Climatic niche divergence in allopatry shapes the speciation among select scolopendrid centipedes in Peninsular India

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Sudhanshu Kumar

Indian Institute of Science Education and Research Pune

Dr. Homi Bhabha Road,

Pashan, Pune 411008, INDIA.

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Under the guidance of

Dr. Jahnavi Joshi,

Senior Scientist, CSIR-CCMB, Hyderabad

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Certificate

This is to certify that this dissertation entitled **"Climatic niche divergence in allopatry shapes the speciation among select Scolopendrid centipedes in Peninsular India"** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Sudhanshu Kumar** at the Department of Wildlife Conservation and Ecology, Centre for Cellular and Molecular Biology (CSIR-CCMB), Hyderabad under the supervision of **Dr. Jahnavi Joshi**, Senior Scientist, CSIR-CCMB during the academic year 2023-2024.

Dr. Jahnavi Joshi

Senior Scientist

CSIR-CCMB, Hyderabad

Committee:

Guide: **Dr. Jahnavi Joshi**, Senior Scientist, CSIR-CCMB, Hyderabad

TAC: **Dr. Deepak Barua**, Associate Professor, Department of Biology, IISER Pune

This thesis is dedicated to Hemo & Nana.

Declaration

I hereby declare that the matter embodied in the report entitled **"Climatic niche divergence in allopatry shapes the speciation among select Scolopendrid centipedes in Peninsular India"** are the results of the work carried out by me at the Department of Wildlife Conservation and Ecology, Centre for Cellular and Molecular Biology (CSIR-CCMB), Hyderabad, under the supervision of **Dr. Jahnavi Joshi** (Senior Scientist, CSIR- CCMB, Hyderabad), and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgment of collaborative research and discussions.

Sudhanshu Kumar

Registration # **20191199**

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Abstract

Deciphering the role of abiotic factors in the speciation process has been a major question in ecology and evolution. With its high spatial variation in geography and climate, peninsular India is an ideal setting to study the role of abiotic factors on the speciation process. Scolopendrid centipedes are a group of predatory soil arthropods with morphologically cryptic species pairs, indicating that abiotic factors could play an important role in their speciation process. This study assesses the geographic mode of speciation and the role of climate in two Scolopendrid genera, *Ethmostigmus* and *Rhysida*. Within these two genera, we ask if speciation has occurred via allopatry, sympatry, or parapatry and if we observe the signature of phylogenetic niche conservatism or niche divergence. We reconstructed robust species hypotheses for both genera using phylogenetic and species delimitation analyses. Based on the species trees, we selected four sister species pairs from *Rhysida* and a peninsular Indian clade of *Ethmostigmus*. We detected an allopatric mode of speciation within *Ethmostigmus* and three species pairs in *Rhysida,* driven by climatic niche divergence. These results are in concordance with the global pattern seen in small vertebrates. It will be interesting to test for the generality across clades and ecosystems.

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Contributions

Contributor name	Contributor role
JJ, SK, SSD, AM	Conceptualization Ideas
Contributed equally -SK* and SSD*, PD, AM, JJ	Methodology
SK, SSD, PR, AM, JJ	Software
SK, SSD, PR, AM, JJ	Validation
SK, SSD, PR, AM, JJ	Formal analysis
JJ, AM, PR	Investigation
JJ	Resources
SK, SSD, PD, AM, JJ	Data Curation
SK, SSD, AM, JJ	Writing - original draft preparation
SK, SSD, AM, JJ	Writing - review and editing
SK, SSD, AM, JJ	Visualization
JJ	Supervision
JJ	Project administration
JJ	Funding acquisition

SK: Sudhanshu Kumar, IISER Pune

SSD: SS Dhanush, IISER Pune

AM: Aniruddha Marathe, CSIR- CCMB, Hyderabad

PR: Pragyadeep Roy, CSIR- CCMB, Hyderabad

PD: Payal Dash, Jagiellonian University, Kraków, Poland

JJ: Jahnavi Joshi, CSIR- CCMB, Hyderabad

Chapter 1: Introduction

There is immense biodiversity on Earth. We can measure biodiversity at different levels, from genes to biomes, but the most common level at which biodiversity is measured is at the level of species. Currently, there are approximately 1.5 million described species, but the actual number of species has been estimated to be anywhere between 1 to 6 billion (Larsen *et al.*, 2017). To generate this amount of biodiversity, the origin of new species is of fundamental importance. This process, which leads to the origin of new species, is known as speciation. The term speciation was coined by Orator F. Cook in 1906. He described speciation as the origin and multiplication of species by subdivision, and he said that it is usually due to environmental incidents (Cook, 1906). There are many different definitions and concepts for species, but the common theme among them is that a species is an independently evolving metapopulation lineage or the largest lineage connected by gene flow. Speciation is a process that splits such a lineage into two due to a barrier to gene flow (Wiens, 2004b; De Queiroz, 2007).

There are multiple biotic and abiotic factors known to act as barriers to gene flow, leading to the formation of two distinct species. Among abiotic factors, geography and climate are two prominent factors responsible for creating barriers to gene flow that can eventually lead to speciation (Barraclough and Vogler, 2000; Hua and Wiens, 2013). Three distinct geographic modes of speciation are observed- allopatric, sympatric, and parapatric (ed. JB Losos et al., 2013). Allopatric speciation occurs when a species' range becomes divided into separate areas by geographic or climatic barriers, disrupting gene flow between populations and promoting evolutionary divergence (Mayr, 1999). Allopatric speciation, which could be driven primarily by biogeographic processes such as vicariance and dispersal processes, is widely recognized as the most common form of speciation (Hernández-Hernández *et al.*, 2021). Vicariance suggests the presence of physical barriers that divide populations, while dispersal entails the movement of a subset of individuals across a pre-existing barrier, both leading to allopatric speciation (Stigall, 2018). The allopatric species pairs could either show niche similarity due to phylogenetic niche conservatism, where species tend to maintain their ancestral niches over time or

niche divergence, wherein species occupy distinct ecological niches compared to their ancestors (Wiens, 2004a; Hua and Wiens, 2013).

Sympatric speciation denotes the divergence of an ancestral species into two or more distinct species while coexisting within the same geographic region. Thus, despite lack of a geographical barrier to gene flow, the species splits into two new species as other reproductive barriers maintain distinct populations (Foote, 2018). Therefore, sympatric species will show dissimilar niches, as niche divergence can maintain reproductive isolation (Wiens, 2004a; Hua and Wiens, 2013).

Parapatric speciation involves populations residing in neighbouring but not entirely overlapping habitats. Limited gene flow between these adjacent populations facilitates evolutionary divergence, allowing them to evolve distinct characteristics and eventually become new species. This partial isolation maintains the integrity of separate species despite their respective habitats' spatial proximity (Gavrilets *et al.*, 2000).

In this thesis, I focus on understanding the role of the climatic niche in speciation, specifically temperature and precipitation conditions in which an organism can occur (Soberón, 2007). To test the geographic mode of speciation and the role of climate in it, the geographic range and niche of the species formed by the splitting up of ancestral species, known as sister species, must be compared. Their range overlap must be determined to find the geographic mode of speciation. Complete overlap, where one species is nested within the other, will mean sympatric speciation; partial overlap is parapatric speciation, and no overlap will mean allopatric speciation. However, the range overlap of species could have changed since they split from the last common ancestor, so it is possible that currently, sympatric speciation could have arisen via allopatric speciation and, over time, have become sympatric. So, a regression analysis needs to be performed between the range overlap and time since the species split from the most recent common ancestor (MRCA). If non-allopatric species arose via an allopatric mechanism, their range overlap would increase over time and vice versa (Barraclough and Vogler, 2000).

After establishing the geographic mode of speciation, the next step is determining if allopatric speciation was driven by niche conservatism or divergence. If the driving force is niche conservatism, then the geographic ranges of the sister species will have more similar niches. On the other hand, if allopatric speciation is driven by niche divergence, then the sister species' niches will be less similar (Barraclough and Vogler, 2000; Jezkova and Wiens, 2018). Given this, I will first assess the geographic mode of speciation and then evaluate if the sister species pairs/closely related species occupy similar or dissimilar climatic niches.

With their immense biodiversity, tropical regions offer an ideal setting to investigate speciation (Richards, 1969; Mittelbach *et al.*, 2007). Within tropical areas, mountains have been shown to have high speciation rates, narrower thermal niches, and low dispersal ability (Polato *et al.*, 2018; García-Rodríguez *et al.*, 2021). Within peninsular India, one of the global biodiversity hotspots, the Western Ghats, is a continuous chain of mountains which runs ~ 1600 kilometres along the western coast (8°N to 21°N) (Myers *et al.*, 2000). The Western Ghats are subdivided into three subregions: Northern Western Ghats (NWG), Central Western Ghats (CWG), and Southern Western Ghats(SWG) (Subramanyam and Nayar, 1974; Biswas and Karanth, 2021). Within this complex landscape, the Palghat Gap (PG) (11°N), spanning about 40 kilometres, emerges as a significant biogeographic barrier, shaping the distribution and evolution of species within the Western Ghats. Two other minor barriers are Shencottah Gap (SG) at 9° N and Goa Gap (GG) at 15.8° N. The GG is a climatic rather than a geographic barrier (Biswas and Karanth, 2021).

Geologically, the Western Ghats has a rich history, having once been part of the ancient Gondwanan supercontinent approximately 200 million years ago, later amalgamating with the Asian landmass around 50 million years ago (Ali and Aitchison, 2008). The southern reaches of the Western Ghats have maintained a degree of climatic stability throughout geological epochs, particularly during Cretaceous volcanism around 65 million years ago. This climatic stability has rendered the southern Western Ghats a refuge for numerous plant and animal species, preserving pockets of ancient biodiversity amidst shifting environmental dynamics (Joshi and Karanth, 2013). Furthermore, the Western Ghats exhibit a pronounced gradient in seasonality, characterised by variations in temperature and

precipitation across its northwestern and southwestern extents. The Northern Western Ghats experience greater seasonality in temperature and precipitation compared to their counterparts in the Southern Western Ghats, reflecting the diverse ecological niches and microclimatic conditions across the mountain range (Pascal, 1988). Similar to the gradient in climate, there also exists a gradient in biodiversity, as demonstrated in plants and centipedes (Davidar *et al.*, 2005; Joshi and Karanth, 2013; Bharti *et al.*, 2021; Gopal *et al.*, 2023).

Given this intricate interplay of geological history, climatic variability, and ecological heterogeneity, the Western Ghats emerge as an ideal natural laboratory for investigating the role of geographic and climatic factors underlying speciation processes. By delving into the complex interactions between geological events, climatic regimes, and ecological dynamics within this region, researchers can gain valuable insights into the mechanisms driving the remarkable biodiversity and evolutionary divergence observed in tropical ecosystems. There is also another mountain chain in peninsular India, the Eastern Ghats, on the east coast of India, which is much more broken and has isolated hill ranges (Mani, 1974). Due to its fragmented nature, there could be more barriers leading to many opportunities for speciation.

Arthropods represent the majority of Earth's biodiversity, yet non-insect arthropods remain relatively understudied in the context of speciation (May, 1986; Hernández-Hernández *et al.*, 2021). Predatory soil-dwelling species pose a particular challenge within the diverse arthropods due to their morphological crypticity and often low population densities (Bharti *et al.*, 2021). Among these, centipedes, belonging to the class Chilopoda, stand out as a group with a rich and long evolutionary history dating back approximately 420 million years (Giribet and Edgecombe, 2019). Within the Chilopoda, the family Scolopendridae is particularly interesting due to its well-characterized taxonomy and phylogeny in the Western Ghats of peninsular India. Originating in the late Cretaceous period around 100 million years ago, the Scolopendridae family constitutes one of the oldest soil-dwelling arthropod communities in the Western Ghats (WG) region (Joshi and Karanth, 2011).

The Scolopendridae family is one of the most diverse groups of centipedes found in tropical Asian forests, and around 35 species belonging to seven genera occur in the Western Ghats (Bharti *et al.*, 2021). This diversity and their endemism render Scolopendridae an intriguing subject for investigations into speciation processes. It is worth mentioning that speciation in centipedes, particularly within the Scolopendridae family, is not typically accompanied by significant morphological diversification but is instead believed to be driven primarily by climatic and geographic factors (Joshi and Edgecombe, 2019; Joshi *et al.*, 2020).

Within the Scolopendridae family, the genus *Rhysida* is one of the most diverse groups, particularly in the Indian context. Indian species within this genus segregate into two distinct clades: the *immarginata* clade, comprising eight species and the *longipes* clade, comprising four species, of which nine are endemic to peninsular India. The two clades diversified from the Late Cretaceous to the Early Miocene (105-20 Mya) (Joshi *et al.*, 2020). Another noteworthy genus, *Ethmostigmus*, also found in peninsular India, exhibits an endemic lineage of five species. The diversification of *Ethmostigmus* dates back to the end of the Cretaceous period approximately 72.2 million years ago, with species-level diversification occurring at the onset of the Cenozoic era around 47.3 to 30.3 million years ago. Morphologically, *Ethmostigmus* species tend to be larger, exceeding 30 centimetres, compared to the genus *Rhysida*, whose members typically reach body lengths of around 20 centimetres (Joshi and Edgecombe, 2018, 2019).

Using these two genera as my model system, I want to explore the role of geography and climate in speciation in the mountains of peninsular India. I have two main questions:

1. What is the geographic mode of speciation in the two genera of centipedes- *Rhysida* and *Ethmostigmus*?
2. What is the role of geography and climate in the speciation process? If it's allopatric speciation, was it driven by niche conservatism or niche divergence? If sympatric, on what climatic niche axes have the species diverged?

Based on the earlier studies on centipedes, I expect allopatry to be the most common geographic mode of speciation in both genera. It is also the most common geographic mode globally in animals (Hernández-Hernández *et al.*, 2021). Squamates, especially the fossorial ones like snakes occupying a similar niche as centipedes, show allopatry as the major mode of speciation (Jezkova and Wiens, 2018; Alencar and Quental, 2023). The conserved morphology of centipedes is another factor that might lead to allopatry as the major mode of speciation. The conserved morphology will not allow centipedes of different species that are closely related to coexist, as they will be occupying almost the same niche. This will lead to allopatric speciation, which is more probable than sympatric speciation. The low dispersal ability of centipedes will also lead to more allopatric speciation. Due to low dispersal ability, once centipede populations are sufficiently distant, they won't be able to have much gene flow. This can eventually lead them to split into different species. The geography of the Western and Eastern Ghats, consisting of mountains with biogeographic barriers and climatic gradients, could also lead to allopatric speciation. Eastern Ghats being more disjunct from the Western Ghats might support more allopatric speciation.

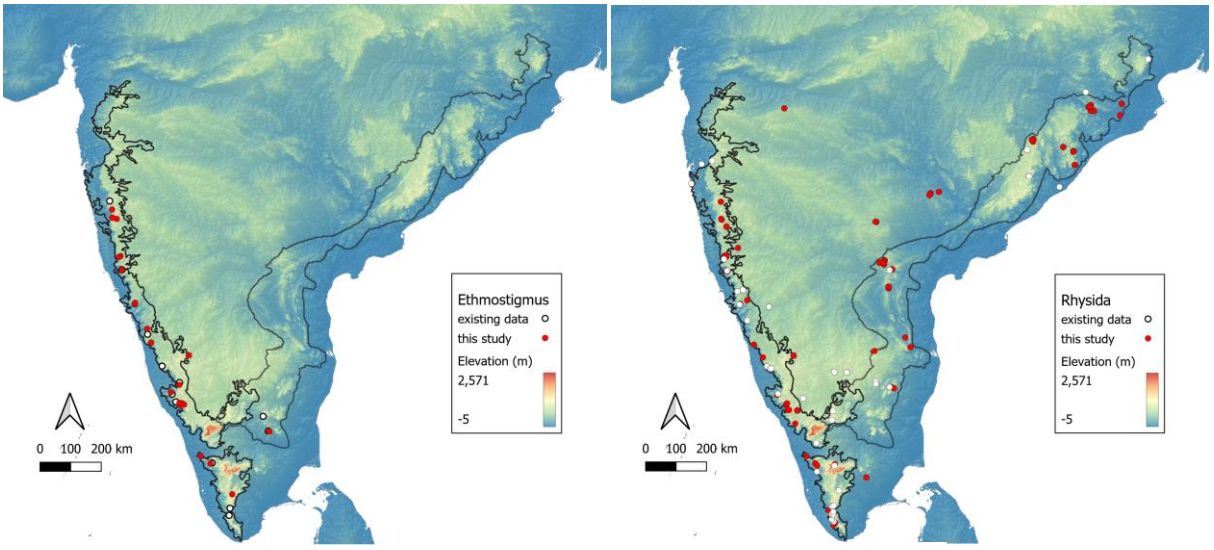
Therefore, geography and climate are expected to play major roles in the speciation process of these two genera. The effect of climate might be more pronounced in the Western Ghats than in the Eastern Ghats due to its stark climatic gradient. The effect of geography might be similar in both ranges, with Eastern Ghats having smaller barriers and Western Ghats having fewer but larger barriers. Also, centipedes have less dispersal ability, so climate and geography become important for their speciation process. Climatic Niche divergence is the most common driver for allopatric speciation in snakes and lizards (Jezkova and Wiens, 2018). Since centipedes occupy similar niches to many of the squamates, they are also expected to show a similar trend.

Chapter 2: Materials and Methods

2.1 Taxon Sampling

Sampling was done by Evolutionary Ecology Lab members over a period of three years from 2021 to 2024. Centipedes from the family Scolopendridae were sampled

in protected as well as non-protected areas from the states of Maharashtra, Karnataka and Kerala covering the Western Ghats and Odisha, Telangana, Andhra Pradesh and Tamil Nadu covering the Eastern Ghats. The sampling covered different habitats, such as tropical rainforests, deciduous forests, shola grasslands, savannah grasslands, and lateritic plateaus. Systematic sampling was conducted across the elevational and latitudinal gradients in the Western and Eastern Ghats. Centipedes were actively searched in forest areas, mostly under stones, leaf litter, and inside dead logs. The samples are preserved in 70-75% ethanol.



1a 1b

Figure 1: Map of sampling locations for the genus *Ethmostigmus* (1a) and *Rhysida* (1b). Red dots indicate locations for which molecular data has been generated in this study, and white dots indicate molecular data sourced from literature.

2.2 Morphological Identification

The samples are observed under a stereo zoom microscope and are identified up to the species level using diagnostic characters provided in keys (Joshi and Edgecombe, 2018; Joshi *et al.*, 2020). In some cases, diagnostic characters might not be identifiable. In those cases, the samples are identified up to the genus level, and further identification is done only using genetic data.

2.3 Molecular Data Generation

2.3.1 DNA Extraction

We performed DNA extraction on preserved specimens consisting of 154 samples of the genus *Rhysida* and 35 samples of *Ethmostigmus.* The MN (Macherey Nagel) blood and tissue kit (https://www.mn-net.com/) was used for DNA extractions. We mainly used tissue from the leg and sometimes from the segment in case the legs were too small. The number of legs used for extraction was determined according to the size and the quality of the preserved sample. On average, we used about 4-5 legs for most samples of the genus *Rhysida* and 1 leg for most samples from the genus *Ethmostigmus*. We cut the tissue using a scalpel and transferred it to a 1.5 ml tube with 180 µl of lysis buffer (T1 buffer). The legs are crushed using a micro-pestle to expose the tissue inside the chitin layer to buffer. 25 µL proteinase K is added, and the reagent mixture is incubated overnight in a thermal shaker at 56°C at 800 r.p.m. Further, the DNA extraction was carried out following the MN kit protocol. Finally, the samples are eluted in 50µl of the kit elution buffer and stored at 4° C for further downstream steps.

2.3.2 DNA Quantification

We quantified the extracted DNA in a NanoDrop® ND-1000 spectrophotometer (https://www.marshallscientific.com/Nanodrop-ND-1000-Spectrophotometer-p/nd-1000.htm), and samples with satisfactory absorbance ratios (260/280(~1.8) and 260/230(2-2.2)) and high DNA concentrations(>80 ng/ul) are diluted to 30ng/ul by adding the kit elution buffer. The extractions are repeated for samples with low yield (<20ng/µL) or for samples with many impurities indicated by erroneous 260/280 or 260/230 ratios.

2.3.3 Polymerase Chain Reaction (PCR)

DNA amplification was done for CO1 (~650 bp)(Folmer *et al.*, 1994), 16S (~550 bp) (Xiong and Kocher, 1991; Edgecombe *et al.*, 2002) (mitochondrial), and 28S (~400 bp)(Whiting *et al.*, 1997) (nuclear) DNA markers using a 3-step DNA PCR reaction in Eppendorf thermal cycler (https://www.eppendorf.com/us-en/eShop-Products/PCR/Thermocyclers/Mastercycler-nexus-p-PF-14698) and in Bio- Rad T100 Thermal cycler (https://www.bio-rad.com/en-us/product/t100-thermal-

cycler?ID=LGTWGIE8Z). The PCR conditions (Table S3, S4) have been standardised for these markers in previous studies on centipedes (Joshi and Karanth, 2011). These markers have also been used in previous studies, so using them will be useful for comparing data across studies and creating a common database.

2.3.4 Gel Electrophoresis

To check whether our PCR has worked, we run gel electrophoresis for the PCR products. We used agarose gel (2%). The gel image was visualised using Ethidium Bromide (EtBr) under UV light in a gel camera. In a DNA gel electrophoresis, the DNA moves according to the charge/mass ratio from the negative to the positive node of the electrophoresis machine. Since all DNA fragments have the same charge per unit mass, smaller fragments move faster than the larger ones, and the fragments get separated based on size. If our DNA fragment of interest has amplified, we will see a clear band in the gel at the region of its expected size(Stellwagen, 1998). We run a DNA ladder to check for size of the amplified fragment. If a sample does not give clear bands we repeat the PCR, sometimes with minor adjustments in PCR conditions.

2.3.5 PCR product purification and DNA sequencing

For DNA sequencing, we send the PCR products to the sequencing facility Eurofins Genomics India Pvt Ltd. at CCMB , Hyderabad (https://www.eurofins.in/). PCR product purification is also done by them. Sanger sequencing is performed after that(Sanger *et al.*, 1977).

2.3.6 Cleaning DNA sequences

The chromatograms of DNA sequences were visualized in Chromas (v2.6.6) (https://technelysium.com.au/wp/chromas/) . Individual sequences were edited in MEGA7(Kumar *et al.*, 2016) using CLUSTALW(Thompson *et al.*, 1994), and multiple sequence alignments (MSAs) were performed using MUSCLE(Edgar, 2004). MSAs for individual markers were made first and then those were concatenated using MEGA11(Tamura *et al.*, 2021) software.

2.4 Secondary Data Collection

Apart from the data we generated, we also used sequence data from existing literature about *Rhysida* (Joshi *et al.*, 2020) and *Ethmostigmus* (Joshi and Edgecombe, 2018). From these two studies, we had 70 *Rhysida* sequences belonging to 12 species and 20 *Ethmostigmus* sequences belonging to 6 species. Thus, our dataset consists of 224 *Rhysida* individuals and 55 *Ethmostigmus* individuals belonging to a peninsular Indian *Ethmostigmus* clade and *Rhysida longipes* and *immarginata* clades (Table 1). We also used sequences for species within *Rhysida* and *Ethmostigmus* found outside India and outgroups from different clades within the family Scolopendridae (Table S1).

Genus	*Species*	*Total no. of samples*	*Molecular data generated*			*Molecular data from existing literature*		
			CO1	*16S*	*28S*	*CO1*	*16S*	*28S*
Rhysida	*longipes*	13	5	5	6	7	7	7
Rhysida	*crassispina*	3	0	1	1	2	2	1
Rhysida	*pazhuthara*	24	11	15	15	8	9	8
Rhysida	*konda*	32	21	26	26	3	4	3
Rhysida	*trispinosa*	72	43	49	52	17	18	18
Rhysida	*aspinosa*	5	0	1	1	2	4	3
Rhysida	*sada*	7	0	5	5	1	2	2
Rhysida	*immarginata*	5	0	1	1	4	4	4
Rhysida	*lewisi*	28	13	16	16	11	12	12
Rhysida	*ikhalama*	3	0	0	0	2	3	2
Rhysida	*sp.1*	4	1	1	0	3	3	3
Rhysida	*sp.2*	14	5	10	12	2	2	2
Rhysida	*sp.3*	2	1	2	2	0	0	0

Rhysida	*sp.4*	10	6	10	10	0	0	0
Rhysida	*sp.5*	2	2	2	2	0	0	0
Ethmostigmus	*agasthyamal-aiensis*	4	1	1	1	3	3	3
Ethmostigmus	*sahyadrensis*	20	15	16	13	3	3	3
Ethmostigmus	*coonooranus*	13	6	9	8	3	4	1
Ethmostigmus	*praveeni*	4	7	6	4	4	4	4
Ethmostigmus	*tristis*	6	2	1	1	4	4	3

Table 1: Summary of molecular dataset for the two genera *Rhysida* and *Ethmostigmus*

2.5 Phylogenetic Analyses

2.5.1 Maximum Likelihood Tree

Maximum likelihood trees were reconstructed using the IQ tree web server (http://iqtree.cibiv.univie.ac.at/) (Trifinopoulos *et al.*, 2016). The trees were run for 1000 ultrafast bootstraps. We let the program choose the best model for sequence evolution for our partitions. We did not provide any partition scheme for 16S and 28S, whereas a 3-partition scheme was given for CO1. We built trees for individual markers, 16S, for CO1, then for combined mtDNA- CO1 and 16S concatenated and lastly mtDNA and nuDNA combined for CO1,16S and 28S. We reconstructed the maximum likelihood phylogenetic trees for the genus *Rhysida*, *Ethmostigmus,* and the whole Otostigminae subfamily. The trees were visualised using FigTree v1.4.4 (https://github.com/rambaut/figtree/releases).

2.5.2 Species delimitation

Species delimitation was done using Multi-rate Poisson tree processes (mPTP) (http://github.com/Pas-Kapli/mptp) that uses a single locus for species delimitation (Kapli *et al.*, 2017). It has been empirically shown to be better than other methods like General Mixed Yule Coalescent (GMYC) (Fujisawa and Barraclough, 2013) and the Poisson tree process (PTP) (Zhang *et al.*, 2013), and it consistently provides species estimates similar to what taxonomy suggests. mPTP is an advancement of

PTP that uses a two-parameter model- one for speciation (Yule, 1925) and the other for the coalescent process (Hudson, 1990). It assumes that each substitution has a small probability of generating a branching event, and the branching event will be more frequent within species than among species. So, the probability of observing n speciations for k substitutions follows a Poisson process. Therefore, an exponential distribution can model the number of substitutions until the next speciation event. Whereas PTP assumes one exponential distribution for the speciation events and only one for coalescent events across all species in the phylogenetic tree, mPTP fits the branching events of each delimited species to a distinct exponential distribution. The assumption is that each species evolves at a different rate. It also uses the Markov Chain Monte Carlo (MCMC) sampling approach for inferring delimitation support values (Kapli *et al.*, 2017).

We used the markers 16S and CO1 to run mPTP. We applied mPTP for both the genera *Rhysida* and *Ethmostigmus*. We provided the aligned FASTA file and the rooted phylogenetic tree to estimate the minimum branch length. After finding the minimum branch length, we provided the rooted phylogenetic tree, entered the minimum branch length, outgroups, MCMC set to 10000000, MCMC sample to 1000, 4 MCMC runs and burnin to 1000000.

2.6 Spatial Analyses

2.6.1 Spatial distribution and Geographic overlap

We used Minimum Convex Polygons (MCPs) to cover all occurrences and represent the geographic distribution for each species. MCP is created by finding the outermost points from occurrence data and then joining them to form a polygon. To estimate geographic overlap between the sister species pairs, we used these MCPs and estimated the area of intersection (Jezkova and Wiens, 2018). We did this in R v4.3.2 (https://www.r-project.org/) using the package sf v1.0 (simple features) (https://cran.r-project.org/web/packages/sf/index.html).

2.6.2 Climatic niche overlap

As a measure of the abiotic niche of the species, we used climate data at the occurrence locations. We downloaded the 19 bioclimatic variables for the current

climate at the resolution of 30 arc seconds (approximately 1kmx1km) for the whole of peninsular India from WorldClim (https://www.worldclim.org/). We summarized the climate for peninsular India using principle component analyses (PCA) using the command dudi.pca in the package ade4 v1.7 in R v4.3.2 (https://rdrr.io/cran/ade4/man/dudi.pca.html). We then selected the first two PCA axes, which explained 59% of the variation to represent the climatic conditions in the entire Peninsular India. We then represented the climate space for each species with respect to the same two PCA axes using the climate conditions at the occurrence locations and within a region around the occurrences that is assumed to be available to the species. We estimated the available climate by masking the climate layers based on the observed distribution of each species. For species within the WG, the climate layers were masked by the latitudinal extents of the species, as well as the WG boundary. For species within the EG, the climate layers were masked by the latitudinal extents of the species, as well as the EG boundary. We used a 50km buffer around each occurrence point to mask the climate layer for species that have any occurrences outside of the western or the eastern ghats.

Then, we projected the climate at the occurrence points and the available climate space on the first two principal components using the function 'suprow' in the package ade4 v1.7. We used Schoener's D index as a measure of niche overlap. Schoener's D index represents the difference between the climate conditions (axes scores), with 0 being no overlap and 1 being complete overlap. We tested the significance of the niche overlap metric using a randomization test, where the occurrences of the two sister species were randomly reshuffled for 100 iterations. We used the function 'ecospat.niche.equivalency.test' in the package ecospat v4.0 for the randomization test. (Warren *et al.*, 2008).

Chapter 3: Results

3.1 Phylogenetic Analyses

3.1.1 Maximum Likelihood Tree

In the concatenated (CO1, 16S, and 28S) Maximum Likelihood tree of the subfamily Otostigminae, both Indian *Ethmostigmus* and *Rhysida* were monophyletic, with

bootstrap supports being 100% and 96%, respectively. We discuss the results based on the concatenated tree, which was resolved well with high bootstrap support. Also, a combined tree based on mitochondrial and nuclear markers helps resolve deep and shallow-level relationships(Hwang and Kim, 1999). The individual and concatenated gene trees are shown in the supplementary material (S5-S16). Within the genus *Ethmostigmus,* all the species except *E. sahyadrensis* were monophyletic. *E. sahyadrensis* was paraphyletic, wherein one of the clades was sister to *E. coonoranus. E. agathyamalaiensis* was a sister to the rest of the *Ethmostigmus* species with 94% bootstrap support*. E. tristis* was sister to a clade comprised of *E. praveeni, E. sahyadrensis,* and *E. coonoranus*. In the genus *Rhysida,* the monophyly of *longipes* and *immarginata* clades was not recovered, where *R. ikhalama* and a newly emerged clade (*R.sp.5)* were sister to the rest of the peninsular Indian *Rhysida* clade. Within the *longipes* clade, *R. longipes* and *R. crassispina* were sisters but with a low bootstrap support value of 56%. *R. pazhuthara* and *R.konda* were sister species with bootstrap support of 96%. *R. sp.2* and *R. aspinosa* was another species pair recovered with high bootstrap support of 80%. *Rhysida trispinosa,* a widespread species, was polyphyletic and was split into five clades.

3.1.2 Species delimitation

For the genus *Ethmostigmus* mPTP analysis based on 16S suggested the presence of seven distinct clades, whereas CO1 suggested the presence of eight distinct clades. In species delimitation analyses of 16S and COI datasets, *E. agasthyamalaiensis, E. tristis, and E. preveeni* were recovered as a single species. *E. coonoranus* was split into two species in both 16S and COI, and *E. sahyadrensis* was split into two in 16S and three in CO1.

For the genus *Rhysida,* species delimitation analyses (mPTP) based on the 16S dataset suggested the presence of 18 species, whereas estimates based on CO1 were 27 species. In species delimitation analyses based on 16S data, *R. longipes, R. crassispina, R. pazhuthara, R. ikhalama, R. trispinosa, R. sada, R. immarginata, R. lewisi* and *R. sp.1* were retrieved as distinct species*.* However*, R. konda*, *R. aspinosa* and *R. sp.2* were split into two distinct clades. Three new putative species were identified, which I have referred to as *R. sp.3* (Northern Eastern Ghats), R. sp.4 (Northern Western Ghats) and *R. sp.5* (Northern Eastern Ghats).

In this thesis, I will use conservative estimates of species, even though some recognised species have been further split and formed distinct clades within them. To confirm their status as distinct species, these distinct clusters need to be further tested with more samples and validation tools such as BPP (Yang, 2015).

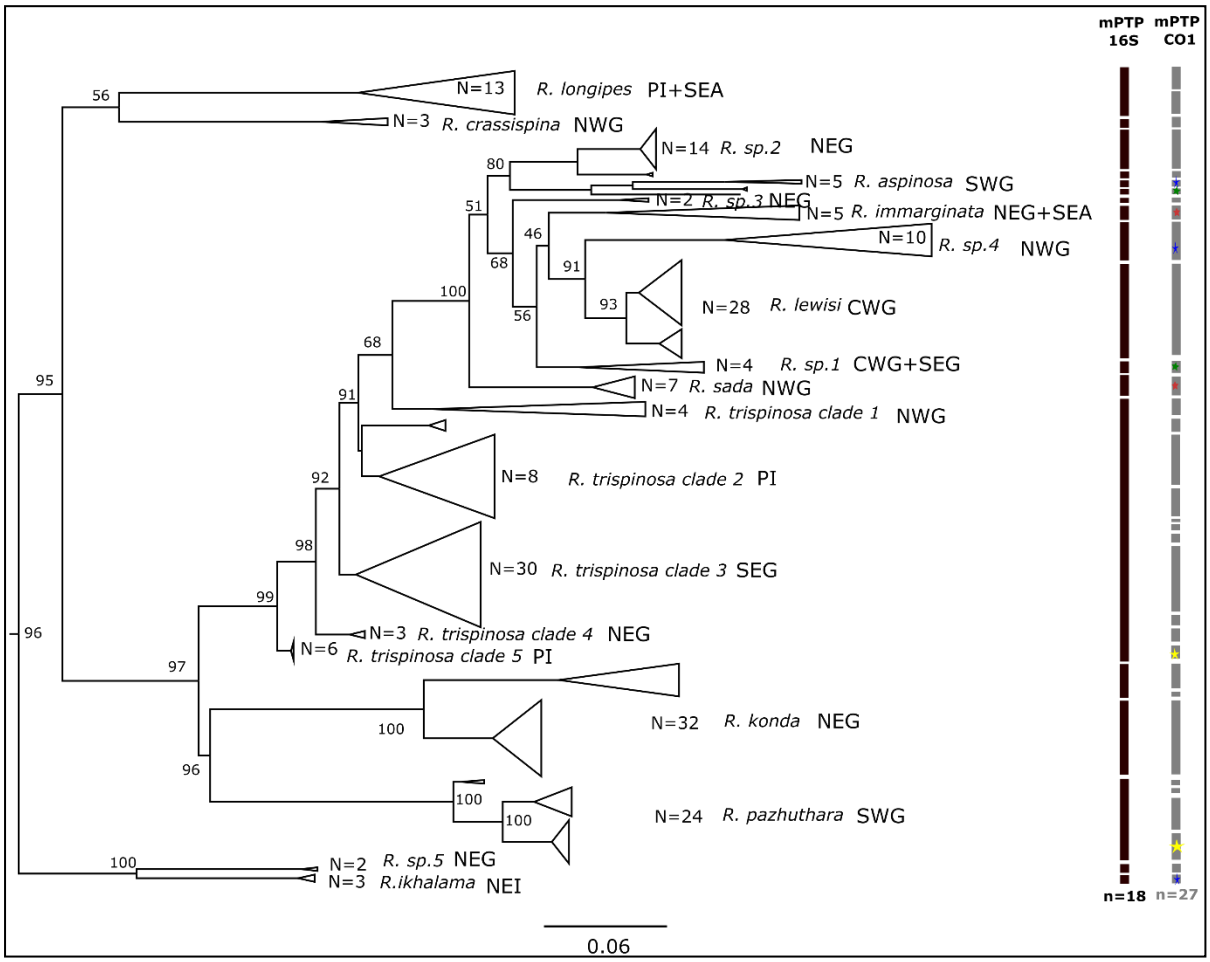


Figure 2a: Phylogenetic tree using the Maximum Likelihood method for the genus *Rhysida*. Numbers at the nodes indicate bootstrap support values. Dark and light bands on the right-side show species delimitation (mPTP) results for the two mitochondrial markers, 16S and CO1, respectively. Stars indicate the placement of the respective samples in the same species. The geographical area occupied by each species has been added (NWG= Northern Western Ghats, CWG= Central Western Ghats, SWG= Southern Western Ghats, EG= Eastern Ghats, NEG= Northern Eastern Ghats, SEG= Southern Eastern Ghats, NEI= North East India, PI= Peninsular India, SEA= South East Asia)

3.1.3 Species pairs

Based on the likelihood tree and the species delimitation analyses, I have selected four sister species pairs from the genus *Rhysida*—*R. pazhuthara* and *R. konda*, *R. crassispina* and *R. longipes*, *R.* sp.2 and *R. aspinosa*, and *R. lewisi* and *R.* sp. 4—for

spatial analyses. For the genus *Ethmostigmus*, analyses was done at the peninsular Indian clade level, including all five closely related species.

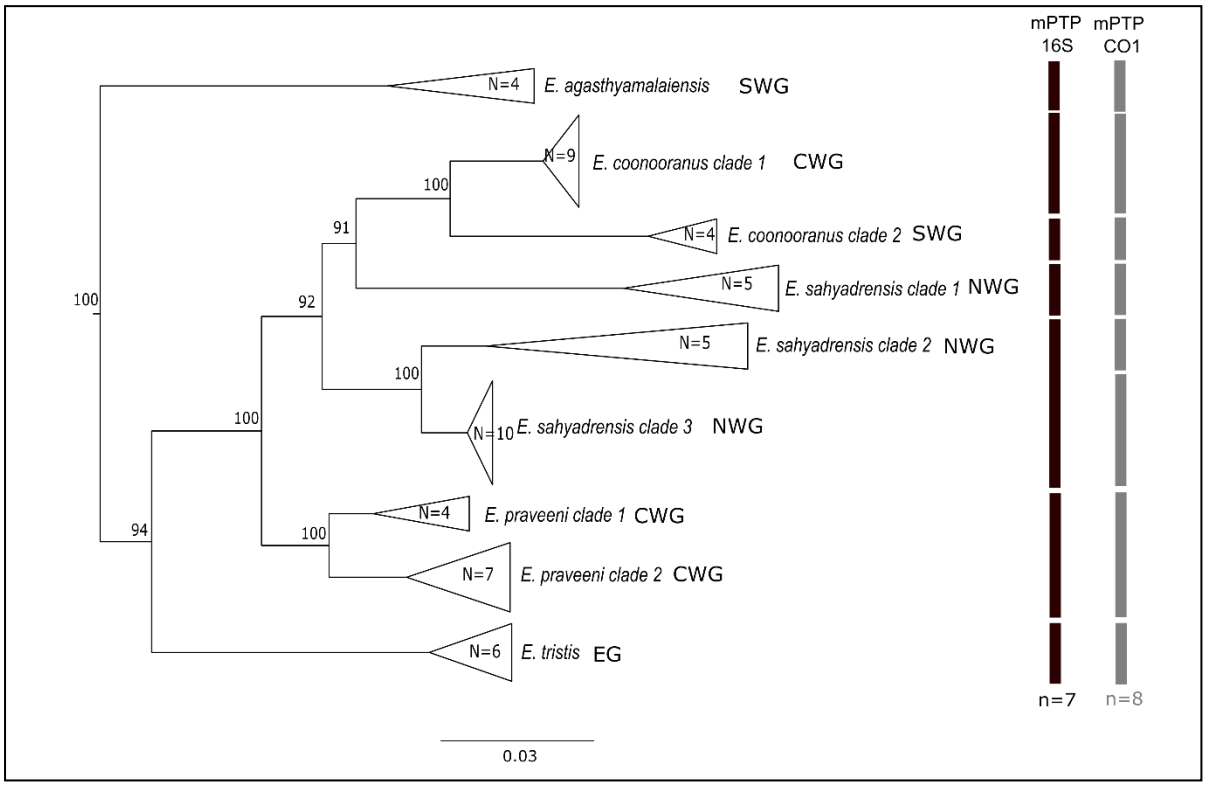


Figure 2b: Phylogenetic tree using the Maximum Likelihood method for the genus *Ethmostigmus*. Numbers at the nodes indicate bootstrap support values. Dark and light bands on the right-side show species delimitation (mPTP) results for the two mitochondrial markers, 16S and CO1, respectively. The geographical area occupied by each species has been added (NWG= Northern Western Ghats, CWG= Central Western Ghats, SWG= Southern Western Ghats, EG= Eastern Ghats)

3.2 Spatial Analyses

3.2.1 PCA

Summarising the variation in climate across the entire peninsular India, using PCA, resulted in the first two axes explaining 59% of the total variation. Variables relating to temperature seasonality, particularly the annual temperature range (BIO7), standard deviation of the temperature (BIO4), and mean diurnal temperature range(BIO2), contributed to the first principal component. The annual mean temperature(BIO1), annual precipitation(BIO12), and maximum precipitation (BIO16)

contributed to the second principal component. We used the species scores from these two axes to summarise the climate niche of the species.

3.2.2 Geographical overlap

The MCPs for the genus *Ethmostigmus* show no overlap, with *E. sahyadrensis* occupying NWG*, E. praveeni* occupying CWG*, E. coonoranus* occupying the southern part of CWG and northern part of SWG, *E. agasthyamalaiensis* occupying SWG and *E. tristis* occupying the southern part of EG as well as some part of SWG. The *Rhysida* species pairs also showed no geographic overlap except R. longipes and R. crassispina. *R. lewisi* and *R. sp.4* occupy the adjoining areas in CWG, but no geographic overlap. *R. pazhuthara* and *R. konda* are separated by a large distance, with *R. pazhuthara* being in SWG and *R. konda* being in NEG. We can see the same pattern in *R. aspinosa* and *R. sp.2,* with *R. aspinosa* being found in SWG and *R. sp.2* being found in Northern Eastern Ghats. *R. crassispina* and *R. longipes* show geographic overlap between them (11km^2).

3.2.3 Climatic Niche overlap

Although geographic distributions do not overlap for most species, some species do show some overlap in climate space. The maximum niche overlap in the climate space is between *E. sahyadrensis and E. coonooranus* (Schoener's D= 0.066). Other *Ethmostigmus* species pairs show niche overlap of 0.003 (*E. agasthyamalaiensis and E. tristis)* and 0.002 (*E. agasthyamalaiensis and E. coonooranus)*. All the overlap metrics are significantly lower compared to the random expectations.

The same pattern of niche overlap is seen in *Rhysida* species, with one species pair, *R. lewisi* and *R.* sp.4, showing some overlap in climate space (Schoener's D= 0.004). Except for *R. sp.2* and *R. aspinosa* the overlap metrics for all 3 species pairs are significantly lower than random expectations.

3.2.4 Geographic mode of speciation and its driver

In the genus *Ethmostigmus* none of the species showed geographical overlap. The niche overlaps are also either zero or very low. This suggested that the species' may have speciated through allopatric mode of speciation, which could have been driven

by niche divergence. *E. sahyadrensis* and *E. praveeni* are also separated by the Goa gap, which has been shown to act as a climatic barrier (Biswas and Karanth, 2021). This could have led to their allopatric speciation. However, the relationship between *E. sahyadrensis* and *E. praveeni* was not that of sister species pairs but were part of a clade along with *E. coonooranus*.

There is no geographic overlap in all *Rhysida* sister species pairs except for *R. longipes* and *R. crassispna* and all species pairs show no climatic niche overlap. All of them may have speciated through the allopatric mode of speciation except for *R. longipes* and *R. crassispina,* which might have originated via parapatric mode. Speciation for all the species pairs was driven by niche divergence. For *R. lewisi-R. sp.4* there is no apparent barrier. For *R. aspinosa* - *R. sp.2*, and *R. pazhuthara* - *R. konda*, the separation between EG and SWG could have acted as a barrier.

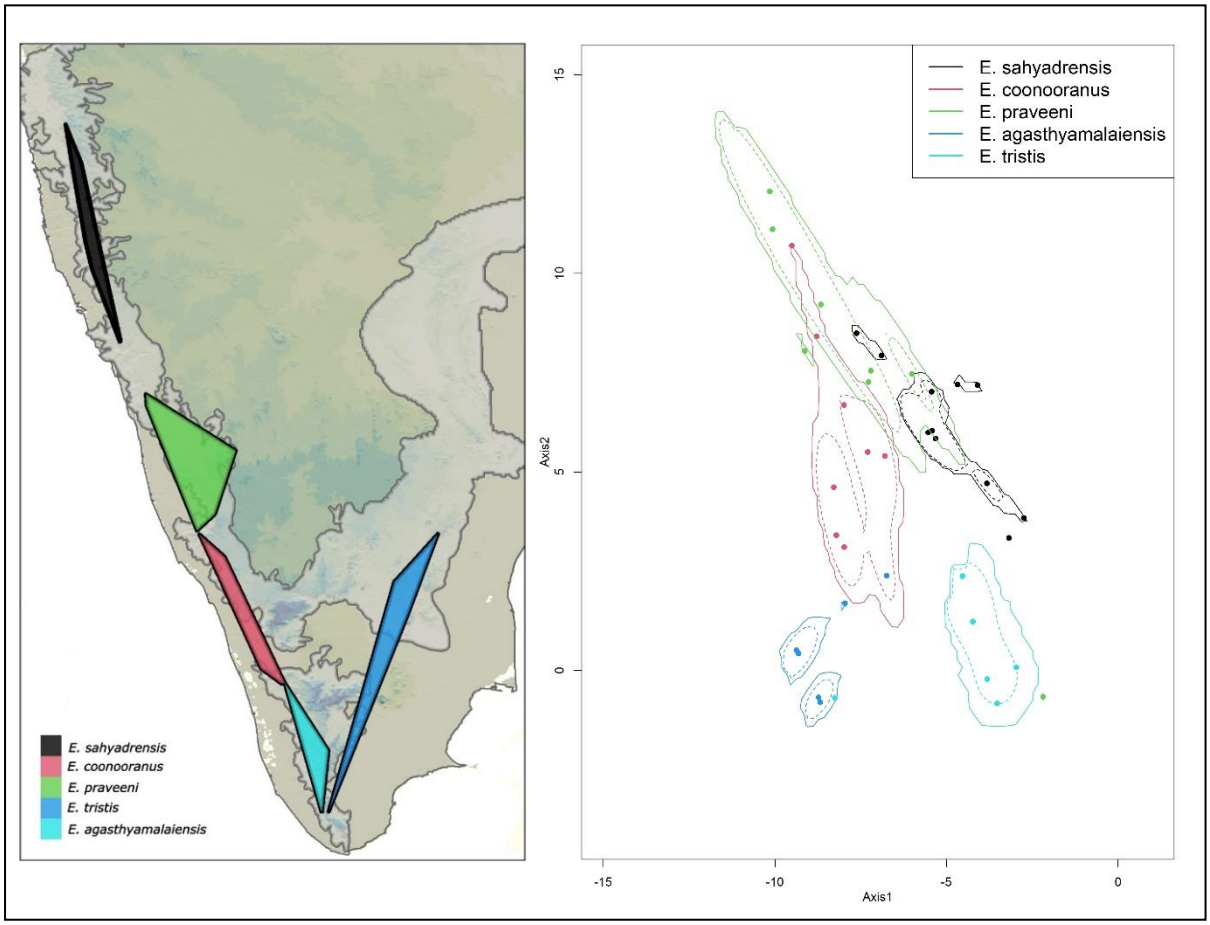


Figure 3a: MCPs (left) of all the peninsular Indian *Ethmostigmus* species and their niche overlaps (right). The colours representing the species are the same in both panels. The lines around occurrence points show the observed climate space using the kernel density estimate.

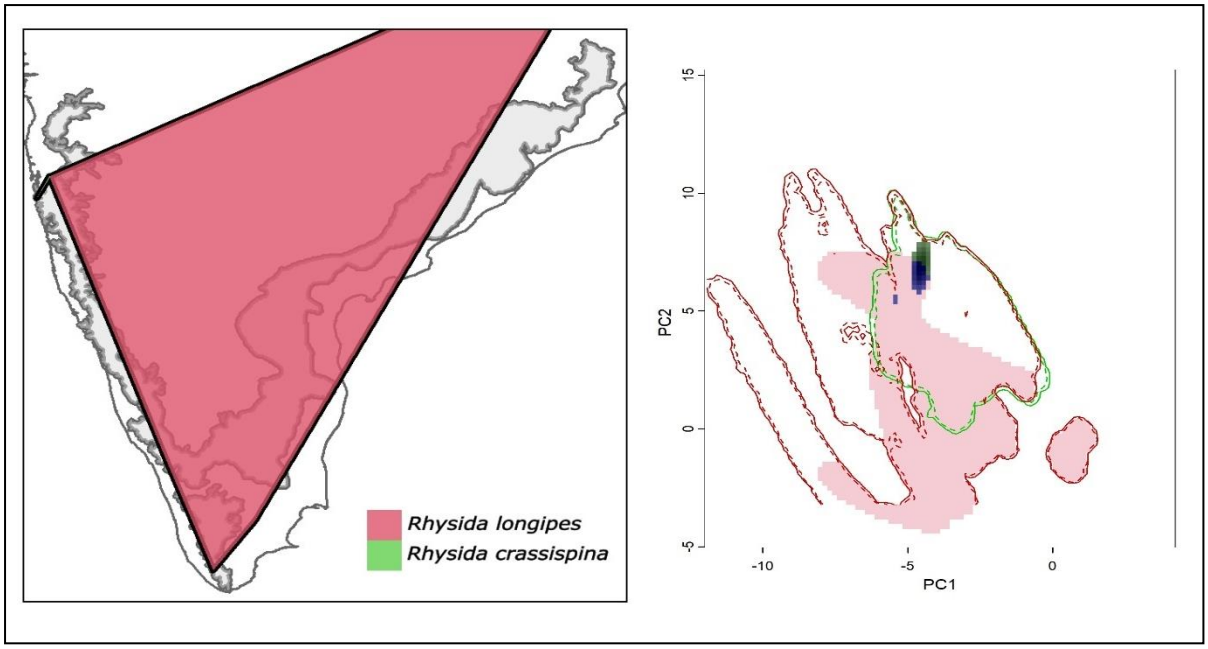


Figure 3b: MCPs (left) of *Rhysida longipes* (red) and *Rhysida crassispina* (green) and their niche overlaps (right). The dotted lines in the left figure represents available climate space and the solid colours (red and green) represent the climate space occupied by the species, blue represents the niche overlap.

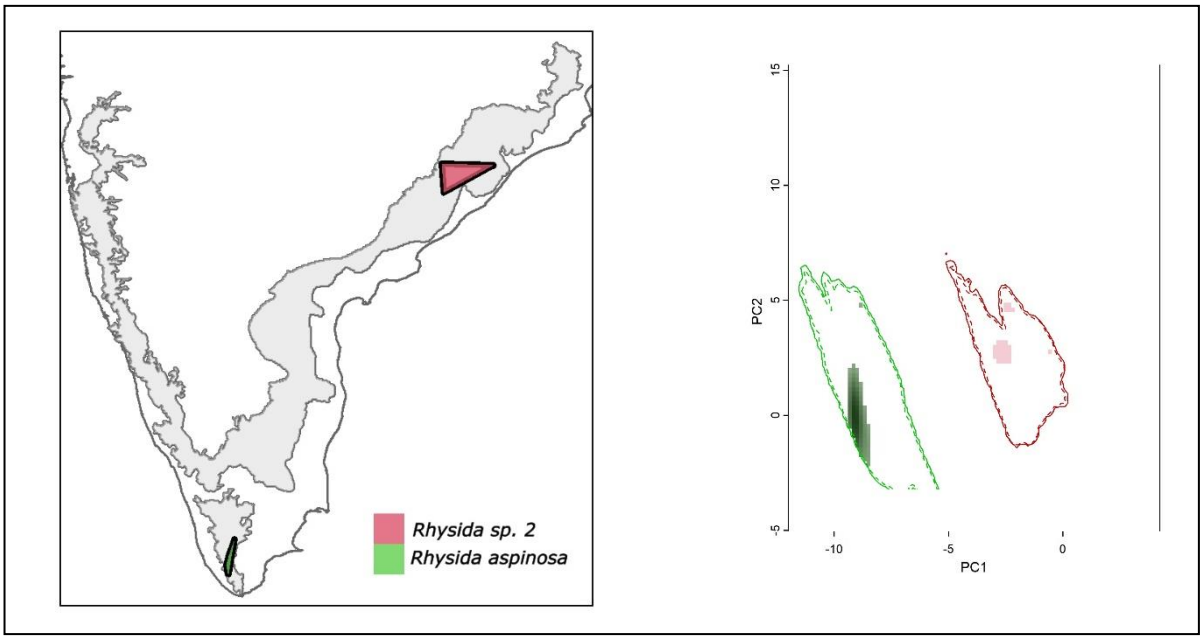


Figure 3c: MCPs (left) of *Rhysida sp.2 (red)* and *Rhysida aspinosa (green)* and their niche overlaps (right). The dotted lines in the left figure represents available climate space and the solid colours (red and green) represent the climate space occupied by the species.

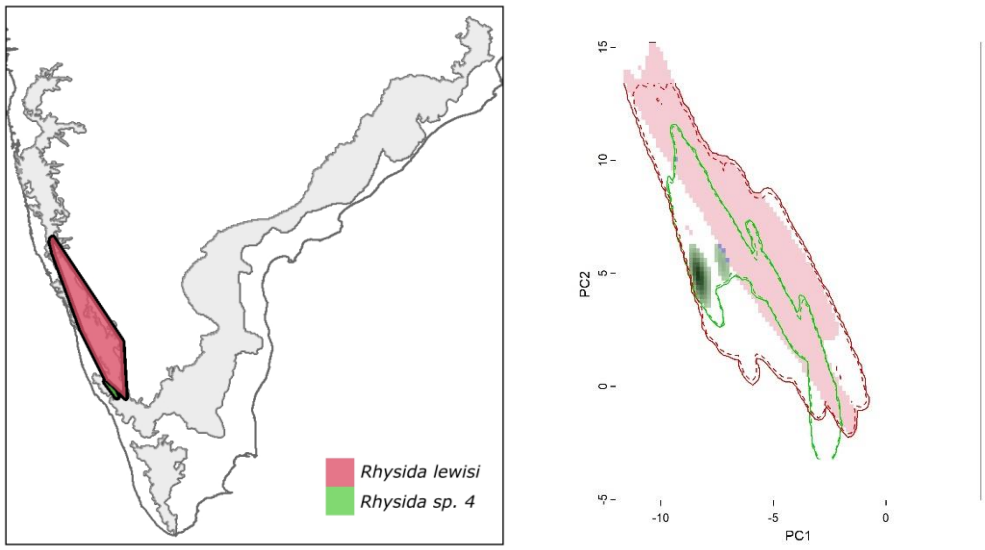


Figure 3d: MCPs (left) of *Rhysida lewisi* (red) and *Rhysida sp.4* (green) and their niche overlaps (right). The dotted lines in the left figure represents available climate space and the solid colours (red and green) represent the climate space occupied by the species, blue represents the niche overlap.

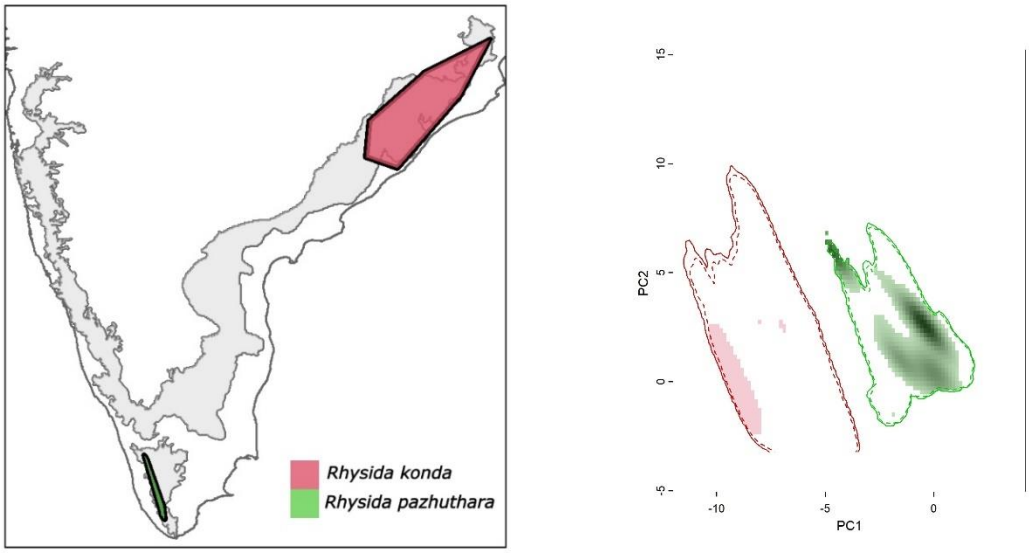


Figure 3e: MCPs of *Rhysida konda* (red) and *Rhysida pazhuthara* (green) and their niche overlaps. The dotted lines in the left figure represents available climate space and the solid colours (red and green) represent the climate space

3.3 Genus wise result summary

3.3.1 *Rhysida*

The peninsular Indian *Rhysida* clade was monophyletic with a high bootstrap support value of 96%. The monophyly of the two clades within *Rhysida- longipes* and *immarginata* were not recovered. Species delimitation analysis (16S) split some species within it but retained most of them as single species (9 out of 12 existing species). Spatial and climatic analyses showed no geographic or niche overlap between the sister species pairs selected except for *R. longipes* and *R. crassispina,* which showed geographic overlap.

3.3.2 *Ethmostigmus*

The peninsular Indian *Ethmostigmus* clade was monophyletic with a high bootstrap support value of 100%. Except for *E. sahyadrensis*, the monophyly of all other species was recovered. Species delimitation analysis (16S) split some species within it but retained most of them as single species (3 out of 5 existing species). Spatial and climatic analyses showed no geographic and niche overlap among all species.

Species 1	Species 2	Geographic overlap (km^2)	Mode of speciation	Niche overlap(Schoener's D)	Driver
Rhysida longipes	*Rhysida crassispina*	11.13	Parapatric	0*	Niche Divergence
Rhysida sp.2	*Rhysida aspinosa*	0	Allopatric	0	Niche Divergence
Rhysida lewisi	*Rhysida sp.4*	0	Allopatric	0.0004*	Niche Divergence
Rhysida pazhuthara	*Rhysida konda*	0	Allopatric	0*	Niche Divergence
Ethmostigmus agasthyamalaiensis	*Ethmostigmus coonooranus*	0	Allopatric	0.002*	Niche Divergence
Ethmostigmus agasthyamalaiensis	*Ethmostigmus praveeni*	0	Allopatric	0*	Niche Divergence

Ethmostigmus agasthyamalaiensis	*Ethmostigmus sahyadrensis*	0	Allopatric	0*	Niche Divergence
Ethmostigmus agasthyamalaiensis	*Ethmostigmus tristis*	0	Allopatric	0.0003*	Niche Divergence
Ethmostigmus coonooranus	*Ethmostigmus sahyadrensis*	0	Allopatric	0.066*	Niche Divergence
Ethmostigmus coonooranus	*Ethmostigmus praveeni*	0	Allopatric	0*	Niche Divergence
Ethmostigmus coonooranus	*Ethmostigmus tristis*	0	Allopatric	0*	Niche Divergence
Ethmostigmus praveeni	*Ethmostigmus sahyadrensis*	0	Allopatric	0.025*	Niche Divergence
Ethmostigmus praveeni	*Ethmostigmus tristis*	0	Allopatric	0*	Niche Divergence
Ethmostigmus sahyadrensis	*Ethmostigmus tristis*	0	Allopatric	0*	Niche Divergence

Table 2: Geographic mode of speciation and its driver for selected species (* p< 0.05)

Chapter 4: Discussion

4.1 Geographic mode and driver of speciation in *Rhysida* and *Ethmostigmus*

The monophyly of both genera was recovered with high bootstrap support values. The phylogenetic relationships within *Ethmostigmus* in the previous study (Joshi and Edgecombe, 2018) suggested that there were two sister species pairs (*E. agasthyamalaiensis- E. tristis* and *E. praveeni -E. coonooranus). H*owever, these were not recovered in the current analyses. *E. sahyadrensis* is paraphyletic, with one of its clades being sister to *E. coonooranus.* This could be because our dataset has a larger geographic representation and can better resolve the phylogenetic relationships between the clades. Species delimitation has recovered two distinct clusters in *E. coonooranus* across the Palghat gap (PG). PG, a major biogeographic barrier, could have split *E. coonooranus* into two distinct clades (Biswas and

Karanth, 2021). These clades must be further analysed to confirm if they represent distinct species using validation tools like BPP (Yang, 2015) and more genetic data. Similarly, *E. sahyadrensis* is split into two clusters using 16S and three using CO1, which also have a strong geographic structuring. It must be checked if some kind of barrier is present, specifically in northern Western Ghats. The MCPs for all the *Ethmostigmus* species show no overlap. But did they speciate in allopatry, or did their range change with time? To figure out this, we plan to do regression against the divergence time of the species and the overlap among them. If the overlap decreased with time, then it is possible that they evolved in sympatry and are now allopatric or vice-versa (Barraclough and Vogler, 2000). The time-calibrated tree is necessary for such analyses and will be done in the future. There is no significant niche overlap among the five species. This could mean that divergent selection on climatic axes has driven allopatric speciation. Again, we must ensure that the present niche overlaps represent the niche overlaps at their time of speciation (Jezkova and Wiens, 2018). For this, we need to do a regression of niche overlap against divergence time. If the niche overlap decreased with time, then this would mean that their speciation happened via niche conservatism, and vice-versa. All *Ethmostigmus* species are linearly distributed in WG, the only exception being *E. tristis* found in EG. *E. agasthyamalaiensis* is the most ancient lineage; all others are nested within it. Geographically, it is the southernmost species found in SWG. Then *E. tristis* is a sister lineage to the rest of the *Ethmostigmus* clade. This is supported by a previous study on the evolutionary biogeography of *Ethmostigmus,* which shows that the split between *E. agasthyamalaiensis* and *E. tristis* was the oldest (Joshi and Edgecombe, 2019).

Rhysida is much more diverse, and the phylogenetic relationships are more complex. The two clades of *longipes* and *immarginata* are not monophyletic, as was reported in an earlier study (Joshi et al., 2020). Three new species have emerged in our analyses, two in EG and one in CWG. The branch containing *R. ikhalama* and its sister *R.* sp. 5 is the oldest, with a peninsular Indian clade nested inside it. After that, *R. longipes* and *R. crassispina* are sister to the rest of the *Rhysida* clade. The monophyly of the two clades needs to be examined through the Bayesian phylogenetic analyses as the bootstrap support value for the branch leading to the clade containing *R. longipes* and *R. crassispina* are quite low (56%). *R. trispinosa*, a

widespread species, has split into five clades. Geographical and niche analyses suggest similar results as *Ethmostigmus.* Three sister species pairs are allopatric to each other, and there is no significant niche overlap between them. One species pair, *R. longipes* and *R. crassispina*, shows parapatry and no niche overlap. Thus, all species pairs except the last pair could have evolved by allopatric speciation and also have niche divergence. A regression of divergence time with their range overlap and niche overlap needs to be done to confirm this. Since *R. longipes* is very widely distributed and *R. crassispina* is a point endemic species, *R. crassispina's* range may be nested within that of *R. longipes*, and they might be sympatric.

Thus, in both genera, allopatric speciation is the prominent mode of speciation driven by niche divergence. In squamates, allopatry is the major mode of speciation driven by niche divergence (Jezkova and Wiens, 2018). In small mammals such as those of the family Geomyidae and Viverridae, allopatry is a major mode of speciation (Skeels and Cardillo, 2019). Within the Western Ghats, amphibians and reptiles have shown an allopatric mode of speciation (Vijayakumar *et al.*, 2016; Shameer *et al.*, 2022). Allopatric speciation has been demonstrated to be the most common mode in animals globally (Hernández-Hernández *et al.*, 2021).

4.2 Limitations and Future Work

A more robust phylogenetic tree using the Bayesian approach should be made to get a well-resolved phylogenetic tree. The species delimitation results need to be validated using tools such as BPP (Yang, 2015). If new species are confirmed, the same analyses could be performed on them. Divergence time estimation needs to be done, and regression between time since the split of sister species and their geographic and niche overlap, respectively, should be performed to test if the current geographic and niche overlap represents their respective overlaps at the time of their split (Jezkova and Wiens, 2018).

Supplementary material

https://drive.google.com/file/d/1E0bHPYs3VfJOslfuQM-ynuhHqDH8W3HN/view?usp=drive_link

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