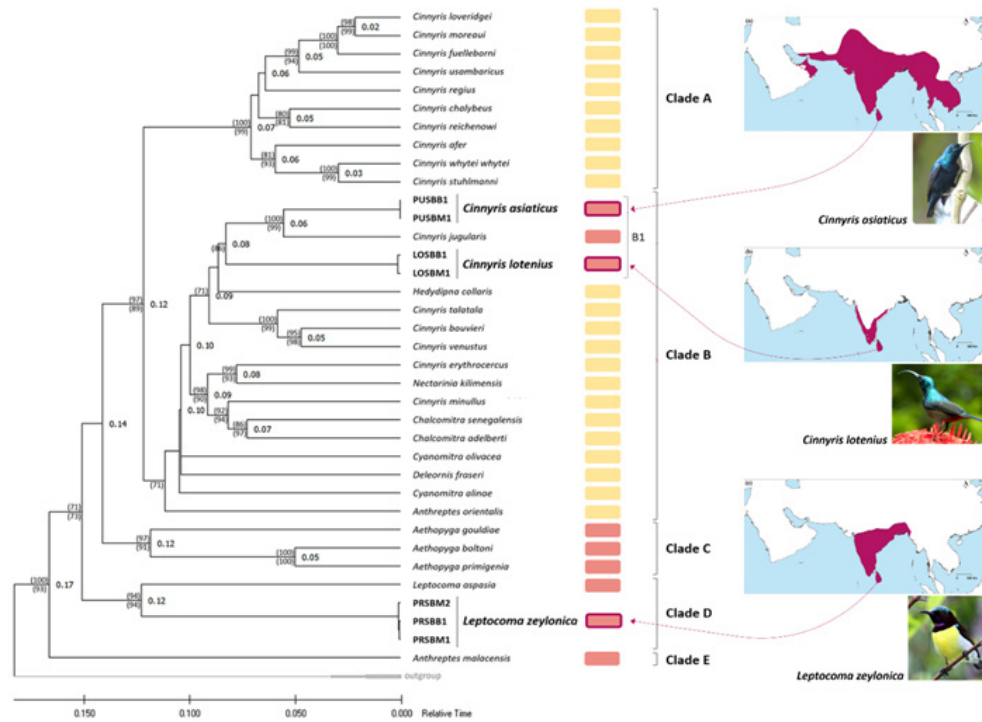


RESEARCH ARTICLE

mtDNA-based phylogeny of sunbirds (Passeriformes: Nectariniidae) found in Sri Lanka

C. D. Wickramasinghe, M. Samaraweera, N. Jayasena and L. G. S. Lokugalappatti*



Highlights

- In Sri Lanka, *Cinnyrus asiaticus* is the most recently speciated sunbird.
- *Leptocoma zeylonica* is evolutionary the oldest sunbird found in Sri Lanka.
- *Cinnyrus asiaticus* and *Cinnyrus lotenius* have a sister-group relationship.
- Genus *Leptocoma* is reciprocally monophyletic.
- Genera *Cinnyrus* and *Anthreptes* are polyphyletic.

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mtDNA-based phylogeny of sunbirds (Passeriformes: Nectariniidae) found in Sri Lanka

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Received: 30/06/2021; Accepted: 01/02/2022

Abstract: Sunbirds are small Passerines in Family Nectariniidae that generally live in the Old World. The evolutionary relationships among most of the sunbird species of this Family are yet to be understood. Sri Lanka is home to three species of sunbirds: Loten's Sunbird (*Cinnyris lotenius*), Purple Sunbird (*Cinnyris asiaticus*), and Purple-rumped Sunbird (*Leptocoma zeylonica*), but no phylogenetic hypothesis exists for them. Therefore, the objective of this study was to explore the molecular phylogenetic relationships of Sri Lankan sunbird species within the Family Nectariniidae using partial mitochondrial ATPase subunit 6 (ATPase 6) and NADH dehydrogenase subunit 3 (ND3) gene sequences. Our data set included the three sunbird species in Sri Lanka from Mannar and Bundala, and 29 reference sequences of sunbirds from Asia, Indian Ocean islands, and Africa obtained from National Centre for Biotechnology Information. Phylogenetic trees were constructed using Maximum Likelihood and Bayesian Inference. The resulting trees exhibited 100% congruent topologies. In the phylogeny, *C. asiaticus* showed a sister-group relationship with *C. lotenius*, and was the most recently speciated sunbird in Sri Lanka. Species of genus *Leptocoma* including Purple-rumped sunbird formed a reciprocally monophyletic clade. Our findings revealed that *L. zeylonica* was evolutionarily the oldest sunbird found in Sri Lanka. Even though the precise region of sunbird origin is unresolved, the resulted phylogenetic tree suggests an Indo-Malayan origin. Further, our results reaffirmed the polyphyly of genera *Cinnyris* and *Anthreptes*. We believe that our findings will generate novel research approaches on evolution of avifauna in Sri Lanka.

Keywords: ATPase 6; *Cinnyris asiaticus*; *Cinnyris lotenius*; *Leptocoma zeylonica*; ND3

INTRODUCTION

Sunbirds are small nectar-feeding pollinators (Brown *et al.*, 1978) in the Family Nectariniidae (Clements *et al.*, 2019), showing strong sexual dimorphism. Over 125 species of sunbirds are currently recognized in 14 genera (Clements *et al.*, 2019), that occur throughout continental Africa across Asia to Australasia (Clements *et al.*, 2019). Due to the complexity of species, taxonomy has been revised from time to time. Recent molecular studies have broadened our understanding of the genetic diversity, population genetics, and phylogeography of some sunbird species in this Family (Warren *et al.*, 2003; Bowie *et al.*, 2004; Smith *et al.*, 2011; Hosner *et al.*, 2013; Bowie *et al.*, 2016; Wang and Liang,

2016), yet the systematics of most are still poorly known.

Sri Lanka is home to 5% of the global avian diversity with more than 34 endemic bird species (Rasmussen and Anderton, 2005; Fernando *et al.*, 2016). The diverse Sri Lankan avifauna includes three species of sunbirds, Loten's Sunbird - *Cinnyris lotenius*, (Linnaeus, 1766), Purple Sunbird - *Cinnyris asiaticus*, (Latham, 1790), and Purple-rumped Sunbird - *Leptocoma zeylonica*, (Linnaeus, 1766; del Hoyo *et al.*, 2016), but none are endemic to the island (Clements *et al.*, 2019). *Cinnyris lotenius* lives in Sri Lanka and southern India (Clements *et al.*, 2019); *C. asiaticus* inhabits a large range from the Arabian Peninsula through the Indian subcontinent and Southeast Asia (Clements *et al.*, 2019), and *L. zeylonica* is endemic to the Indian Subcontinent (Clements *et al.*, 2019). From their subspecies the ones residing in Sri Lanka are *L. z. zeylonica*, *C. l. lotenius*, and *C. a. asiaticus*, with the latter, is also found in India (Clements *et al.*, 2019).

Previous molecular and vocalization studies hypothesized that the origin of sunbirds is in the Indo-Malayan region of Asia (Warren *et al.*, 2003; Iddi, 2008; Bowie *et al.*, 2016). However, none of the previous molecular studies have considered Sri Lankan sunbirds in testing the molecular phylogeny of sunbirds. Therefore, the objective of this study was to determine the phylogenetic relationships of three species of sunbirds that occur in Sri Lanka within the Family Nectariniidae using partial mitochondrial ATPase subunit 6 (ATPase 6) and NADH dehydrogenase subunit 3 (ND3) gene sequences.

MATERIALS AND METHODS

Sample collection

Seven blood samples representing all three sunbird species found in Sri Lanka were collected from Talaimannar, Mannar Vankalai Bird Sanctuary, and Bundala National Park under the collection permit WL/3/2/58/17 issued by the Department of Wildlife Conservation. Blood samples were preserved in Queen's lysis buffer (Warren *et al.*, 2003) at -80 °C and were deposited at the avian tissue collection of the Department of Basic Veterinary Sciences, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya.

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DNA extraction, PCR amplification, and sequencing

Following an additional overnight proteinase K digestion at 55 °C (Bowie *et al.*, 2016), DNA was extracted using MasterPure™ DNA Extraction Kit (Epicentre, Madison, WI) following the manufacturer's standard protocol. Extracted DNA was quantified using NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA) and was then electrophoresed in a 1% agarose gel to ensure the success of DNA extraction and quality of the extracted DNA.

Primer pairs;

A8PWL (5'-CCTGAACCTGACC-ATGAAC-3') and CO3HMH (5'-CATGGGCTGGGGTCTACTATGTG-3') were used to amplify the 609 bp region of mitochondrial ATPase subunit 6 (ATPase 6) gene and L10755 (5'-GACTTCCAATCTTTAAAATCTGG-3') and H11151 (5'-GATTTGTTGAGCCGAAATCAAC-3') to amplify the 351 bp region of mitochondrial NADH subunit 3 (ND3) gene (Warren *et al.*, 2003). PCR conditions are detailed in Table 1. PCR products were electrophoresed in Ethidium bromide-stained 1% agarose gel with a 100/1000 bp ladder (Vivantis Technologies, Malaysia) and subjected to capillary sequencing by Big Dye Terminator Chemistry. All sequences generated anew for this study have been archived in GenBank (Table 2).

Sequence alignment and phylogenetic analysis

Raw sequences were initially subjected to Basic Local Alignment Search Tool (BLAST) in the open-access nucleotide database (GenBank) of the National Centre for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/>) to verify the sequence with the respective Family. In order to examine the relationships between Sri Lankan sunbirds and their continental congeners, we have included 29 GenBank sequences of sunbirds from Asia, the Indian Ocean islands, and Africa (Table 2). Though Hosner *et al.* (2013) used more species in their phylogenetic study, not all those species have both ND3 and ATPase 6 gene sequences. Our dataset included all the species in Family Nectariniidae for which both the ATPase 6 and ND3 gene fragments were available in GenBank at the time of analysis. Sequence from *Pycnonotus barbatus* Desfontaines, 1789

was used as outgroup (Table 2). All raw sequences were checked using Sequencher 5.4.6 (Gene Codes Corp., Ann Arbor, Michigan) for the presence of insertions, deletions, or stop codons. The two sets of sequences; ATPase 6 and ND3, were then edited and assembled separately using the same software program. The resultant assemblies were aligned using the MAFFT sequence alignment server (Katoh and Standley, 2013). The ends of the two alignments were trimmed accordingly (ATPase 6, 603 bp; ND3, 324 bp). Since all genes in mitochondrial DNA are maternally inherited as one unit, ND3 and ATPase 6 gene alignments were concatenated (927 bp) using Geneious Prime (<https://www.geneious.com/>) to implement Maximum likelihood (ML) and Bayesian inference (BI).

General Time Reversible model - Gamma distributed with Invariant Sites (GTR+G+I) was the best fit substitution model selected by JModeltest 2.1.10 (Posada, 2008) according to the Akaike Information Criterion (AIC). Maximum likelihood analysis for the concatenated data set was carried out with 1000 bootstrap replicates using W-IQ-TREE (Trifinopoulos *et al.*, 2016). Bayesian inference was estimated using MrBayes software v 3.2.5 (Ronquist and Huelsenbeck, 2003) with the GTR+G+I model. Four Markov Chains (one cold and three heated) were run two times for 20,000,000 generations, with trees sampled every 10 generations. The log-likelihood values, posterior probabilities, and average deviation of split frequencies were checked to ascertain whether the chains had reached convergence using Tracer v 1.7.1 (Rambaut *et al.*, 2018). The resultant phylogenetic trees were visualized, formatted, and written to Newick file format using FigTree v 1.4.4 software (McAllister *et al.*, 2019). Relative times of divergence were estimated using the Timetree tool implemented in MEGA X software (Mello, 2018).

RESULTS AND DISCUSSION

As this study constituted the first analysis of global relationships among sunbird species in Family Nectariniidae considering Sri Lankan sunbirds, we have included all the Asian species and *Leptocoma aspasia* (Lesson, 1827) in order to compare our results with published information.

Table 1: Primers and thermal cycle conditions used in this study

Gene region	Primer names	Reference	PCR conditions			Number of cycles
			Denaturation	Annealing	Extension	
ATPase 6	A8PWL (forward), CO3HMH (reverse)	Warren <i>et al.</i> , 2003	95 °C for 30 s	52 °C for 30 s	72 °C for 45 s	36
ND3	L10755 (forward), H11151 (reverse)	Warren <i>et al.</i> , 2003	94 °C for 30 s	54 °C for 30 s	72 °C for 45 s	36

Table 2: Accession numbers of sequences obtained from GenBank and sequences generated for this study

Species	Voucher/ Sample Ref	Locality	GenBank Accession Numbers	
			ATP6	ND3
<i>Hedydipna collaris</i> *	GenBank	Burundi	AY234003	AY233987
<i>Deleornis fraseri</i> *	GenBank	Ghana	AY234004	AY233988
<i>Anthreptes malacensis</i>	GenBank	Philippines	AY234005	AY233989
<i>Anthreptes orientalis</i>	GenBank	Kenya	AY234006	AY233990
<i>Chalcomitra adelberti</i> *	GenBank	Ghana	AY234007	AY233991
<i>Cyanomitra alinae</i> *	GenBank	Uganda	AY234008	AY233992
<i>Cinnyris bouvieri</i> *	GenBank	Cameroon	AY234009	AY233993
<i>Cinnyris erythrocercus</i> *	GenBank	Uganda	AY234010	AY233994
<i>Cinnyris jugularis</i> *	GenBank	Australia	AY234011	AY233995
<i>Nectarinia kilimensis</i>	GenBank	Uganda	AY234012	AY233996
<i>Cinnyris minullus</i> *	GenBank	Uganda	AY234013	AY233997
<i>Cyanomitra olivacea</i> *	GenBank	Central African Republic	AY234014	AY233998
<i>Chalcomitra senegalensis</i> *	GenBank	Cameroon	AY234015	AY233999
<i>Cinnyris talatala</i> *	GenBank	South Africa	AY234016	AY234000
<i>Cinnyris venustus</i> *	GenBank	Cameroon	AY234017	AY234001
<i>Cinnyris afer</i>	GenBank	South Africa	KX904376	KX904364
<i>Cinnyris stuhlmanni</i>	GenBank	Uganda	KX904377	KX904365
<i>Cinnyris chalybeus</i>	GenBank	South Africa	KX904378	KX904366
<i>Cinnyris reichenowi</i>	GenBank	Burundi	KX904380	KX904367
<i>Cinnyris regius</i>	GenBank	Burundi	KX904381	KX904368
<i>Cinnyris usambaricus</i>	GenBank	Tanzania	KX904382	KX904370
<i>Cinnyris fuelleborni</i>	GenBank	Tanzania	KX904383	KX904371
<i>Cinnyris moreaui</i>	GenBank	Tanzania	KX904384	KX904372
<i>Cinnyris loveridgei</i>	GenBank	Tanzania	KX904385	KX904373
<i>Cinnyris whytei whytei</i>	GenBank	Malawi	KX904436	KX904474
<i>Aethopyga boltoni</i>	GenBank	Philippines	AY234002	AY233986
<i>Aethopyga primigenia</i>	GenBank	Philippines	KX904375	KX904363
<i>Aethopyga gouldiae</i>	GenBank	China	KP772257**	
<i>Leptocoma aspasia</i>	GenBank	New Guinea	NC_051024**	
<i>Cinnyris lotenius</i>	FVMAS-BVS- LOSBM1	Sri Lanka, Mannar	MT732387	MT732389
	FVMAS-BVS- LOSBB1	Sri Lanka, Bundala	MT732388	MT732390
<i>Cinnyris asiaticus</i>	FVMAS-BVS- PUSBM1	Sri Lanka, Mannar	MT683491	MT683496
	FVMAS-BVS- PUSBB1	Sri Lanka, Bundala	MT683492	MT683497
	FVMAS-BVS- PRSBM1	Sri Lanka, Mannar	MT670429	MT683493
<i>Leptocoma zeylonica</i>	FVMAS-BVS- PRSBM2	Sri Lanka, Talaimannar	MT683489	MT683494
	FVMAS-BVS- PRSBB1	Sri Lanka, Bundala	MT683490	MT683495
<i>Pycnonotus barbatus</i>	GenBank	Cameroon	JX259156	JX259249

* Latest nomenclature by del Hoyo et al. (2016) and Clements et al. (2019), ** Complete mitochondrial genome.

The topologies of ML and BI trees were congruent (Figure 1). The phylogeny of sunbirds was split into five clades (Clades A, B, C, D, and E) supported by high posterior probability values and bootstrap values. In both the ML and BI trees that we obtained, the two genera found in Sri Lanka occupied different phylogenetic positions but were placed together with other Asian species.

Similarly, *Aethopyga primigenia* of the Philippines (Clements *et al.*, 2019) creates the basal lineage in the Maximum likelihood tree of African sunbird taxa constructed by Bowie *et al.* (2016). Further, based on vocalization analyses, Iddi (2008) states that sunbirds might have originated in the Indian Subcontinent.

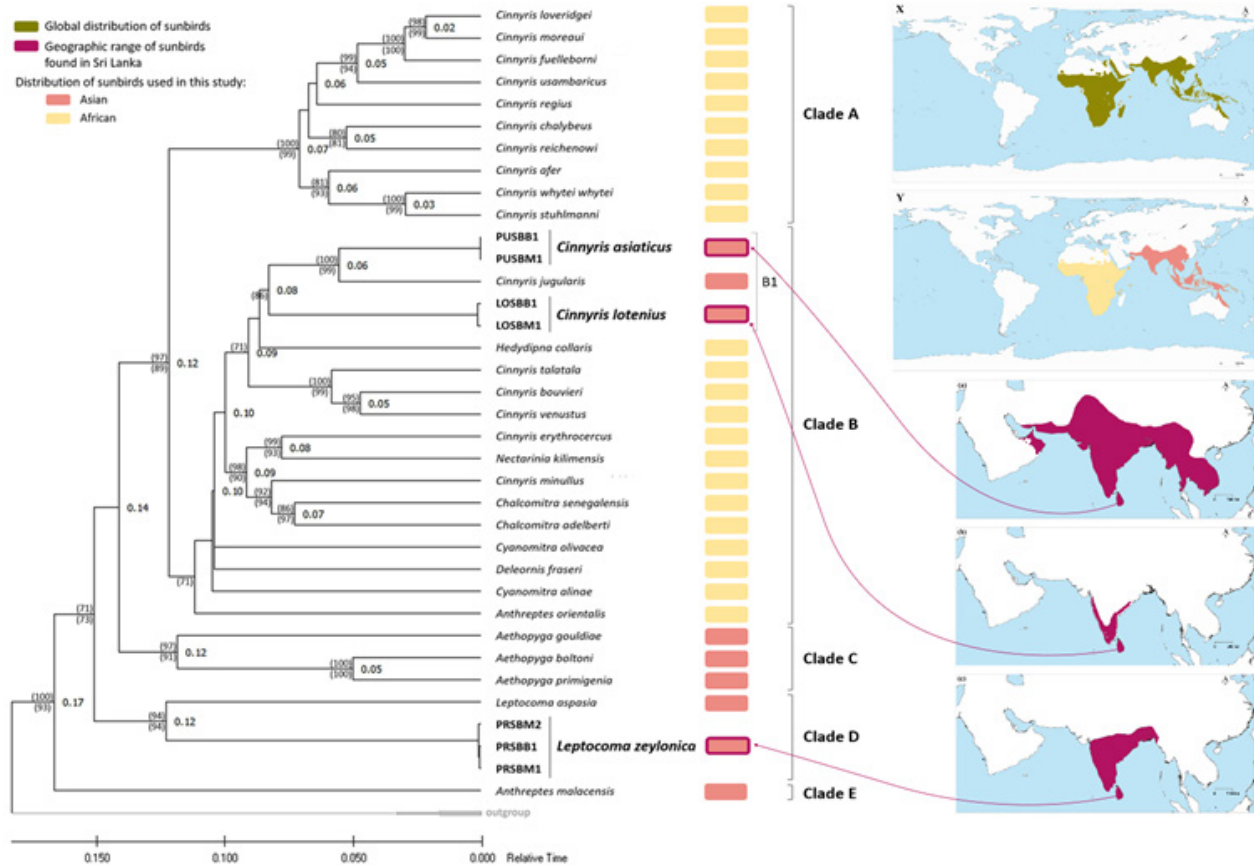


Figure 1: Bayesian inference and ML analyses of family Nectariniidae based on a concatenated dataset of mitochondrial ATPase 6 and ND3 gene sequences. The three sunbird species found in Sri Lanka are indicated in bold. Numbers within brackets at the nodes indicate the BI posterior probability and the ML bootstrap values (BI/ML). The decimal values at the nodes indicate the relative times of divergence. Colors in front of each taxa denote its geographic locality which is illustrated in the map “Y”. Global distribution of sunbirds is shown in map “X”. Geographic range of *Cinnyris asiaticus*, *Cinnyris lotenius* and *Leptocoma zeylonica* are shown in maps (a), (b), and (c) respectively (modified from <https://ebird.org/>).

Our analysis revealed that *Leptocoma* (Clade D) inclusive of Purple-rumped Sunbird *L. zeylonica* and Black Sunbird *L. aspasia* of New Guinea (Clements *et al.*, 2019), formed a distinct clade that is ancestral to all other sunbird species in Family Nectariniidae, excluding the Asian species *Anthreptes malacensis* (Clades E).

Aethopyga boltoni Mearns, 1905, *Aethopyga gouldiae* Vigors, 1831, *Aethopyga primigenia* Hachisuka, 1941, *Anthreptes malacensis* Scopoli, 1786, and *Cinnyris jugularis* (Linnaeus, 1766) are the Asian sunbird species used in previous phylogenetic studies based on both ATPase 6 and ND3 gene sequence data (Warren *et al.*, 2003; Bowie *et al.*, 2016; Wang and Liang, 2016). In the Bayesian analysis by Warren *et al.* (2003), *Aethopyga boltoni* and *Anthreptes malacensis* which inhabit the Philippines and Southeast Asia, respectively (Clements *et al.*, 2019), form the basal lineages for the Indian Ocean and

Clade B was split into sub-clades, one of which (Sub-clade B1) included the Asian species Loten’s Sunbird *C. lotenius*, Purple Sunbird *C. asiaticus*, and Olive-backed Sunbird *C. jugularis*. Others in Clade B were African species. Strong nodal support united *C. asiaticus* and *C. jugularis* as sister taxa (100% posterior probability and 99% bootstrap support), with *C. lotenius* as sister to both of them. Therefore, this study strongly suggests a sister-group relationship between *C. asiaticus* and *C. lotenius*. Clustering of *C. asiaticus* with *C. jugularis* of Southeast Asia to northeastern Australia agreed with findings of a previous study which also showed their sister relationship (Wang and Liang, 2016). The species of genus *Cinnyris* appeared in both Clades A and B, indicating its polyphyletic nature as previously described by Warren *et al.* (2003). Genus *Anthreptes* also showed polyphyly (Clades B and E), while the genus *Leptocoma* appeared to be monophyletic.

It is widely believed that faunal exchange that occurred through the number of dispersal events between mainland India and Sri Lanka at different geological time periods has shaped the present faunal diversity in the island (Meegaskumbura *et al.*, 2002; Bossuyt *et al.*, 2004; Biswas and Pawar, 2006). The three species found in Sri Lanka have various levels of endemism, from near-endemic to widespread species in Indo-Malaya. Unavailability of sequence data from the rest of the range of these three species does not allow us to test the possible means of speciation stated by Wickramasinghe *et al.* (2017), that could explain the faunal exchange between mainland India and Sri Lanka (multiple colonization or independent colonization) or in situ speciation event that took place within Sri Lanka. As the results suggest, all three Sri Lankan sunbird species or some would have evolved in the Indian Peninsula.

CONCLUSION

In Sri Lanka, *L. zeylonica* is evolutionarily the oldest while *C. asiaticus* being the most recently speciated sunbird. *Cinnyris asiaticus* showed a sister-group relationship with *C. lotenius*. Genera *Cinnyris* and *Anthreptes* are polyphyletic, and genus *Leptocoma* is reciprocally monophyletic. The resulted phylogeny suggests an Indo-Malayan origin of sunbirds which is in line with the currently accepted hypothesis. However, this study urges the need for a detailed phylogenetic analysis using nuclear genes and more species in Family Nectariniidae, from a wider geographic range to unravel the evolutionary history of Sri Lankan sunbirds and to explore the precise region of sunbird origin in Asia.

ACKNOWLEDGMENT

The authors acknowledge the financial assistance given by the National Research Council, Sri Lanka (Grant No. NRC/17/094).

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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