Historic black rat invasions into Sri Lanka lead to hybridization forming two sub-lineages in the *Rattus rattus* species complex

P.P.C. Hemamali and S.H. Boyagoda

**Highlights**

- All Sri Lankan black rats fall into *Rattus rattus* complex lineage I of the phylogeny.
- Four subspecies in Sri Lanka form a unique Sri Lankan sub-lineage within the lineage I.
- Subpopulations of *R. r. alexandrinus* and *R. r. kandianus* are in the main sub-lineage within the lineage I.
- There is evidence of introgression of *R. r. kandianus* mitochondrial DNA into other three subspecies.
- There is evidence of introgression of *R. r. alexandrinus* mitochondrial DNA into a subpopulation of *R. r. kandianus*. 
Historic black rat invasions into Sri Lanka lead to hybridization forming two sub-lineages in the *Rattus rattus* species complex

P.P.C. Hemamali\(^1\)\(^2\) and S.H. Boyagoda\(^1\)

\(^1\)Department of Zoology, Faculty of Science, University of Peradeniya, Peradeniya, Sri Lanka.  
\(^2\)Postgraduate Institute of Science, University of Peradeniya, Peradeniya, Sri Lanka.

Received: 19/01/2020; Accepted: 07/11/2020

**Abstract:** In this work, we used two mitochondrial (16S rRNA and cytochrome-b) and one nuclear (Rag 1) gene fragments to analyse phylogeographic history of black rats in Sri Lanka at subspecies level. Of the five subspecies recorded in Sri Lanka three (*Rattus rattus kelaarti, R.r. rufescens, R. r. kandianus*) are believed to have invaded Sri Lanka in prehistoric times and two (*R.r. alexandrinus, R. r. rattus*) in the recent past through trade ships from Europe and India. All subspecies, except *R. r. kandianus*, have restricted distribution with none occurring sympatrically. *Rattus r. kandianus* is widely distributed throughout the country. In the phylogeny, all black rats from Sri Lanka fell into the *Rattus rattus* complex lineage I. But, *R. r. kelaarti, R.r. rufescens* most of *R. r. kandianus* and one individual of *R. r. alexandrinus* formed a unique Sri Lankan sub-lineage within the lineage I. The results suggest hybridization with mitochondrial introgression of the endemic *R. r. kandianus* with all other subspecies separately. Introgression of *R. r. alexandrinus* mitochondrial DNA into a subpopulation of *R. r. kandianus* is responsible for placing few individuals of the latter in the widespread sub-lineage in the Lineage I with *R. r. alexandrinus* and *R. r. rattus* from other parts of the world. The fifth subspecies, *R.r. rattus* which was reported in small numbers from ports in 1930s, has most likely been replaced by *R. r. alexandrinus*.

**Keywords:** subspecies; mitochondrial introgression; murid rodents; phylogeography.

**INTRODUCTION**

Genus *Rattus*, comprised of 66 species, is cosmopolitan in distribution and may probably have originated in mainland Asia (Watts & Baverstock 1994; Chaimanee & Jaeger 2001). The black rat, *R. rattus* (Linnaeus 1758), well known as a pest and a disease carrier, is native to the Indian Peninsula and has been introduced world-wide (Musser & Carleton 2005). There are more than 80 subspecies listed under *R. rattus* (Musser & Carleton 2005) however, the subspecies level diversification is largely ignored by taxonomists (Corbet & Hill 1991; Wilson & Reeder 2005).

In general, subspecies are phenotypically distinct, but not distinct enough to prevent hybridization among them. Subspecies has had a long history in taxonomy, dating back to the 19\(^{th}\) century. Initially called “varieties”, taxonomists placed any distinct natural population that was not sufficiently different to be called a species in a subspecies (Mayr 1982). But, with the advent of molecular studies, the failure to identify subspecies as phylogenetically distinct entities started a long-running controversy of recognizing subspecies as a valid taxonomic unit (Mayr 1982; O’Brien & Mayr 1991; Ball & Avise 1992; Burbrink et al. 2000; Zink 2004). However, since by definition subspecies interbreed in the zone of contact, it may not always be possible to see phylogenetic distinctness (Patten 2010). Studies which included island subspecies have recovered high level of phylogenetic distinctness, explained by divergence in isolation and smaller population size (Hastings & Gavrilets 1999; Gavrilets 2004; Phillimore & Owens 2006). In addition, some studies have proved that subspecies are useful in estimating historic patterns of divergence among populations (O’Brien & Mayr 1991), populations which may become species with time. Many argue that geographic variation of species can be better understood with use of subspecies (Mayr 1982; Barrowclough 1982). Well-defined subspecies may also serve to track migration, dispersal and geographic origins of introduced populations (Zusi 1982).

With respect to *R. rattus*, numerous studies carried out throughout the world have tried to understand migratory patterns, hybridization or mitochondrial introgression and phylogeography of the group (Chinen et al. 2005; Robins et al. 2007; Aplin et al. 2011; Lack et al. 2012; Conroy et al. 2013; Yasuda et al. 2014). However, only few studies even mention subspecies (Robins et al. 2007; Aplin et al. 2011; Robins et al. 2014).

In Sri Lanka there are three species of *Rattus* (*R. rattus, R. norvegicus* and *R. montanus*), of which *R. rattus* is subdivided into 5 subspecies: *R. r. rattus, R. r. alexandrinus, R. r. rufescens, R. r. kandianus* and *R. r. kelaarti*, with the latter two endemic to the country (Phillips 1980). *Rattus r. rattus*, commonly known as the black rat, is a slender, medium-sized rat with a slender tail longer than head and body and large naked ears. Colour is black or blackish grey on dorsal, which pales in to grey black in lower parts. It has been reported in small numbers around dock areas in sea-port towns: Colombo, Galle and Trincomalee. *Rattus r. alexandrinus*, commonly known as Egyptian house-rat or ship rat, can be readily distinguished by its brown or brownish grey dorsal fur and dingy grey lower parts. Size and build is similar to *R. r. rattus*. It is recorded in

*Corresponding Author’s Email: suyamam@pdn.ac.lk*

https://orcid.org/0000-0002-3555-3153

This article is published under the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
moderate numbers in and around the docks and densely populated areas in Colombo and Trincomalee. Both these subspecies are known to have imported in ships from Europe and Indian ports (Phillips 1980). *Rattus r. rufescens* is a medium-sized long-tailed house rat found commonly in the dry zone. It can be easily distinguished from other subspecies by rusty tinge of grey fur of the belly. It has undoubtedly spread from South India to Sri Lanka (Phillips 1980). *Rattus r. kandianus*, is a subspecies endemic to Sri Lanka but found commonly almost everywhere on the island. Its upperparts are generally dark brown and underparts creamy white to light brown. But the colour is very variable among the individuals that occur in different altitudes. Fur texture, though very variable, is usually short, harsh and spiny in lowland populations and long and soft in highland populations. *Rattus r. kelaarti*, commonly known as Ceylon highland rat, is also endemic to Sri Lanka (Phillips 1980). Its dorsal colour is dark olive brown and underparts are greyish white. It can be distinguished from other subspecies by its longer, softer and darker fur and relatively short tail. It is found commonly but confined to jungles of higher hills of central mountains. Except *R. r. kandianus*, which occur sympatrically with all other subspecies, none of the others cross each other’s paths.

*Rattus rattus*, being a species widely distributed in the world, has diversified into a complex, forming four phylogenetic lineages (Aplin et al. 2011). Lineage RrC LI (*Rattus rattus* Complex: Lineage I), mainly centred in western India has a broad distribution throughout Europe, Americas, Africa, Madagascar, Australia and Pacific islands. RrC LI is distributed in Himalayan foothills of Pakistan and Nepal. Lineages RrC LI and RrC LIV are found in Indo Malayan region. Aplin et al. (2011) has included a single sequence of *R. r. kandianus* from highlands of Sri Lanka in their phylogeny, which fell in the lineage RrC LIV. This lineage is typically found in lower Mekong river catchment in southern Laos, Thailand and southern Vietnam. Considering the unique faunal diversity in Sri Lanka, Aplin et al. (2011) deduced a prehistoric invasion of Sri Lanka by *R. rattus*. However, a later study based on cytochrome-b gene by Yasuda et al. (2014) revealed that specimens collected from Kandy district, Sri Lanka, fall in RrC LI not RrC LIV, forming two distinct sub-lineages. One sub-lineage (RrC Lla), which is widely distributed in the world, included few Sri Lankan specimens with *R. rattus* from other parts of the world. The other sub-lineage (RrC Llb), closely related to RrC Lla sub-lineage, included only specimens from Sri Lanka. They inferred two migration events to Sri Lanka, one ancient and the other recent. They also suggested a possible hybridization between the two sub-lineages.

Considering the report of two migration events of *R. rattus* subspecies to Sri Lanka by Phillips (1980), we aimed to determine their phylogenetic relationships and phylogeographic history. Based on distribution of subspecies reported by Phillips (1980) and our own field experience, we hypothesised hybridization or mitochondrial introgression between the widespread *R. r. kandianus* with the other subspecies, including ones introduced through trade ships.

**MATERIALS AND METHODS**

*Rattus rattus* subspecies were collected from selected sites throughout the country during field surveys from 2003 to 2005, and 2015 to 2016 (Fig.1; Table 1). One hundred mesh traps and Sherman traps baited with pieces of roasted coconut were used to collect specimens. They were identified using the descriptions given by Phillips (1980). All specimens are deposited in the Department of Zoology, Faculty of Science, University of Peradeniya.

A muscle sample was taken from each specimen and preserved in 90% ethanol for DNA extraction. Two mitochondrial (16S rRNA and cytochrome-b) and one nuclear (Rag 1) gene fragments were sequenced for molecular analysis. DNA was extracted from ethanol-preserved tissues using Promega wizard blood and tissue extraction kit following manufacturer’s protocols. DNA was amplified by PCR using 25 µl reactions containing 2.0 µl of template, 1.0 µl of each primer (10 µM), and 12.5 µl GoTaq Green master mix and 8.5 µl nano water. Thermal cycling for the cytochrome-b fragment was as follows: 35 cycles of denaturation at 94° C for 30 s, annealing at 45° C for 30 s, and extension at 72° C for 1 min, with a final extension of 72° C for 5 min. The same conditions were used to amplify 16S gene, except for the annealing temperature, which was 48° C. Sequence length of cytochrome-b and 16S were 1140 and 547 bp, respectively. Thermal cycling for Rag 1 gene (819 bp) was as follows: 35 cycles of denaturation at 95° C for 45 s, annealing at 55° C for 45 s, and extension at 72° C for 1 min, with a final extension of 72° C for 5 min. Cytochrome-b and 16S were amplified using the primers MVZ 05/ MVZ 14 and 16S ar/ 16S br, respectively. Primer sequences are as follows: MVZ 05 5’ CGA AGC TTG ATA TGA AAA ACC ATC GTTG 3’; MVZ 14 5’ GGT GTT CAT CTY HGG YTT ACA AGAC 3’; 16S ar 5’ CGC CTG TTT ATC AAA AAC AT 3’; 16S br 5’ CCG GTC GTA ACT CAG ATC AC GTG 3’. Primers used for Rag 1 PCR were: AmpRAG1 F 5’ AGC TCGAGY CAR TAC CAY AAR ATG TA 3’; Amp RAG1R1 5’ AAC TCA GCT GCA TTK CCA ATR TCACA 3’. Sequences were cleaned using ChromasPro 1.7.7, aligned using Clustal W in MEGA 6 software and phylogenies were generated using MEGA 6 software. Two species of *Bandicota* Gray, 1873 and two species of *Mus* Linnaeus, 1758 were also used in the analysis as they are closely related to *Rattus* species (Yasuda et al. 2014; Pages et al. 2010). *Tatera indica* (Hardwicke 1807) was used as the outgroup because of its suggested close relationship to murine rodents (Steppan et al. 2004; Jansa and Weksler 2004). Genetic distance values for cytochrome-b gene were calculated using MEGA 6 software. The GenBank accession numbers for the sequences generated anew for the study and sequences downloaded from the GenBank are given in the Table 1.
**Table 1**: The details of the rodent specimens subjected to phylogenetic analysis.

| Voucher number | GenBank accession number of cytochrome-b/16S sequences/Rag 1 | References |
|----------------|------------------------------------------------------------|------------|
| **Bandicota indica** | | |
| Galle; 06°03’N, 80°22’E, 7.2m | PDZ 43 | KY697990/KY673247 | This study |
| **Bandicota bengalenis gracilis** | | |
| Peradeniya; 07°25’N, 80°59’E, 488m | WHT 6831 | KY697993/KY673251 | This study |
| **Rattus norvegicus** | | |
| Galle; 06°03’N, 80°22’E, 7.2m | PDZ 44 | KY697996/KY673255 | This study |
| Japan | — | AB211039 | Chinen et al., 2005 |
| Zambia | — | AB752996 | Nakamura et al., 2013 |
| South Africa | — | DQ439852 | Bastos et al., 2011 |
| Tanzania; Oman; India | — | HM217365; HM217366; HM217367 | Pagès et al., 2010 |
| France; Europa island | — | JF718277; JF718278 | Russell et al., 2011 |
| California USA | — | JQ814227 | Conroy et al., 2013 |
| Costa Rica; Argentina; Egypt; Philippines | — | JQ823417; JQ823422; JQ823431; JQ823524; JQ823534 | Lack et al., 2012 |
| Mali | — | JX292875 | Schwan et al., 2012 |
| Madagascar | — | LC147016 | Sakuma et al., 2016 |
| India- Langol; India- Iroisemba | — | AB973103; AB973101 | Chingangbam et al., 2015 |
| Nepal | — | KY002803; KY002804 | Unpublished data |
| **Rattus rattus kandianus** | | |
| Peradeniya; 07°25’N, 80°59’E, 488m | PDZ 9 WHT 6830 WHT 6832 | MH253704/MH253733 MH253718/MH253748 MH253719/MH253749 | This study |
| Horton Plains; 06°80’N, 80°83’E, 2150m | PDZ 21 PDZ 25 PDZ 28 PDZ 34 WHT M 159 | MH253705/MH253734; MH253714/MH253735; MH253706/MH253736; MH253707/MH253737/ MN160105 MH253716/MH253746 | This study |
| Location                  | Coordinates                | GenBank Accession Numbers | Study Reference |
|---------------------------|-----------------------------|---------------------------|-----------------|
| Loolwatta; 06°37'N, 80°84'E, 980m | WHT 6805, WHT 6821, WHT 6901 | MH253717/MH253747, KY986756/KY986809/MN160103, MH253724/MH253754 | This study |
| Kitulgala; 06°99'N, 80°42'E, 110.2m | WHT 6856, WHT 6857, WHT 6859, WHT 6861 | MH253720/MH253750, KY986758/KY986810/MH253721/MH253751, MH253722/MH253752 | This study |
| Kottawa; 06°10'N, 80°32'E, 38.6m | WHT 6885 | MH253723/MH253753 | This study |
| Kudawa; 06°41'N, 80°43'E, 360m | WHT 6902, WHT 6903 | MH253725/-/MN160111, MH253726/MH253755 | This study |
| Anuradhapura; 08°21'N, 80°22'E, 108m | WHT 6916, WHT 6919 | KY986757/MH253756/MN160104, MH253727/MH253757/MN160102 | This study |
| Udawalawe; 06°47'N, 80°90'E, 112.2m | WHT 6926, WHT 6927 | KY697997/KY763254, MH253728/MH253758 | This study |
| Hindagolla; 07°48'N, 80°41'E, 142m | WHT 6932, WHT 6933, WHT 6935 | MH253729/MH253759, MH253730/MH253760, MH253731/MH253761 | This study |
| Morningside; 06°41'N, 80°63'E, 900 m | WHT 6938 | MH253732/MH253762 | This study |

**Rattus rattus kelaarti**

| Location                  | Coordinates                | GenBank Accession Numbers | Study Reference |
|---------------------------|-----------------------------|---------------------------|-----------------|
| Horton Plains; 06°80'N, 80°83'E, 2150m | WHT M 029, WHT M 140, PDZ 32, PDZ 39 | K Y 9 8 6 7 5 5 / K Y 9 8 6 8 1 7, KY986750/MH253738/MN160109, MH253727/MH253757/MN160102 | This study |

**Rattus rattus rufescens**

| Location                  | Coordinates                | GenBank Accession Numbers | Study Reference |
|---------------------------|-----------------------------|---------------------------|-----------------|
| Peradeniya; 07°25'N, 80°59'E, 488m | PDZ 5, PDZ 6 | K Y 9 8 6 7 5 5 / K Y 9 8 6 8 1 7, MH253708/MH253740 | This study |
| Nugegoda; 06°86'N, 79°90'E, 7.1m | PDZ 10 | K Y 9 8 6 7 5 5 / K Y 9 8 6 8 1 8/ MN160108 | This study |
| Rathmalkuduwa; 07°17'N, 80°56'E, 504m | PDZ 12, PDZ 15, PDZ 16 | M H 2 5 3 7 0 9 / M H 2 5 3 7 4 1, M H 2 5 3 7 1 0 / M H 2 5 3 7 4 2, MH253711/MH253743 | This study |

**Rattus rattus alexandrinus**

| Location                  | Coordinates                | GenBank Accession Numbers | Study Reference |
|---------------------------|-----------------------------|---------------------------|-----------------|
| Galle; 06°03'N, 80°22'E, 7.2m | PDZ 3 | K Y 9 8 6 7 5 4 / K Y 9 8 6 8 1 9/ MN160107 | This study |
| Galle; 06°03'N, 80°22'E, 8.1m | PDZ 42, PDZ 46 | K Y 9 8 6 7 5 5 / K Y 9 8 6 8 2 0/ MN160106, MH253713/MH253744 | This study |

**Mus booduga**

| Location                  | Coordinates                | GenBank Accession Numbers | Study Reference |
|---------------------------|-----------------------------|---------------------------|-----------------|
| Agarapathana; 06°87'N, 80°72'E, 1378.0m | WHT 6873 | KY697998/KY673256 | This study |

**Mus musculus**

| Location                  | Coordinates                | GenBank Accession Numbers | Study Reference |
|---------------------------|-----------------------------|---------------------------|-----------------|
| Agarapathana; 06°87'N, 80°72'E, 1378.0m | WHT 6886 | KY697999/KY673257 | This study |

**Millardia meltada**

| Location                  | Coordinates                | GenBank Accession Numbers | Study Reference |
|---------------------------|-----------------------------|---------------------------|-----------------|
| Udawalawe; 06°47'N, 80°90'E, 112.2m | WHT 6925 | KY986803/KY986866 | This study |

**Tatera indica**

| Location                  | Coordinates                | GenBank Accession Numbers | Study Reference |
|---------------------------|-----------------------------|---------------------------|-----------------|
| Pakistan | — | AJ430563/ — Chevret P.D. (unpublished) | |
| Yala; 06°43'N, 81°31'E, 29.4m | WHT 6893 | —/KY673258 | This study |
RESULTS

Only four subspecies of *R. rattus* were collected during the study (*R. r. kandianus, R. r. alexandrinus, R. r. rufescens* and *R. r. kelaarti*). In spite of 1875 trap nights in Colombo, Galle and Trincomalee, we were not able to capture *R. r. rattus*.

Mitochondrial gene trees and mitochondrial gene combined tree (cytochrome-β and 16S) resulted in the same topology except the placement of *R. norvegicus* in the 16S gene tree. Rag1 gene was not included in the phylogeny because there were no sequence differences between any subspecies of *R. rattus*. In cytochrome-β gene tree, *R. rattus* subspecies fell into four distinct lineages. *Rattus rattus* from Sri Lanka fell into one lineage (RrC LI), which is reciprocally monophyletic and had two distinct sub-lineages. One sub-lineage (RrC Llb) included only Sri Lankan specimens with representatives from all four subspecies, whereas the other sub-lineage (RrC LIa) included two specimens of *R. r. alexandrinus* and three *R. r. kandianus* with *R. rattus* from other parts of the world (Fig. 2). All *R. r. rufescens* (*n=6*), all *R. r. kelaarti* (*n=4*), one out of three *R. r. alexandrinus* and 23 out of 27 *R. r. kandianus* in our collection fell into Sri Lankan unique RrC Llb sub-lineage. All relationships had high bootstrap support. Sri Lankan population of *R. r. alexandrinus* and *R. r. kandianus* which were in the sub-lineage RrC LIa fell together in a distinct clade with 97% bootstrap support.

There were 13 cytochrome-β haplotypes in RrC Llb sub-lineage. Of 34 individuals in RrC Llb sub-lineage, 18 were of a single haplotype which was common to all four subspecies. One haplotype was only found in *R. r. rufescens* and two others were shared by *R. r. kandianus* and *R. r. rufescens*, and *R. r. kandianus* and *R. r. kelaarti*. All other nine haplotypes were found from *R. r. kandianus* (Table 2). All Sri Lankan samples in RrC LIa were of a single unique haplotype.

Uncorrected percentage pairwise distances for cytochrome-β gene between the four main lineages of *Rattus rattus* (RrC LI, RrC LII, RrC LIII and RrC LIV) were 3.72-6.79%. That of between individuals of two sub-lineages of RrC LII was 1.79-2.61%. Individuals within RrC LIa and RrC Llb had genetic distances of 0.00-0.98% and 0.00-0.89%, respectively. Percentage pairwise distances among the subspecies present in Sri Lanka, regardless of the placement in the phylogeny, were between 0.00-2.52%, with some individuals of all four subspecies having 0.00 genetic difference for cytochrome-β. Sri Lankan subspecies in the RrC LIa formed a separate clade in the phylogeny with a genetic distance of 0.53-0.89% from others in the same sub-lineage.

**Table 2**: The cytochrome-β haplotypes of the sub-lineage RrC Llb (*Rattus rattus* Complex: Lineage I b), number of individuals found with each haplotype, their subspecies representation, localities and single-nucleotide polymorphism (SNPs)/ insertions and deletions (INDELs) compared with common haplotype 1.

| Haplotype number | Number of individuals | Subspecies | Locality                      |
|------------------|------------------------|------------|--------------------------------|
|                  |                        |            | C    | T   | C    | C    | G    | C    | A    | C    | T    | A    | A    | A    | G    | C    | T    | A    | C    |        |
| 1                | 18                     | All four subspecies | 39   | 63  | 165  | 174  | 297  | 303  | 318  | 348  | 383  | 477  | 573  | 694  | 702  | 703  | 768  | 771  | 807  | 816  | 924  |
| 2                | 1                      | *R. r. rufescens* | Peradeniya | .   | .   | .   | A    | .   | T    | C    | G    | G    | .    | .    | .    | .    | .    | .    | .    | .    | .    | .    |       |
| 3                | 1                      | *R. r. kandianus* | Peradeniya | .   | .   | .   | .    | A   | T    | C    | G    | G    | .    | .    | A    | .    | .    | .    | .    | .    | .    | .    |       |
| 4                | 1                      | *R. r. kandianus* | Agarapathana | T  | .   | .   | .    | .   | .    | .    | .    | .    | .    | .    | .    | .    | .    | .    | .    | .    | .    | .    |       |
| 5                | 1                      | *R. r. kandianus* | Agarapathana | .  | C   | T   | .    | .   | T    | C    | G    | G    | .    | C    | A    | .    | .    | .    | .    | .    | .    | .    |       |
| 6                | 1                      | *R. r. kandianus* | Knuckles    | .   | .   | .   | .    | .   | .    | T    | C    | C    | G    | G    | .    | .    | .    | .    | .    | .    | .    | .    |       |
| 7                | 1                      | *R. r. kandianus* | Kitulgala   | .   | .   | .   | .    | .   | .    | .    | .    | .    | .    | .    | C    | .    | .    | .    | .    | .    | .    | .    |       |
| 8                | 1                      | *R. r. kandianus* | Hindagolla | C   | T   | .   | .    | .   | T    | C    | G    | G    | .    | .    | .    | .    | .    | .    | .    | .    | .    | .    |       |
| 9                | 1                      | *R. r. kandianus* | Hindagolla | .   | .   | T   | .    | A   | .    | G    | T    | C    | G    | G    | .    | .    | .    | .    | .    | .    | .    | .    |       |
| 10               | 2                      | *R. r. kandianus* | Horton Plains, Hindagolla | .   | T   | A   | .    | G    | T    | C    | G    | G    | C    | .    | .    | .    | .    | .    | .    | .    | .    | .    |       |
| 11               | 2                      | *R. r. kandianus* | Horton Plains, Udawalawe | .   | .   | .   | .    | .   | .    | .    | .    | .    | .    | .    | .    | .    | .    | .    | .    | G    | A    | .    |       |
| 12               | 2                      | *R. r. rufescens*/ *R. r. kandianus* | Peradeniya | T   | .   | .   | .    | A   | .    | .    | .    | .    | .    | .    | .    | .    | .    | .    | .    | .    | .    | .    |       |
| 13               | 2                      | *R. r. kelaarti*/ *R. r. kandianus* | Horton Plains, Morningside | C   | T   | .   | .    | .   | T    | C    | G    | G    | .    | .    | .    | .    | .    | .    | .    | .    | .    | .    |       |

Position according to PCR product where nucleotides vary
Figure 2: Maximum likelihood phylogram of cytochrome-\(b\) gene depicting four lineages of \textit{Rattus rattus} in the world and the placement of the \textit{R. rattus} subspecies of Sri Lanka. Bootstrap values are presented on the branches.
DISCUSSION

Only four subspecies of R. rattus were captured (R. r. kandianus, R. r. alexandrinus, R. r. rufescens and R. r. kelaarti) during this study. We were not able to capture R. r. rattus during this study. Early surveys by Phillips (1980) reported the presence of R. r. rattus in small numbers around the dock areas in Colombo, Galle and Trincomalee. At present, it is probable that this subspecies is entirely replaced by R. r. alexandrinus that occur in docks.

Even though the four subspecies are morphologically distinguishable, genetic differences among them were not substantial (0.00–2.52%). This range does not exactly fall within the average genetic distance value of 0.09-2.34% reported for currently identified subspecies taxa (Bradley & Baker 2001). There were individuals of all four subspecies with zero genetic divergence for cytochrome-b. Accordingly, we can deduce that there is hybridization or mitochondrial introgression among all four subspecies. However, this is not the case for these four subspecies.

We have included 40 R. rattus individuals collected from around the country representing four subspecies in the phylogeny. They all fell in two sub-lineages of lineage RrC L1 as reported earlier (Yasuda et al. 2014). The Sri Lankan unique sub-lineage, RrC Lib, contained only the four R. rattus subspecies from Sri Lanka, with all individuals of R. r. kelaarti and R. r. rufescens falling in it. All four subspecies having individuals with zero cytochrome-b divergence indicate that each subspecies interbreed with R. r. kandianus which is the widespread and common subspecies throughout the island. Rattus r. kandianus sharing two cytochrome-b haplotypes with R. r. rufescens and R. r. kelaarti also supports this. Rattus r. kandianus is the only subspecies occurring sympatrically with all the other subspecies, facilitating its hybridization with the others. All other subspecies have restricted distributions and none of them occurs sympatrically with each other.

Other sub-lineage, RrC L1a, included four R. r. kandianus and two R. r. alexandrinus individuals, with R. rattus from other parts of the world (Fig. 2). With respect to R. r. kandianus, both sub-lineages had individuals from different localities in the country, from lowland dry zone to highland wet zone. All three R. r. alexandrinus specimens were from Galle. Rattus r. rattus and R. r. alexandrinus have been introduced to the island through trade vessels from Europe and India and were reported from in and around the docks in Galle, Colombo and Trincomalee (Phillips, 1980). Other three subspecies had been in the island for a longer period of time and may have invaded Sri Lanka during the glacial sea-level lowstands when there were terrestrial connections between Sri Lanka and India (most recently until ca 10,000 ybp) or with the help of ancient trade boats. Hence, according to our phylogeny, most likely scenario is hybridization or mitochondrial introgression between sub-populations of R.r. kandianus and R. r. alexandrinus. Introgression appears to have happened in both directions. Rattus r. kandianus being a subspecies widespread around the country, occur sympatrically with R. r. alexandrinus.

Hence, may have interbred with R. r. alexandrinus. Since we did not find R. r. rattus in any of the ports, it is not possible to evaluate its relationship with the other Sri Lankan subspecies. However, it is possible that they also may have hybridized with sub-populations of R. r. kandianus and R. r. alexandrinus.

Hybridization and mitochondrial introgression between different Rattus rattus lineages due to human intervened invasions has been reported in other parts of the world (Chinen et al. 2005; Lack et al. 2012; Conroy et al. 2013). Global picture of invasions and hybridization will be useful in identifying disease transfer paths, pest control methods and even prehistoric human migrations (Matisoo-Smith & Robins 2004).

Rattus rattus rufescens was previously reported only from the dry zone of Sri Lanka (Phillips 1980). During our field collections we found two specimens from Giampola (Rathmalkaduwa) and Peradeniya, indicating the spread of this subspecies towards upland. Though R. r. rufescens is reported from Trincomalee, they are not reported to occur sympatrically with R. r. alexandrinus and R. r. rattus in sea ports. During our field surveys also we did not find them living sympatrically.

Biological invasions result in novel interactions among taxa and can have significant evolutionary implications on both native and invading taxa (Chinen et al. 2005; Lack et al. 2012). The genetic distance between individuals of the Sri Lankan unique sub-lineage, RrC Lib is 0.00-0.89%. The genetic distance of cytochrome-b among the R. rattus living in Sri Lanka has increased up to 2.52% due to mitochondrial genes being introgressed from the later introduced R. r. alexandrinus into a sub-population of R. r. kandianus. A sub-population of R. r. alexandrinus has also changed due to mitochondrial introgression from R. r. kandianus.

ACKNOWLEDGMENTS

We gratefully acknowledge the National Science Foundation (NSF) of Sri Lanka (Research Grant No.: RG/2014/EB/04) for financial support; the Department of Wildlife Conservation, Sri Lanka, for granting permits to collect specimens, and Mohamod Bahir, Sudhath Nanayakkara and Lahiru Nanayakkara for field assistance.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Aplin, K.P., Suzuki, H., Chinen, A.A., Chesser, R.T., Ten Have, J., Donnellan, S.C., Austin, J., Frost, A., Gonzalez, J.P., Herbreteau, V. and Catzeflis, F. (2011). Multiple geographic origins of commensalism and complex dispersal history of black rats. PloS one 6(11):26357.

Ball, Jr, R.M. and Avise, J.C. (1992). Mitochondrial DNA phylogeographic differentiation among avian populations and the evolutionary significance of subspecies. The Auk 109(3):626–636.
Barrowclough, G.F. (1982). Geographic variation, predictiveness, and subspecies. *The Auk* 99(3): 601–603.

Bastos, A.D., Nair, D., Taylor, P.J., Brettschneider, H., Kirsten, F., Mostert, E., Von Maltitz, E., Lamb, J.M., Van Hooft, P., Belmain, S.R. and Contrafatto, G. (2011). Genetic monitoring detects an overlooked cryptic species and reveals the diversity and distribution of three invasive Rattus congener in South Africa. *BMC genetics* 12(1): 26.

Bradley, R.D. and Baker, R.J. (2001). A test of the generic species concept: cytochrome-b sequences and mammals. *Journal of Mammalogy* 82(4): 960–973.

Burrbrink, F.T., Lawson, R. and Slowinski, J.B. (2000). Mitochondrial DNA phylogeography of the polytypic North American rat snake (Elaphe obsoleta): a critique of the subspecies concept. *Evolution* 54(6): 2107–2118.

Chaimanee, Y. and Jaeger, J.J. (2001). Evolution of *Rattus* (Mammalia, Rodentia) during the plio-pleistocene in Thailand. *Historical Biology* 15(1–2): 181–191.

Chinen, A.A., Suzuki, H., Aplin, K.P., Tsuchiya, K. and Suzuki, S. (2005). Preliminary genetic characterization of two lineages of black rats (*Rattus rattus* sensu lato) in Japan, with evidence for introgression at several localities. *Genes & genetic systems* 80(5): 367–375.

Chingangbam, D.S., Laishram, J.M. and Suzuki, H. (2015). Molecular phylogenetic characterization of common murine rodents from Manipur, Northeast India. *Genes & genetic systems* 90(1): 21–30.

Conroy, C.J., Rowe, K.C., Rowe, K.M., Kamath, P.L., Aplin, K.P., Hui L., James, D.K., Moritz, C. and Patton, J.L. (2013). Cryptic genetic diversity in *Rattus* of the San Francisco Bay region, California. *Biological invasions* 15(4): 741–758.

Corbet, G.B. and Hill, J.E. (1991). A World List of Mammalian Species, Third ed. Natural History Museum Publications & Oxford University Press, London and Oxford. V–vi, pp. 1–243.

Gavrillets, S. (2004). Fitness landscapes and the origin of species (MPB-41) (Vol. 41). Princeton University Press.

Gray, J.E. (1873). LII.—Notes on the rats; with the description of some new species from Panama and the Aru Islands. *Journal of Natural History* 12(71): 416–419.

Hardwicke, T. (1807). IX Description of a species of Jerboa found in the upper provinces of Hindustan, between Benares and Hurdwar. *Transactions of the Linnean Society of London* 08(1): Pp. 279–281.

Hastings, A. and Gavrillets, S. (1999). Global dispersal reduces local diversity. *Proceedings of the Royal Society B: Biological Sciences* 266: 2067–2070.

Jansa, S.A. and Weksler, M. (2004). Phylogeny of murid rodents: relationships within and among major lineages as determined by IRBP gene sequences. *Molecular Phylogenetics and Evolution* 31: 256–276.

Lack, J.B., Greene, D.U., Conroy, C.J., Hamilton, M.J., Braun, J.K., Mares, M.A. and Van Den Bussche, R.A. (2012). Invasion facilitates hybridization with introgression in the *Rattus rattus* species complex. *Molecular Ecology* 21(14): 3545–3561.

Linnaeus, C. (1758). Systema naturae, per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Tomus I. Editio decima, reformata. Holmiae, ii + 824 pp.

Mayr, E. (1982). Of what use are subspecies? *The Auk* 99(3): 593–595.

Matisoo-Smith, E. and Robins, J.H. (2004). Origins and dispersals of Pacific peoples: evidence from mtDNA phylogenies of the Pacific rat. *Proceedings of the National Academy of Sciences* 101(24): 9167-9172.

Musser, G.G. and Carleton, M.D. (2005). Super family Muroidea. In: D.E. Wilson, D.M. Reeder (Eds), *Mammal species of the world*, a taxonomic and geographic reference, Johns Hopkins Press, Baltimore Pp. 894–1531.

Nakamura, I., Hang’ombe, B.M., Sawa, H., Kobayashi, S., Orba, Y., Ishii, A., Thomas, Y., Isozumi, R., Yoshimatsu, K., Mweene, A.S. and Takada, A. (2013). Cross-reactivity of secondary antibodies against African rodents and application for sero-surveillance. *Journal of Veterinary Medical Science* 12-0471.

O’Brien, S.J. and Mayr, E. (1991). Bureaucratic mischief: recognizing endangered species and subspecies. *Science* 251(4998): 1187-1189.

Pagès, M., Chaval, Y., Herbreteau, V., Vaengssothorn, S., Cosson, J.F., Hugot, J.P., Morand, S. and Michaux, J. (2010). Revisiting the taxonomy of the Rattini tribe: a phylogeny-based delimitation of species boundaries. *BMC evolutionary Biology* 10(1): 184.

Patten, M.A. (2010). Null expectations in subspecies diagnosis. *Ornithological Monographs* 67(1): 35-41.

Philhimore, A.B. and Owens, I.P. (2006). Are subspecies useful in evolutionary and conservation biology?. *Proceedings of the Royal Society B: Biological Sciences* 273(1590): 1049-1053.

Phillips, W.W.A. (1980). A manual of the mammals of Sri Lanka.Wildlife and Nature Protection Society of Sri Lanka, Colombo, 389+xxxv pp.

Robins, J.H., Hingston, M., Matisoo-Smith, E. and Ross, H.A. (2007). Identifying Rattus species using mitochondrial DNA. *Molecular Ecology Notes* 7(5): 717–729.

Robins, J.H., Tintinger, V., Aplin, K.P., Hingston, M., Matisoo-Smith, E., Penny, D. and Lavergy, S.D. (2014). Phylogenetic species identification in Rattus highlights rapid radiation and morphological similarity of New Guinean species. *PloS one* 9(5): 98002.

Russell, J.C., Gleeson, D.M. and Le Corre, M. (2011). The origin of Rattus rattus on the Îles Éparses, Western Indian Ocean. *Journal of biogeography*. 38(9): 1834-1836.

Sakuma, Y., Ranorosoa, M.C., Kinoshita, G., Shimoji, H., Tsuchiya, K., Ohdachi, S.D., Arai, S., Tanaka, C., Ramino, H. and Suzuki, H. (2016). Variation in the coat-color-controlling genes, Mc1r and Asip, in the *Rattus* species and reveals the diversity and distribution of three invasive Rattus congener in South Africa. *BMC genetics* 12(1): 26.
of the tick-borne relapsing fever spirochete Borrelia crocidurae in Mali, West Africa, and the potential for human infection. *PLoS Neglected Tropical Diseases* 6(11): 1924.

Steppan, S.J., Adkins, R. and Anderson, J. (2004). Phylogeny and divergence date estimates of murid rodents based on multiple nuclear genes. *Systematic Biology* 53(4): 533–553.

Watts, C.H.S. and Baverstock, P.R. (1994). Evolution in some South-east Asian Murinae (Rodentia), as assessed by microcomplement fixation of albumin, and their relationship to Australian murines. *Australian Journal of Zoology* 42(6): 711-722.

Wilson, D.E. and Reeder, D.M. (Eds.) (2005). *Mammal species of the world: a taxonomic and geographic reference* (Vol. 1). JHU Press.

Yasuda, S.P., Gamage, C.D., Koizumi, N., Nishio, S., Isozumi, R., Shimizu, K., Koma, T., Amada, T., Suzuki, H., Yoshimatsu, K. and Arikawa, J. (2014). Distinct genetic characteristics of Sri Lankan *Rattus* and *Bandicota* (Murinae, Rodentia) inferred from mitochondrial and nuclear markers. *Genes & genetic systems* 89(2):71-80.

Zink, R.M. (2004). The role of subspecies in obscuring avian biological diversity and misleading conservation policy. *Proceedings of the Royal Society of London B: Biological Sciences* 271(1539): 561-564.

Zusi, R.L. (1982). Intraspecific geographic variation and the subspecies concept. *The Auk* 99(3): 606-608.