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Molecular Phylogenetic relationships among the species of geckos in Adams'peak in Sri Lanka

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ABSTRACT

In the current study we attempt to identify the relationship of among geckos species that are surviving in Adam's peak Sri Lanka. A ten month survey was undertaken to document the diversity and abundance of geckos at the Adam's peak in Sri Lanka. By using molecular data, the aim was to genetically characterize the species of geckos occurring along directions in Adam's peak and phylogenetically relate these to other different parts of the world. A total of 79 individuals of gecko's tails and eggs were captured in four different areas in Adam's peak. We use 373 bp of RAG1 to detect the gene location and DNA sequence data to recover phylogenetic relationships and elucidate a biographic pattern and close relationship among geckos. According to the morphological identification suggested that gecko's tail specimens belonged to several species and the molecular detection method confirmed the gene pool is differentiated among the population. Analysis of sequences of the RAG1 region from gecko species confirmed that the samples belonged to family *Gekkonidae* with the different gene population.

***Keywords*:** Geckos species; Adams peak; lizard tails; RAG1gene

1. INTRODUCTION

Geckos have undergone million years of evolution, spreading across the planet and adapting to a diversity of habitats both on land and in the water. There are thought to be over 2,000 different species of geckos found around the world. They are selectively bred and have different body colours, mouth colours and various markings on their bodies (Meegaskumbura *et al*., 2002). They have chosen several places to live due to their different inhabiting patterns (Meegaskumbura *et al*., 2002).

The gecko is a small to medium species of lizard that is found in the more temperate and tropical regions of the world and more commonly found around the Equator (Bossuyt *et al*., 2005). Therefore they can be found in a wide variety of habitats in the warmer parts of the world including rocky deserts, mountains, jungles, rainforests, grasslands and even in urban areas where it is common to find geckos (Meegaskumbura *et al*., 2002).

They evolved in a terrestrial niche where selection favored smaller body sizes, agility, and nocturnal habits (Bossuyt *et al*., 2005). The particular brightly colored patch of skin on the throat and usually hide between scales. It can erect the hyoid bone of its throat, resulting in a large vertical flap of brightly colored skin beneath the head which can be then used for communication (Meegaskumbura *et al*., 2002). Geckos are well known for their amazing ability to walk up vertical surfaces even those as smooth as glass (Meegaskumbura *et al*., 2002). The feet of the gecko are covered in tiny hairs that stick to surfaces like sucker pads.

This adaptation means that the gecko is a very agile animal (Meegaskumbura *et al*., 2002). Some species can change colour and may be lighter in colour at night time. Some species of lizards also use bright colors and these colours would be highly visible to predators. Therefore they often hide on the underside or between scales and only revealed when necessary (Meegaskumbura *et al*., 2002). Some gecko species have bright colours inside the mouth, it can be orange, deep blue, purple or grey. The colours inside the mouth are divided into two characters as the skin lining the lower jaw and the tongue. Each of these is used separately. The skin lining the upper jaw and the throat is not used (Gunathilleka *et al*., 1995). Geckos have very strong jaw muscles that any attempt to prise open the mouth of a live gecko may inflict a serious injury on the individual. Wild geckos will often bite when initially picked up and this is an ideal opportunity to observe the mouth colour (Gunathilleka *et al*., 1995).

Geckos have the ability to autotomize their tails which has the ability to shed the tail when threatened and regrow it back. The regenerated tails lacks the color, shape or the ornamentations of the previous tails (Das & de Silva *et al*., 2005). Most geckos are able to scale walls and ceilings thanks to adhesive toe pads with microscopic, hair-like projections called setae (Bossuyt *et al*., 2005). Geckos can range in size from just a few centimetre to more than 50 cm in length. The largest species of gecko which is now believed to be extinct but the native to New Zealand and it grows to nearly 60 cm in length. The smallest species of gecko is less than 2 cm in length and found Dominican Republic in South America (Bossuyt *et al*., 2005).

Most geckos are egg laying, though in some species the eggs are retained until the live young emerge. After mating, the female gecko lays 2 sticky eggs that have a soft shell and are white in colour. The gecko eggs quickly harden so that the developing gecko inside is more protected. The eggs of the gecko can take between 1 and 3 months to hatch but the incubation period is largely dependent on the species of gecko and the area in which it inhabit.

The female gecko is not known to nurse or look after the baby geckos after they hatch. (Meegaskumbura *et al*., 2002). Some species are parthenogenic, this improves the gecko's ability to spread to new islands. However, in a situation where a single female gecko populates an entire island, the island will suffer from a lack of genetic variation within the geckos that inhabit it (Bossuyt *et al*., 2005).

Today many species of gecko are considered to be threatened with extinction due to habitat loss and pollution. Geckos are also popular pets around the world and many are caught in the wild to be sold into the exotic pet trade. All Sri Lankan geckos are fully protected and only be handled, collected or kept in captivity under permit. It is illegal to deliberately harm them. The current distribution of geckos are not only influenced by factors such as biogeography and geology in Sri Lanka.

In this project, we will use our current knowledge to predict the extent of probable past distributions of the several species of Lizards. We will then compare these in detail with the currently known distributions of several species around the world. This will allow us to identify those species that can respond to management and act as useful indicators of the effectiveness of management. We cannot conserve species if we do not know that they exist, or if we cannot tell them apart from other, related species. More than half of the known species of Sri Lanka geckos have not been formally described. This project will lead to the formal identification of features that will allow their reliable classification.

Sri Lanka, despite its small size of 65,610 km^2 has been ranked as one of the biologically richest countries in South Asia. Sri Lanka is also being considered as a biodiversity hotspot together with western Ghats of India (Bossuyt *et al*., 2005; Gunathilleka *et al*., 1995; Gunewardena *et al*., 2007; Meegaskumbura *et al*., 2002). The soil properties, topography and climatic conditions have resulted in the variety of forest types which provides habitats to wide range of fauna including reptiles.

The twenty species of gecko in Sri Lanka, nine species and sub species are considered to be endemic to the country (de Silva *et* al, 2001). They are *Calodactylodes illingworthorum* (Deraniyagala 1953), *Cnemaspis podihuna* (Deraniyagala, 1944), Cyrtodactylus fraenatus (Gunther, 1864), Geckoellatriedra (Gunther, 1864), *Geckoellayak huna* (Deraniyagala, 1945), *Hemidactylus brookii* and *parvimaculatus* (Deraniyagala, 1953), *Hemidactylus depressus* (Gray 1842) and *Hemidacty lustriedruslankae* (Deraniyagala1953).

There are three main climatic zones in Sri Lanka, namely wet zone, intermediate zone and dry zone (Bambaradeniya, 2006; De Silva, 1996). The high level of rainfall, humidity and temperature has contributed to the high herpato fauna diversity. Adam's peak is one such mountain belonging to the wet zone. Adam's peak belongs to the Samanala Nature Reserve (SNR) which is a large forest area with endemic biodiversity.

Gecko tail loss and regrowth

Some species of geckos have an interesting defense mechanism where they will drop their tail if they feel threatened or if their tail is grabbed by a predator. The dropped tail will actually wiggle and twitch on the ground as though it were still attached to the body of the gecko. Many gecko owners experience this tail loss when they try to grab their gecko by the tail or if they are holding their gecko too tightly and they are trying to escape. Tail loss can happen for a number of reasons and tends to be more common in younger geckos. Some reasons for a tail to be dropped are tail being grabbed, being bullied by other geckos sharing waters, stress, fear, illnesses or infections (Bambaradeniya *et al.*, 2006; De Silva *et al.*, 1996).

The tails are designed to do this and have special connective tissue inside them that creates a location where the tail breaks off readily. If a gecko drops their tail the blood vessels to the tail will constrict and very little blood loss occurs. Eventually, the gecko will grow a new tail though it is likely to have a different appearance than the original tail. The new tail is usually shorter than the original one, colored differently and more blunt at the end but it can vary from species to species and depending on how long the regrown tail has been present. The setae of geckos are mostly made of proteins called beta-keratins. During this study, tails which were shed on the Adam's peak pathway was used to identify the molecular and phylogenetic relationships among different species of geckos.

2. STUDY AREA

Adams' peak has been relished for its religious importance as well as its divine natural beauty since centuries. The center of the western ridge of central highlands is situated on latitudes 6'44" and 6'54" North and longitudes 80'25" and 80'49" East in the Kandy (Central province), Rathnapura and Kegalle (Sabaragamuwa province) districts covering an area of 55,300 acres. The average temperature varies from 27 °C in Ratnapura to around 15 °C in Nuwera Eliya. The area receives an average annual rainfall of 2000-5000 mm (DWC 1998). Adams' peak wilderness is one of the few areas in Sri Lanka having a variation of lowland mixed Dipterocarp forest to montane cloud forest. It was considered as a sanctuary since 25th October 1940.

3. THE GENETICS OF ANALYZING LIZARDS SPECIES

Recombination activating gene 1 (RAG-1)

Recombination activating gene 1 is known as RAG-1 which is a protein that living beings is encoded by the RAG1 gene. The protein encoded by this gene is involved in activation of immunoglobulin recombination. The encoded protein is involved in recognition of the DNA substrate, but stable binding and cleavage activity also requires RAG2. Defects in this gene can be the cause of mutations. Catalytic component of the RAG complex mediates the DNA cleavage phase during recombination.

V(D)J Recombination assembles a diverse repertoire of immunoglobulin and T-cell receptor genes in developing B and T-lymphocytes through rearrangement of different V (variable), in some cases D (diversity), and J (joining) gene segments. In the RAG complex, RAG1 mediates the DNA-binding to the conserved recombination signal sequences (RSS) and catalyzes the DNA cleavage activities by introducing a double-strand break between the RSS and the adjacent coding segment. RAG2 is not a catalytic component but is required for all known catalytic activities. DNA cleavage occurs in 2 steps, a first nick is introduced in the top strand immediately upstream of the heptamer, generating a 3'-hydroxyl group that can attack the phosphodiester bond on the opposite strand in a direct transesterification reaction, thereby creating 4 DNA ends.2 hairpin coding ends and 2 blunt, 5'-phosphorylated ends.

The chromatin structure plays an essential role in the V(D)J recombination reactions and the presence of histone H3 trimethylated at 'Lys-4' stimulates both the nicking and haipinning steps. The RAG complex also plays a role in pre-B cell allelic exclusion, a process

leading to expression of a single immunoglobulin heavy chain allele to enforce clonality and monospecific recognition by the B-cell antigen receptor (BCR) expressed on individual B-lymphocytes. The introduction of DNA breaks by the RAG complex on one immunoglobulin allele induces ATM-dependent repositioning of the other allele to pericentromeric heterochromatin, preventing accessibility to the RAG complex and recombination of the second allele. In addition to its endonuclease activity, RAG1 also acts as a E3 ubiquitin-protein ligase that mediates monoubiquitination of histone H3. Histone H3 monoubiquitination is required for the joining step of V(D) J recombination. The analyzing of the particular genetic sites has been done as follows. The three particular special sites have mentioned, namely Cytochrome (CYT F and CYT R) as the mitochondrial genes and RAG1 and Phosducine as the nuclear genes. Further, this study has experimented with RAG1gene.

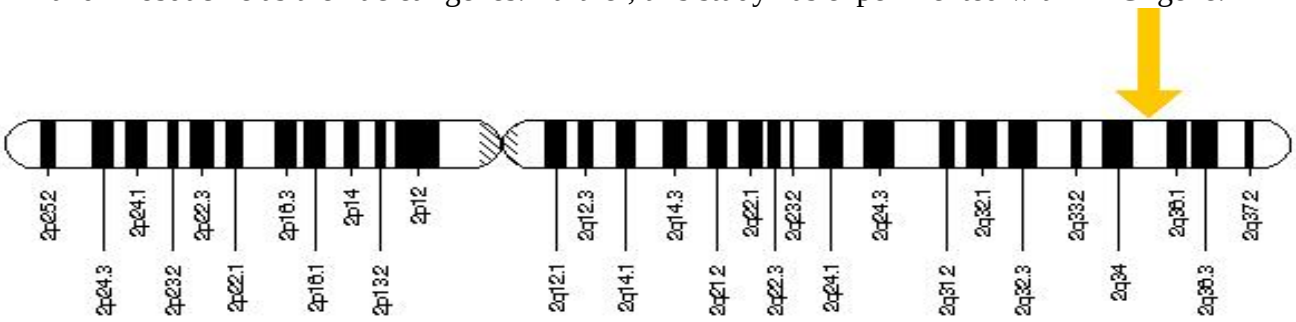

Figure 1. RAG1 gene

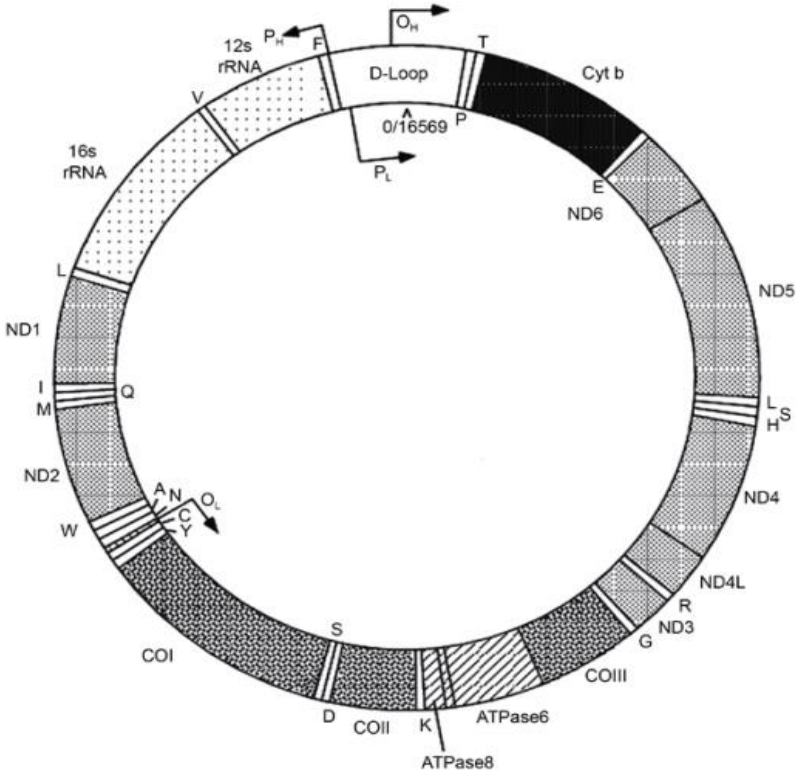

Figure 2. Rag1 has been widely used as the nuclear markers in squmate phylogentic analysis.

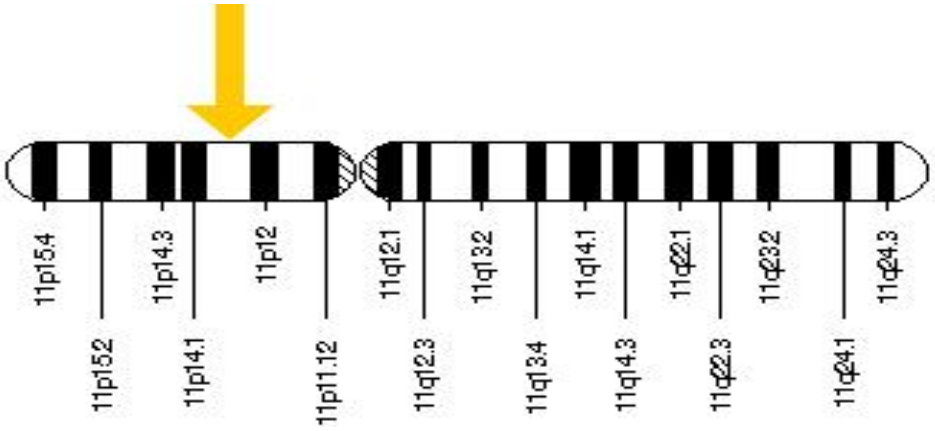

Figure 3. The activity of the gene

It is expressed in high levels in retinal photo receptor and pineacells. RAG1 has a role of a transcriptional activator.

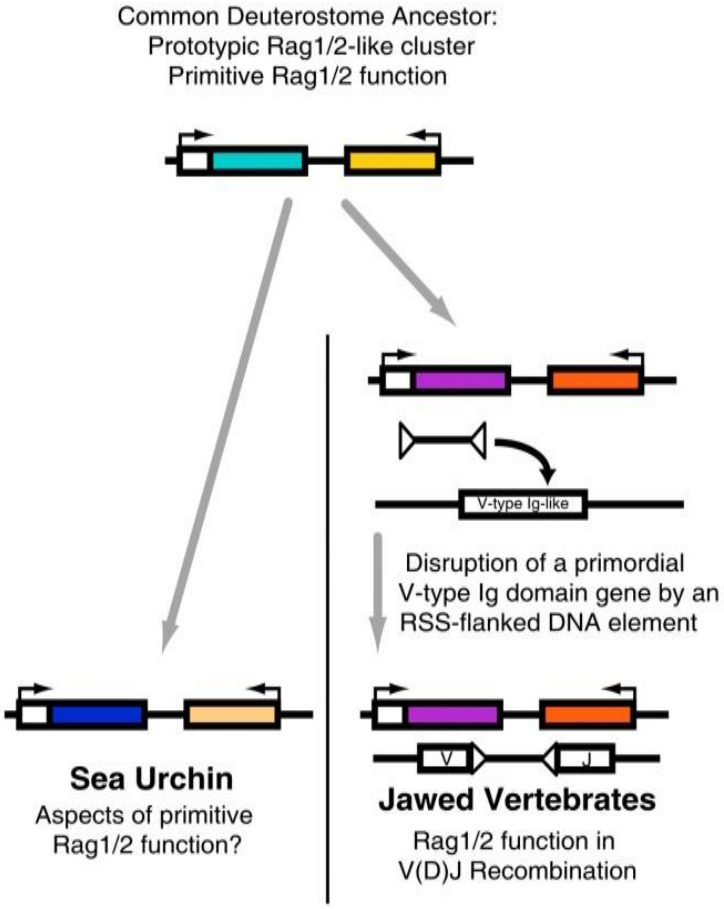

Figure 4. Evaluation of gene

4. MATERIAL AND METHODS

Sampling

Seventy nine samples of lizards were collected at Adam's peak and divided them into two main groups known as "AA" and "BB". Most of the samples were collected in the dawn time. All the sample were preserved under absolute alcohol in separate containers, until they were transferred to the laboratory. As well there are some several samples have been stored under -70 °C without the preservatives. Due to the collection of egg samples should be stored under particular conditions thus it dissolves in absolute alcohol.

5. DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

Sample Collection and DNA amplification

Tissues samples from the tails and eggs (n = 79) were collected from every parts from Adam's peak in Sri Lanka and preserved in 95-100 % ethanol. Genomic DNA was extracted individually for each tail or eggs from specimens by using the DNeasy® Tissue Kit (Qiagen) following manufacturer's protocols.

We used double stranded PCR to amplify 373bp aligned bases of RAG 1 genes used as the primers. Amplification of 25μl PCR reaction were executed on gradient thermo cycler added to the extension per 35 cycles for genomic DNA.

A fragment of the genomic DNA from tissues of specimens was amplified with universal primers, RAG F700 and RAG R 700 (Bauer et al. 2007) using the following PCR protocol: 4 min at 94 ºC/32 cycles of 94 ºC for 20 s, 56 ºC for 40 s and 72 ºC for 1 min/72 ºC for 1min with 25 mL reactions with, 2.5U of AmpliTaq DNA Polymerase (Promega), 1X of PCR Buffer, 3 mM of $MgCl_2$, 0.4 mM of dNTPs, 0.4 µM of each primer, 2 ng/µL of extract of DNA and double-distilled water to complete total volume.

6. GENE LOCATIONS

RAG1 F700	RAG1	Bauer et al. (2007)	50-GGAGACATGGACACAATCCATCCTAC-30
RAG1 R700	RAG1	Bauer et al. (2007)	50-TTTGTACTGAGATGGATCTTTTTGCA-30

When needed annealing temperatures were adjusted to increase or decrease the specified on a case by case basis. Products were visualized using 1.5% agrose gel electrophosis. Target products were purified with the AMP pure magnetic beads solution and sequenced with the Big Dye© terminator v3.1 cycle sequencing kit. Positive reactions were purified using the Minelute (Qiagen, Germany) kit and cycle sequencing was carried out using the following final concentrations: 0.16 µM of each primer, 0.25X reaction Buffer, 0.5 µL of BigDye v.3 (Applied Biosystems), and 0.2-0.3 ng/µL of template DNA. Thermocycling conditions included an initial denaturation of 1 min at 96 ºC, followed by 35 cycles of 10s at 96 ºC, 5s at 50 ºC, and 4min at 60 ºC. Products were purified using Sephadex^{TM} G-50 medium (GE Healthcare Bio-Sciences AB) and both strands were sequenced on an ABI 3130 Automatic Sequencer.

DNA was successfully amplified from 79 specimens. Purified PCR products were sequenced at the Genetech, Colombo. Sequences of the forward and reverse reads were aligned using the Geneious Pro 5.5 software (Drummond *et al.*, 2009).

Additional sequences of t region that were generated in previous studies were downloaded from Genbank (http://www.ncbi.nlm.nih.gov /genbank/). The accuracy of sequences was ensured by incorporating negative controls and sequencing complementary strands. The analysis was done using bio edit software programme.

7. PHYLOGENETIC ANALYSIS

The phylogenetic relationships among the species were assessed using parsimony likelihood. Maximum parsimony analyses were conducted in PAUP* 4.0blo. The heuristic search algorithm was used with the following conditions. 25 random addition replicates, accurate characters transformation three bisection reconnection, TBR branching swapping, zero length branches collapsed to yield polytomies and gaps treated asthe missing data. RAG1 was analyzed separately with ML using a general time reversible modified with variable sites and gamma distributions. All estimated from the MP tree of the RAG gene data.

8. RESULTS

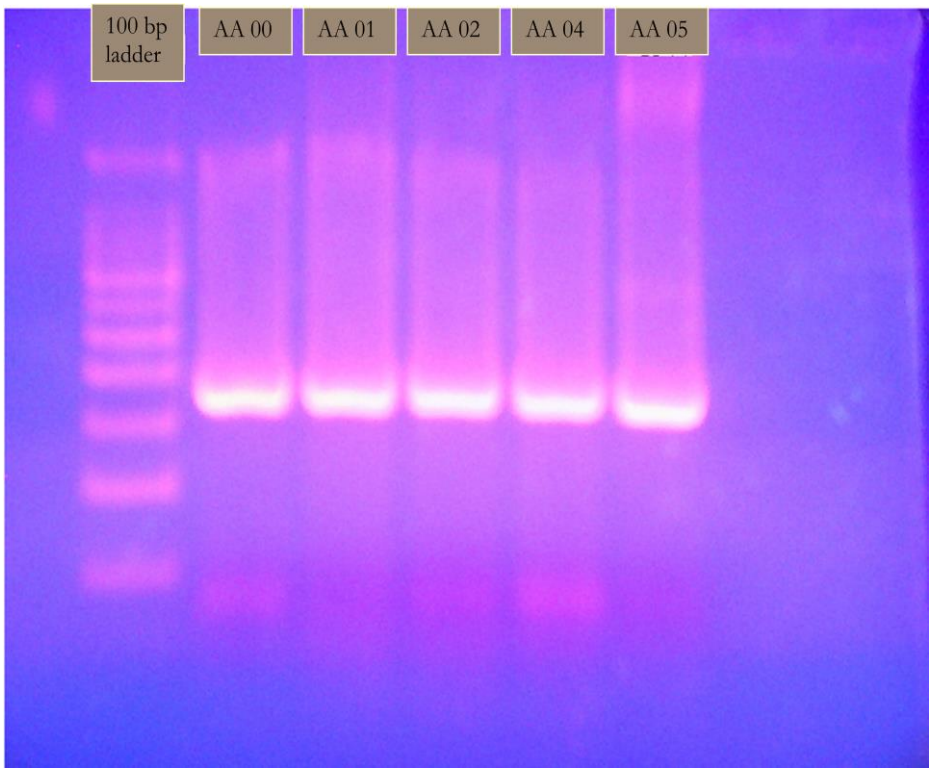

Figure 5. Band size is determined by using the universal primer. The above gel picture shows the molecular observations. Banding pattern is used for the identification of species and size is 373 bp.

Using the RAG1 primer pair, we produced sequences for the majority of the Sri Lankan species of geckos. The success rate of the PCR amplification and DNA sequencing was constantly high even when only degraded DNA or minimal amounts of tissue samples were available. The results that has given is for five samples. All the 79 samples have extracted and results are available.

ID No	BAND SIZE	RESULTS	DATE
AA 00	**373bp**	**Positive**	**20. 02. 2008**
AA 01	**373bp**	**Positive**	**20. 02. 2008**
AA 02	**373bp**	**Positive**	**20. 02. 2008**
AA 04	**373bp**	**Positive**	**20. 02. 2008**
AA 05	**373bp**	**Positive**	**20. 02. 2008**

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The analyzing of the samples have been done by using Bayesian interference analysis. The combined RAG1 data set analysis by using Bayesian interference yields a well-supported pattern in a relationship.

9. DISCUSSION AND CONCLUSIONS

Molecular genetics are being increasingly used in identifying unidentifiable species. DNA barcoding has now expanded to identifying unrecognized living beings, detecting of mutations among the species, producing genetically modified food, animal and plant embryology and many streams throughout the world. Among all these motivations, one of the most widely used applications of DNA barcoding is the identification of wild life species identification. In the current study, this method was used to identify *Gekkonidae* in Sri Lanka. Further, attempts were made to identify the putative population the individual samples belonged to, since it is well documented that populations could be identified from DNA sequences (Avise, 2000). The genetic analyses suggested that the 79 samples were indicating that our initial identification which was based on external morphology was accurate. Using combined morphological, ecological and genetic methods, establishing the precise lineage of *Gekkonidae* in Sri Lanka is of paramount importance with regard to establishing the ecology of the species and subspecies as well as devising conservation measures.

Biologists have used morphological data for centuries to describe and infer relationships among species. The advent of molecular tools has drastically changed this activity (Wiens,

2007) and molecular data overcame the use of traditional characters to reconstruct lineage relationships.

RAG1 as a phylogenetic marker in lizards

In this analysis RAG1, provided strong support for basal relationships within lizard species. Further, the CI for RAG1 in combination with Bayesia tree was much higher for the RAG1 gene. It is also roughly comparable to that of RAG1, which has been widely used in squmate phylogentic and has been regarded as a valuable marker for both interspecific and higher order relationships in gecko species. The small size (373 bp) and concomitant case and cheapness of sequencing the RAG1 combined with its empirical consistency with RAG1 derived result.

Phylogenetic relationships of lizards

The majority of species in which the rostral is exhibit the alternative condition and other that exhibit the *Picta* condition, but occur in other clades. However, several clades retrieved in our analysis are supported by other morphological and chromatid characters. The life coloration of adult species is known and due to intensive field surveys and breeding, even the juvenile's coloration of most species. All these species, expect the most basal in which highly hatched juveniles strongly resembles the adults in color and patterns are characterized by juveniles with more colourful and contrasting patterns than the adults. A comparably distinct juvenile coloration is unknown for each and individual. The species in Sinharaja share a blackish dorsal ground colour and even blackish irish colour, but apparently do not repeat a nonphylogenetic group. The tail shape, colour of the body, colour of the mouth and body patterns are the main character that has been used for analyizing data comparing with molecular data. The tail sample has been used as the main specimen. The distribution of these several other characters states indicates a relatively high congruence of genetic and non-molecular data. The relationship among the species do not correspond to the convenient wet dry biogeography division. Some biographic patterns and coloration sequences has been identified by phylogenetic analysis. Further, every character can be analyzed by using unique primers for each character.

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