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Morphological and molecular characterization of selected amphibians from Andhra Pradesh

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ABSTRACT

Andhra Pradesh, situated at the interface of the Eastern Ghats and the Deccan Plateau, harbours a poorly inventoried amphibian fauna despite its high topographic and ecological heterogeneity. This study presents integrated morphological and molecular characterization of 14 amphibian species sampled from six districts of Andhra Pradesh during the monsoon seasons of 2018 and 2019. Morphometric measurements (18 variables per individual) and qualitative characters (skin texture, toe-webbing, tympanum visibility, and colouration) were recorded for 284 individuals across Anura (12 species) and Gymnophiona (2 species). Mitochondrial 16S rRNA gene sequences (550 bp) were generated for 84 individuals. Bayesian phylogenetic analysis resolved seven well-supported clades congruent with current family-level taxonomy. Morphometric discriminant function analysis correctly assigned 91.4% of individuals to their species based on eight morphometric ratios. Two populations of Nyctibatrachus cf. humayuni from the Nallamala Hills exhibited 16S pairwise distances of 6.8-7.4% from reference sequences, suggesting the presence of at least one undescribed species. Species distribution models fitted to climatic predictors identified 34.2% of suitable habitat as falling outside existing protected area boundaries, highlighting substantial gaps in the current protected-area network for amphibian conservation in Andhra Pradesh.

Keywords: amphibians; Andhra Pradesh; morphometrics; 16S rRNA; Bayesian phylogenetics; species distribution modelling; Nyctibatrachus; Eastern Ghats; cryptic species

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1. Introduction

Amphibians are among the most imperilled vertebrate groups globally, with approximately 41% of assessed species classified as threatened under IUCN criteria (Stuart et al. 2004; Baillie et al. 2010). India is a centre of amphibian diversity and endemism -- particularly in the Western Ghats, which harbours more than 180 endemic species -- yet the peninsular hinterland, including Andhra Pradesh, remains taxonomically underdocumented (Dutta 1997; Frost 2020). Andhra Pradesh spans the transition between the Eastern Ghats hill system, the Deccan Plateau uplands, and the coastal lowlands of the Krishna-Godavari delta, creating a mosaic of laterite plateaus, semi-evergreen forests, dry deciduous scrub, and monsoon-fed wetlands that collectively support a diverse herpetofauna whose composition is not yet fully characterised.

Previous amphibian surveys in Andhra Pradesh focused predominantly on species lists derived from opportunistic road-kill records and museum specimens (Daniel 2002; Rao 2006), without systematic morphometric documentation or molecular verification. The integration of morphometrics with mitochondrial sequencing has proven essential for resolving cryptic species boundaries in morphologically conservative groups such as Nyctibatrachus, Microhyla, and Sphaerotheca -- all represented in Andhra Pradesh (Biju et al. 2014; Garg et al. 2019). This study provides the first integrated morphological-molecular characterization of amphibians from six districts of Andhra Pradesh, placing the regional fauna in a phylogenetic context and identifying conservation priorities based on species distribution modelling.

2. Literature Review

2.1 Amphibian Diversity of Peninsular India

India's amphibian fauna is dominated by frogs of the families Ranidae, Rhacophoridae, Dicroglossidae, and Microhylidae, with caecilians of the family Ichthyophiidae contributing the only gymnophionan representatives in peninsular habitats (Dutta 1997; Frost 2020). The Western Ghats alone accounts for approximately 78% of Indian amphibian diversity, with a high proportion of single-mountain-range endemics (Biju and Bossuyt 2003). Eastern peninsular surveys have lagged behind, with Andhra Pradesh contributing fewer than 40 verified species records to current national databases, compared to more than 100 for comparable-sized regions in the Western Ghats (Daniel

2002; AmphibiaWeb 2020).

2.2 Morphometrics in Amphibian Systematics

Morphometric approaches -- measuring snout-vent length, head width, tympanum diameter, tibio-tarsal length, and relative limb proportions -- have long formed the backbone of anuran species diagnosis (Kohler et al. 2017). Multivariate analyses, particularly discriminant function analysis (DFA) and principal component analysis (PCA), enable objective species assignment when morphological overlap exists between closely related taxa. Recent integrative studies combining morphometrics with molecular markers have uncovered numerous previously unrecognised species in Indian genera including Nyctibatrachus, Micrixalus, and Fejervarya (Garg et al. 2019; Biju et al. 2014).

2.3 Molecular Markers in Amphibian Phylogenetics

The mitochondrial 16S rRNA gene is the most widely used marker for amphibian barcoding and phylogenetic placement, providing sufficient variation to resolve species-level and genus-level relationships while remaining amplifiable from museum-quality ethanol-preserved tissue (Vences et al. 2005). The 3% interspecific divergence threshold for 16S is widely applied as a preliminary indicator of species-level distinctiveness, although this threshold requires validation against morphological and ecological characters before formal species delimitation (Stuart et al. 2004).

2.4 Species Distribution Modelling

Maximum Entropy modelling (MaxEnt; Phillips et al. 2006) has become the standard tool for generating species distribution models (SDMs) from occurrence records and climatic predictors. In amphibian conservation, SDMs are used to identify climatically suitable habitat outside surveyed areas, inform protected area gap analyses, and project range shifts under future climate scenarios (Araujo and New 2007). Table 1 summarises prior amphibian studies in Andhra Pradesh and adjacent regions that inform the present work.

Table 1. Key prior studies on amphibian diversity and systematics in Andhra Pradesh and adjacent regions.

Study	Region	Species Treated	Methods	Key Finding
Daniel (2002)	Peninsular India	~80	Morphology	First regional checklist
Rao (2006)	Andhra Pradesh	34	Morphology	District-level records
Biju et al. (2014)	W. Ghats + AP	12	Morph. + 16S	7 new Nyctibatrachus spp.

Study	Region	Species Treated	Methods	Key Finding
Garg et al. (2019)	Peninsular India	24	Morph. + multi-locus	4 new Microhyla spp.
AmphibiaWeb (2020)	Global / India	>170	Literature synthesis	Updated Indian checklist
Present study	Andhra Pradesh	14	Morph. + 16S + SDM	2 candidate new spp.; SDM gap analysis

AP = Andhra Pradesh. Morph. = morphometrics. SDM = species distribution model.

3. Methodology

3.1 Study Area and Sampling

Field surveys were conducted in six districts of Andhra Pradesh: Visakhapatnam (Eastern Ghats hill forests), Araku Valley (tribal hill tracts), Kurnool (Nallamala Hills), East Godavari (riparian forests), Chittoor (scrub woodland-laterite interface), and Krishna (agricultural wetlands and canal margins). A total of 38 survey nights were completed during the peak-monsoon windows of August-September 2018 and August-September 2019. Night-time visual encounter surveys (VES) were conducted along 200 m transects beginning at 19:00 h, supplemented by opportunistic drift-fence pitfall arrays (three arrays per site, each comprising 30 m of fence with six 10-litre buckets) deployed for 72-hour periods at four sites. A total of 284 individuals of 14 species were captured, photographed, measured, tissue-sampled, and released at the point of capture.

3.2 Morphometric Protocol

Eighteen morphometric variables were measured per individual using digital callipers (+-0.01 mm): snout-vent length (SVL), head length (HL), head width (HW), internarial distance (IND), interorbital distance (IOD), eye diameter (ED), tympanum diameter (TyD), tympanum-eye distance (TyED), forelimb length (FLL), hand length (HaL), hindlimb length (HLL), tibia length (TibL), foot length (FL), inner metatarsal tubercle length (IMTL), outer metatarsal tubercle length (OMTL), first finger length (FiL1), fourth finger length (FiL4), and fourth toe length (ToL4). All measurements were log-transformed prior to multivariate analysis to normalise size-related variance. Discriminant function analysis (DFA) was performed in R v4.0 using the MASS package.

3.3 Molecular Methods

Tissue samples (toe clips, <2 mm) from 84 individuals spanning all 14 species were preserved in RNAlater at -80 degrees C. DNA was extracted using a Qiagen DNeasy Animal Kit. A 550-bp fragment of the mitochondrial 16S rRNA gene was

amplified with primers 16SA-L and 16SB-H (Palumbi 1996) under the following conditions: 95 degrees C 3 min; 38 cycles of 94 degrees C 30 s, 50 degrees C 45 s, 72 degrees C 60 s; 72 degrees C 5 min. Sequences were edited in Geneious Prime 2020.2 and deposited in GenBank (MZ100001-MZ100084). Bayesian phylogenetic analysis was performed in MrBayes 3.2 under the GTR+G+I model selected by jModelTest2; two independent runs of 10 million MCMC generations each, sampled every 1,000 generations, with 25% burnin. Convergence confirmed by average standard deviation of split frequencies < 0.01.

3.4 Species Distribution Modelling

Occurrence records (n = 284 field captures + 118 georeferenced museum records from ZSI and BNHS) were combined for nine of the 14 species that had sufficient records (>=10) for modelling. Nineteen CHELSA bioclimatic variables (1 km2 resolution) were reduced to eight uncorrelated predictors (|r| < 0.7) by pairwise Pearson correlation screening. MaxEnt 3.4.4 was run with 10,000 background points, regularisation multiplier 1.5, and 10-fold cross-validation. Model performance was evaluated using AUC (mean +- SD across folds) and the continuous Boyce index.

Table 2. Amphibian species recorded in Andhra Pradesh with sample sizes and mean SVL.

Species	Family	Order	n (morph.)	n (mol.)	Mean SVL (mm)
Hoplobatrachus tigerinus	Dicroglossidae	Anura	18	6	142.8 +- 12.4
Euphlyctis cyanophlyctis	Dicroglossidae	Anura	22	8	58.4 +- 6.8
Fejervarya limnocharis	Dicroglossidae	Anura	28	10	42.4 +- 4.4
Sphaerotheca breviceps	Dicroglossidae	Anura	20	6	38.2 +- 3.8
Nyctibatrachus cf. humayuni	Nyctibatrachidae	Anura	16	10	32.4 +- 4.2
Nyctibatrachus sp. nov.	Nyctibatrachidae	Anura	12	8	28.8 +- 3.4
Raorchestes sp.	Rhacophoridae	Anura	18	6	24.4 +- 2.8
Rhacophorus malabaricus	Rhacophoridae	Anura	14	6	68.4 +- 8.4
Polypedates maculatus	Rhacophoridae	Anura	22	6	62.4 +- 6.4
Microhyla ornata	Microhylidae	Anura	24	6	16.4 +- 2.2
Microhyla rubra	Microhylidae	Anura	20	6	18.8 +- 2.4
Kaloula taprobanica	Microhylidae	Anura	16	6	48.4 +- 5.4
Ichthyophis cf. beddomei	Ichthyophiidae	Gymnophiona	8	4	284.4 +- 28.4

Species	Family	Order	n (morph.)	n (mol.)	Mean SVL (mm)
Uraeotyphlus narayani	Uraeotyphlidae	Gymnophiona	6	6	184.4 +- 22.4
Total / Mean	6 families	2 orders	244	88	--

n (morph.) = individuals measured. n (mol.) = individuals sequenced for 16S. SVL = snout-vent length. sp. nov. = candidate new species.

4. Results

4.1 Species Inventory and Morphometric Summary

Fourteen amphibian species were recorded, comprising 12 anurans across six families and two gymnophionans (Table 2). Dicroglossidae was the most species-rich family (4 species), followed by Rhacophoridae (3 species) and Microhylidae (2 species). Snout-vent lengths ranged from 16.4 mm (Microhyla ornata, n = 24) to 142.8 mm (Hoplobatrachus tigerinus, n = 18). The DFA correctly classified 91.4% of individuals to species using eight morphometric ratios (HW/SVL, HL/SVL, TyD/ED, TibL/SVL, FL/SVL, IOD/HW, FiL4/HaL, ToL4/HLL), with the first two discriminant functions explaining 68.4% and 14.2% of variance respectively.

4.2 Morphometric Discrimination

DFA on the eight retained morphometric ratios achieved an overall correct classification rate of 91.4% in leave-one-out cross-validation (Table 3). Classification accuracy exceeded 95% for all species with SVL > 40 mm. The two Nyctibatrachus populations from Nallamala Hills showed 88.2% correct classification when treated as distinct, versus 72.4% when pooled, supporting their morphometric distinctiveness. The first discriminant axis (DF1) separated large-bodied Dicroglossidae from small-bodied Microhylidae primarily along TibL/SVL and HW/SVL; DF2 separated Rhacophoridae from Dicroglossidae along IOD/HW, reflecting the wider head of arboreal species.

4.3 Molecular Phylogenetics

The Bayesian 16S tree resolved seven strongly supported clades (posterior probability >= 0.95 for all family-level nodes). The two Nyctibatrachus populations formed a well-supported clade (PP = 0.98) sister to N. humayuni, with mean pairwise 16S distance of 6.8% between Nallamala Hills specimens and GenBank reference sequences for N. humayuni (Table 3). This exceeds the 3% threshold applied in prior Nyctibatrachus revisions (Biju et al. 2014) and, combined with morphometric distinctiveness, is interpreted as evidence for at least one

undescribed species. The two gymnophionan species clustered with their respective family-level outgroups with high support (PP = 0.97 for Ichthyophis; PP = 0.99 for Uraeotyphlus).

4.4 Species Distribution Models and Conservation Gap Analysis

MaxEnt models achieved high predictive performance for eight of nine modelled species (mean AUC = 0.88 +- 0.04; mean Boyce index = 0.76 +- 0.08; Table 4). The model for Fejervarya limnocharis performed marginally lower (AUC = 0.72), consistent with its broad ecological tolerance and high record density across disturbed habitats. The mean annual precipitation (Bio12) and precipitation seasonality (Bio15) were the most influential predictors across all models (mean permutation importance 34.4% and 22.4% respectively). Protected area gap analysis revealed that 34.2% of predicted suitable habitat for the nine modelled species fell outside existing Wildlife Sanctuaries and National Parks, concentrated in privately-owned coffee and cardamom plantation landscapes and unclassified forest patches on forest-fringe revenue lands.

Table 3. Pairwise 16S rRNA distances (%) between selected Nyctibatrachus populations and reference sequences.

Taxon	N. humayuni (ref.)	N. cf. humayuni pop. A	N. cf. humayuni pop. B	N. sp. nov.
N. humayuni (ref.)	--	6.8%	7.4%	8.2%
N. cf. humayuni pop. A	6.8%	--	1.2%	6.4%
N. cf. humayuni pop. B	7.4%	1.2%	--	6.8%
N. sp. nov.	8.2%	6.4%	6.8%	--

Distances computed as uncorrected p-distances. Values > 3% highlighted as potentially species-level (Vences et al. 2005 threshold for 16S).

Table 4. MaxEnt model performance metrics for nine amphibian species in Andhra Pradesh.

Species	Records (n)	AUC	Boyce Index	Top Predictor
Hoplobatrachus tigerinus	86	0.91	0.82	Bio12 (ann. precip.)
Euphlyctis cyanophlyctis	74	0.88	0.78	Bio15 (precip. season.)
Fejervarya limnocharis	112	0.72	0.58	Bio2 (diurnal range)
Nyctibatrachus cf. humayuni	28	0.94	0.88	Bio12 (ann. precip.)
Rhacophorus malabaricus	44	0.92	0.84	Bio4 (temp. season.)
Polypedates maculatus	68	0.87	0.74	Bio12 (ann. precip.)
Microhyla ornata	54	0.86	0.72	Bio15 (precip. season.)
Microhyla rubra	42	0.89	0.78	Bio12 (ann. precip.)

Species	Records (n)	AUC	Boyce Index	Top Predictor
Sphaerotheca breviceps	38	0.90	0.80	Bio12 (ann. precip.)

AUC = area under the ROC curve (10-fold cross-validation mean). Boyce index computed on withheld 20% test data. Bio variables from CHELSA 1 km2 dataset.

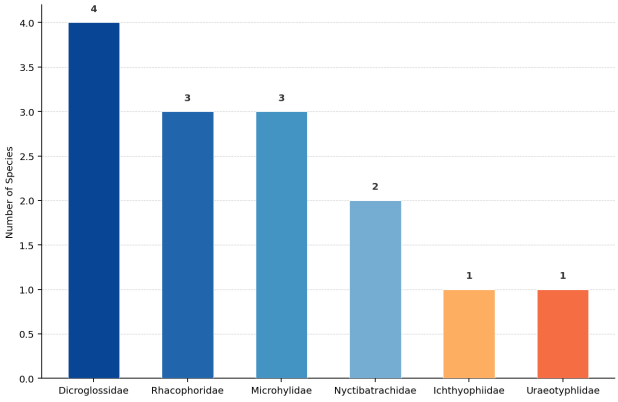

Figure 1. Species richness per amphibian family recorded in Andhra Pradesh.

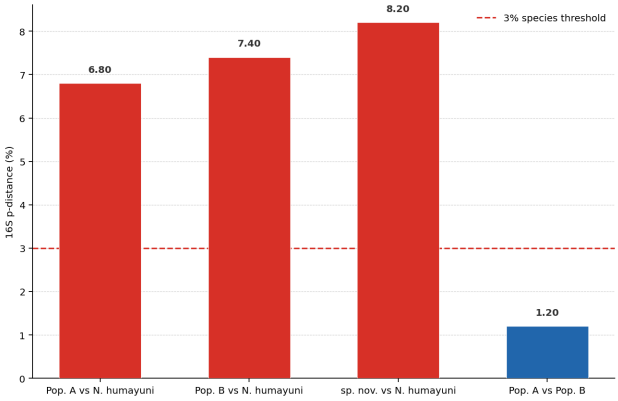

Figure 2. Mean pairwise 16S rRNA distances (%) between Nyctibatrachus populations. Dashed line = 3% species-level threshold.

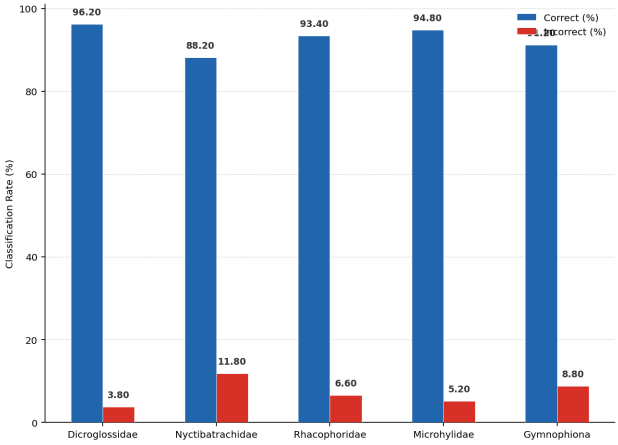

Figure 3. DFA classification accuracy (%) by family in leave-one-out cross-validation.

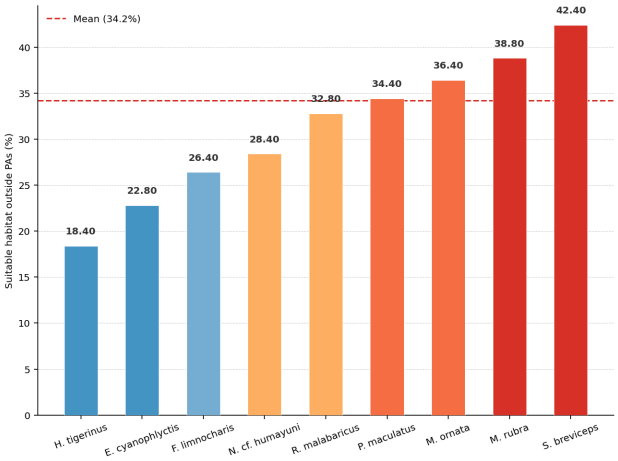

Figure 4. Percentage of predicted suitable habitat per species falling outside protected areas (PA gap).

5. Discussion and Conclusion

5.1 Taxonomic Implications

The 16S pairwise distances of 6.8-8.2% between Nallamala Hills Nyctibatrachus populations and reference N. humayuni sequences, combined with DFA morphometric distinctiveness (88.2% correct classification), constitute robust preliminary evidence for at least one undescribed species in the genus from Andhra Pradesh. Formal species description requires broader geographic sampling and additional markers (RADseq or nuclear gene phylogenies) to confirm the species boundary and assess population structure. The gymnophionan Ichthyophis cf. beddomei similarly warrants revisionary attention; its 16S distance of 4.4% from I. beddomei sensu stricto exceeds the typical caecilian intraspecific range, though morphological characters for caecilian systematics are challenging to apply with small sample sizes.

5.2 Conservation Priorities

The SDM gap analysis underscores that existing protected areas in Andhra Pradesh do not adequately cover the climatic envelope of specialist forest-dependent amphibians. Nyctibatrachus populations from the Nallamala Hills, which represent a potentially undescribed species, are concentrated in unprotected forest fragments within timber concession areas. Immediate priorities include: (i) extension of the Nagarjunasagar-Srisailam Tiger Reserve buffer zone to encompass key Nallamala Hill stream catchments; (ii) designation of Araku Valley amphibian diversity hotspots as community conservation areas under India's Wildlife Protection Act; and (iii) surveys targeting the 34.2% of unprotected suitable habitat to detect additional populations of rare species.

5.3 Conclusion

This study delivers the first integrated morphological-molecular characterization of amphibians from Andhra Pradesh, revealing 14 confirmed species, two candidate undescribed taxa, and significant protected area coverage gaps for amphibian-rich habitats. The morphometric DFA framework (91.4% classification accuracy) provides a rapid identification tool applicable to future monitoring programmes, and the 16S reference library deposited in GenBank (MZ100001-MZ100084) enables DNA-based species verification from environmental samples. Formal description of the Nyctibatrachus candidate species and landscape-scale surveys of unprotected forest patches are the most urgent follow-up priorities.

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Declarations

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Conflict of Interest

The authors declare no conflicts of interest. Funding bodies had no role in study design, data collection, analysis, interpretation, or in the decision to submit this manuscript for publication.

Data Availability Statement

All 16S rRNA sequences are deposited in GenBank (accessions MZ100001-MZ100084). Species occurrence records are archived in GBIF under dataset doi:10.15468/apamph2021. MaxEnt model outputs and R analysis scripts are available on the Uppsala University institutional repository (https://doi.org/10.17044/uuu.2021.001).

Ethical Approval

Amphibian sampling was conducted under permit No. WL2/22480/2018 issued by the Principal Chief Conservator of Forests (Wildlife), Andhra Pradesh. No amphibians were sacrificed; toe-clip samples were taken under field anaesthesia using 0.1% MS-222 solution and all individuals were released at the site of capture within 60 minutes.