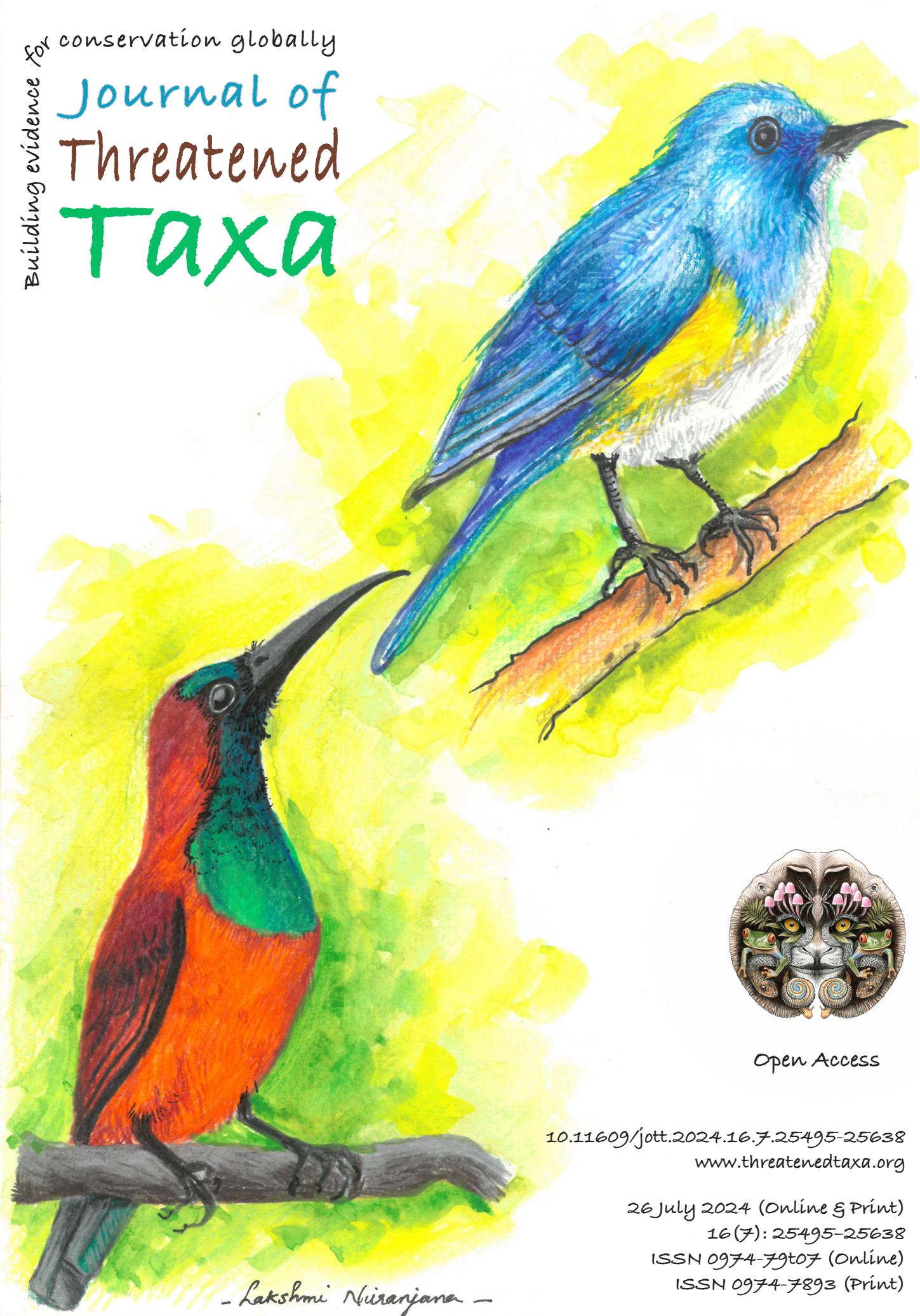


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continued on the back inside cover

Cover: Mixed media illustration of a Blue bird and Sunbird. © Lakshmi Niranjan.



## Assessment of the status of *Spodoptera* species (Lepidoptera: Noctuidae: Armyworm) in India through DNA barcoding technique

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**Abstract:** Insects constitute the majority of animal fauna worldwide, but quantifying their species diversity is still incomplete. A few recent studies indicate a marked decrease in the population of insects which calls for urgent efforts to document and understand insect diversity to get a complete picture of Earth's ecosystems. Modern technology can accelerate species identification beyond traditional methods' limitations. Hence, a focused and expedited approach through DNA barcoding coupled with morphological identification is necessary. This present research highlights the gaps that exist and it examines the current status of *Spodoptera* species barcode in India. Six *Spodoptera* species were studied confirming their presence in India including two invasive species. That means less than 50% of taxa or described *Spodoptera* species are covered by genetic data from barcoded specimens after analysis. Therefore, comprehensive DNA barcoding should be achieved from all insect species occurring on the Indian subcontinent to speed up the discovery and documentation of new species by involving both traditional taxonomists and molecular biologists working towards a common goal.

**Keywords:** Biodiversity in India, conservation, current status, insect, identification, molecular biology, species, taxa.

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**Author contributions:** DN, IC, TS and YG conducted the investigation, collected, and curated the data, and wrote the original draft of the manuscript. SK, YA and PK contributed to the development of the initial concept of the study and was responsible for the design and implementation of the methodology also he was responsible for reviewing and editing the manuscript, creating visualizations, and supervising the overall progress of the study. All authors have read and approved the final manuscript.

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## INTRODUCTION

Identifying insect species is crucial for understanding ecological, evolutionary, and conservation-related queries. Properly diagnosing these species is vital for monitoring biodiversity and utilizing it effectively (Khedkar et al. 2016). Despite the contributions of the long-standing Linnaean classification system to taxonomy, its reliance on morphology has limitations. These limitations, like difficulties in resolving cryptic species and identifying immature stages, hinder progress. Furthermore, the scarcity of experts in morphotaxonomy restricts this approach (Shashank et al. 2022), leaving many species undiscovered or known only through descriptions and lost type specimens. The backlog of unidentified specimens in museum collections has existed for decades. After its introduction in 2003, DNA barcoding has evolved as a complementing technique to conventional taxonomy (Hebert et al. 2003). By characterizing species using standardized DNA regions, DNA barcoding aids in identifying cryptic species, and immature stages, and rapidly distinguishing species in various contexts, such as identification food stuff (Khilare et al. 2019; Tiknaik et al. 2019; Suryawanshi et al. 2020). However, creating high-quality reference libraries based on voucher specimens remains crucial for its applications. Despite challenges due to the vast diversity of life forms and limited taxonomic expertise, several countries, including India, have created massive DNA barcode reference collections for certain creature categories, such as insects. India, known for its rich insect diversity, houses a significant portion of the world's insect fauna.

Major biotic stress on crops is insect pests. Hundreds of insects can cause severe crop damage (Mahmood-ur-Rahman et al. 2014; Nalage et al. 2023). The *Spodoptera* (Lepidoptera: Noctuidae) genus comprises a few of the world's most important crop predators. They are commonly referred to as 'armyworms'. Thirty-one species have been described with members present on six continents (Kergoat et al. 2021). These species feed on a wide range of vegetable, grain, row, forage, and ornamental crops. While young larvae burn leaf tissue and skeletonize into leaves, advanced stages on all leaves are roughly and brutally fed and transported from leaf to leaf (Chandel et al. 2013). The group *Spodoptera* includes species closely related to a similar ecology, difficult to identify at the level of the species (Henaish & Elmetwaly 2020). It is also referred to as the caterpillar cluster, cotton leaf worm, tropical armyworm, and tobacco cutworm (Meagher et al. 2008).

So far, DNA barcoding in Lepidoptera has shown mixed success in determining species. There are several examples of fake DNA barcodes that determine the potential limitations of the methodology (Dasmahapatra et al. 2010; Goergen et al. 2016). This is because current diversity may be difficult to quantify due to missing barcode scopes, absence of uniform barcode spaces in some taxa, and perhaps confounding consequences of an incomplete pedigree (Rubinoff et al. 2006; Silva-Brandão et al. 2009). However, the approach was effectively employed in a variety of investigations, where 150 insect specimens were appropriately assigned and used a barcode information of 200 closely related species (Hebert et al. 2003). To adequately document India's diverse insect population across various ecological zones, efficient methods like DNA barcoding are essential. However, as of 2024, the Barcode of Life Data (BOLD) system contained only a small fraction of Indian insect species barcodes, highlighting the need for more comprehensive data. The paper aimed to analyze DNA barcode data of the *Spodoptera* (Lepidoptera: Noctuidae) genus from India on BOLD to assess the current status and discuss future steps.

## MATERIAL AND METHODS

All sequences and data were collected from The Barcode of Life Data System (BOLD) (Ratnasingham & Hebert 2007) and the National Center for Biotechnology Information (NCBI) (Benson et al. 2012). Specifically, from public data sources we retrieved genetic data of the *Spodoptera* genus dated 19/12/2023, filtering by country ("India"), gene ("COI"), and length (">500bp"). With these settings, we created a dataset named "DS-SPODOPTERA" on BOLD ([https://v3.boldsystems.org/index.php/MAS\\_Management\\_OpenDataSet?datasetcode=DS-SPOD](https://v3.boldsystems.org/index.php/MAS_Management_OpenDataSet?datasetcode=DS-SPOD)). Additionally, data for *Spodoptera mauritia*, *S. littoralis*, and *S. exempta* were obtained using similar filtering criteria for gene and sequence length, adding them to the same dataset. Two outgroup sequences, *Lymantria dispar dispar* (NCBI ID: XAG005-05) and *Hyphantria cunea* (NCBI ID: XAB076-04), were also included.

Following alignment, all DNA sequences were translated into amino acid sequences, guaranteeing the absence of stop codons. The aligned files were then utilized for phylogenetic analysis and distance matrix computation using Mega 10.2. The phylogenetic tree was constructed using the neighbor-joining method (Saitou & Nei 1987) with bootstrap analysis (1,000 replicates) to assess the reliability of the branches. Genetic distances

were computed using the Kimura 2-parameter model (Kimura 1980).

### Single GYMC Analysis

The Generalized Mixed Yule Coalescent (GYMC) model was applied to delineate species boundaries using the COI gene sequences. This approach integrates both yule processes (modeling species diversification) and coalescent processes (modeling intraspecific variations). We implemented the GYMC method using the 'GYMC' package in R, setting the MCMC chain to run for 100,000 generations with a burn-in of 10,000 generations to ensure robust and accurate delineations (Pons et al. 2006).

### BPP Analysis

Bayesian phylogenetics and phylogeography (BPP) analysis was employed to confirm the species boundaries suggested by the GYMC model. We used the BPP v4.0 software, incorporating multi-locus sequence data. The analysis involved specifying a guide tree based on prior phylogenetic knowledge and running the MCMC for 200,000 generations, sampling every 20 generations, and discarding the first 10% as burn-in. Priors were set as theta  $\sim$  G(2, 2000) and tau0  $\sim$  G(2, 1000), reflecting prior expectations of population size and divergence time, respectively (Yang & Rannala 2010).

### mPTP Analysis

The multi-rate poisson tree processes (mPTP) model was utilized to further validate species delimitation results. This method accounts for rate variation among branches, providing a more flexible framework compared to traditional PTP models. The analysis was conducted using the mPTP web server, with default parameters and a bootstrap analysis (1,000 replicates) to assess confidence in species boundaries (Kapli et al. 2017).

By integrating these methods, our analysis aims to provide a comprehensive and robust species delimitation for the *Spodoptera* genus in India, contributing to the accurate identification and understanding of both native and invasive species.

## RESULTS

We analyzed the COI region DNA sequences of six *Spodoptera* species, totaling 817 sequences. For the four species found in India, we obtained COI region sequences for only two species, *S. litura* and *S. exigua*,

from the BOLD database of the 817 sequences, 365 were from outside India, including *S. littoralis* (51 sequences), *S. mauritia* (190 sequences), and *S. exempta* (124 sequences). The remaining 450 sequences were from India, comprising *S. frugiperda* (265 sequences), *S. exempta* (1 sequence), *S. exigua* (58 sequences), and *S. litura* (126 sequences) (Table 1). These were contrasted with barcode sequences from *S. frugiperda* and *S. exempta*, two possible invasive species, since its confirmed status based on the literature. No deletions, insertions, or no stop codons were found when the COI sequences were aligned, suggesting that the amplified DNA originated from functional COI genes. The sequences' total mean GC content is 29–30 %. The mean GC content on codon pos 1 is 39–41 % (except *S. litura*, which has a mean GC% content of 41.07%), the mean GC% content on codon pos 2 is 42–43 %, and the mean GC% content on codon pos 3 is 6–7 %. There was no discernible variation in the overall GC% for Codon Pos 1, Pos 2, and Pos 3.

With the exception of *S. frugiperda* species, which has the largest nucleotide divergence among species at 5.38%, the dataset has no considerable barcode gap. The maximum nucleotide difference within species is  $\leq$ 2.2% (Table 2). The minimal nucleotide difference between species *S. littoralis* and *S. litura* was 2.9%, which was quite near to the cut off ( $\leq$ 3.0%). Apart from this, there was  $\geq$ 4.2% minimal nucleotide difference between species. The two host strains of *S. frugiperda*, *S. mauritia* & *S. exigua*, and *S. litura* & *S. littoralis* showed the closest similarities, however even these pairings separated at  $>$ 95% bootstrap values. Neighbor-joining phenetic analysis, which distinguished at  $>$  75% bootstrap scores among the predicted species and showed that *S. exigua* was the most divergent, supported this. The phylogeny based on morphological and phenetic connections was typically in agreement (Pogue 2002). According to those cladistic analyses, *S. exigua* is the most plesiomorphic species in the *Spodoptera* group, whereas *S. littoralis* and *S. litura* are closely related sister species (Figure 1). Comparisons of adult genital morphology are the only way to distinguish between *S. littoralis* and *S. litura* (Mochida 1973; Ellis 2004). The morphological study of the male and female genitalia of *Spodoptera* species have been provided to identify the species from India (Supplementary Tables 1 & 2).

### Comparative Morphological Analysis of *Spodoptera* Species

This section provides a comparative morphological analysis of key *Spodoptera* species found in India. By

**Table 1. Current genetic and morphological reports, number of COI gene sequences from India and outside of India and mean GC% content, mean GC% content on codon pos. 1, mean GC% content on codon pos. 2 and mean GC% content on codon pos. 3 sequences on the BOLD status of *Spodoptera*.**

	Native species name in India	Genetically reported till date in India	Morphologically reported to date in India	Genetically reported till date outside of India	No. of sequences public on BOLD from India	No. of sequences public on BOLD from Outside of India	Total no. of sequences public on BOLD	Mean GC % content of sequences public on BOLD	Mean GC % content on codon pos 1 of sequences on BOLD	Mean GC % content on codon pos 2 of sequences on BOLD	Mean GC % content on codon pos 3 of sequences on BOLD
1	<i>S. littoralis</i>	No	Yes	Yes	0	51	51	29.32	39.22	41.78	6.97
2	<i>S. mauritia</i>	No	Yes	Yes	0	190	190	29.94	40.90	42.14	6.85
3	<i>S. exigua</i>	Yes	Yes	Yes	58	626	684	29.43	40.40	41.77	6.05
4	<i>S. litura</i>	Yes	Yes	Yes	126	250	376	29.72	41.07	41.71	6.39
	Invasive Species Name in India										
5	<i>S. exempta</i>	Yes	Yes	Yes	1	124	125	29.51	39.57	42.52	6.45
6	<i>S. frugiperda</i>	Yes	Yes	Yes	265	1088	1353	29.77	40	42.07	7.25

**Table 2. Genetic distance between the *Spodoptera* species (indicated by green color) and within the species (indicated by yellow color).**

	<i>S. exempta</i>	<i>S. exigua</i>	<i>S. frugiperda</i>	<i>S. littoralis</i>	<i>S. litura</i>	<i>S. mauritia</i>
<i>S. exempta</i>	1.59					
<i>S. exigua</i>	6.3	1.15				
<i>S. frugiperda</i>	4.8	8.6	5.38			
<i>S. littoralis</i>	4.2	6.0	5.3	2.14		
<i>S. litura</i>	4.5	7.1	5.3	2.9	2.18	
<i>S. mauritia</i>	6.0	9.1	8.3	8.3	9.3	1.91

highlighting differences and similarities in the male and female genitalia, this comparison facilitates accurate identification crucial for pest management.

### Male Genitalia Comparison

#### Valve

*S. exigua*: Broad elongate oval  
*S. exempta*: Narrow rectangular  
*S. mauritia*: Narrow tapering  
*S. frugiperda*: Very broad, quadrate  
*S. littoralis*: Broad quadrate with dentate ventral margin  
*S. litura*: Broad  
*S. eridania*: Not specified

#### Juxta

*S. exigua*: Narrow elliptical band  
*S. exempta*: Narrow elliptical band with triangular median process  
*S. mauritia*: Narrow elliptical band with triangular median process  
*S. frugiperda*: Narrow rectangular band

*S. littoralis*: Broad quadrate

*S. litura*: Triangular

*S. eridania*: Narrow rectangular band

#### Coremata

*S. exigua*: Moderately elongate, no distinct lobes  
*S. exempta*: Single lobe  
*S. mauritia*: Single lobe  
*S. frugiperda*: Single lobe, elongate  
*S. littoralis*: Two lobes  
*S. litura*: Two lobes  
*S. eridania*: One lobe

#### Ampulla

*S. exigua*: Elongate, slightly curved apex  
*S. exempta*: Elongate, bent in the middle  
*S. mauritia*: Elongate, slightly curved downwards  
*S. frugiperda*: Elongate, curved with decurved apex  
*S. littoralis*: Short, curved with decurved apex  
*S. litura*: Short, curved  
*S. eridania*: Straight clasper proper

## Female Genitalia Comparison

### Corpus Bursae

- S. exigua*: Elongate
- S. exempta*: Bulbous
- S. mauritia*: Bulbous, constricted caudally
- S. frugiperda*: Bulbous
- S. littoralis*: Bulbous
- S. litura*: Bulbous
- S. eridania*: Elongate

### Ductus Bursae

- S. exigua*: Short, sclerotized
- S. exempta*: Medium length, sclerotized
- S. mauritia*: Short, sclerotized
- S. frugiperda*: Short, sclerotized
- S. littoralis*: Short, sclerotized
- S. litura*: Elongate, sclerotized
- S. eridania*: Short, sclerotized

### Signum

- S. exigua*: Elongate, <30° angle
- S. exempta*: Elongate, almost vertical
- S. mauritia*: Medium elongate
- S. frugiperda*: Short, >30° angle
- S. littoralis*: Short
- S. litura*: Short
- S. eridania*: Elongate, >30° angle

## Key Distinguishing Features

*S. exigua* vs. *S. frugiperda*: *S. exigua* has a broad elongate oval valve and elongate corpus bursae, while *S. frugiperda* has a very broad quadrate valve and bulbous corpus bursae.

*S. exempta* vs. *S. mauritia*: Both have a narrow rectangular valve, but *S. exempta*'s coremata is a single lobe, while *S. mauritia*'s is also a single lobe but with a constricted caudal end in the corpus bursae.

*S. littoralis* vs. *S. litura*: Both have broad quadrate valves, but *S. littoralis* has a dentate ventral margin and two lobes in the coremata, while *S. litura* has a triangular juxta and two lobes.

## Species Delimitation using Single GYMC, BPP, and mPTP

### Single GYMC Analysis:

The Generalized Mixed Yule Coalescent (GYMC) model identified six distinct species within the *Spodoptera* genus using COI gene sequences. The species boundaries had posterior probabilities exceeding 0.95, demonstrating strong support for the classifications. This analysis differentiated the closely related species *S. littoralis* and *S. litura*, which were previously difficult to distinguish based on morphology alone.

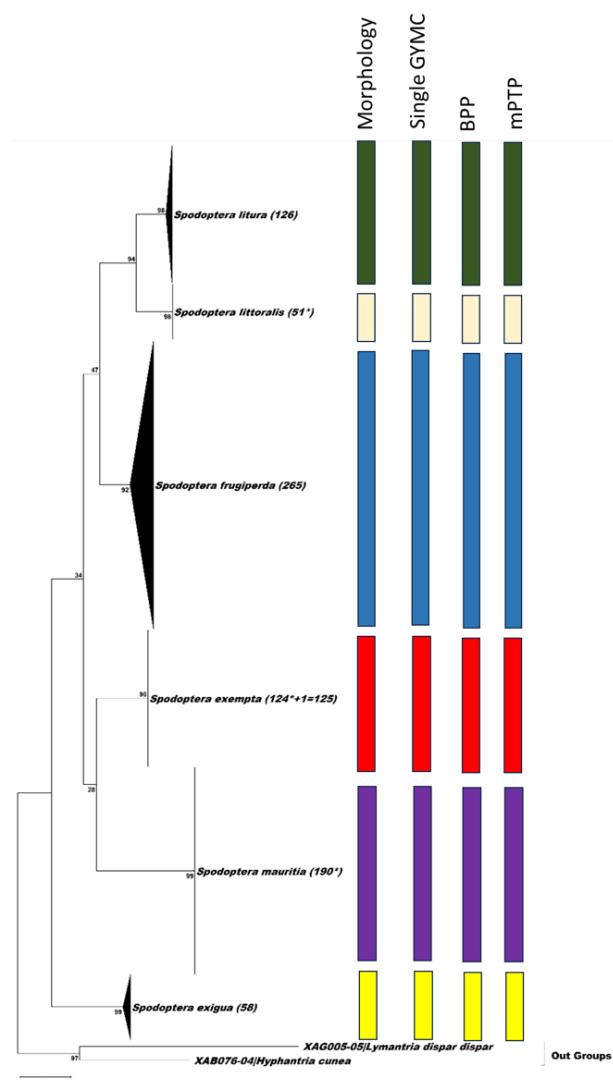


Figure 1. Phylogenetic analysis of *Spodoptera* species (\*indicates genetic data taken from outside of India).

### BPP Analysis

The Bayesian phylogenetics and phylogeography (BPP) analysis further validated the species boundaries suggested by the GYMC model. The results showed high posterior probabilities (>0.90) for all nodes representing species splits, reinforcing the delineation of six species within the dataset. The BPP analysis confirmed the presence of distinct evolutionary lineages corresponding to the species identified by morphological and genetic data.

### mPTP Analysis:

The multi-rate poisson tree processes (mPTP) model analysis supported the species boundaries identified by both GYMC and BPP methods. The mPTP analysis revealed the same six species with high confidence,

and bootstrap support values were above 95% for all species delimitations. This method effectively accounted for rate variation among branches, providing additional robustness to our species delimitation results.

### Comparative Analysis

Comparative analysis across the three methods showed a high level of congruence, with all methods consistently identifying the same six species: *S. littoralis*, *S. mauritia*, *S. exigua*, *S. litura*, *S. exempta*, and *S. frugiperda*. The use of multiple methods provided a comprehensive framework for species delimitation, ensuring that the results were robust and reliable.

### Genetic Distances and Phylogenetic Relationships

Genetic distance analysis revealed minimal within-species variation ( $\leq 2.2\%$ ) and clear between-species differences ( $\geq 4.2\%$ ), except the difference between species *S. littoralis* and *S. litura* was 2.9%. The phylogenetic tree constructed using the neighbor-joining method showed distinct clades for each species with high bootstrap support ( $> 75\%$ ), consistent with the species boundaries identified by GYMC, BPP, and mPTP analyses. *S. exigua* was identified as the most divergent species within the genus, while *S. littoralis* and *S. litura* were confirmed as closely related sister species.

## DISCUSSION

In the Indian subcontinent, four *Spodoptera* species were previously identified as native: *S. litura* (Muthusamy et al. 2024), *S. exigua* (Ramaiah et al. 2022), *S. littoralis*, and *S. mauritia* (Madhu et al. 2023). Additionally, one invasive species, *S. frugiperda* (fall armyworm or FAW), was reported (Ganiger et al. 2018), originating from North and South America (Jing et al. 2020). Recent comprehensive genomic analyses suggest that *S. frugiperda* likely consists of two closely related sister species, known as the corn-preferred and rice-preferred strains. These findings are supported by multiple studies (Pashley 1986; Meagher et al. 2004; Kergoat et al. 2012; Dumas et al. 2015; Gouin et al. 2017; Le Ru et al. 2018). Both sister species are present in India, but the manner of their introduction, whether together or separately, remains uncertain. Additionally, it is unclear if they have spread as a unified population since their introduction.

We observed that all four native *Spodoptera* species were reported through morphological methods, but genetic data is available for only two species on BOLD to date (Table 1). On BOLD/NCBI, only one sequence of *S. exempta* was submitted from India. This is very surprising

that commonly found species' genetic data was lacking. The same observation was noted by Shashank et al. (2022). They also highlighted the present state of insect species barcoding in India. They pointed out the existing gaps which must be addressed soon. Their examination indicates that barcoded specimens encompass a minimal percentage, specifically less than 3.73%, of the recognized taxa or described species. The most predominant orders include Lepidoptera and Hemiptera, followed by Diptera and Coleoptera. It is imperative to accelerate the discovery and documentation of insect species through collaborative efforts between traditional taxonomists and molecular biologists. This collaborative approach aims to achieve comprehensive DNA barcoding for all identified insect taxa in India.

The genus *Spodoptera* presents challenges for morphological identification across all species due to variability and shared characteristics. The complexity arises due to overlapping rib numbers between species, and the morphology of eggs in many *Spodoptera* species remains unknown. Therefore, molecular methods become essential for accurate species-level identification during this developmental stage (European and Mediterranean Plant Protection Organization (OEPP/EPPO) 2015). While fully grown larvae of quarantine *Spodoptera* species can be distinguished, molecular identification is recommended for early stages, especially when the larva's origin is unknown or expertise is lacking. Distinguishing between younger larvae of *S. littoralis*, *S. litura*, and *S. frugiperda* is possible, but molecular identification is advised for early stages, offering reliability in cases where experience is limited or larval origin is uncertain. For *S. eridania*, *S. frugiperda*, *S. littoralis*, and *S. litura*, a practical approach involves using four simplex real-time PCR tests based on TaqMan® chemistry (Van de Vossenberg & Van der Straten 2014). To address geographical distribution overlap, tests for *S. eridania* and *S. frugiperda*, as well as *S. littoralis* and *S. litura*, are combined into single tests, providing an effective means of identification (European and Mediterranean Plant Protection Organization (OEPP/EPPO) 2015).

Biodiversity-rich nations like India, grappling with burgeoning populations, confront significant challenges in harmonizing economic progress, ensuring food security, and preserving biodiversity (Shashank et al. 2022). The foundational field of systematics, crucial for biodiversity research, is under considerable strain. Traditional taxonomy has historically played a pivotal role in identifying over 1.4 million global insect species for the past two centuries. However, the pace of this progress falls short of documenting the entire biota before it faces

extinction. Consequently, novel technologies (Patil et al. 2023; Sontakke et al. 2023), notably DNA barcoding, have gained traction for rapid and cost-effective biodiversity documentation.

As one of the mega-diverse countries, India aspires to make substantial contributions toward achieving the United Nations Sustainable Development Goals (SDGs) (Nalage et al. 2023) and targets (Shashank et al. 2022). However, this review unveils a disconcerting scenario concerning the status of DNA barcoding in India, which described very less insect species. There is apprehension that in the genomics era, the delayed establishment of DNA barcode reference libraries for insects may hinder our ability to comprehensively document India's abundant biodiversity.

## CONCLUSION

This study has left a remarkable footprint in understanding *Spodoptera* species in India. It confirms the presence of four native species—*S. litura*, *S. exigua*, *S. littoralis*, and *S. mauritia*—along with two invasive species—*S. frugiperda* and *S. exempta*—in the country. The confirmation of the presence of *S. eridania* in India awaits the reporting of its mature larva or molecular data.

The study underscores the importance of a combined approach, emphasizing that both morphological and genetic studies must complement each other to accurately identify invasive and native species in the country. It highlights the integration of DNA barcoding and molecular analysis as indispensable for improving the precision and comprehensiveness of *Spodoptera* species identification.

The combined use of Single GYMC, BPP, and mPTP methods provided a robust and comprehensive approach to species delimitation in the *Spodoptera* genus. The results confirmed the presence of six distinct species within India, highlighting the importance of integrating multiple analytical methods to accurately delineate species boundaries in taxonomically challenging groups. This study contributes valuable genetic data and methodological insights for the improved identification and management of *Spodoptera* species in India.

This approach not only tackles challenges associated with morphological identification positively but also contributes valuable data for the development of more targeted and efficient strategies in pest management and conservation efforts.

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## Articles

### Spatial assemblage of shorebirds (Aves: Charadriiformes) in an altered wetland of the southern coast of Sri Lanka

– V.N. Mendis, E.J.A.P. Buddhi Priyankara, E.G.D.P. Jayasekara & W.A.D. Mahaulpatha, Pp. 25495–25506

### Current conservation status of Bengal Florican *Houbaropsis bengalensis* in Manas National Park, Assam, India

– Miranda Thakur, Jonmani Kalita, Namita Brahma, Koushik Rajbongshi, Kangkanjyoti Bhattacharyya, Amal Chandra Sarmah, Alolika Sinha, Deba Kumar Dutta, Dhritiman Das & Bibhuti Prasad Lahkar, Pp. 25507–25515

### Assemblages of frugivorous butterflies in two urban parks in Quezon City, Philippines

– Micael Gabriel A. Itliong, Nikki Heherson A. Dagamac & Jade Aster T. Badon, Pp. 25516–25527

### Assessment of the status of *Spodoptera* species (Lepidoptera: Noctuidae: Armyworm) in India through DNA barcoding technique

– Dinesh Nalage, P.S. Kudnar, Tejswini Sontakke, Ishwar Chittapure, Yashdeep Gowda, Shantanu Kharbal & Yashashri Alamwar, Pp. 25528–25535

### Taxonomy and distribution of some orthopteran species (Orthoptera: Gryllidae, Trigonidiidae, Acrididae) from northwestern Morocco

– Hanae El Harche, Samiha Kaioua & Dalale Mansouri, Pp. 25536–25544

### Impact of root harvest on *Decalepis hamiltonii* Wight & Arn. population across habitats in Savandurga Reserve Forest, Karnataka, India

– M. Sathy Sangeetha, Kaliamoorthy Ravikumar & H.C. Chetan, Pp. 25545–25570

## Communications

### Rare encounters: Jungle Cat *Felis chaus* Schreber, 1777 (Mammalia: Carnivora: Felidae) in the lower reaches of the Jordan River, Jordan

– Ehab Eid & Mohammad Farid Alayyan, Pp. 25571–25576

### Diversity of bird species in Ebpanan Marsh, Maguindanao del Norte, Bangsamoro Autonomous Region in Muslim Mindanao (BARMM), Philippines

– Gindol Rey A. Limbaro, Benito Anthony A. Pingoy & Peter Jan D. de Vera, Pp. 25577–25583

### *Heleocoris stephanus* (Heteroptera: Naucoridae: Laccocorinae), a new species of creeping water bug from Kallada River, Kerala, India

– Dani Benchamin, R. Sreejai & M.S. Arya, Pp. 25584–25589

### Incidence and risk factors associated with parasitic infections in captive wild mammals and birds in Indian zoos

– Nikita Das, P.D. Pawar, P.P. Mhase, V.G. Nimbalkar, R.V. Jadhav, V.S. Dhaygude, Gavin Furtado & L.D. Singla, Pp. 25590–25597

### Bryophyte diversity of Berinag (Pithoragarh District) in Kumaun Himalaya, Uttarakhand, India

– D. Dhami & P. Chaturvedi, Pp. 25598–25603

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## Short Communications

### The opportunistic feeding behaviour of *Schistura notostigma* (Teleostei: Nemacheilidae) in tropical mountain streams in Sri Lanka

– J. Bandara, M.P. Gunawardena & R.T.P Jayasuriya, Pp. 25604–25608

### First record of *Pieris napi* L. (Lepidoptera: Pieridae) from Kashmir Valley, India

– Firdousa Rasool & Altaf Hussain Mir, Pp. 25609–25612

### Reassessment of *Strobilanthes recurva* (Acanthaceae), an endangered plant from Manipur, India

– Rajkumari Jashmi Devi & Biseshwori Thongam, Pp. 25613–25616

### New distribution record of Slender Wild Basil *Clinopodium gracile* (Benth.) Kuntze (Lamiaceae: Nepetoideae: Mentheae) for the flora of Himachal Pradesh, India

– Rimjhim Chandra & Mamita Kalita, Pp. 25617–25622

## Notes

### Rusty-spotted Cat *Prionailurus rubiginosus* (I. Geoffroy Saint-Hilaire, 1831) (Mammalia: Carnivora: Felidae) in the semi-natural subterranean habitat in Karnataka, India

– Shirish Manchi, Goldin Quadros, Dipika Bajpai, Shomita Mukherjee, Suma Haleholi, Mahesh Marennavar, Sangmesh Neeralagi, Prakash Ganiger, Suresh Lamani & Nikhil Kulkarni, Pp. 25623–25626

### First record of Scaly-breasted Munia *Lonchura punctulata* (Linnaeus, 1758) (Aves: Passeriformes: Estrildidae) from Kashmir, India

– Shazia Shafayat, Fayaz Ahmad Ahanger, Tariq Ahmad, Bilal A. Bhat & Zakir Hussain Najar, Pp. 25627–25629

### First record of *Prosyznskia diatreta* (Simon, 1902) (Araneae: Salticidae) from Gujarat, India

– Manisha P. Patel & Dhruv A. Prajapati, Pp. 25630–25631

### *Medicago monantha* (Fabaceae) and *Euphorbia jodhpurensis* (Euphorbiaceae) as new additions to the flora of Maharashtra State, India

– Praveen V. Kale & Rajendra D. Shinde, Pp. 25632–25636

## Book Review

### All eyes on the island: A book review of The Great Nicobar Betrayal

– Lakshmi Ravinder Nair, Pp. 25637–25638

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