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RESEARCH ARTICLE

Species boundaries, biogeography and evolutionarily significant units in dwarf toads: *Duttaphrynus scaber* and *D. atukoralei* (Bufonidae: Adenominae)

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Abstract: Species boundaries and patterns of gene flow in Dwarf toads, *Duttaphrynus scaber* and *D. atukoralei,* were assessed using mitochondrial DNA markers. Samples from four populations in Sri Lanka (Mihintale, Ampara, Yala, Galle) were analyzed for three mitochondrial gene fragments (*16S rRNA*, *COI* and *Cyt b*) along with four Genbank sequences of *16S rRNA* from Indian samples (Thiruvananthapuram, Maharashtra, Mudigere). Phylogenetic trees and haplotype networks were generated, and morphology was assessed. Analyses suggest a single species (*Duttaphrynus scaber*) with three major clades: a widespread clade shared between India and Northern Sri Lanka, an Eastern and Southeastern Sri Lankan clade (previously referred to as *D. atukoralei,* the validity of which, however, our analysis disputes), and a distinct Southern wet-zone clade from Galle (referred previously to as *D. atukoralei*). *Duttaphrynus atukoralei* (topotypes from Yala, Sri Lanka) is genetically too close to *D. scaber* (Indian and northern Sri Lankan clade) to be distinguished as a species; these two clades have a genetic distance of 0.95 – 1.55% for the *16S rRNA fragment*. The haplotype networks for the *16S rRNA* gene suggest incomplete lineage sorting between the Ampara and Yala populations; *COI* and *Cyt b* show complete sorting for all populations analyzed, suggesting strong population structure. All analyses suggest substantially restricted gene flow to the southern wet-zone population (Galle). This population also assumes a basal phylogenetic position, suggesting that *D. scaber* first evolved in southern Sri Lanka's wet zone and dispersed across the lowland areas of the island and to India. Here, we provisionally recognize this population (Galle) as an evolutionarily significant unit of *D. scaber*; future analyses using multiple criteria may indicate this to be a new Dwarf toad species. External morphology is largely uninformative as the Yala, Ampara and Galle populations cannot be distinguished from each other; the morphological distinction between Yala, Ampara, Galle versus Mihintale is restricted to only the shape of the parotid glands – slightly oval versus rounded – a minor difference. Both genetic and morphological evidence so far suggest that there is only a single Dwarf toad species in Sri Lanka, which is also shared with India, namely *Duttaphrynus scaber*; however, with strong population structure, including an evolutionarily significant unit (Southern wet-zone population).

Keywords: gene flow, haplotype networks, phylogenetics, conservation genetics.

INTRODUCTION

Sri Lanka is a global amphibian hotspot with many unique and threatened taxa, especially of the genus *Pseudophilautus* (shrub frogs), which have undergone a large endemic radiation resulting in some 100 species (Meegaskumbura *et al*., 2002). Bossuyt *et al*. (2004), in an analysis of several vertebrate (shrub frogs, caecilians, earth snakes and freshwater fish) and invertebrate (freshwater crabs and shrimps) groups representing a diversity of life-history strategies showed that the wet-adapted taxa are characterized by clade level endemism. For wet-adapted frogs, this notion was reinforced with the recognition of a Sri Lankan endemic genus, *Taruga*, (Meegaskumbura *et al*., 2010), delineation of reciprocal endemicity of Hylarana species of India and Sri Lanka (Biju *et al.,* 2014), endemicity of *Nannophrys* (which exhibits several unique features (Senevirathne & Meegaskumbura, 2015), and the endemicity of the genus *Adenomus* (Meegaskumbura *et al.,* 2015; Van Bocxlaer *et al.,* 2009). However, it is not known if this pattern is representative of the other anuran taxa, especially for some of the dry-adapted species, which are putatively shared with India.

Members of the family Bufonidae, commonly known as true toads, with over 500 species, show an extensive global distribution (Pramuk *et al.,* 2008). Using molecular data, Van Bocxlaer *et al.* (2009) allocated some of the South and Southeast Asian bufonids to the subfamily Adenominae Cope, 1861. Although Indian bufonids comprise six genera, Sri Lanka has only two: *Adenomus* Cope, 1861, endemic to the island, and *Duttaphrynus* Frost, Grant, Faivovich, *et al.,* 2006, of which some species are shared with India (Meegaskumbura *et al.,* 2015; Van Bocxlaer *et al.,* 2009). The Dwarf Toads, a non-taxonomic term used to recognize the small toads inhabiting India and Sri Lanka include *Duttaphrynus scaber* (Schneider, 1799), a species shared with India, and *D. atukoralei*, a species thought to be endemic to Sri Lanka. The Dwarf Toads, according to published work (Van Bocxlaer *et al*., 2009; Meegaskumbura *et al.,* 2015) form a well-supported clade.

In the original description of *Duttaphrynus atukoralei,* Bogert & Senanayake (1966) considered *D. scaber* (referred to as *Bufo fergusonii* Boulenger, 1892, until

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Dubois and Ohler (1999) showed it to be a junior subjective synonym of *D. scaber*) to be its sister species. The type locality of *Duttaphrynus scaber* is Thiruvananthapuram, Kerala, South India, while that of *D. atukoralei* is Yala, southeastern Sri Lanka (Bogert and Senanayake, 1966). Within Sri Lanka, however, the two species are thought to overlap in distribution across the lowland dry zone of the eastern region: *D. scaber* shows a northwestern, northern and eastern distribution while *D. atukoralei* appears to be restricted to the eastern and southeastern segments of the island despite the habitat being uniform, without apparent barriers to dispersal (Bogert and Senanayake, 1966).

The two species are morphologically similar, distinguished largely by the shape of the parotid glands: lobulated in *D. atukoralei,* rounded in *D. scaber* (Bogert and Senanayake, 1966). Though Bogert and Senanayake (1966) noted that the call of *D. atukoralei* was different from that of *D. scaber* (= *B. fergusonii*), they only sampled the call of *D. scaber* from Sri Lanka.

The partial sympatry of *Duttaphrynus scaber* and *D. atukoralei*, the shared distribution of *D. scaber* between the Indian mainland and Sri Lanka, and the occurrence of *D. atukoralei* in the wet zone (Galle), in the context of the clade-level endemicity of wet-adapted species observed by Bossuyt *et al.* (2004), gives rise to several questions regarding the taxonomy and biogeography of these taxa, such as: is there congruity between molecular and morphological data in the context of species boundaries? Is there clade-level endemism shown by dry zone elements? What are the patterns of haplotype structures for these species?

Here we examine the species boundaries and patterns of gene flow of *D. scaber* and *D. atukoralei* in Sri Lanka using three mitochondrial genes (16S ribosomal RNA – *16S rRNA*, Cytochrome *b* – *Cyt b* and Cytochrome c oxidase subunit 1 – *COI*).

MATERIALS AND METHODS

This study has been cleared by the Ethical Clearance Committee, Postgraduate Institute of Science, University of Peradeniya at its 18th meeting held on 19th May 2015.

Sample localities and tissue collection

Specimens of *Duttaphrynus atukoralei* and *D. scaber* were collected from four populations (Mihintale, Galle, Ampara and Yala) between January and December 2014 (Table 1). Three samples each from Mihintale, Galle and Yala, and two from Ampara, were collected during the course of the survey. The specimens from Galle, Yala and Ampara were identified as *D. atukoralei* on the basis of the shape of their parotid glands (Bogert and Senanayake, 1966) while those from Mihintale were assigned to *D. scaber.* Tissue samples taken from either thigh muscles or toe tips and were preserved in 100% ethanol. All specimens and tissue samples were deposited in the natural history collection of the Department of Zoology (DZ), University of Peradeniya.

Two sequences (*16S rRNA*) of *D. scaber* from Mudigere, India (AB530643, AB530644), one from Maharashtra, India (JQ898086) and one from Thiruvananthapuram (the type locality for the species), India (FJ882785), were downloaded from GenBank; a sequence (*16S rRNA*) of *D. atukoralei* (FJ882835) which, however, lacks specific locality data was also downloaded.

DNA extraction, PCR amplification, DNA sequencing and sequence alignment

DNA was extracted from ethanol-preserved tissues using Qiagen DNeasy tissue extraction kits following manufacturer's protocol. Mitochondrial *16S rRNA*, *Cyt b* and *COI* gene fragments were amplified using standard PCR conditions and primers; 16Sar and 16Sbr (Palumbi, 1996), which amplified a ~550 bp region of *16S rRNA* gene; CB-J-10933 and BSF4 (Bossuyt and Milinkovitch, 2000), which amplified a ~ 590 bp of *Cyt b* gene; chmf4 and chmr4 (Che *et al.,* 2012), which amplified a ~ 640 bp of *COI* gene. PCR conditions for *16S rRNA* and *Cyt b* were as follows: denaturation at 95° C for 30 s, annealing at 45° C for 40 s and extension at 72° C for 40 s, 35 cycles, with a final extension of 72° C for 7 min and PCR conditions for *COI* as; denaturation at 95° C for 1 min, annealing at 48° C for 1 min and extension at 72° C for 1 min, 35 cycles, with a final extension of 72° C for 10 min. PCR Products were purified using QIAquick (Qiagen) PCR purification kit and sequenced on an ABI 3500 automated sequencer following manufacturer's protocols.

Phylogenetic analyses

Our study included 38 species, representing Adenominae. The data set contained 16 samples of *Duttaphrynus atukoralei* and *D. scaber* sampled from the four locations (Table 1). Additionally, 22 sequences of *16S rRNA* were obtained from the GenBank (Table 1). Four species of the genus *Ansonia* (*Ansonia spinulifer*, *A. malayana*, *A. hanitschi*, *A. fuiginea* and *A. leptopus*) served as the outgroup; this is the closest sister group of the family Adenominae (Van Bocxlaer *et al.,* 2009). Sequences were aligned using ClustalW as implemented in MEGA v. 5.0 (Tamura *et al.,* 2011), and ambiguous regions were identified. A DNA fragment of *16S rRNA* ca. 467 bp was used in the subsequent phylogenetic analyses. Bayesian (BA) and maximum likelihood (ML) analyses were performed to infer relationships among clades and assess node support. The best-fit model for the dataset was chosen using jModelTest v. 2.1.4 (Darriba *et al.,* 2012; Guindon and Gascuel, 2003). Maximum likelihood analysis was performed using the software GARLI (Zwickl, 2006) on the Cipres Science Gateway (Miller *et al.,* 2010), using the model selected by jModeltest (GTR+I+G) with all parameters estimated. The run was repeated twice to ascertain the tree topology. The tree was visualized using FigTree v. 1.3.1 (Rambaut and Drummond, 2010). Bayesian inference as implemented in MrBayes v. 3.1.2 (Huelsenbeck & Ronquist, 2001) was used to assess posterior probability values for each node with the parameters of a GTR model of sequence evolution with gamma-distributed rate variation among sites and a proportion of invariant sites (GTR+I+G) estimated as obtained from the jModelTest. Four Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) chains were run for ten million generations. Burn-in of 5 million generations was estimated using Tracer v. 1.6 (Rambaut *et al.,* 2014).

Table 1: Taxa included in the study, along with sampling localities, tissue or voucher references and GenBank accession numbers (GBS: to be submitted to GenBank).

Genus	Species	Voucher /Tissue Ref	Locality	*16S rRNA*	*COI*	*Cyt b*
Duttaphrynus	*dhufarensis*	GenBank	Oman	FJ882837	_	_
Duttaphrynus	*hololius*	GenBank	India	FJ882781	_	_
Duttaphrynus	*parietalis*	GenBank	India, Western Ghats	FJ882784	_	_
Duttaphrynus	*melanostictus*	GenBank	India, Western Ghats	FJ882791	_	_
Duttaphrynus	*brevirostris*	GenBank	India, Western Ghats	FJ882786	_	_
Duttaphrynus	*crocus*	GenBank	Myanmar	FJ882789	_	_
Duttaphrynus	*himalayanus*	GenBank	India	FJ882790	_	_
Duttaphrynus	*stuarti*	GenBank	Myanmar	FJ882788	_	_
Duttaphrynus	*stomaticus*	GenBank	India, Western Ghats	FJ882787	_	_
Xanthophryne	*koynayensis*	GenBank	India, Western Ghats	FJ882782	_	_
Pedostibes	*tuberculosus*	GenBank	India, Western Ghats	FJ882793	_	_
Adenomus	*kelaartii*	DZ1120	Sri Lanka, Panwila	KM921782	_	_
Adenomus	*kandianus*	DZ1206	Sri Lanka, Moray	KM921795	_	_
Ghatophryne	*ornata*	GenBank	India, Western Ghats	FJ882797	_	_
Duttaphrynus	*atukoralei*	DZ1463	Sri Lanka, Ampara	GBS	GBS	GBS
Duttaphrynus	*atukoralei*	DZ1464	Sri Lanka, Ampara	GBS	GBS	GBS
Duttaphrynus	*scaber*	DZ1488	Sri Lanka, Mihintale	GBS	GBS	GBS
Duttaphrynus	*scaber*	DZ1489	Sri Lanka, Mihintale	GBS	GBS	GBS
Duttaphrynus	*scaber*	DZ1490	Sri Lanka, Mihintale	GBS	GBS	GBS
Duttaphrynus	*atukoralei*	DZ1524	Sri Lanka, Yala	GBS	GBS	GBS
Duttaphrynus	*atukoralei*	DZ1565	Sri Lanka, Yala	GBS	GBS	GBS
Duttaphrynus	*atukoralei*	DZ1565	Sri Lanka, Yala	GBS	GBS	GBS
Duttaphrynus	*atukoralei*	DZ1169	Sri Lanka, Galle	GBS	GBS	GBS
Duttaphrynus	*atukoralei*	DZ1170	Sri Lanka, Galle	GBS	GBS	GBS
Duttaphrynus	*atukoralei*	DZ1362	Sri Lanka, Galle	GBS	GBS	GBS
Duttaphrynus	*atukoralei*	GenBank	Sri Lanka	FJ882835	_	_
Duttaphrynus	*scaber*	GenBank	India, Maharashtra	JQ898086	_	_
Duttaphrynus	*scaber*	GenBank	India, Thiruvananthapuram	JQ882785	_	_
Duttaphrynus	*scaber*	GenBank	Mudigere, India	AB530643	_	_
Duttaphrynus	*scaber*	GenBank	Mudigree, India	AB530644	_	_
Duttaphrynus	*sp_FB2009a*	GenBank	India, Western Ghats	FJ882792	_	_
Duttaphrynus	*sp_FB2009b*	GenBank	India, Western Ghats	FJ882839	_	_
Bufo	*sp_FB2009*	GenBank	India, Western Ghats	FJ882783	_	_
Ansonia	*malayana*	GenBank	Malaysia, Borneo	AB331712	_	_
Ansonia	*hanitschi*	GenBank	Malaysia, Borneo	FJ882794	_	_
Ansonia	*spinulifer*	GenBank	Malaysia, Borneo	FJ882798	_	_
Ansonia	*fuliginea*	GenBank	Malaysia, Borneo	AB331709	_	_
Ansonia	*leptopus*	GenBank	Malaysia, Borneo	FJ882795	_	_

Bootstrapping was done in a ML framework using RAxML 8 (Stamatakis, 2014). GTRGAMMA model was used with one thousand RAxML searches and 1000 iterations. Clade support was assessed using posterior probability (PP) and ML bootstrapping values.

Phylogenetic inference

Three independent mtDNA alignments using *16S rRNA* (467 bp), *Cyt b* (482 bp) and *COI* (614 bp) genes were created in MEGA 5.0 (Tamura *et al.,* 2011). For each dataset, a Neighbor Joining tree was constructed using the Kimura 2-parameter (K2P) distance model. Uncorrelated pairwise genetic distances were also calculated separately using PAUP* 4.0b10 (Swofford, 2002).

Haplotype network reconstruction

The TCS 1.21 (Clement *et al.,* 2000) in PopArt (Leigh & Bryant, 2014) was used to reconstruct the haplotype networks for *16S rRNA*, *Cyt b*, and *COI* gene sequences. TCS is a computer program that implements the estimation of gene genealogies from DNA sequences as described by Templeton *et al.* (1992).

Molecular diversity indices

Nucleotide diversity (Nd) of Nei (1987), the average number of nucleotide differences per site between sequences, and haplotype diversity (Hd) were calculated for the all the three genes and separately for each population using the program DnaSP, version 5.10.01 (Librado & Rozas, 2009).

RESULTS

Phylogenetic analysis

Sequence data for 467 bp of *16S rRNA* were obtained for a total of 38 species of Adenominae, including *D. scaber* and *D. atukoralei*. jModelTest results shows that GTR+I+G is the best fit model for the data set. Maximum Likelihood inference was performed using the GTR+I+G model with the following parameters applied: Rate matrix: R (G-T) = 1, R (C-T) = 663.6677, R (C-G) = 0.0113, R (A-T) = 68.2184, R (A-G) = 143.1083, R (A-C) 55.5275 Nucleotide frequency: A = 0.3644, C = 0.2047, G = 0.1887, T = 0.2423; Rate variation: shape parameter for gamma distributed rate variation among sites (alpha) = 0.3110; proportion of invariable sites = 0.4280.

The uncorrected pairwise genetic distance between *D. scaber* (Indian and Mihintale population) and *D. atukoralei* (Yala population; topotypes) were between 0.97 – 1.55% for the *16S rRNA* fragment (Fig. 1C). The highest average pairwise genetic distances (*16S rRNA*; 2.90% *COI*; 5.37% *Cyt b*; 7.68%) were between the population from Galle and *D. scaber* from Mihintale. The average pairwise genetic distance between Galle and Yala populations was 2.32% for *16S rRNA*, 4.72% for *COI*, 6.64% for *Cyt b* (Figures 1C and 2). The maximum genetic distance between samples from Yala (type locality of *D. atukoralei*) and other *D. scaber* samples was 1.55% for *16S rRNA*, 3.96% for *COI* and 4.36% for *Cyt b*. The average pairwise genetic distance between the Indian *D. scaber* and the Sri Lankan forms are 1.51% (n=48) for *16S rRNA*.

Dwarf toads (*D. scaber* and *D. 'atukoralei'*) form a highly supported clade (posterior probability, PP = 100; bootstrap = 96) (Figure 1A). All samples from Galle form a well-supported (PP= 94; bootstrap= 98) haplotype clade while other samples from Ampara and Yala form a well-supported monophyletic clade (PP=100, bootstrap = 65), which is sister (PP = 100, bootstrap = 88) to *D. scaber* clade (PP = 89, bootstrap = 73) from India and Northern Sri Lanka (i.e. Mihintale).

Haplotype Networks

The four populations from Sri Lanka, for the most part, did not share haplotypes. Haplotype sharing only occur for the 16S rRNA gene fragment between the Ampara and Yala populations.

(Figures 1B and 3A-C). Haplotype networks for the *COI* and *Cyt b* genes showed an absence of haplotype sharing (sequence data for *D. scaber* from India, however, were not available for these two genes: Figure 3B,C). All genetic markers also indicated high intraspecific genetic diversity between different localities (Figures 1B and 3). *Duttaphrynus scaber* had 7, 2 and 3 unique haplotypes, while *D. atukoralei* had 4, 5 and 6 unique haplotypes for the *16S rRNA*, *COI* and *Cyt b* mitochondrial markers, respectively (Figures 1B and 3B,C). In all three markers, *D. 'atukoralei'* samples from Galle were deeply divergent from their conspecifics from other locations (Figures 1B and 3B,C). The molecular differences between *D. scaber* and *D. 'atukoralei'* in *16S rRNA* were low (maximum number of changes = 11) compared to intraspecific differences among *D. 'atukoralei'* from different localities (maximum number of changes = 11) (Figure 1B).

DISCUSSION

Our molecular phylogeny suggests a single species of Dwarf toad (*D. scaber*) with three closely related clades: a widespread clade shared between India and Northern dry zone of Sri Lanka, a clade restricted to the Eastern and Southeastern dry zone of Sri Lankan (previously referred to as *D. atukoralei;* referred to as Ampara-Yala population), and a clade known from the southern wet zone of Sri Lanka (Galle population), which is the most distinct form from all currently recognized taxa. The genertic distance between *Duttaphrynus scaber* and *'D. atukoralei'* is sufficiently low (Figs. 1C, 2) for the latter to be considered a synonym of the former. For the examined populations, the phylogeny and haplotype networks for the *16S rRNA* gene suggests incomplete lineage sorting between two populations (Ampara and Yala); *COI* and *Cyt b* show complete sorting. The incomplete lineage sorting of *16S rRNA* and the close genetic distance suggests that *D. atukoralei,* for which the type specimen is from Yala (Southeastern dry zone of Sri Lanka) to be synonymous with *D. scaber;* complete lineage sorting of *COI* and *Cyt b* suggests that this species is a distinct population of *D. scaber.* However, all analyses suggest restricted gene flow to the Southern wet-zone population (Figure 1A).

The clades from Ampara-Yala are resolved as the sister to the Indian and Northern Sri Lankan *D. scaber* clade rather than the clade from Galle. Although the locality data for the *D. 'atukoralei'* from Sri Lanka for Van Bocxlaer *et al.*'s (2009) analysis was not provided, it is 100% similar to the Galle population.

In our analysis, the uncorrected pairwise genetic distances calculated for Mihintale and Galle were 2.51-2.90% for *16S rRNA*, 4.75-5.37% for *COI* and 6.91-7.68% for *Cyt b.* These are the highest pairwise genetic distances observed among all populations studied by us. Earlier studies have proposed 3% and 4-8% as threshold values for species discrimination using *16S rRNA* and *COI*, respectively (Fouquet *et al*., 2007; Smith *et al.,* 2008). Indeed, Fouquet *et al.* (2007) show that the levels of divergence between lineages, populations and even most sister species in temperate areas for the *16S rRNA* gene are well below the 3% threshold limit. For *Cyt b*, species-level benchmarks have not been suggested so far. Therefore, these benchmarks only come close to the distances between Mihintale and Galle populations. Given that the Galle population is morphologically indistinguishable from the Mihintale and Ampara-Yala populations, at present we recognize the Galle population also as *D. scaber.*

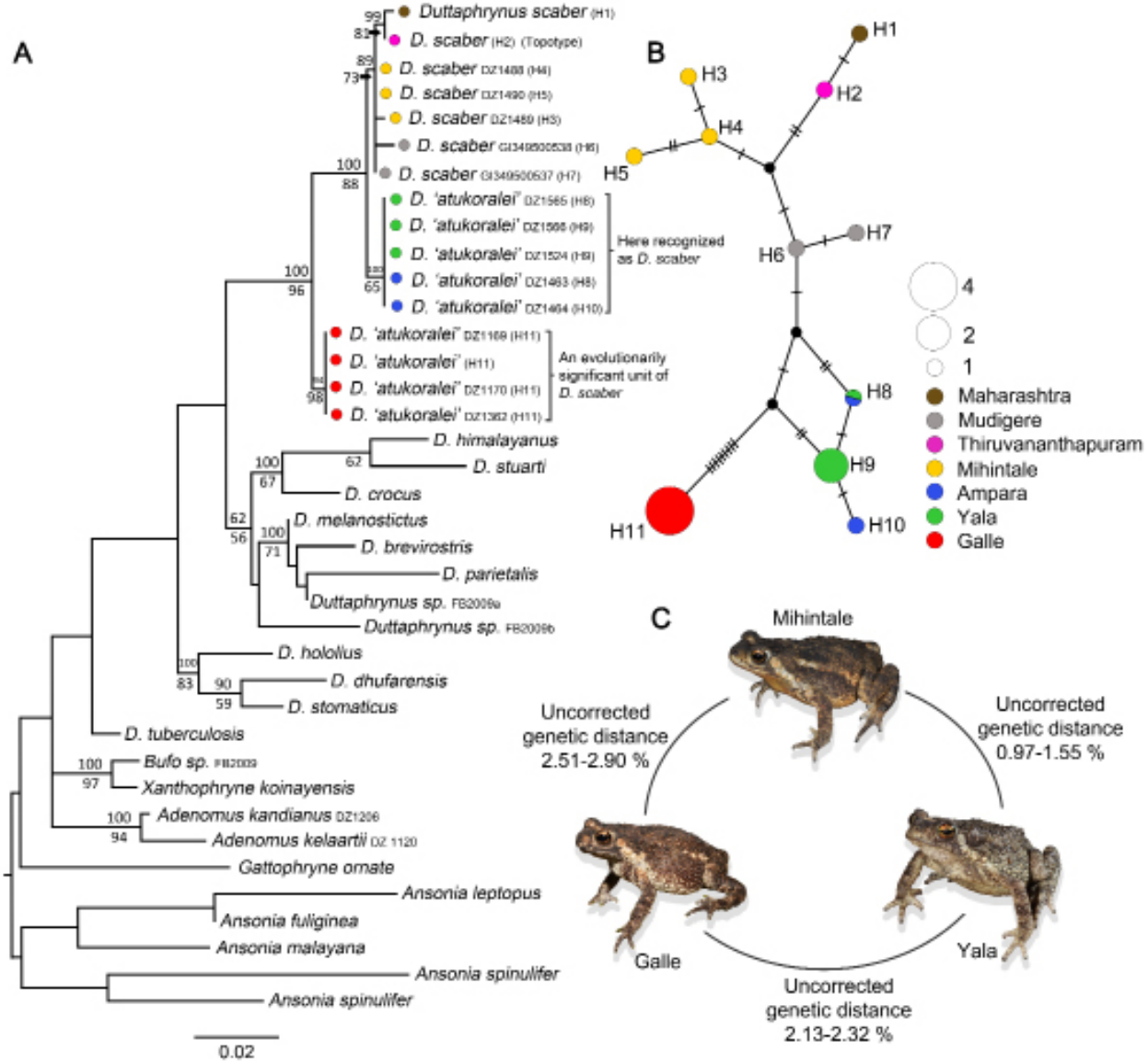

Figure 1: Results of the phylogenetic analysis and haplotype network analyses of *16S rRNA* gene for the Dwarf Toads. (A) Maximum Likelihood (ML) tree of the 38 putative species of Adenominae analyzed in this study. Values above nodes – percent Bayesian posterior probabilities; values below nodes – ML bootstrap; highlighted according to colours allocated to particular location. (B) Haplotype network of 11 unique haplotypes of *Duttaphrynus scaber* and *D. 'atukoralei'* (now *D. scaber*), based on the analysis of 517 bp fragment of the *16S rRNA* gene. Size of the circles proportional to the number of individuals sharing a given haplotype. Number of mutational steps are indicated by black dashes. (C) Habitus of *D. scaber* (Mihintale), *D. atukoralei* (Topotypes; Yala; now *D. scaber*), and *D. 'atukoralei'* (now *D. scaber*), (Galle) showing uncorrected p-distance range between populations.

The pairwise genetic distance calculated for the two clades (Ampara + Yala) and (Mihintale + India) were 0.19-1.55% for *16S rRNA*, 0.32-4.08% for *COI* and 0.21-4.36% for *Cyt b*. The highest genetic distance within the Mihintale + India *D. scaber* cluster is 1.16% for 16*S rRNA* (*COI* and *Cyt b* gene sequence data for Indian *D. scaber* were unavailable in the GenBank; we therefore used only *16S rRNA* data); the Mihintale samples are morphologically and genetically consistent with *D. scaber*.

A rough molecular divergence estimate based on the largest genetic distances of the *Cyt b* gene between suggests that northern and southern populations of *D. scaber* diverged about 4.7-7.6 million years ago in the Pliocene. When two rates of change of 0.69% (Martin and Palumbi, 1993) and 1.0% (Tan and Wake, 1995) are applied to the *Cyt b* molecular distances for the Galle and Mihinthale populations, a range of 4.7-7.6 million years since divergence is given. Though we do not have *Cyt b* material from an Indian population to compare with the Mihintale population, the patterns that we see from *16S rRNA* suggests that their separation is more recent. This suggest recent gene exchange between India and Sri Lanka's northern population. The more basal position of the Galle population in the context of the phylogeny, suggests that Dwarf toads evolved in Sri Lanka and moved across the Sri Lanka's lowland areas to India, however with later migrations (genetic interchanges). For a better understanding of the biogeography and phylogeography of these toads, dense population sampling and gene sampling is needed, especially from India.

The morphology of the Dwarf toads portrays a more complicated situation. The samples we collected from the

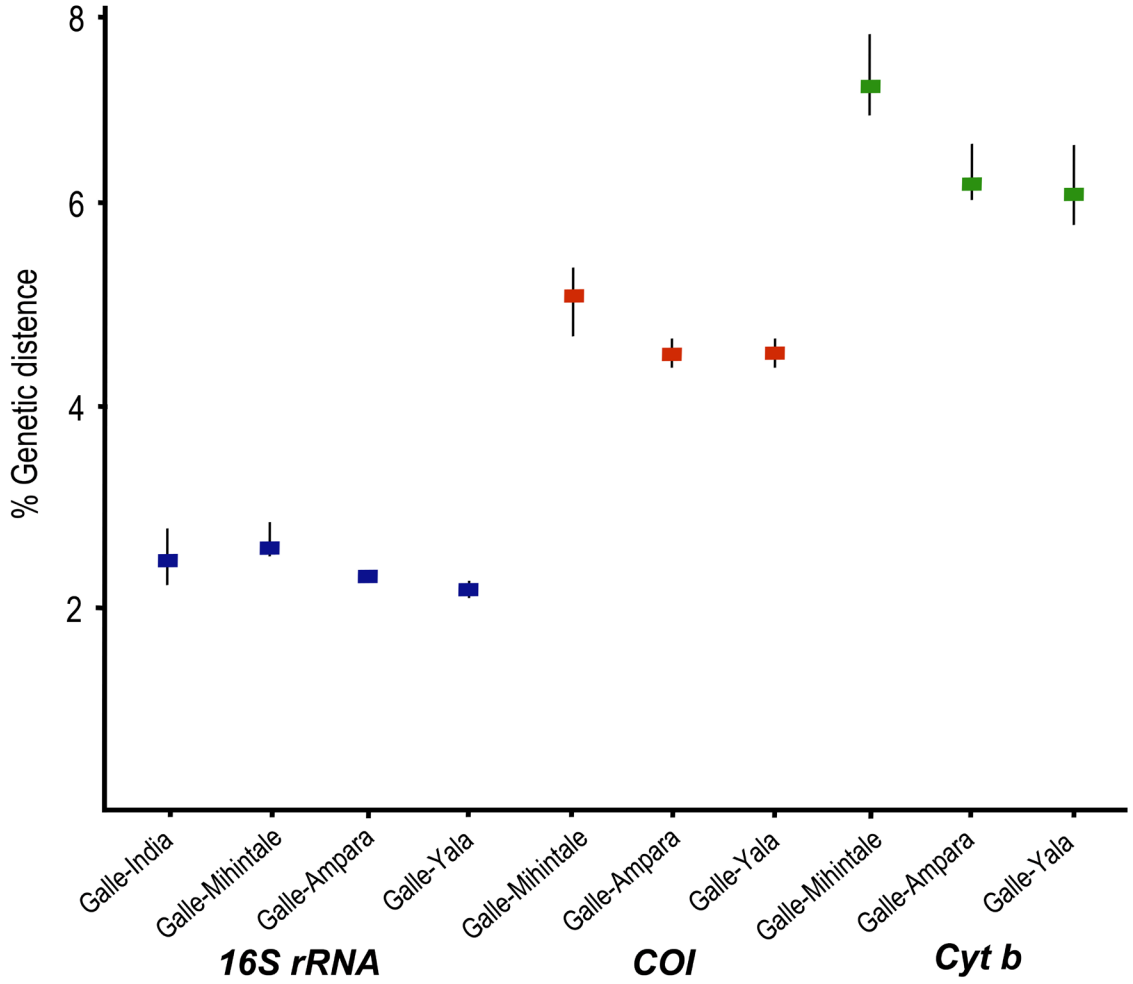

Figure 2: Mean, Minimum and maximum uncorrected pairwise genetic distance values of Galle samples with other dwarf toad samples (*D. scaber* from India and Mihintale, *D. 'atukoralei'* (now *D. scaber*) from Yala, Ampara and Galle) for *16S rRNA* (Blue), *COI* (Red) and *Cyt b* (Green) gene fragments.

type locality of *D. atukoralei* (Yala, southeastern Sri Lanka) appear morphologically similar to *D. atukoralei* (by the presence of a lobulated parotid gland), though genetically they form a clade with *D. scaber,* whose type locality is Thiruvananthapuram, Kerala State, India. The population from Galle also show a lobulated parotid gland, though it is genetically distinct from the Yala population.

Despite the small sample sizes, high mitochondrial haplotype diversity was present among all three markers. Interestingly, none of the *16S rRNA* haplotypes of *D. scaber* was shared between the Indian and Sri Lankan populations. All three mitochondrial markers showed similar patterns of genetic structure though *COI* and *Cyt b* were similar to each other. Haplotype networks of *16S rRNA, COI* and *Cyt b* corroborate the patterns of the phylogenetic analysis. According to our *Cyt b* haplotype network, no haplotype-sharing was observed between the four populations (Mihintale, Ampara, Yala and Galle). In contrast, haplotype sharing was observed only between two samples of the Ampara population and two of the Yala population. The *COI* TCS haplotype network is comparable with the *Cyt b* TCS network. When considering both the *COI* and the *Cyt b* gene sequences, the *Cyt b* sequences seems to be more suitable for constructing haplotype networks because of the greater number of variations.

16S rRNA haplotype networks indicated that there is no haplotype sharing between Mihintale (yellow), Thiruvananthapuram (pink), Maharashtra (brown), Galle (red) and Mudigere (India); (grey) samples. Ampara (blue) and Yala (green) samples share the same haplotype and all the Galle samples within the population share the same haplotype (Figure 1B). Although Indian and Sri Lankan populations do not share any haplotypes, there are only a few mutational steps among them. Bogert & Senanayake (1966), considering the external morphology, also noted that these toads in peninsular India are larger but identical with those in northern Sri Lanka despite the seawater barrier that prevents gene exchange between toads in the two areas. In both the *Cyt b* and *16S rRNA* haplotype networks, haplotypes from Yala show only a few mutational steps with *D. scaber* haplotypes, though the Galle haplotypes show significant differences from other *D. scaber* haplotypes.

Within Sri Lanka, there are no apparent extrinsic barriers to gene flow and *D. scaber* populations, except for the Galle population which is isolated by a climatic barrier. According to our results, the intra-population genetic diversity (*16S rRNA* and *COI*) of *D. scaber* (*'atukoralei'*) is low for the Galle population, suggesting a small population persisting in relative isolation. Hence our results suggest

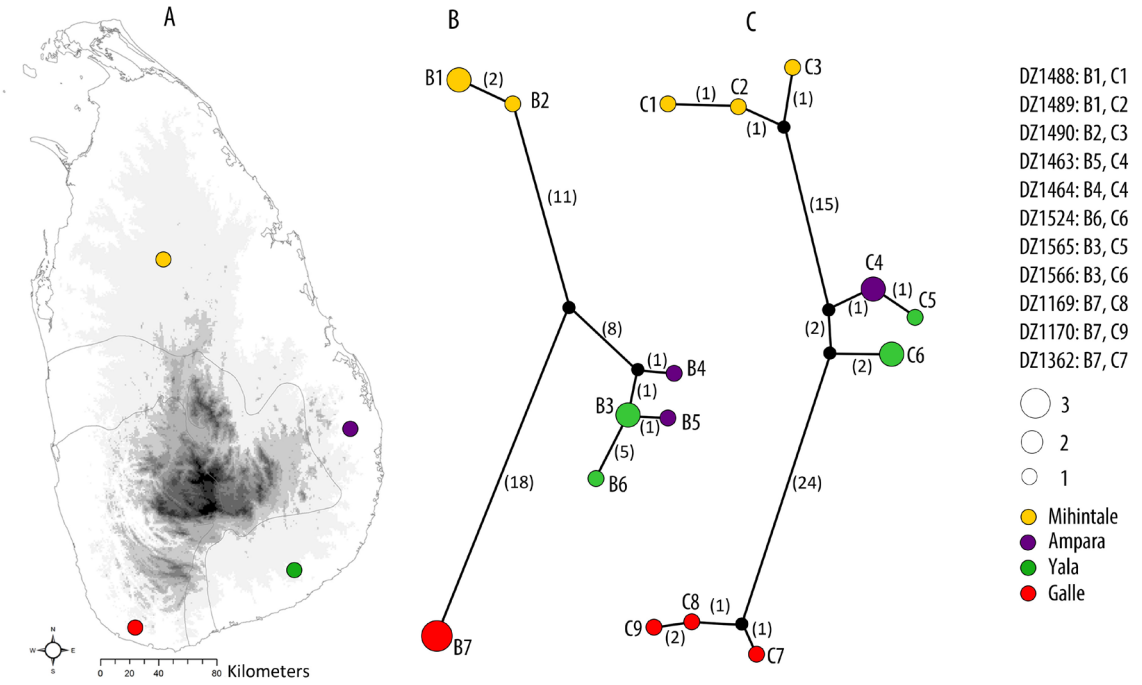

Fig. 3. (A) Sampling localities of *Duttaphrynus scaber* and *D. atukoralei* (B) TCS Haplotype network of 7 unique haplotypes of *D. scaber* and *D. 'atukoralei'* (now *D. scaber*), based on the analyses of 614 bp fragment of the *COI* gene (C) TCS Haplotype network of 9 unique haplotypes of *D. scaber* and *D. 'atukoralei'* (now *D. scaber*), based on the analysis of 482 bp fragment of the *Cyt b* gene. Size of the circles are proportional to the number of individuals sharing a given haplotype. Numbers of mutational steps are indicated by numbers within brackets.

that the Galle population is an evolutionarily significant unit (ESU) worthy of special conservation attention.

CONCLUSIONS

Our molecular analysis suggests that there is only one Dwarf toad species in Sri Lanka, which is shared with India – *Duttaphrynus scaber.* However, there is strong population structure within the populations analyzed. The analysis of the *16S rRNA* gene fragment shows incomplete lineage sorting, suggestive of a recent divergence event; the other two gene fragments analyzed, *COI* and *Cyt b,* show complete lineage sorting. The wet-adapted population from Galle is here considered to be *D. scaber,* which stands out as a distinct clade that we recognize as an evolutionarily significant unit that does not share any haplotypes with any other population. Deeper analyses using multiple criteria are necessary to ascertain if the Galle population of *D. scaber* can be considered a distinct species.

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Appendix A: Uncorrected pairwise distances (%) for *16S rRNA* gene fragments.
(localities: Ma - Maharashtra, Mu - Mudigere, T - Thiruwananrhapuram, Mi - Mihintale, A - Ampara, Y - Yala, gb - GenBank, G - Galle)

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*D. scaber* Ma															
2	*D. scaber* Mu	0.84														
3	*D. scaber* Mu2	1.05	0.21													
4	*D. scaber* T	0.19	0.63	0.83												
5	*D. scaber* Mi	0.77	0.42	0.62	0.58											
6	*D. scaber* Mi2	0.96	0.63	0.83	0.77	0.19										
7	*D. scaber* Mi3	1.16	0.84	1.04	0.96	0.38	0.58									
8	*D. atukoralei* A	1.74	1.05	0.84	1.55	1.35	1.54	1.74								
9	*D. atukoralei* A2	1.35	0.63	0.84	1.16	0.97	1.16	1.35	0.39							
10	*D. atukoralei* Y	1.55	0.84	1.05	1.35	1.16	1.35	1.55	0.19	0.19						
11	*D. atukoralei* Y2	1.35	0.63	0.84	1.16	0.97	1.16	1.35	0.39	0.00	0.19					
12	*D. atukoralei* Y3	1.55	0.84	1.05	1.35	1.16	1.35	1.55	0.19	0.19	0.00	0.19				
13	*D. 'atukoralei'* gb	2.71	2.32	2.52	2.51	2.51	2.51	2.90	2.32	2.32	2.13	2.32	2.13			
14	*D. 'atukoralei'* G	2.71	2.32	2.52	2.51	2.51	2.51	2.90	2.32	2.32	2.13	2.32	2.13	0.00		
15	*D. 'atukoralei'* G2	2.71	2.32	2.52	2.51	2.51	2.51	2.90	2.32	2.32	2.13	2.32	2.13	0.00	0.00	
16	*D. 'atukoralei'* G3	2.71	2.32	2.52	2.51	2.51	2.51	2.90	2.32	2.32	2.13	2.32	2.13	0.00	0.00	0.00

Appendix B: Uncorrected pairwise distances (%) for *COI* and *Cyt b* gene fragments.
Values above the diagonal are distances based on the COI fragment; values below the diagonal are based on the *Cyt b* fragment (Localities: Mi - Mihintale, A - Ampara, Y - Yala, gb - GenBank, G - Galle)

	Species	1	2	3	4	5	6	7	8	9	10	11
1	*D. scaber* Mi		0.00	0.49	4.08	3.91	4.08	3.91	3.91	5.22	5.37	5.37
2	*D. scaber* Mi2	0.21		0.33	3.95	3.78	3.94	3.78	3.78	5.10	5.25	5.25
3	*D. scaber* Mi3	0.63	0.42		3.60	3.43	3.60	3.43	3.43	4.75	4.89	4.89
4	*D. atukoralei* A	3.73	3.53	3.56		0.49	0.98	0.16	0.16	4.57	4.72	4.72
5	*D. atukoralei* A2	3.94	3.73	3.56	0.21		1.14	0.32	0.32	4.40	4.56	4.56
6	*D. atukoralei* Y	4.36	4.15	4.20	1.04	1.24		0.81	0.81	4.57	4.72	4.72
7	*D. atukoralei* Y2	3.94	3.73	3.77	0.21	0.41	1.24		0.00	4.40	4.56	4.56
8	*D. atukoralei* Y3	4.36	4.14	4.20	1.04	1.24	0.00	1.24		4.40	4.56	4.56
9	*D.'atukoralei'* G	7.26	7.05	6.92	6.02	6.22	5.81	6.22	5.81		0.00	0.00
10	*D.'atukoralei'* G2	7.68	7.47	7.34	6.43	6.64	6.24	6.64	6.22	0.41		0.00
11	*D.'atukoralei'* G3	7.26	7.05	6.91	6.02	6.02	5.81	6.22	5.81	0.41	0.83	