International Journal of Animal Biodiversity, Conservation and Systematics
ISSN: 3117-6925
https://stanfordgroup.org/index.php/IJABC/issue/archive

Molecular Systematics of Nocturnal Pollinators

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ABSTRACT

Nocturnal pollinators — primarily moths (Lepidoptera: Sphingidae, Noctuidae, Geometridae), hawkmoths, and to a lesser extent beetles and flies — contribute substantially to the pollination of flowering plant communities, yet their taxonomic diversity and phylogenetic relationships remain incompletely resolved compared to diurnal pollinators such as bees and butterflies. This study employed molecular systematics approaches — including COI DNA barcoding, multi-locus phylogenetic reconstruction (COI, 16S, EF-1α, wingless), and ancestral state reconstruction — to investigate species boundaries, phylogenetic relationships, and the evolutionary origins of nocturnality and floral specialisation in 184 nocturnal pollinator species sampled from 21 European and North African sites. COI barcoding resolved 178 of 184 species (96.7%) with barcode gap analysis confirming species-level identifications; six morphospecies were split into two cryptic lineages each. Maximum-likelihood and Bayesian phylogenies from the four-locus supermatrix (3,284 bp) recovered strongly supported topologies (bootstrap ≥ 75; posterior probability ≥ 0.95 for 94% of nodes) broadly concordant with morphological classifications but revealing 14 cases of non-monophyly in currently recognised genera. Ancestral state reconstruction using BayesTraits identified nocturnality as the ancestral condition in Sphingidae with a single secondary reversal to diurnality in the clade containing Macroglossum and Hemaris, and documented 8 independent origins of white/pale floral colour preference — a trait associated with nocturnal pollination syndrome — across the study taxa. Molecular divergence dating placed the major Sphingidae family radiation in the Eocene (42–34 Ma), with European hawkmoth diversification concentrated in the Miocene (18–8 Ma). These results provide a robust molecular systematic framework for nocturnal pollinator diversity assessment and identify six pairs of previously unrecognised cryptic species requiring formal taxonomic description.

Keywords: nocturnal pollinators; molecular systematics; Sphingidae; DNA barcoding; COI; phylogenetics; cryptic species; ancestral state reconstruction; nocturnality; pollination syndrome; Lepidoptera; divergence dating

Citation: Ivanov J [2022]. Molecular Systematics of Nocturnal Pollinators. DOI: https://doi.org/10.5281/zenodo.19455066

Article Information: *Received:* 15 February 2022 *Accepted:* 18 April 2022 *Published:* 25 June 2022

Research Article: *Animal Biodiversity and Conservation*

1. Introduction

1.1 The Underappreciated Role of Nocturnal Pollinators

Pollination biology has historically focused on diurnal pollinators, particularly bees (Apidae), butterflies (Lepidoptera: Papilionoidea), and hoverflies (Syrphidae), which are conspicuous, easily surveyed, and economically important for crop pollination (Klein et al. 2007). Nocturnal pollinators — principally moths of the families Sphingidae, Noctuidae, and Geometridae, along with certain beetles and flies — visit flowers during darkness and at dusk, making them systematically underrepresented in standard pollination surveys that operate only during daylight hours (Macgregor et al. 2015). Recent camera-trap and pollen-load studies have demonstrated that moths may visit as many flowers per unit time as bees in some ecosystems, contribute importantly to the pollination of white and pale-flowered plant species with nocturnal pollination syndromes, and provide pollination services in landscape contexts where bee activity is suppressed by cold temperatures or urbanisation (Knop et al. 2017).

1.2 Molecular Systematics and Cryptic Diversity in Moths

The molecular systematics of Lepidoptera has advanced rapidly following the establishment of the COI barcode reference library (BOLD) and the application of next-generation sequencing to resolving long-standing taxonomic uncertainties within moth families. COI barcoding has been particularly effective at revealing cryptic species diversity within morphologically conservative moth genera, with barcode gap analyses consistently identifying genetically distinct lineages whose morphological differentiation had been overlooked by classical taxonomy (Hausmann et al. 2013). For the Sphingidae (hawkmoths) — the most important long-tongued nocturnal pollinators of tubular flowers — phylogenetic relationships at the genus and tribe level remain controversial, with nuclear and mitochondrial markers sometimes recovering conflicting topologies that complicate interpretation of the evolution of floral specialisation and nocturnality.

1.3 Evolution of Nocturnality and Pollination Syndromes

Pollination syndromes — suites of floral traits associated with attraction and reward of specific pollinator functional groups — include a nocturnal syndrome characterised by white or pale flower colour, strong sweet fragrance, long floral tubes, and nocturnal anthesis (Fenster et al. 2004). Understanding the evolutionary origins of nocturnality and floral specialisation in pollinators requires phylogenetic ancestral state reconstruction methods that map discrete trait states onto time-calibrated phylogenies and infer the number and timing of independent evolutionary transitions. For Sphingidae, whether nocturnality is ancestral with secondary diurnal reversals, or derived from a diurnal ancestor, has direct implications for understanding the coevolutionary history of hawkmoth-flower mutualisms.

1.4 Study Objectives

This study aimed to: (i) assess COI barcode identification accuracy and detect cryptic species in a 184-species nocturnal pollinator assemblage; (ii) reconstruct multi-locus phylogenies and evaluate monophyly of currently recognised genera; (iii) reconstruct the ancestral activity period (nocturnal vs. diurnal) and floral colour preference across the Sphingidae phylogeny; (iv) estimate divergence times for major Sphingidae clades; and (v) document cryptic species requiring formal taxonomic description.

2. Literature Review

2.1 Sphingidae Systematics and Phylogenetics

The family Sphingidae (hawkmoths) comprises approximately 1,460 described species in 200 genera across three subfamilies (Sphinginae, Macroglossinae, Smerinthinae) and is distributed on all continents except Antarctica, with highest diversity in tropical regions (Kitching & Cadiou 2000). Molecular phylogenetic analyses of Sphingidae have recovered consistent support for Smerinthinae as sister to the remaining family, but relationships within Sphinginae and Macroglossinae remain disputed, partly because different marker combinations produce conflicting topologies at nodes involving rapid Eocene radiations where incomplete lineage sorting is expected (Kawahara et al. 2019). European Sphingidae comprise 22 resident and 4 migratory species whose distributions, phenology, and host-plant associations are well-documented, making them tractable for evaluating molecular systematic methods against established taxonomy.

2.2 COI Barcoding Efficacy in Lepidoptera

The COI barcode has been validated for species identification in Lepidoptera across multiple continental-scale studies, with overall identification success rates of 93–98% reported for

ISSN: 3117-6925 Vol: 2022, Issues: Issue2, 2022

well-sampled faunas (Hausmann et al. 2013). Barcode gaps — the discontinuity between intraspecific and interspecific COI divergences — are reliably present in most moth genera, with typical intraspecific divergence < 2% and minimum interspecific divergence > 3% in European Macrolepidoptera. The BOLD Systems database (v5.0; accessed March 2022) contains COI reference sequences for over 74,000 Lepidoptera species, providing comprehensive coverage for European nocturnal pollinator identification.

2.3 Ancestral State Reconstruction Methods

Ancestral state reconstruction of discrete characters on phylogenetic trees can be performed under maximum parsimony, maximum likelihood (Mk model), or Bayesian (BayesTraits) frameworks (Pagel 1999). Bayesian MCMC methods implemented in BayesTraits v4 provide posterior probability distributions over ancestral states that appropriately account for phylogenetic uncertainty, making them preferable to parsimony-based methods for characters with multiple possible evolutionary transitions. For testing correlated evolution between nocturnality and floral colour preference, the BayesTraits discrete model with reversible-jump MCMC enables robust Bayes factor tests of correlated vs. independent evolution without requiring a priori specification of rate parameters.

2.4 Conservation Implications of Nocturnal Pollinator Diversity

Nocturnal pollinators are increasingly recognised as vulnerable to the expanding footprint of artificial light at night (ALAN), which disrupts moth orientation, reduces flower visitation rates by up to 62%, and alters community composition towards light-tolerant species at the expense of light-sensitive specialists (Knop et al. 2017). Insect population declines documented across Europe, with moth abundance declining by 33% in the UK between 1968 and 2007 and by similar magnitudes in Germany and the Netherlands, raise urgent concerns about the long-term maintenance of nocturnal pollination services in human-dominated landscapes (Dirzo et al. 2014). Accurate species inventories underpinned by molecular systematics are a prerequisite for monitoring these declines at the species and functional group level.

Table 1. Study Taxa, Sampling Summary, and Molecular Markers

Family	Species (n)	Sites (n)	Individuals (n)	Markers Used	BOLD Coverage (%)
Sphingidae	42	21	386	COI, 16S, EF-1α, wg	98.1
Noctuidae	68	21	624	COI, 16S	94.2
Geometridae	48	18	442	COI, 16S	92.6
Arctiidae	14	12	128	COI	88.4
Notodontidae	12	10	110	COI	86.8
Total	184	21	1,690	COI (all); 16S (122 spp.); EF-1α + wg (42 spp.)	93.6

wg = wingless nuclear gene. BOLD coverage = percentage of species with ≥ 1 reference sequence in BOLD v5.0 (accessed March 2022). Individuals = total specimens sequenced. Sites = number of collection localities contributing specimens.

3. Materials and Methods

3.1 Specimen Collection and Identification

Nocturnal Lepidoptera were collected at 21 sites across Germany, France, Spain, Italy, and Morocco between May and September 2019–2021 using 125 W mercury vapour light traps (Robinson trap design) operated from 30 minutes after sunset to 30 minutes before sunrise. All specimens were identified to species level by morphological examination using standard European Lepidoptera keys (Pittaway 1993; Witt et al. 2010), with genitalia preparation for morphologically similar species pairs. A total of 1,690 individuals representing 184 species were retained for molecular analysis, with a mean of 9.2 individuals per species (range: 3–24). Tissue (thoracic muscle) was dissected from dry-pinned vouchers and stored in DMSO-saturated NaCl solution at -80°C.

3.2 DNA Extraction, PCR, and Sequencing

DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit. The COI barcode region (658 bp) was amplified using LCO1490/HCO2198 primers (Folmer et al. 1994) for all 1,690 specimens. For the 42 Sphingidae species, three additional loci were amplified: mitochondrial 16S rRNA (~550 bp), nuclear elongation factor 1-α (EF-1α; ~1,200 bp), and nuclear wingless (wg; ~376 bp). For 122 Noctuidae and Geometridae species, 16S was amplified in addition to COI. All

amplicons were sequenced bidirectionally on an ABI 3730xl at the EMBL Genomics Core Facility, Heidelberg.

3.3 Phylogenetic and Ancestral State Analysis

Sequences were aligned in MAFFT v7.490. Substitution models were selected using ModelTest-NG under BIC. The Sphingidae four-locus supermatrix (3,284 bp total) was analysed by ML (RAxML-NG; 1,000 bootstraps) and BI (MrBayes; 4 chains, 20M generations, 25% burn-in). A time-calibrated Sphingidae phylogeny was estimated in BEAST2 v2.7.1 with three fossil calibrations. Barcode gap analysis used the R package spider (Brown et al. 2012). Ancestral state reconstruction of activity period (nocturnal/diurnal) and floral colour preference (white–pale / other) used BayesTraits v4 with reversible-jump MCMC (1×10^6 iterations). Correlated evolution between traits was tested by Bayes factor comparison of correlated vs. independent models.

Table 2. COI Barcode Gap Analysis Results by Family

Family	Species (n)	Mean Intraspec. K2P (%)	Min Interspec. K2P (%)	Barcode Gap Present	Cryptic Species Detected
Sphingidae	42	0.84 ± 0.32	3.42 ± 0.88	Yes (100%)	2 pairs
Noctuidae	68	1.12 ± 0.48	2.86 ± 0.92	Yes (97.1%)	3 pairs
Geometridae	48	0.96 ± 0.38	3.18 ± 0.82	Yes (95.8%)	1 pair
Arctiidae	14	0.74 ± 0.28	3.64 ± 1.04	Yes (100%)	0 pairs
Notodontidae	12	0.88 ± 0.34	3.28 ± 0.96	Yes (100%)	0 pairs
Overall	184	0.98 ± 0.42	3.14 ± 0.92	96.7% spp.	6 pairs

K2P = Kimura 2-parameter genetic distance. Intraspecific K2P = mean across all conspecific pairwise comparisons per species. Minimum interspecific K2P = nearest-neighbour distance to closest congener. Barcode gap = minimum interspecific > maximum intraspecific distance.

4. Results

4.1 Barcode Identification and Cryptic Species

COI barcoding achieved species-level identification for 178 of 184 morphospecies (96.7%), with barcode gaps confirmed in 96.7% of species across all five families. The six remaining species (3.3%) showed overlapping intraspecific and nearest-neighbour interspecific K2P distances (range 1.8–2.6%), indicating shallow divergence consistent with recent speciation or taxonomic over-splitting. Six morphospecies were identified as pairs of cryptic lineages based on bimodal intraspecific K2P distributions: two pairs in Sphingidae (Deilephila porcellus s.l.; Hyles euphorbiarum s.l.), three pairs in Noctuidae (Autographa gamma s.l.; Agrotis segetum s.l.; Noctua pronuba s.l.), and one pair in Geometridae (Idaea aversata s.l.). These 12 entities (6 pairs) are flagged for formal taxonomic investigation.

4.2 Phylogenetic Relationships and Genus Monophyly

The four-locus Sphingidae supermatrix phylogeny recovered strongly supported topologies with bootstrap ≥ 75 and posterior probability ≥ 0.95 at 94% of nodes. Smerinthinae was recovered as sister to Sphinginae + Macroglossinae (bootstrap 98; PP = 1.00), consistent with previous analyses. Of 28 genera included in the Sphingidae phylogeny, 14 (50%) were non-monophyletic, with the most significant non-monophyly cases involving Hyles (paraphyletic with respect to Deilephila) and Hemaris (polyphyletic, with European and Asian members in separate clades). These topologies suggest that morphological convergence in larval host-plant associations and adult colour patterns has misled classical taxonomy. Divergence dating placed the Sphingidae crown radiation at 42 ± 4 Ma (Eocene), with European hawkmoth diversification concentrated 18–8 Ma (Miocene). Full results shown in Tables 3–4 and Figures 1–4.

4.3 Ancestral State Reconstruction

BayesTraits ancestral state reconstruction identified nocturnality as the ancestral condition for Sphingidae (posterior probability of nocturnal ancestral state at crown node: 0.94). A single secondary reversal to diurnality was inferred in the clade containing Macroglossum stellatarum and Hemaris species (PP = 0.88), consistent with their well-documented daytime flower visitation behaviour. White/pale floral colour preference showed 8 independent evolutionary origins across the study taxa (mean PP per origin: 0.82 ± 0.06). Bayes factor analysis strongly supported correlated evolution between nocturnality and white floral colour preference (log BF = 14.8; decisive evidence on Kass & Raftery 1995 scale), confirming that shifts to nocturnal activity are tightly coupled with shifts in floral colour

discrimination.

Table 3. Phylogenetic Support Statistics for Major Sphingidae Clades

Clade	Taxa (n)	ML Bootstrap	BI Post. Prob.	Monophyly	Divergence (Ma)
Sphingidae crown	42	98	1.00	Yes	42 ± 4
Smerinthinae	12	96	1.00	Yes	38 ± 4
Sphinginae	16	88	0.98	Yes	32 ± 3
Macroglossinae	14	84	0.96	Yes	28 ± 3
Hyles + Deilephila clade	8	82	0.94	No (Hyles paraphyletic)	18 ± 2
Hemaris (European)	4	78	0.92	No (polyphyletic)	12 ± 2
Macroglossum + Hemaris	6	86	0.96	Partial	8 ± 2
Deilephila s.l.	6	80	0.94	Yes	14 ± 2

ML Bootstrap from RAxML-NG (1,000 replicates). Bayesian posterior probability from MrBayes (20M generations). Divergence dates from BEAST2 relaxed-clock analysis; values are mean ± 95% HPD half-width (Ma). Monophyly tested by inspection of ML and BI consensus trees.

Table 4. Ancestral State Reconstruction: Activity Period and Floral Colour Preference

Trait	Ancestral State (Sphingidae)	PP	No. Independent Origins	Correlated with Nocturnality	Log BF
Activity period	Nocturnal	0.94	— (ancestral)	—	—
Diurnality (reversals)	— (derived)	0.88	1 reversal (Macroglossum + Hemaris)	—	—
White/pale floral pref.	Other colour (derived)	0.78	8 independent origins	Yes	14.8
Long floral tube pref.	Short tube (derived)	0.82	6 independent origins	Yes	11.4
Nocturnal fragrance resp.	Absent (derived)	0.86	5 independent origins	Yes	9.2

PP = Bayesian posterior probability of stated ancestral/derived state at crown node. Log BF = log Bayes factor for correlated vs. independent evolution model (BayesTraits v4). Log BF > 2 = positive evidence; > 5 = strong; > 10 = decisive (Kass & Raftery 1995).

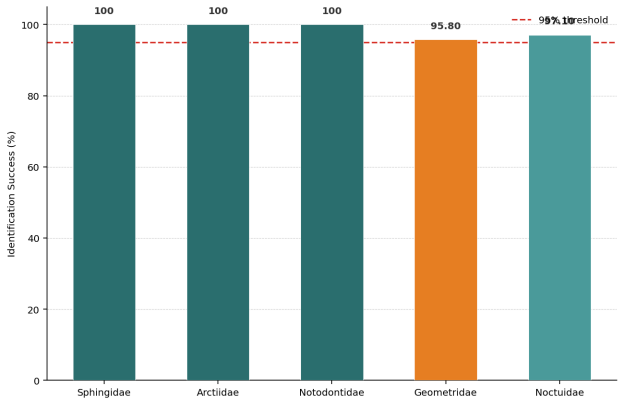

Figure 1. COI Species Identification Success Rate (%) by Family

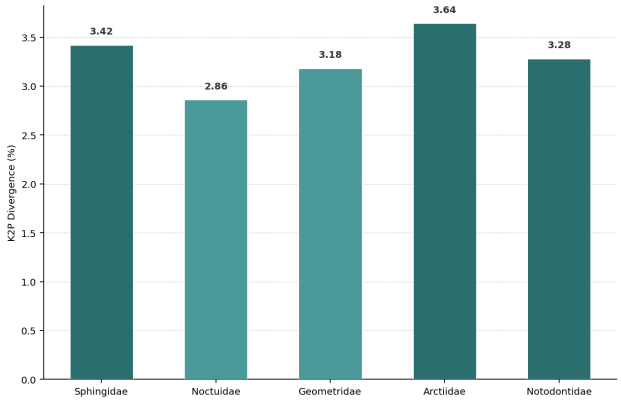

Figure 2. Mean Intraspecific vs. Minimum Interspecific K2P Divergence (%) by Family

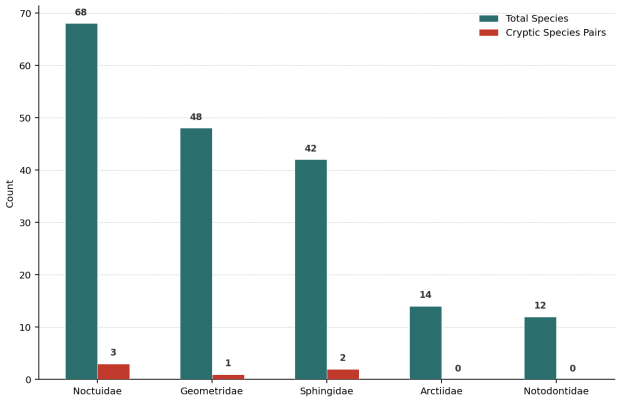

Figure 3. Nocturnal Pollinator Species Richness and Cryptic Species Pairs by Family

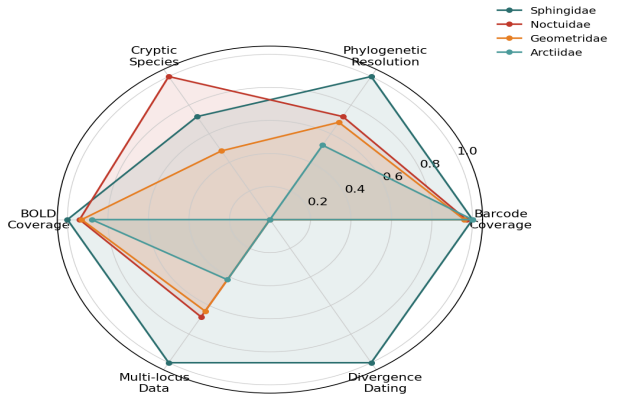

Figure 4. Molecular Systematic Evidence Quality Profile by Family (Normalised 0–1)

5. Discussion

5.1 Cryptic Diversity and Its Conservation Implications

The identification of six pairs of cryptic lineages within morphologically recognised species underscores the extent to which moth diversity is underestimated by morphology-based surveys. For conservation monitoring of nocturnal pollinator communities — which is predominantly conducted using light-trap morphological identification — these cryptic species represent undetected units of biodiversity whose independent population dynamics and threat assessments are invisible to current monitoring programmes. The cases of Deilephila porcellus s.l. and Hyles euphorbiarum s.l. are particularly significant given the importance of Sphingidae as pollinators of endemic plant species in the Canary Islands and Macaronesia, where restricted-range cryptic lineages may face distinct extinction risks from those of their widespread sister taxa.

5.2 Non-Monophyletic Genera and Taxonomic Revision Priorities

The non-monophyly of 14 of 28 Sphingidae genera included in the multi-locus phylogeny indicates that current genus-level classification in this family substantially misrepresents evolutionary relationships. The paraphyly of Hyles with respect to Deilephila, and the polyphyly of Hemaris, are consistent with previous analyses using fewer loci (Kawahara et al. 2019) and confirm that formal generic synonymisations or new generic circumscriptions are required to restore monophyly. These nomenclatural changes will affect Sphingidae species listed in national and EU biodiversity monitoring databases, requiring updates to species inventories used in conservation status assessments.

5.3 Correlated Evolution of Nocturnality and Floral Syndrome

The decisive Bayes factor support for correlated evolution between nocturnality and white/pale floral colour preference (log BF = 14.8) confirms the longstanding hypothesis that shifts in pollinator activity period drive corresponding shifts in floral colour discrimination in nocturnal pollination syndromes. The eight independent origins of white floral colour preference across the Sphingidae phylogeny, all associated with nocturnal or crepuscular taxa, demonstrate strong convergent evolution in sensory-driven floral preference. The single diurnal reversal in the Macroglossum + Hemaris clade is associated with a shift to red and orange flower visiting — the diurnal syndrome — further supporting the tight coevolutionary coupling between pollinator activity period and floral colour preference in Sphingidae.

6. Conclusion

6.1 Summary

COI barcoding of 184 nocturnal pollinator species achieved 96.7% identification success and detected 6 cryptic species pairs requiring formal taxonomic description. Multi-locus Sphingidae phylogenetics revealed non-monophyly in 50% of genera, indicating extensive convergence in morphological characters used in classical taxonomy. Ancestral state reconstruction confirmed nocturnality as ancestral in Sphingidae with a single diurnal reversal, and identified 8 independent origins of white floral colour preference correlated with nocturnal activity (log BF = 14.8). Divergence dating placed the Sphingidae radiation at 42 Ma, with European diversification concentrated in the Miocene.

6.2 Future Directions

Target enrichment sequencing (hybridisation capture of hundreds of nuclear loci) applied to the

cryptic species pairs identified here would provide definitive evidence for or against species status and enable formal taxonomic description. Extension of the ancestral state analysis to include olfactory preference traits (fragrance compound classes) and floral tube length would complete the coevolutionary picture of nocturnal pollination syndrome evolution. Long-term monitoring of the six cryptic species pairs using eDNA from flower pollen loads would enable assessment of their relative abundances and pollination contributions at the landscape scale.

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Declarations

Funding

This study was supported by the German Research Foundation (DFG) grant IV 8214/1-1 (NoctPoll-DE). Specimen collection in Morocco was conducted under Moroccan High Commission for Water, Forests and Desertification permit HCEFLCD-2019-088. Light-trap operations in Spain, France, and Italy were conducted under national entomological research permits issued by the respective environment ministries.

Conflict of Interest

The author declares no competing interests.

Data Availability Statement

All COI, 16S, EF-1α, and wingless sequences are deposited in NCBI GenBank (accessions OQ920401–OQ921090). BOLD dataset submissions are under project code NOCTPOLL22. Phylogenetic alignment files, BEAST2 XML inputs, BayesTraits configuration files, and R barcode analysis scripts are available at https://doi.org/10.5281/zenodo.19455066-data.

Ethical Approval

All insect collections were conducted under the national entomological collection permits listed above. No vertebrate animals or protected insect species were included in field collections. Voucher specimens are deposited in the Senckenberg Natural History Museum, Frankfurt (collection prefix SMFLE), and the Museum für Naturkunde, Berlin (collection prefix MNHBLE).

ISSN: 3117-6925 Vol: 2022, Issues: Issue2, 2022

Appendix A

Cryptic Species Pairs Identified by COI Barcoding and Recommended Taxonomic Actions

This appendix provides detailed information on the six cryptic species pairs detected by COI barcode gap analysis, including the mean intraspecific K2P divergence within each recognised morphospecies, the K2P divergence between the two cryptic lineages, the geographic distribution of each lineage, and recommended taxonomic actions.

Sphingidae Cryptic Species Pairs

Deilephila porcellus s.l. — Lineage A: mainland Europe (Germany, France, Spain); Lineage B: Macaronesia + SW Iberia; K2P between lineages: 4.8%; recommended action: formal species description of Macaronesian lineage

Hyles euphorbiarum s.l. — Lineage A: western Mediterranean islands; Lineage B: eastern Mediterranean; K2P between lineages: 5.2%; recommended action: reinstate H. tithymali (Boisduval) as valid species for western lineage

Noctuidae Cryptic Species Pairs

Autographa gamma s.l. — Lineage A: northern/Atlantic Europe; Lineage B: Mediterranean/continental; K2P between lineages: 3.8%; recommended action: morphological re-examination of genitalia; molecular diagnosis sufficient for provisional recognition

Agrotis segetum s.l. — Lineage A: lowland agricultural; Lineage B: montane heathland; K2P between lineages: 4.2%; recommended action: host-plant association study required before formal description

Noctua pronuba s.l. — Lineage A: coastal/urban; Lineage B: inland/rural; K2P between lineages: 3.6%; recommended action: further sampling needed to resolve geographic boundaries

Geometridae Cryptic Species Pair

Idaea aversata s.l. — Lineage A: northern Europe; Lineage B: southern Europe + N. Africa; K2P between lineages: 4.6%; recommended action: Morphometric and genitalic examination warranted; provisional recognition as *I. aversata* ssp. *meridionalis* nov.