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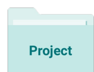

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Mitochondrial Phylogeny of Namib Day Geckos (*Rhoptropus*) Based on Cytochrome *b* and 16S rRNA Sequences

Trip Lamb and Aaron M. Bauer

We examined phylogenetic relationships among the Namib day geckos (genus *Rhoptropus*) using DNA sequence data from the mitochondrial cytochrome *b* (cyt*b*) and 16S ribosomal RNA genes. Maximum-parsimony analysis of the cyt*b*, 16S, and combined (cyt*b*/16S) datasets each recovered an identical single most-parsimonious tree, revealing two well-supported clades: (1) *Rhoptropus afer* + *Rhoptropus bradfieldi* ssp and (2) *Rhoptropus boultoni* + (*Rhoptropus barnardi* + *Rhoptropus biporosus*). Maximum-likelihood analysis identified the same two species groups and corroborated patterns of relationship within respective parsimony clades. Moreover, the mitochondrial DNA trees were congruent with parsimony trees derived from morphological and allozymic characters. Congruence observed among these different data offers strong evidence for our molecular phylogeny of *Rhoptropus*, bringing taxonomic stability to a genus whose species-level relationships were widely debated throughout the last century.

THE Namib day geckos (genus *Rhoptropus*) form an unusual assemblage of strictly diurnal gekkonines endemic to the arid regions of western Namibia and southern Angola. In addition to diurnal activity, *Rhoptropus* exhibits several apomorphic features that are otherwise rare or unique among gekkonine geckos. These diagnostic characters include the following: (1) a reduced number of presacral vertebrae (24–25 vs 26 in other African gekkonines); (2) ventrolateral fat deposits; and (3) a ligamentous binding between the metatarsals of digits II and III, which causes these elements to lie parallel (Bauer and Good, 1996). Conversely, other digital features, most notably the hyperphalangy of digit I, reflect close evolutionary ties of the genus to the *Pachydactylus* group, a large assemblage of Afro-Mediterranean gekkonines (Russell, 1972; Haacke, 1976; Kluge and Nussbaum, 1995). We recently generated molecular phylogenetic support from mitochondrial sequence data for this systematic interpretation, identifying *Rhoptropus* as the sister taxon to *Pachydactylus* sensu lato (unpubl.).

Intrageneric relationships among the Namib day geckos have been less tractable. Centered largely in the mid-1900s, the systematic history of *Rhoptropus* entailed extensive debate over species boundaries, resulting in taxonomic flux and frequent nomenclatural misapplication. By the time the last of six currently recognized species was described (Laurent, 1964), each taxon (except *Rhoptropus afer*) had been linked to every other taxon through misidentification, synonomy, or presumed affinity (for a review, see Bauer and Good, 1996). Russell (1972) addressed scalation features in a preliminary assessment of relationships within *Rhoptropus*. Bauer and Good (1996) provided a more definitive phylogenetic hypothesis in a cladistic analysis of 16 morphological and six allozymic characters (Fig. 1). Herein, we present a phylogenetic analysis of sequence data from the mitochondrial cytochrome *b* and 16S ribosomal RNA genes. We compare the phylogenetic estimates from these different datasets to assess levels of congruence, from which we infer a *Rhoptropus* phylogeny.

Materials and Methods

Liver tissue was obtained for five of the six species of *Rhoptropus* (samples of the Angolan endemic *Rhoptropus taeniostictus* could not be secured). We processed 2–3 specimens per species, choosing localities that maximized distance between collection sites to assess levels of intraspecific variation. Two of the six species, *Rhoptropus boultoni* and *Rhoptropus bradfieldi,* are polytypic. We secured both subspecies of *R. bradfieldi* (*Rhoptropus bradfieldi bradfieldi, Rhoptropus bradfieldi diporus*) but caught only the nominate subspecies for *R. boultoni.* The remaining subspecies *Rhoptropus bradfieldi benguellensis* and *Rhoptropus bradfieldi montanus,* both Angolan endemics, were not accessible. Two basal species within the sister taxon *Pachydactylus* (*Pachydactylus bibronii* and *Pachydactylus tetensis*) were selected as outgroups.

Tissue samples were processed at field collection sites and preserved in liquid nitrogen (for subsequent storage at −80 C) or in a saturated salt-DMSO buffer (Amos and Hoelzel, 1991). Genomic DNA was extracted from liver using

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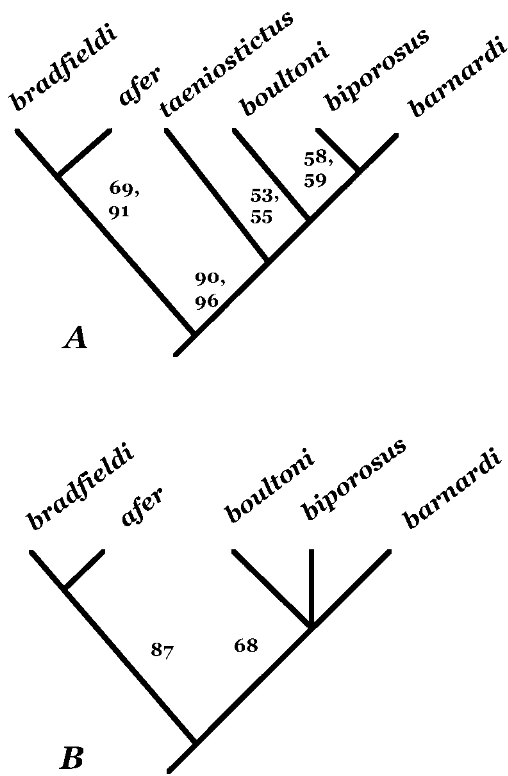

Fig. 1. Phylogenetic hypotheses for the genus *Rhoptropus* depicted by (A) majority rule consensus of three equally parsimonious trees based on 16 morphological characters and (B) strict consensus of two trees based on six allozymic characters (Bauer and Good, 1996). The combined data yield a majority rule consensus (from three trees) identical to the morphology tree. Numbers indicate proportion of 1000 bootstrap replicates supporting given nodes; paired values for tree A are bootstrap estimates for morphological (top) and combined (bottom) data.

the Qiagen QIAamp DNA Mini kit. Portions of the mitochondrial cytochrome *b* (cyt*b*) and 16S rRNA genes provided some 830 nucleotide bases for phylogenetic analysis. The primers L14724 and H15149 (Meyer et al., 1990) were used to amplify a 400 bp segment of the cyt*b* gene. DNA samples from *Rhoptropus biporosus* and *R. boultoni* failed to amplify using the cyt*b* primers above, necessitating the design of a new heavy stand primer (5′-GTG CCR ATG TTT CAT GTT TCT AYG TT-3′) as a substitute for H15149. The resulting amplification yields a slightly smaller fragment (~ 340 bp). A 600 bp segment of the 16S rRNA gene was amplified using the primers LGL 286 and LGL 381 (Bickham et al., 1996). For both cyt*b* and 16S segments, 50 μl reactions were amplified for 32 cycles at 90 C for 45 sec, 50–55 C for 35 sec, and 72 C for 1 min.

Amplification products were purified over Centri-sep columns and served as templates in cycle-sequencing reactions employing dye-labeled terminators (PRISM kit, Applied Biosystems, Inc.). PRISM reaction products were analyzed on an Applied Biosystems 373A automated DNA sequencer. Forward and reverse sequences were generated for each sample and their complementarity confirmed using the Sequence Navigator software (Applied Biosystems, Inc.). Sequences were initially aligned using the CLUSTAL X program, applying default settings (Thompson et al., 1997). Given the indel variation commonly observed in rDNA sequences, we examined 16S alignments in greater detail, exploring gap placements for a series of gap opening (= 5, 10, 15, and 20) and extension costs (= 0.10 and 5.0) with the Multiple Alignment Parameters option in CLUSTAL X. Regions of 16S sequence whose nucleotide position homologies varied across different gap parameters were considered alignment-ambiguous (Gatesy et al., 1993), and any regions so disclosed were excluded from phylogenetic analysis. We also aligned the 16S sequences against those for the iguanids *Petrosaurus mearnsi* and *Uta stansburiana,* two lizards for which the secondary structure of 16S rRNA has been characterized (Wiens and Reeder, 1997). Using the gap cost variables outlined above, we examined gap placement in gecko sequences relative to stem versus nonstem regions identified in the iguanid sequences. We assessed levels of intra- versus interspecific sequence divergence using estimates derived from the Hasegawa-Kishino-Yano model (HKY85; Hasegawa et al., 1985), which assumes different evolutionary rates for transitions and transversions and unequal base frequencies.

Phylogenetic inference was based on maximum parsimony (MP) and maximum likelihood (ML) analyses implemented in PAUP* (vers. 4.0, D. L. Swofford, Sinauer, Sunderland, MA, 1998, unpubl.). For MP, we conducted two exhaustive searches, employing the cyt*b* and 16S datasets, respectively. Given the distinct post-transcriptional functions of these two genes (and different evolutionary constraints thus imposed), we performed the incongruence length difference (ILD) test (Farris et al., 1994) to assess combinability of the cyt*b* and 16S datasets. ILD analysis involved 1000 replicates using the partition homogeneity test in PAUP*; parsimony-uninformative characters were excluded from the respective datasets prior to analysis. Results of the ILD test showed no significant

TABLE 1. PAIRWISE GENETIC DISTANCE VALUES (HKY85) FOR THE 16S (ABOVE DIAGONAL) AND CYT*b* (BELOW DIAGONAL) GENE SEQUENCES.

	1	2	3	4	5	6	7	8
1 *R. biporosus*	—	0.1016	0.1300	0.1455	0.1365	0.1409	0.3011	0.2835
2 *R. barnardi*	0.1931	—	0.0893	0.1308	0.1172	0.1266	0.2614	0.2692
3 *R. boultoni*	0.2109	0.2183	—	0.1258	0.1028	0.1166	0.2527	0.2770
4 *R. afer*	0.2656	0.2599	0.2577	—	0.0909	0.1044	0.2635	0.2881
5 *R. b. bradfieldi*	0.2497	0.2193	0.2291	0.1853	—	0.0394	0.2859	0.2843
6 *R. b. diporus*	0.2255	0.2370	0.2409	0.2021	0.0709	—	0.2712	0.2754
7 *P. tetensis*	0.3752	0.3512	0.3821	0.3611	0.3252	0.3582	—	0.2642
8 *P. bibronii*	0.4138	0.4408	0.4305	0.3964	0.3660	0.3929	0.3875	—

conflict between the two mitochondrial datasets ($P = 0.958$), which were combined in a third MP analysis. All character state changes were weighted equally in the initial parsimony searches.

Prior to ML analysis, we used the MODELTEST program (vers. 3.04; Posada and Crandall, 1998) to identify the substitution model most appropriate for the combined (cyt*b*/16S) dataset. MODELTEST selects one of up to 56 models that best fit the DNA data, executing a hierarchical series of likelihood ratio tests ($\delta = -2 \log \Lambda$) to compare likelihood scores between increasingly complex models for a given user tree. We used the program's default settings to generate a neighbor-joining tree as a test tree and subsequently to compare substitution models ranging from simple (Jukes-Cantor) to parameter rich (general time-reversible; Rodríquez, et al. 1990; Yang, 1994). Bootstrap analyses involving 1000 pseudoreplicates were conducted to estimate confidence limits for topological patterns revealed by MP and ML procedures.

RESULTS

Sequence variation.—Sequences 315 nucleotides (nt) in length were obtained for the cyt*b* gene; for the 16S gene, sequences ranging 506–511nt were compiled as 515 aligned positions. Four ambiguously aligned regions (totaling 19 nt positions) were identified among the 16S sequences and excluded from subsequent analysis. Each of the four excluded regions lies within loops associated with the stems S27, S30–2 (two regions), and S32, as identified in the iguanid 16S sequences (stem nomenclature follows Gutell and Fox, 1988). Sequences are deposited in GenBank (accession numbers AY026920–026935). A total of 159 variable sites was detected for the cyt*b* segment, 49 of which involved changes in the first codon position, 18 in the second, and 92 in the third. Of the 159 variable sites, 92 were informative under the conditions of parsimony. The 16S segment had 186 variable sites, 98 of which were parsimony-informative. The two gaps retained in the 16S alignments (a two base deletion in the outgroup taxon *P. bibronii,* and a single base deletion shared among all *Rhoptropus*) were treated as missing data.

Intraspecific sequence divergence was minimal overall. Identical gene sequences were observed for populations of *R. afer*; similarly, no genetic variation was detected between geographic locales for *R. boultoni.* Population samples for *Rhoptropus barnardi* and *R. diporus* differed by one silent, third position transition in cyt*b* for each species. Genetic distances between the two subspecies of *R. bradfieldi* were more substantive, 7.1% for cyt*b* and 3.9% for 16S. Divergence values among *Rhoptropus* species ranged from 9–15% for 16S, whereas those for cyt*b* ranged from 19–27% (Table 1). The latter values reflect the higher end of congeneric genetic distances assembled in a recent comparative summary of cyt*b* sequence divergence in reptiles (and other vertebrates; Johns and Avise, 1998). The divergence levels and patterns of substitution observed for the cyt*b* sequences suggested saturation might be problematic at third position sites. To examine substitutional saturation, we compared uncorrected pairwise divergences with corrected (HKY85) divergences for first, second, and third position partitions. In this qualitative assessment, the degree to which scatter point plots deviate from the $x = y$ line reflects saturation levels. The results of these scatterplots indicated third position sites appear to be approaching saturation at higher levels of sequence divergence (Fig. 2).

MP and ML analyses.—The exhaustive searches of separate (cyt*b,* 16S) and combined (cyt*b*/16S) datasets each recovered an identical single most-parsimonious tree (Fig. 3). Results of these parsimony analyses clearly indicate the division

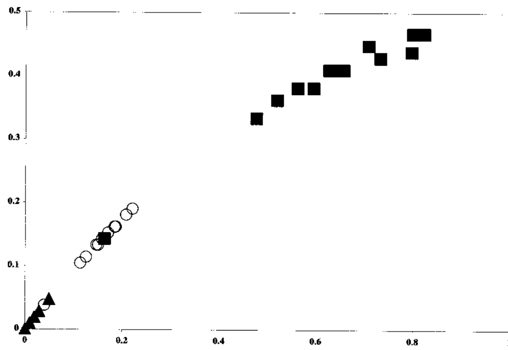

Fig. 2. Plots of uncorrected versus corrected (HKY85) pairwise divergences for each codon position for cyt*b*. First position comparisons are represented by open circles, second position by triangles, and third position by squares.

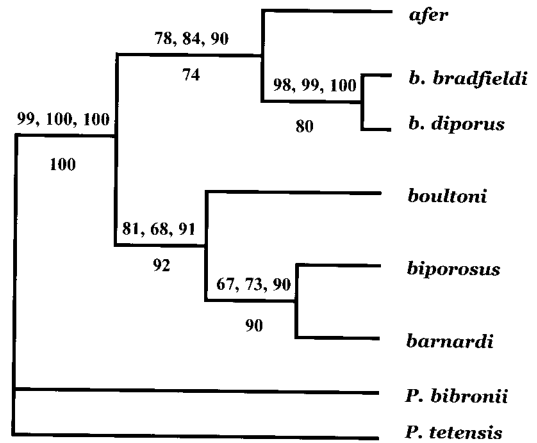

Fig. 3. Single most-parsimonious tree for cyt*b*, 16S, and combined (cyt*b*/16S) sequence datasets for the genus *Rhoptropus*. Numbers above branches indicate proportion of bootstrap replicates supporting nodes for (left to right) cyt*b*, 16S, and combined data. Values below branches are bootstrap estimates for ML analysis of the combined data.

of *Rhoptropus* into two well-supported clades: (*afer* + *bradfieldi* ssp) and (*boultoni* + (*barnardi* + *biporosus*)) (Table 2). Bootstrap support for the two clades was high (> 70% for each analysis; Hillis and Bull, 1993), especially for the combined data (> 90%; Fig. 3). Given the possibility of some substitutional saturation at third position sites for cyt*b* (particularly transitions), we performed an additional MP analysis of the cyt*b* data. This analytical permutation, which involved weighting transversions 2.4 times transitions for all substitutions [the tv:ts ratio derived from a maximum likelihood (HKY85) estimate], yielded a single most-parsimonious tree identical to those of the equal weighting analyses, with comparable tree statistics (Table 2). Results of the MODELTEST identified the transversional model with variable sites assumed to follow a gamma distribution (TVM + Γ) as the best fit for the combined dataset. The ML analysis recovered a topology identical to that of the MP analyses, with a $-\ln L_{TVM + \Gamma} = 3705.23$ and high bootstrap support for the patterns of relationship observed for respective parsimony clades (Fig. 3).

Discussion

Much of the systematic history of *Rhoptropus* has centered on issues regarding species descriptions and species boundaries. Part of this debate can be attributed to limited material available for study; even the most geographically widespread species were not described until the mid-1900s. FitzSimons (1938) drew attention to the confounding effects of significant variation in scalation, calling to question characters that were previously considered diagnostic for *Rhoptropus* species. Overall, preoccupation with these alpha-level issues hindered efforts to resolve phylogenetic affinities within *Rhoptropus* as a whole.

The first attempt to evaluate patterns of intrageneric relationship in *Rhoptropus* was that of Russell (1972). He grouped *R. afer* and *R. bradfieldi* on the basis of certain scalation features (no preanal pores, no postmental scales, large gular shields, more scansors) and, in turn,

Table 2. Tree Statistics for Parsimony Analyses of mtDNA Sequence Datasets.

	Cyt*b*	Cyt*b* tv 2.4 X ts	16S	Cyt*b*/16S
Tree length	309	478	312	621
Consistency index	0.7443	0.7383	0.7660	0.7552
Retention index	0.4733	0.5170	0.5629	0.5205
Rescaled consistency index	0.3523	0.3817	0.4312	0.3931

linked *R. barnardi* and *R. boultoni* using alternative states for these characters (preanal pores, small postmentals, imbricate gulars, fewer scansors). Subsequently, Bauer and Good (1996) conducted a cladistic analysis of morphological (skeletal and scalation) and allozyme characters, recovering the consensus tree illustrated in Figure 1. Bauer and Good identified two clades within the genus that corresponded with Russell's (1972) preliminary groupings. *Rhoptropus afer* and *R. bradfieldi* were retained as sister species in a distinct clade, whereas *R. biporosus* and *R. taeniostictus* were incorporated in the *barnardi* + *boultoni* clade. Although relationships among *barnardi, boultoni,* and *biporosus* could not be resolved definitively by allozyme data alone, Bauer and Good's analysis of the morphological and, subsequently, combined datasets depicted *barnardi* and *biporosus* as sister taxa. Our mtDNA trees are consistent with both Russell's preliminary findings and Bauer and Good's cladistic analysis. Topologically, trees from these different datasets are (with the noted absence *R. taeniostictus*) perfectly congruent. The placement of *R. taeniostictus* as the basal taxon to *boultoni* + (*barnardi* + *biporosus*) rests solely on morphological data (Fig. 1). Nonetheless, its affinity with the *boultoni/barnardi* clade is unambiguously supported by three morphological synapomorphies (Bauer and Good, 1996).

Phylogenetic estimates derived from different datasets allow insightful comparisons of evolutionary patterns and the impact they impose on recovering a credible phylogeny. For example, functional convergence is often disclosed when estimates, particularly those between morphological versus molecular data, disagree (McKracken et al., 1999). Conversely, congruence among independent data sets underscores strong evidence of phylogeny (Hillis, 1987). Identical trees observed for the mtDNA sequence, morphological, and morphological + allozymic datasets offer a robust phylogenetic hypothesis for the genus *Rhoptropus*.

Materials Examined

Collection abbreviations: AMB = A. M. Bauer field series, CAS = California Academy of Sciences, SMW = National Museum of Namibia. Abbreviations in square brackets indicate ultimate repository for specimens not yet accessioned into public museum collections.

Pachydactylus bibronii: CAS 200002 (10 km S Steinkopf, Northern Cape, South Africa—29°20′16″S, 17°47′31″E).

Pachydactylus tetensis: AMB 6180 [CAS] (N bank Rwenya River, Mashonaland East, Zimbabwe—17°15′19″S, 33°00′12″E).

Rhoptropus afer: CAS 193868 (30 km N Swakopmund on Hentiesbaai Rd., Namibia), CAS 206949 (17.3 km E Hentiesbaai on Usakos Rd., Namibia—22°03′04″S, 14°26′29″E).

Rhoptropus barnardi: AMB 5938 [SMW] (Palmwag Lodge, Namibia), CAS 206953 (62.5 km NE of Ugab River mouth, Namibia).

Rhoptropus biporosus: CAS 193822 (37 km W Orupembe, Namibia), CAS 206976 (E border of Skeleton Coast Park, Namibia at 17°51′S).

Rhoptropus boultoni: CAS 193661 (62.1 km E Kamanjab on Outjo Rd., Namibia), CAS 206975 (E border of Skeleton Coast Park, Namibia at 17°51′‘S).

Rhoptropus bradfieldi bradfieldi: CAS 193897, 193868 (30 km N Swakopmund on Hentiesbaai Rd., Namibia), CAS 206951 (11.3 km S. Cape Cross on Hentiesbaai Rd., Namibia—21°49′54″S, 14°04′13″E).

Rhoptropus bradfieldi diporus: AMB 6368 (near Gai-as, Namibia—20°47′18″S, 14°06′44″E).

Acknowledgments

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