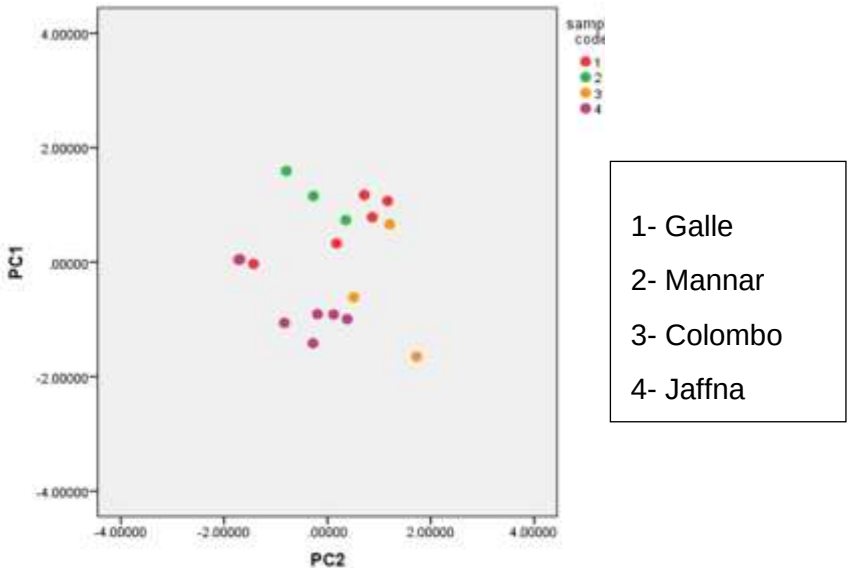

Figure 2: PCA for Plumage traits**.**

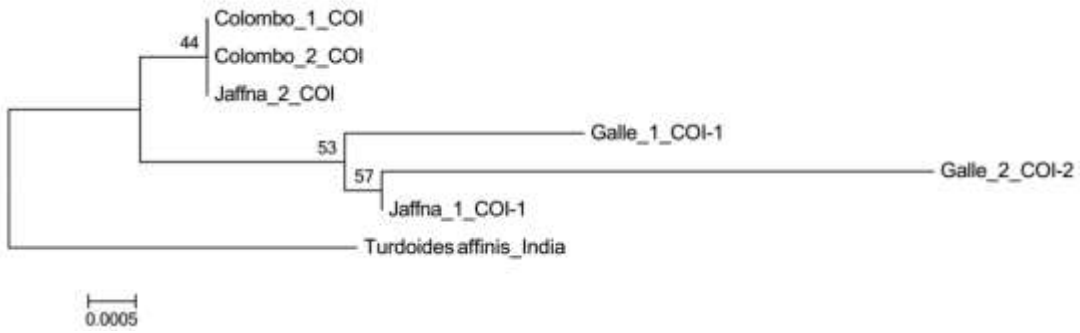

Figure 3: Molecular phylogenetic analysis by maximum likelihood method for COI gene showing regional populations of Yellow-billed babblers clustering with the Indian forms

Table 1: Morphological features measured in regional populations of Yellow- billed babblers (mean ± SEM; with data range in parentheses)

	Field sample (n = 18)			
	Jaffna group (n = 6)	**Mannar group (n = 3)**	**Colombo group (n =3)**	**Galle Group (n =6)**
Morphometric traits				
Head length	29.66± 1.93	30.52±0.97	29.42±0.75	30.42±1.67
Head width	21.82± 0.76	21.26±0.55	21.00±0.72	21.28±0.90
Beak length	34.15±1.63	33.50± 2.07	32.50± 2.00	32.61±2.50
Bill height	8.96± 1.00	8.91± 0.54	8.71± 0.62	8.76±0.85
Bill width	9.11±0.81	8.36±0.81	8.45±0.61	9.16±0.70
Flat-wing length	**138.59± 6.22***	133.60±2.82	135.50±1.78	131.64±2.34
Tarsus length	27.50±1.99	24.14±1.01	23.22±1.05	25.90±2.03
Tail length	**94.70± 1.48***	82.92±3.10	83.62±2.20	86.80±0.83
1st claw length	8.94±0.25	8.96±0.33	8.66±0.53	8.97±0.54

CONCLUSION

The molecular phylogenetic analysis implies that the Northern and Southern populations have no significant genetic divergence in mitochondrial CO1 gene because all the Sri Lankan populations are placed in two clades, each with more than 98% bootstrap support, though they showed a significant morphological and plumage difference. However, there is a considerable genetic difference between the Indian subspecies and Sri Lankan species since they are in two different major clades in the phylogenetic tree. In conclusion, this study demonstrates a clear illustration of local adaptation of regional populations of *Turdoides affinis taprobanus* in Sri Lanka leading to isolation by distance and resulting in phenotypic variation.

Acknowledgements
The Faculty Research Grant 2020 of the Faculty of Natural Sciences, The Open University of Sri Lanka is acknowledged.

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