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Decoding Lepidopteran Biodiversity through DNA Barcodes

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Abstract

Lepidoptera, one of the largest insect orders, holds significant ecological and economic importance for human society. Many moths and butterflies within this order contribute to livelihoods, habitat conservation, and pollination, and serve as ecological indicators. Evolutionarily, they greatly impact host plants, adapting to and circumventing their defense mechanisms. Environmentalists benefit from their role in understanding atmospheric gases, land mass migration, and periodic ice ages. However, taxonomists face challenges in classifying lepidopterans due to reliance solely on morphological traits, resulting in many species lacking proper placement in superfamilies and families, with some misassigned altogether. Addressing these issues, the Barcode of Life Database (BOLD) provides a valuable platform for recording and tracking species globally. Utilizing Barcode Index Numbers (BINs) and associated software facilitates molecular genetic identification and classification using mitochondrial markers like COI (Cytochrome Oxidase I), Cyt b (Cytochrome b), and NAD (Nicotinamide Adenine Dinucleotide) genes, garnering acceptance from researchers worldwide. While BOLD encompasses certain lepidopteran families from various continents, it still lacks representation for a significant portion of existing species in the order. In order to fix this knowledge gap this paper predominantly explores the efficacy of DNA barcoding as a leading methodology for unraveling the species diversity of lepidopterans on a global scale which would aid for devising mechanisms of conservation of this ecologically and economically important animal order worldwide.

Keywords: DNA barcoding; Lepidoptera; COI; Cyt b; BOLD

Lepidoptera is among the three largest insect orders, encompassing approximately 160,000 known species of moths and butterflies. Following Hemiptera, Lepidoptera is the most diverse, widespread, and easily recognized insect order within the class Insecta and the phylum Arthropoda. Between 1707 and 1778, Linnaeus categorized Lepidoptera into three groups: butterflies, skippers, and micro and macro moths. This order includes 46 superfamilies and 126 families. They can be distinguished by their morphological, anatomical, behavioral, and ecological traits (Miller et al., 2003). Of the 5,00,200 Lepidoptera species described, 70,820 are butterflies, 3,700 are skippers, and 165,000 are micro and macro moths (Shield, 1989; Sutton and Sutton, 1999).

Origin and evolution of Lepidoptera:

Lepidopterans constitute one of the most extensive evolutionary herbivore radiations (Scoble 1992, Wahlberg et al. 2013). Alongside Coleoptera, Diptera, Hymenoptera, and Hemiptera, they form one of the five mega-diverse insect orders originating in the Jurassic (Goldstein, 2017). Predominantly, lepidopteran superfamilies emerged in the Cretaceous, with major Macro-lepidopteran superfamilies arising in the Late Cretaceous or Early Tertiary, concurrent with the diversification of flowering plants. Despite their narrow dietary range focused on vegetative substrates, their phylogenetic diversity expanded alongside the evolution of flowering plants and the spread of insect parasitoids and mammalian predators (Powell et al. 1999, Grimaldi and Engel 2005; Goldstein 2017). Climatic shifts, such as changes in atmospheric gases, landmass migration, and ice ages, influenced their diapause behavior and phenology. Species radiation might have been driven not only by ancestral food plant evolution but also by habitat changes due to climatic shifts and the rise of C4 grasses in the Miocene (Toussaint et al. 2012; Goldstein and Fibiger, 2005). The debate over whether lepidopterans evolved during the Cretaceous or Paleogene is hampered by the scarcity of fossils (Sohn et al. 2015). While molecular data could aid phylogenetic reconstruction, acceptance within the community hinges on the integration of morphological clarity (Goldstein 2017).

Ecological Roles of Lepidoptera:

Lepidopterans, like their sister order Trichoptera (Caddisflies), undergo complete metamorphosis. Insights into sexual selection have been gained from the discovery of lock-and-key mechanisms in moth genitalia (Eberhard 1985, Shapiro and Porter 1989, Mikkola 2008) and "Chasity belts" in butterflies for sperm competition. Studies on pheromones, phenology, and diurnality contribute to understanding disruptive selection and allochronic

speciation. Examples of co-evolution include the mimicry complexes in Heliconis butterflies (Gilbert 1984, conner 1996) and Dioptine moths (Miller 1996, 2009), representing diffuse co-evolution, while strict co-evolution is exemplified by Yucca and Yucca moths (Pellmyr 2003). Lepidopteran research has elucidated diet breadth, dietary specialization, and the chemical basis of aposematism (Edger et al, 2015). Evolutionary studies reveal that butterfly vision is under positive selection, while wing patterns are influenced by kin recognition and Müllerian mimicry. DeVries (1991) described how caterpillars use acoustic signals to communicate with ants, providing defense against parasitoids. Sphingid and Saturniid moths have evolved sonar-jamming abilities to evade bat predation (Barber and Kawahara 2013, Barber et al. 2015), and tiger moths have developed acoustic aposematism (Barber and Conner 2007, Conner and Corcoran 2012, Corcoran et al. 2009). Recent observations indicate lepidopteran species are migrating towards the North Pole, increasing voltinism, and clustering in high-altitude habitats, reflecting adaptation to climatic changes (Parmesan 2006). Lepidopterans, sensitive to environmental changes, serve as bioindicators, providing early warnings of ecological degradation (Chowdhury et al 2023).

Many Lepidopterans cause significant damage to forests, agriculture, and stored goods as pests, often leading to severe outbreaks as invasive species. Caterpillars from families like Noctuidae, Tortricidae, and Pyraloidea are major culprits in damaging crops (Bhagat 2018). The introduction of the Gypsy moth (*Lymantria dispar*) (Wu et al. 2020, Keena and Richards 2020) and Browntail moth (*Euproctis chrysorrhoea*) (Boyd et al. 2021) from the Erebidae family, as well as the Winter moth (*Operophtera brumata*) (Vindstad et al. 2022) from the Geometridae family, has resulted in extensive destruction of American forests. Traditional control measures, including pesticides such as DDT and carbaryl and biocontrol parasitoids like *Compsilura concinnata*, have proven ineffective against these pests. Similarly, the accidental introduction of the Pyralid moth (*Cactoblastis cactorum*), originally used as a biocontrol agent for *Opuntia cacti* in Australia, now poses a threat to various cactus species in other countries (Stiling 2002).

Existing Lepidopteran Taxonomy:

The primary research focus for the order Lepidoptera centers on elucidating the higher-level relationships among major clades and addressing problematic taxa, alongside the unexpected taxonomic flux within and among superfamilies. Consequently, many higher-level phylogenetic arrangements remain unstable (Regier et al. 2013). In truth, only a few major

lepidopteran groups have maintained stable higher-rank classifications. For example, the Gelechioidea superfamily has been particularly unstable over the past 25 years as researchers have scrutinized its families and superfamilies (Wahlberg et al. 2009). Similarly, the placement of butterflies has remained uncertain across families. The term "Macrolepidoptera" has fallen out of use, with these groups now classified under "Macroheterocera". Traditionally, Lepidoptera have been categorized into large and small moths, butterflies, and skippers. Pre-Hennigian classifications placed butterflies and skippers with clubbed antennae in Rhopalocera and moths in the paraphyletic Heterocera. However, Heterocera contains many imperfect characteristics, such as wing coupling, venation, and the separation of the gonopore from copulatory organs, along with misnomers like ‘macro’ and ‘micro’. Historically, primitive superfamilies like Hepialoidea, thought to be micro moths, included large moths, while small moths like Micronoctuinae were classified within multiple superfamilies in higher Ditrysia. One such superfamily, Pyraloidea, remains terminologically contentious and is not fully accepted by either Macro-lepidopterans or Micro-Lepidopterans (Regier et al. 2009).

Presently, numerous species remain unassigned to specific families or superfamilies due to ongoing challenges in classification and character categorization encountered by taxonomists (refer: Table 1). Taxonomists have recognized approximately 131 families across 42 superfamilies in the current context, with 8 families yet to be assigned to any superfamily. Among these, five families belong to early lepidopterans, one to early Ditrysians, and two to Apoditrysians (see Table 1) (Goldstein 2017). The phylogenetic relationships among these superfamilies are depicted in Figure 3.

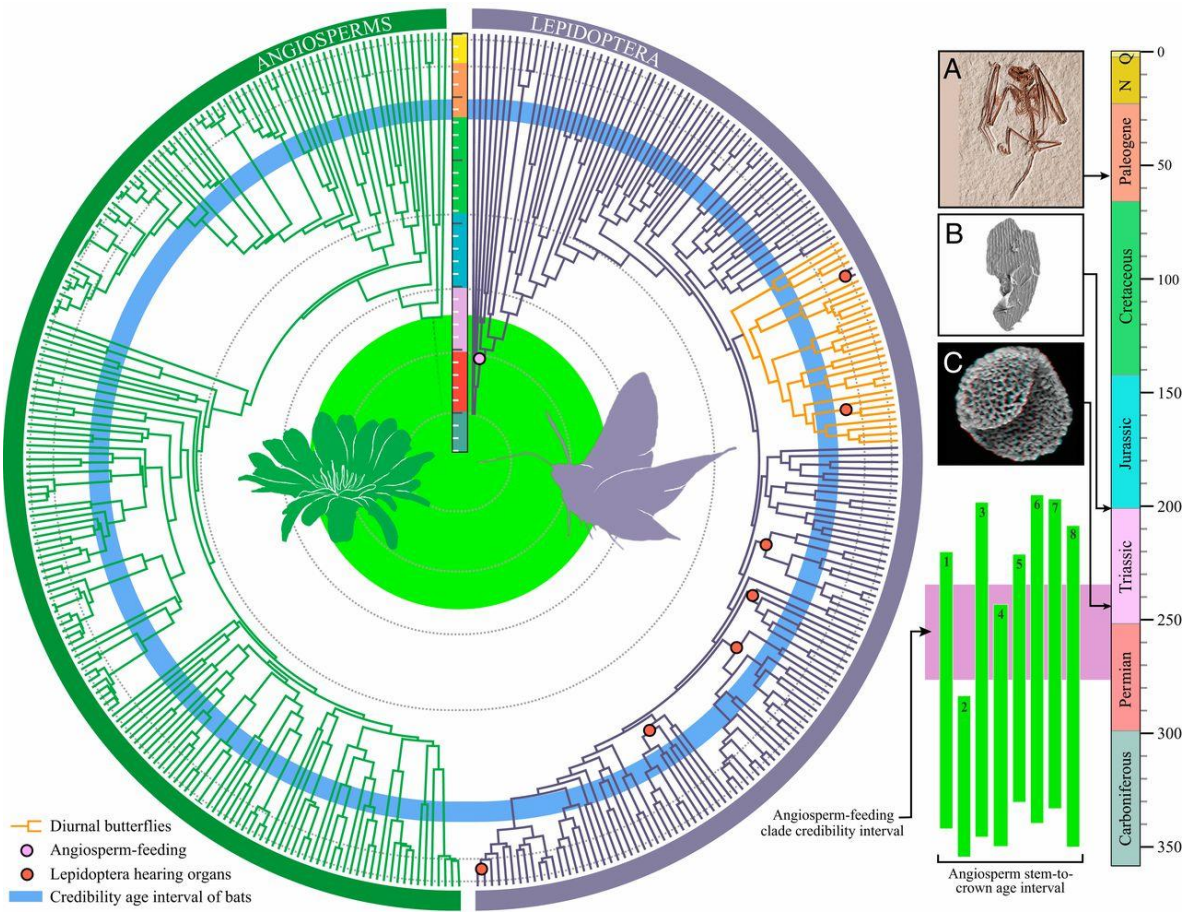

Fig 1: Comparative phylogenetic tree showing Angiosperm plant evolution (green) and the Lepidopteran evolution (Purple) (Kawahara et al. 2019)

Table 1 The Existing Classification of The Members of the Lepidopterans

Superfamily	**Family**	**No. of Genus**	**No. of Species**
Micropterigoidea	Micropterigidae	21	265
Agthiphagoidea	Agathiphagidae	1	2
Heterobathmioidea	Heterobathmiidea	1	10
Eriocranioidea	Eriocraniidae	5	30
Acanthopterocteoidea	Acanthopteroctetide	2	5
Lophocoronoidea	Lophpcoronidae	1	6
	Aenigmatineidae	1	1
Neopseustoidea	Neopseustidae	4	14

Hepialoidea	Mnesarchaeidae	1	14
	Hepialidae	69	652
Nepticuloidea	Nepticulidae	13	852
	Opostegidae	7	194
Andesianoidae	Andesianidae	1	3
Adeloidea	Heliozelidae	12	124
	Adelidae	5	294
	Incurvariidae	11	51
	Cecidosidae	5	16
	Prodoxidae	9	97
	Tridentaformidae	1	1
Palaephatoidea	Palaephatidae	7	57
Tischerioidea	Tischeriidae	3	112
Tineoidea	Ericottidae	6	80
	Psychidae	211	1246
	Tineidae	321	2110
	Meissiidae	35	248
	Dryadaulidae	1	35
Gracillarioidea	Roeslerstammiidae	13	53
	Bucculatricidae	4	297
	Gracillariidae	100	1855
Yponomeutoidea	Yponomeutidae	94	362
	Ypsolophidae	7	163
	Plutellidae	48	150
	Glyphipterigidae	28	535
	Argyresthiidae	1	157
	Lyonetiidae	32	204
	Atteridae	1	52
	Praydidae	3	47
	Heliodinidae	13	69
	Bedelliidae	1	16
	Scythropiidae	1	1

(Unassigned superfamily)	Douglasiidae	2	29
Simaethistoidea	Simaethistidae	2	4
Gelechioidea	Autostichidae	72	650
	Lecithoceridae	100	1200
	Xyloryctiae	60	524
	Oecophoridae	313	3400
	Depressariidae	114	2300
	Cosmopterigidae	135	1792
	Gelechiidae	507	4700
	Elachistidae	47	901
	Coelophoridae	5	1400
	Batrachedridae	10	99
	Scythrididae	30	669
	Blastobasidae	24	430
	Stathmopodidae	44	408
	Momphidae	6	115
	Pterolonchidae	2	30
(Unassigned)	Lypusidae	3	150
	Schistonoeidae	1	1
Alucitoidea	Tineodidae	12	19
	Alucitidae	9	216
Pterophoroidea	Pterophoridae	90	1318
Carposinoidea	Copromorphidae	9	43
	Carposinidae	19	283
Schreckensteinioidea	Schreckensteiniidae	2	8
Epermenioidea	Epermeniidae	10	126
Urodoidea	Urodidae	3	66
Immoidea	Immidae	6	245
Choreutoidea	Choreutidae	18	406
Galacticoidea	Galacticidae	3	19
Tortricoidea	Tortricidae	1071	10387

Cossoidea	Brachodidae	14	137
	Dudgeoneidae	6	57
	Metarbelidae	18	196
	Ratardidae	3	10
	Castniidae	34	113
	Sesiidae	154	1397
	Cossidae	151	971
Zygaenoidea	Epipyropidae	9	32
	Cyclotornidae	1	5
	Heterogynidae	1	10
	Lacturidae	8	120
	Phaudidae	3	15
	Dalceridae	11	80
	Limacodidae	301	1672
	Megalopygidae	23	232
	Aididae	2	6
	Somabrachyidae	4	8
	Himantopteridae	11	80
	Zygaenidae	170	1036
Whalleyanoidea	Whalleyanidae	1	2
Thyridoidea	Thyrididae	93	940
Hyblaeoidea	Hyblaeidae	2	18
	Prodidactidae	1	1
Calliduloidea	Callidulidae	7	49
Papilionoidea	Papilionidae	32	570
	Hedylidae	1	36
	Hesperiidae	570	4113
	Pieridae	91	1164
	Riodinidae	146	1532
	Lycaenidae	416	5201
	Nymphalidae	559	6152
Pyraloidea	Pyralidae	1056	5921

	Crambidae	1018	9666
Mimallonoidea	Mimallonidae	27	194
Drepanoidea	Cimeliidae	2	6
	Doidae	2	6
	Drepanidae	122	660
Lasiocampoidea	Lasiocampidae	224	1952
Bombycoidea	Apatelodidae	10	145
	Eupterotidae	53	339
	Brahmaeidae	7	65
	Phiditiidae	4	23
	Anthelidae	9	94
	Carthaeidae	1	1
	Endromidae	12	59
	Bombycidae	26	185
	Saturniidae	169	2349
	Sphingidae	206	1463
Geometroidea	Epicopeiidae	9	20
	Sematuridae	6	40
(Unassigned)	Uraniidae	90	686
	Geometridae	2002	23002
	Pseudobistonidae	1	1
Noctuoidea	Oenosandridae	4	8
	Notodontidae	704	3800
	Erebidae	1760	24569
	Euteliidae	29	520
	Nolidae	186	1738
	Noctuidae	1089	11772

Comprehensive investigations of DNA Barcoding in Phylogenetics study of Lepidoptera:

To explore the potential of DNA barcoding in the tropical lepidopteran populations, which typically exhibit higher ecosystem diversity than others (Odah 2023), Hajibabaei et al. (2006) published a pivotal study in which they employed DNA barcoding to assess species diversity among three families: Hesperiidae (Skipper butterflies), Sphingidae (Sphinx

Butterflies), and Saturniidae (Wild silk moths) in Costa Rica's Area de Conservacion Guanacaste (ACG). Through the analysis of COI sequences from 521 species, they found that 97.9% of these species were identifiable, with their barcodes forming distinct, non-overlapping clusters in a Neighbour-joining tree. Intra-generic divergences were measured at 4.58%, 4.41%, and 6.02%, while intra-species divergences were 0.17%, 0.43%, and 0.46% for Hesperiidae, Sphingidae, and Saturniidae respectively. These results highlighted the marked genetic variances both within and between species, which could be effectively distinguished using this technique. The persistence of species clusters in the N-J tree, even with larger sample sizes, demonstrated the stability of the 13 distinct COI lineages, thereby validating the accuracy of COI barcodes. Additionally, the study found that physical, environmental, and small-scale geographic variations aligned with the distinct clusters identified through barcode analysis. A notable example is *Automeris zugana*, where separate clusters from rainforest and dry forest habitats revealed that the presumed single species comprised three different species. With 97.9% of the studied species successfully identified, DNA barcoding shows significant promise for identifying tropical lepidopteran species. Furthermore, the utilization of barcoding not only enhances the assessment of species richness but also leads to the discovery of previously unrecognized species, as demonstrated by the identification of 13 potential new species groups in this study.

The barcoding data have offered extensive insights into how historical forest fragmentation has driven the evolutionary diversification of the butterflies (Vella et al. 2022) in the *Heteropsis* genus (Subfamily: Satyrinae), particularly in Madagascar (Pena and Wahlberg 2008), where these old-world grass feeders exhibit numerous recently diversified species with distinct morphological differences (Torres et al 2001; Linares et al 2009). Tree-based barcode analysis strongly supports the monophyly of *S. tepahi* and several *Heteropsis* species, including *H. exocellata*, *H. pauper*, *H. subsimilis*, *H. turbata*, and *H. pallida*. The phylogenetic tree indicates that populations in older forest fragments often diverge from those in nearby continuous forests. These older fragments are frequently fixed for a single haplotype derived from sequences in continuous forest patches. In the case of *S. tepahi*, although a similar diversification pattern was observed, the rate of diversification was higher between haplotypes from other continuous forest patches. The mt.DNA sequence data corroborated the identification of certain species based on physical characteristics, though not all.

Pollinators are traditionally believed to have been pivotal in the diversification of flowering plants (Brockerhoff et al. 2017); however, a growing body of evidence suggests that

parasitic insects also play a significant role in flower evolution (McCall and Irwin 2006, Theis 2006). A notable study on the *Mompha* genus, which comprises inconspicuous moth larvae feeding on the coastal dune plant *Camissoniopsis cheiranthifolia* (Family: Onagraceae) distributed from northern Baja California, Mexico, to southern Oregon, has revealed substantial genetic variation in flower size and mating-related traits (Emery et al 2009). The identification of *Mompha* parasites is complicated due to the association of only larval stages with *C. cheiranthifolia* and the lack of comprehensive larval descriptions (Sentil et al. 2021). Thus, a detailed understanding of these parasitic insects necessitated the integration of morphological analysis and DNA barcoding. This dual approach has streamlined the identification of elusive or juvenile insects, thereby facilitating large-scale ecological and evolutionary studies (Miller et al 2005, Ball & Armstrong 2006, Pfenninger et al 2007). In this research, DNA barcoding and phylogenetic analysis were employed to identify and assess the diversity of taxa parasitizing *C. cheiranthifolia*. Of the 228 specimens submitted, 202 were successfully processed. The ABR filter of BOLD identified all 199 larvae and 3 adults as belonging to the *Mompha* genus. The COI haplotypes were accurately classified to the correct infraorders. Among the 15 haplotypes identified, one dominant haplotype was present in 122 larvae (60% of specimens), another common haplotype in 52 larvae (26% of specimens), and the remaining 13 haplotypes in 27 larvae (13% of specimens). A genetic divergence of less than 3% indicated they likely belong to the same species (Herbert et al 2003). All 15 haplotypes of *C. cheiranthifolia* were monophyletic within the *Mompha* species. The absence of morphological gaps in adult moths bred from *C. cheiranthifolia* suggests they do not exclusively represent a single species. Additionally, their genital characteristics differentiate them from all 42 recognized North American *Mompha* species, including the nine identified through barcoding. Despite estimates of nearly 100 *Mompha* species in North America, only nine have been validated in BOLD, encompassing various feeding behaviors: leaf miners (*M. cephalonthiella*) (Wagner et al 2004), stem feeders (*M. eloisella*) (Forbes 1923), root borers (*M. idaei*) (Koster and Sinev 2003), and flower, fruit, and seed feeders (*M. brevivittellaa*, *M. circumscriptella*, and *M. stellella*) (Forbes 1923). Given that both morphological analysis and DNA barcoding yielded consistent species identifications, DNA barcoding emerges as a valuable tool for elucidating and identifying flowering plant parasites (Judaro-Rivera et al. 2009).

A comparable barcoding endeavour aimed at elucidating the biodiversity of Tibetan moth species (Noctuidae) was undertaken by Jin et al. in 2013 on the Qinghai-Tibetan Plateau, a region renowned as one of the globe's foremost biodiversity hotspots (Jin et al. 2013). This

plateau, the largest and highest on Earth, encompasses over 2.5 million square kilometers, extending across China's Qinghai province, the Tibet Autonomous Region, and portions of adjacent countries such as India, Nepal, and Bhutan. Its extreme altitude, unique geographical features, and profound cultural significance render it an area of substantial scientific inquiry, environmental importance, and cultural heritage. The investigation juxtaposed morphology-based methods with DNA barcoding approaches, unveiling a remarkable congruence for Noctuidae moths, thereby affirming that the precision of DNA barcoding closely parallels that of the traditional morphology-based technique. Although both methodologies exhibit subtle discrepancies in their ability to identify correlations between ecological variables and species diversities, their efficacy is contingent upon the specific diversity measure employed. For instance, the Shannon index derived from DNA barcoding demonstrated a superior capacity to identify correlations between environmental variables and species diversity compared to the traditional morphology-based methods. The spatial distribution analysis of seven moth communities, incorporating parameters such as radiation and rainfall, delineated their presence across two distinct ecological zones: the eastern and the western regions. The eastern region is characterized by weak radiation, high rainfall, and elevated humidity, whereas the western region is typified by strong radiation, low rainfall, and reduced humidity. Employing five diversity indices-Shannon index, Simpson index, exponential of Shannon index, transformed Simpson, and α-diversity-the study consistently found higher values in eastern communities compared to western ones in both DNA barcoding and morphology-based assessments. This consistency underscores the substantial utility of DNA barcoding in conducting diversity analyses in regions with high biodiversity, highlighting its effectiveness in capturing the intricate dynamics of ecological variations.

In 2016, Yang et al. conducted a DNA barcoding study on Satyrine Butterflies (Lepidoptera: Nymphalidae) in China, demonstrating the method's efficacy in identifying Satyrine lepidopterans. This subfamily, encompassing approximately 2,500 extant species worldwide, comprises about 80% of the Satyrine tribe (Ackery et al. 1999; Murillo-Ramos et al. 2021), which began diversifying around 32-34 million years ago (Shi et al. 2015). The researchers generated a total number of 214 COI barcode sequences from 90 species, representing nearly 25% of the Chinese Satyrine population. To evaluate the COI barcoding's effectiveness, they had used 54 barcodes from 16 species and an additional 47 sequences independently. Their analysis covered 90 species across 37 genera, with 34 species newly recorded in the BOLD system. Intraspecific genetic divergence ranged from 0.0-5.1%

(average: 0.8%), with significant divergence (>3%) between *Lopinga achine* and *Neope muirheadii*. Interspecific genetic divergence ranged from 1.1-19.6% (average: 12.9%), with low divergence (<2%) between some species pairs. Overall, interspecific divergence was 16 times greater than intraspecific divergence. Species targeted with multiple specimens exhibited monophyly with high bootstrap values in the Neighbour-Joining tree. Even species with high intraspecific divergence, like *L. achine* and *N. muirheadii*, showed monophyly. The study confirmed the presence of a **'barcoding gap'** and reciprocal monophyly in 96% of the 50 species analysed, validating the method's discriminatory power. Additionally, the technique effectively distinguished between subspecies, such as *L. a. achinoides* and *L. a. catena*. Geographic separation within species also resulted in greater genetic divergence, yet these populations remained monophyletic in the Neighbour-Joining tree.

Lepidopteran stem borers, particularly from the Noctuidae, Tortricidae, Crambidae, and Pyralidae families, are highly destructive agricultural pests, notably reducing sugarcane yields by up to 40%. The Poaceae family, which includes essential grasses, suffers annual global crop losses of 20-40% due to pests, with stem borers being significant contributors and quarantine concerns (Moeng et al. 2018). In Australia, only the native *Bathytricha truncata* exists, causing minimal damage thanks to natural controls (Sallam 2006). Sallam (2006) identified 36 critical sugarcane stem borer species, with seven from the Crambidae and Noctuidae families posing high threats to Australia: *Chilo terrenellus*, *Chilo infuscatellus*, *Chilo sacchariphagus*, *Chilo auricilius*, *Scirpophaga excerptalis*, *Sesamia grisescens*, and *Sesamia inferens*.

Lee et al. (2019) conducted a comprehensive DNA barcoding study, generating 508 sequences and analyzing a total of 1,297 sequences, including those from previous studies and the BOLD database. *Chilo orichalcociliellus* was the most frequently sampled species. The study identified 24 misidentified individuals based on FastTree analysis. Phylogenetic analyses using FastTree, MrBayes, RAxML, and BEAST, with Tortricidae as the outgroup, showed that genera like *Acrapex* and *Sesamia* were paraphyletic, consistently splitting into multiple clades. Most species were monophyletic, but *Bathytricha truncata* was paraphyletic due to the inclusion of other *Bathytricha* species. *Sesamia inferens* exhibited two distinct clades and the highest intraspecific genetic distance (11% K2P), suggesting it represents two species. *Scirpophaga nivella* was paraphyletic except in BEAST analyses, indicating potential species complex issues. *Chilo infuscatellus* showed significant intraspecific diversity, leading to its subdivision into at least six species in 15 analyses. *Chilo sacchariphagus* was divided into three groups correlating with its subspecies, supported by genitalia dissections (morphology). This

study highlights the effectiveness and future potential of DNA barcoding for accurate species identification in stem borers.

To date, limited authentic data has been disclosed regarding the host-plant relationships of Lepidoptera. Research has predominantly focused on caterpillars of economic importance, particularly pests (San Blas 2013; Tay et al. 2016; Zawadneak et al. 2016), while the Microlepidoptera category has been notably under-researched. According to the Barcode of Life Data Systems (BOLD), which analysed approximately 380,000 DNA barcodes of Neotropical lepidopteran species, over 70% of Neotropical moth fauna remains undescribed. It is estimated that accurate feeding records from nature are lacking for over 98% of the presumed 100,000+ Neotropical moth species (Ratnasingham and Herbert, 2007). DNA barcoding facilitates identification even from desiccated integuments post-ecdysis and vacated pupal exuviae after moth eclosion (Lees et al. 2011). A notable study from Peru by Hausmann et al. (2020) utilized barcoding on lepidopteran caterpillars collected from fogged trees. From 47 tree samples, 130 lepidopteran larvae were isolated. DNA barcoding achieved a 91.5% success rate for 119 larvae, which were categorized into 92 unique COI clusters, known as Barcode Index Numbers (BINs), indicating distinct species. Of these larvae, 65 (55%) were identified as belonging to 48 species with close genetic similarity. Another 32 larvae (27%), representing 27 species, showed genus-level matches in the BOLD database, though 5 instances had questionable reliability. Additionally, 19 larvae (16%) were classified into subfamily or family levels. The study concluded that identifying 92 species from 119 larvae underscores how the combination of canopy fogging and molecular analyses can significantly enhance our ecological understanding of Lepidopterans.

The family Geometridae, one of the most diverse among moths, encompasses nearly 24,000 described species within the superfamily (Waugh 2007). The neotropics, a biogeographic region extending from the central plateau of Mexico southward, eastward, and westward, hosts a particularly rich variety of these moths, with approximately 6,500 described species (Mitter et al. 2017, Brehm et al. 2016). Identifying these insects has become increasingly challenging due to a lack of taxonomic expertise and the scattered, outdated, and superficial nature of available taxonomic information online. In 2021, Murillo-Ramos et al. undertook a comprehensive database checklist of Colombian geometrid moths using DNA barcoding techniques (Brehm et al. 2019). This research aimed to contribute to the DNA Barcode library, compiling data over four years from 26 localities in Northeast Colombia. Their findings included significant taxonomic and distributional data. Specimens were provisionally

categorized into putative species based on morphological traits such as wing patterns and abdominal tympanic organs, followed by assignment to Barcode Index Numbers (BINs). The COI gene, alongside two nuclear genes- wingless (*wnt*) and Elongation Factor 1 Alpha (EF-1-Alpha)—were utilized for barcoding purposes. Currently, 21,000 geometrid specimen BINs are available in the Barcode of Life Data Systems (BOLD). Of the 386 Colombian geometrid specimens processed, only 284 were successfully sequenced. Among the 281 available specimens, 157 had been previously identified in other countries like Ecuador, while 115 sequences were assigned to BINs identified only at the genus or tribe level. This revealed that at the time, four out of eight subfamilies of Geometridae were represented in the Colombian DNA Barcode library. Notably, nearly 50% of sequences were attributed to the subfamily Ennominae, represented by 159 BINs assigned to 55 genera. However, over half of the barcodes corresponding to the families Sterrhinae and Larentiinae were not identified at the species level, highlighting the need for taxonomic revisions in South American species. Out of 2,407 records for Colombian Geometridae, only 453 were identified at the species level, 1,619 at the family level, and 335 at the genus level. Phylogenetic analysis of the sequences confirmed the monophyly and taxonomic positions of genera such as *Synchlora, Iridopsis, Glena*, and *Physocleora*. Even though many of the specimens could not be identified at the species level, they were accurately clustered within their respective genera in this study. Conversely, specimens from the genera *Idaea, Scopula, Nephodia, Isochromodes*, and *Macaria* were found to be para-phyletic or poly-phyletic.

In 2020, Kim et al. conducted a comprehensive study on Gelechioidea moth populations in Korea, elucidating the efficacy of DNA barcoding in revealing cryptic diversity within this superfamily. Gelechioidea, encompassing 15-21 families and commonly termed "micromoths," display significant ecological diversity, inhabiting both aquatic and terrestrial environments. This superfamily, which comprises over 18,000 described species, is globally distributed and bears considerable economic importance due to its role as a major pest in agriculture and forestry. The study utilized multiple species delimitation methods, resulting in the identification of the following Molecular Operational Taxonomic Units (MOTUs): 152 via Automatic Barcode Gap Discovery (ABGD), 156 through the Poisson Tree Processes model (PTP), and 213 with the Bayesian Poisson Tree Processes model (bPTP). Notably, only 117 out of 154 morphospecies were consistently identified across all delimitation methods. The researchers determined that a proxy value of 2.5% is effective for preliminary species delimitation within Gelechioidea. The study revealed putative cryptic diversity within three morphospecies—

Neoblastobasis biceratala, *Evippe albidoesella*, and *Promalactis atriplagata*—each exhibiting high intraspecific variability and multiple MOTUs. Additionally, ecological differences were observed in *N. biceratala* and *P. atriplagata*, which were collected from mountainous and urban sites, respectively, suggesting allopatric speciation without morphological differentiation. These findings indicate that geographic isolation, coupled with a high substitution rate in the COI gene, can drive speciation in these species (Pena et al. 2008). The authors advocate for a combined approach integrating morphological analysis with MOTU estimation to accurately identify cryptic species within Gelechioidea, particularly when genetic divergence exceeds 2.5%.

In 2022, Zhan and colleagues conducted a comprehensive diversity investigation in the Xianjiang Wild Fruit Forest, utilizing DNA barcoding to estimate and identify lepidopteran species populations and to assess species richness in the region. Employing both traditional morphological methods and DNA barcoding technology, specifically targeting the COI gene, the study aimed to establish an insect monitoring system and a local gene pool for Xinjiang wild fruit forests, thereby guiding future conservation efforts and resource utilization. A sample of 2,422 individuals revealed 143 morphologically identified Lepidoptera species, spanning 110 genera across 17 families. Noctuidae had the highest number of individuals (1,126), followed by Pyralidae (263) and Crambidae (259), while Cossidae and Lycaenidae were represented by only one individual each. The remaining families, in descending order of abundance, included Erebidae, Notodontidae, Geometridae, Sphingidae, Tortricidae, Pieridae, Nymphalidae, Pterophoridae, Arctiidae, Yponomeutidae, Lasiocampidae, and Limacodidae. Species richness was highest in the Noctuidae family, followed by Geometridae and Crambidae, with proportions of 37.76%, 16.08%, and 9.09%, respectively. The other families had the following proportions: Erebidae (6.99%), Tortricidae (6.29%), Pyralidae (4.90%), Sphingidae (4.90%), Nymphalidae (4.20%), Notodontidae (2.80%), Arctiidae (1.40%), and Yponomeutidae (1.40%). The research produced a set of 196 COI barcodes, representing 67 distinct species distributed among 61 genera across 14 families. Notably, Noctuidae contributed 23 species in 19 genera, Geometridae contributed 8 species in 7 genera, and Erebidae contributed 7 species in 7 genera. The overall mean genetic distance observed was 15.70%, with pairwise genetic distances ranging from 0% to 35.15%. Intraspecific mean sequence divergence varied from 0% to 3.11%, averaging 0.57%, while interspecific divergence ranged from 3.52% to 33.65%, averaging 15.96%. The identification of DNA barcode gaps, indicative of significant differences between species, underscored the effectiveness of DNA barcoding in

species identification. The substantial gaps in both interspecific and intraspecific barcoding distances provided confidence that the sequences generated in this study could effectively distinguish among most species. Additionally, the clustering patterns revealed by the Neighbor-Joining (NJ) tree analysis showed that the majority of the 67 barcoded species formed distinct groups, affirming the practical utility of this DNA barcoding approach for monitoring lepidopteran insects in the natural fruit forests of Xinjiang.

The European lepidopterans are among the most thoroughly researched faunas worldwide, with significant efforts devoted to DNA barcoding (Carlos-Lopez Vaamonde et al. 2021). Of the 10,723 known species in Europe, 7,831 have been barcoded. The Gracillariidae family, the most diverse group of leaf-mining moths, includes over 2,000 described species globally, with 263 species recognized in Europe. Identifying these species based solely on morphology often presents challenges (Heppner 2002; Dincă et al. 2011; Rajaei et al 2022).

Recently in a 2021 study, Lopez-Vaamonde et al. assessed the efficacy of DNA barcoding for the identification and discovery of European Gracillariids. They constructed a comprehensive DNA barcode library comprising 6,791 COI sequences, covering 92% (242 out of 263) of the European species. Their findings showed a strong correlation between morphological identifications and DNA barcodes, with 91.3% (221 out of 242) of the species forming monophyletic clades. This indicates a high accuracy of species identification using barcodes. However, 8.7% of the species exhibited non-monophyly, which introduces some uncertainty in barcode-based identification. Using the Barcode Index Number (BIN) system, species discrimination was successful for 93% of the species, although 7% shared BINs, which posed challenges for precise differentiation. The study uncovered 21 candidate species previously undescribed, and through an integrative approach, validated the existence of six of them. Additionally, 13 species exhibited deep conspecific lineages, representing 27 BINs, without noticeable morphological or ecological differences within the species. These results enhance the reliability of DNA barcoding for identifying lepidopteran species and significantly contribute to the field of taxonomy, setting the stage for future research and conservation efforts.

In identification of new species, from the existing clade, DNA Barcoding data along with the traditional morphology character based identification method gives a more prominent and evident-based clarification to the study (Rach et al 2008). A study by Sohn et al. 2021 on Crambidae and Spilomelinae from Korea also offers a detailed analysis of the *Cotachena* genus

in Korea. Through morphological examinations and DNA barcoding of 23 individuals, the research identified three *Cotachena* species: *C. alysoni, C. brunnealis*, and *C. taiwanalis*, with *C. brunnealis* and *C. taiwanalis* being newly recorded in Korea. The study also discussed the dubious records of *C. pubescens*, suggesting previous misidentifications by taxonomists. DNA barcodes for *C. taiwanalis* and *C. brunnealis* were provided for the first time, facilitating accurate species identification and taxonomic classification. Host plants for *C. taiwanalis* were reported for the first time during the research, adding to the ecological understanding of the species. The genetic analysis revealed significant divergences among East Asian populations, indicating potential cryptic species within the existing genus. The research highlighted the limitations of traditional morphological identification due to overlapping features and proposed DNA barcoding as a more reliable identification method. The resulting NJ tree from the barcoding data revealed eleven distinct clades of *Cotachena* based on the COI sequences of 23 individuals. These clades exhibited an average genetic distance of 10.025% from each other. The highest pair-wise distance was 12.97% between *C. fuscimarginalis* and *C. alysoni*. Intraspecific distances in the COI sequences ranged from 0% to 2%. As for the DNA Barcoding sequence data the DNA barcodes of seven Korean *Cotachena* samples were classified into three Barcode Index Numbers (BINs) as per Ratnasingham & Hebert (2013), aligning with three species: *C. alysoni*, *C. brunnealis*, and *C. taiwanalis*. Notably, no significant genetic divergence was detected between populations of *C. alysoni* from Ulleungdo Island and the Korean mainland. An unidentified *Cotachena* specimen displayed phenetic similarities to *C. taiwanalis* but showed a considerable genetic distance of approximately 3.87%, indicating the presence of two distinct lineages within the East Asian populations of *C. taiwanalis*. Additionally, the COI barcodes revealed around a 2% genetic difference between the Korean and Taiwanese populations of *C. brunnealis*. Similarly, another study by Rosfiansyah and colleagues in the same year on *Agrioglypta* Meyrick revealed the presence of a new species among the population of Japan. DNA sequences of the COI barcode region were successfully obtained for two specimens of *Agrioglypta fulguralis* sp. nov. (658 bp) and one specimen of *A. itysalis* from Tanegashima Island (417 bp). A phylogenetic tree, constructed using the maximum likelihood (ML) method and pairwise genetic distances, confirmed that *Agrioglypta fulguralis* sp. nov. is distinct from *A. itysalis* and other *Agrioglypta* species. The interspecific genetic distances between *A. fulguralis* sp. nov. and *A. itysalis* from various regions ranged from 4.1% to 6.8%, with the closest genetic distance found in *A. itysalis* from Yunnan, China, and the farthest in *A. itysalis* from Java, Indonesia. Conversely, *A. itysalis* from Tanegashima Island, Japan, showed

high genetic similarity (0% to 0.5%) to *A. itysalis* from Papua New Guinea (Milne Bay and Madang) and Australia (Northern Territory).

Another study by Vella and colleagues done in 2023 also demonstrates the resolutional abilities DNA Barcoding process and DNA barcodes possess in biodiversity estimation in the Maltese islands. This study represents the pioneering effort to establish a DNA barcode reference library for Lepidoptera species in Malta. Researchers generated a COI barcode dataset from 374 specimens, encompassing 146 species across 23 families. This dataset covers roughly 25% of the known Lepidoptera species in Malta, significantly enhancing the understanding of the region's lepidopteran diversity. The research uncovered unique haplotypes and genetic sequences, broadening the knowledge of species diversity and distribution in Malta. Among the notable findings were four newly recorded species for the Maltese islands: *Apatema baixerasi, Bostra dipectinialis, Oiketicoides lutea*, and *Phereoeca praecox*. These new records underscore the study's crucial role in documenting and understanding regional biodiversity. The study also addressed the challenge of close morphological similarities among some species, which had led to past misidentifications (Falck et al. 2021). By employing DNA barcoding, these misidentifications were corrected, confirming the presence of newly recorded species through both morphological and genetic analyses. This highlights the importance of incorporating molecular taxonomy into traditional taxonomic methods, as proven before by Misfud and colleagues for clearwing moth and beetle fauna of the islands (Mifsud et al. 2019, Misfud et al. 2021).

DNA Barcoding utilisation in the diversity assessment in India:

Gaikwad et al. (2012) conducted a comprehensive study on Nymphalid butterflies of the Western Ghats, India. They compared the genetic sequences of Western Ghats Nymphalids with those from other regions to assess intraspecific genetic divergence. The study analyzed 124 specimens from 40 species across 27 genera, with 28% of taxa (11 species) represented by single specimens. Fifteen new sequences were added to the DNA Barcoding Meta Data. The average intraspecific nucleotide divergence was 0.26%, ranging from 0-1%, except for Danaus chrysippus and Parantica aglea, which showed divergences of 1.2% (6 haplotypes) and 1% (7 haplotypes), respectively. Approximately 75% of species were resolved to the species level, and 80% (32 species) were discriminated. A clear barcoding gap between intra- and inter-species genetic divergence was observed. Specimens from geographically distant locations or separated by significant barriers showed monophyletic clustering in the N-J Tree. No cases of

misidentification were found, supporting the use of DNA barcoding for taxonomic identification in subtropical regions like India.

Kumar et al. (2019) conducted a study in Namdapha National Park, Eastern Himalaya, on Geometridae moths, generating 13 new barcodes for the BOLD library. Despite 1,558 geometrid moth species reported in India (Kirti et al., 2014), with 879 from the Pan-Indian Himalaya and 70 from Arunachal Pradesh (Sanyal et al., 2018), the absence of a precise DNA barcoding reference library poses challenges in resolving taxonomic uncertainties and biogeographic questions. The study involved generating DNA barcode data for Geometridae moths identified morphologically and analyzing genetic distances, using similarity search tools and Bayesian clustering. Among 44 specimens, 13 could not be classified to species level due to insufficient morphological information. Three were classified within the subfamily Ennominae, and ten were assigned to genera *Cleora, Racotis, Chiasmia,* and *Petelia*. The remaining 31 specimens were identified up to 20 species levels using both morpho-taxonomic and molecular methodologies. New barcode sequences for *C. propulsaria, D. lampasaria, H. lioptilaria, H. coastaria, R. inconclusa, L. erinoma, L. vigens, P. albidior, C. pseudonora, C. moorei, D. calamia, L. acutaria,* and *A. belluria* were added to BOLD from this study. The study highlighted cryptic diversity in *Pelagodes* and its closely related group, *Thalassodes*, due to external morphological variations. Re-evaluation based on male genitalia and the eighth abdominal sternite led to the creation of new genera, *Orothalassodes* and *Pelagodes*, and revealed a new record of *P. bellula* in North-Eastern India. Cleora showed 11.1% intra-generic divergence, with most sequences clustering together except *C. nestiotis*. One specimen, classified under Cleora, showed 6.3-7.1% genetic variance with *C. propulsaria*, indicating it might be another species. A specimen morphologically identified as *Petelia medardaria* exhibited a 1.2% genetic divergence in the database sequence, forming a single clade in the Bayesian tree. Another *Petelia* specimen showed a 2.4-3.4% genetic distance from *P. medardaria*, forming a separate clade, suggesting it may represent a distinct species. *Hypomecis* showed 10.3% intra-generic genetic divergence, with most species forming clear clusters in the Bayesian tree, except for *H. taeniota, H. suasaria, H. proschora, H. atactopa*, and *H. zaloschema*. *Racotis* showed a 6.8% intra-generic genetic divergence, with *R. inconclusa* and *Racotis* sp. exhibiting a 0.2% genetic divergence, suggesting they may be the same species. However, other *R. inconclusa* specimens formed a distinct clade with high intra-species genetic divergence (7.3-9.1%). *Lophophelma vigens* showed a 9.7% genetic divergence compared to *L. erinoma*. *Problepsis* had an 8.5% intra-generic genetic distance, with *P. albidior*

forming a separate clade, suggesting it is a sister species to *P. ocellata* with a 6.2% genetic divergence. The study also revealed genetic diversity and distribution information for *Xerodes*, *Chiasmia, Fascellina, Chorodona, Dalmia, Luxiaria, Antipercnia*, and others, indicating a wide distribution of Geometridae moths in Namdapha National Park. DNA barcoding results were consistent with morphological analysis, demonstrating the accuracy of DNA barcoding and its potential to streamline taxonomic identification efforts.

Recently, Kharwar et al (2024) published their work on one of the majorly biodiversity wise hidden *Theretra* from again one of the most biodiversity rich region of earth, the Western Ghats. In the northernmost part of the Western Ghats, seven species representing the genus *Theretra* were found: *T. alecto, T. castanea, T. clotho, T. gnoma, T. nessus, T. oldenlandiae*, and *T. sumatrensis*, the latter recently reported for the first time in this region. India has reported a total of 12 *Theretra* species, with barcode records available for nine species, while three species (*T. latreillii, T. lycetus,* and *T. pallicosta*) lack records. This barcode analysis included 195 sequences for all 12 Indian species of *Theretra*. Cluster analysis divided the 12 species into 18 Operational Taxonomic Units (OTUs). The sequences of T. *alecto, T. clotho, T. latreillii, T. nessus, T. silhentensis*, and *T. sumatrensis* were split into two OTUs each, while the remaining species each formed a single OTU. A clear barcoding gap was identified, as the maximum intraspecific distances were smaller than the distances to the nearest neighbour. The N-J tree shows tight clustering of individuals of the same species, except for *T. alecto*, *T. latreillii,* and *T. nessus,* demonstrating the applicability of DNA barcoding in resolving the taxonomic status of overlooked taxa. Most species clustered into a single OTU, while six species: *T. alecto, T. clotho, T. latreillii, T. nessus, T. silhentensis,* and *T. sumatrensis* were represented by two OTUs. These specimens have diverse geographic locations, suggesting differential selection pressures due to various geographic and ecological factors. According to Ratnasingham et al. (2007), deep divergence in a clade indicates recent speciation or overlooked taxa, warranting further taxonomic studies. The geographic distribution of the nearest specimen was compared within the OTUs, referring to the nearest specimens as 'Intra-Specific Nearest Specimen (ISNS)' to correlate ecological conditions. Since organisms thrive in specific ecological conditions, comparing the ISNS's ecological conditions can help explain differential OTUs. Similar observations were also reported by Kawahara et al. (2009), indicating a strong correlation between geographic distribution and phylogeny. However, two exceptions were noted: *T. alecto* and *T. nessus* did not follow geographically resolved clustering of the OTUs. This anomaly

might be due to erroneous sample collection data or incorrect base calling during sequence editing.

Biodiversity conservation significantly depends on the utilization of effective tools to characterize and monitor various elements of biological diversity, and often these conservation initiatives are hindered by a shortage of fundamental ecological data and the absence of efficient large-scale monitoring tools . To overcome the limitations of misidentification and inaccurate characterization of Lepidoptera species based solely on morphological traits, DNA barcoding has proven to be an exceptionally effective tool (Herbert 2003, Hajibabei et al 2006, Wilson et al 2013). The accessibility of BOLD data worldwide allows scientists from different regions to accurately verify whether the specimens they are studying belong to a specific species or represent a different morphospecies. While adding sequences from distant geographic regions may increase intraspecific genetic divergence, it has minimal impact on DNA barcoding accuracy based on N-J clustering analysis (Bae et al., 2023). Understanding the phylogeny and evolution of Lepidoptera is crucial for unravelling the mysteries of major climatic shifts, the emergence and extinction of moth and butterfly groups, and the evolution of traits like body size and sound detection. As potent ecological indicators, Lepidoptera offer insights into environmental changes and the impacts of both natural and anthropogenic activities (Choudhary and Chisty, 2020; Rakosy and Schmitt 2011; Gaona et al. 2021). They can serve as flagship species, whose conservation efforts can protect other endemic flora and fauna in biodiverse regions (New 1997). The phylogenetic study of Lepidoptera has traditionally been challenging and controversial (Kim et al. 2010). However, implementing DNA barcoding has proven to be a time-saving and effective method for species identification. Despite some drawbacks, the increasing number of BINs in the BOLD database is likely to mitigate these issues (Antil et al. 2023; Lopex-Vamonde et al. 2021). This necessitates extensive sampling of Lepidoptera populations worldwide. Furthermore, DNA barcoding can significantly aid in the conservation of economically valuable Lepidoptera (Nneji et al. 2020; Ashfaq 2017), such as the silk-producing insects within the Bombycoidea superfamily. Understanding their genetic diversity and distribution through barcoding can enhance conservation strategies and ensure the sustainability of these important species.

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