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Integrative Taxonomic Characterization and first DNA Barcoding of *Chrysozephyrus syla* (Kollar, [1844]) and *C. birupa* (Moore, 1877) for Indian species (Lepidoptera: Lycaenidae)

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Abstract

Genus *Chrysozephyrus* Shirôzu & Yamamoto, 1956 (Tribe: Theclini) contains species with high morphological plasticity, often complicating accurate identification. This study provides an integrative taxonomic assessment of *C. syla* (Kollar, [1844]) and *C. birupa* (Moore, 1877) based on specimens from the Himachal Pradesh region of the Indian Himalayas. Significantly, this investigation provides the first formal description and illustration of the female genitalia of *C. birupa*, alongside the first comprehensive illustrations of both male and female genitalia for *C. syla*. To supplement these morphological findings, mitochondrial cytochrome c oxidase I (COI) sequences were generated, representing the first global DNA barcoding data for these species. Molecular analysis revealed a characteristic A + T bias (average 70.3%), consistent with Lepidoptera mitochondrial evolution. Preliminary phylogenetic reconstruction using Maximum Likelihood confirms the placement of these Indian taxa within the Theclini clade, showing low intergeneric genetic distances (5.0-6.5%) that align with recent genomic hypotheses suggesting potential synonymy within the *Hypaurotis* Scudder, 1876 group. These findings provide essential morphological and molecular baselines for rare high-altitude Lycaenidae and underscore the importance of integrating genital morphology with molecular tools for resolving the complex systematics of the tribe.
Keywords: Lepidoptera, Lycaenidae, Theclini, *Chrysozephyrus*, DNA Barcoding, COI, genitalia, Integrative Taxonomy, India.

Caracterización taxonómica integradora y primer código de barras de ADN de *Chrysozephyrus syla* (Kollar, [1844]) y *C. birupa* (Moore, 1877) para especies indias (Lepidoptera: Lycaenidae)

Resumen

El género *Chrysozephyrus* Shirôzu & Yamamoto, 1956 (tribu: Theclini) contiene especies con una gran plasticidad morfológica, lo que a menudo complica su identificación precisa. Este estudio ofrece una evaluación taxonómica integradora de *C. syla* (Kollar, [1844]) y *C. birupa* (Moore, 1877) basada en especímenes de la región de Himachal Pradesh, en el Himalaya indio. Cabe destacar que esta investigación ofrece la primera descripción formal e ilustración de la genitalia de la hembra de *C. birupa*, junto con las primeras ilustraciones completas de la genitalia del macho y de la hembra de *C. syla*. Para complementar estos hallazgos morfológicos, se generaron secuencias de citocromo c oxidasa I (COI) mitocondrial, lo que representa los primeros datos globales de códigos de barras de ADN para estas especies. El análisis molecular reveló un sesgo característico de A + T (70,3 % de media), en consonancia con la evolución mitocondrial de los Lepidoptera. La reconstrucción filogenética preliminar utilizando la máxima verosimilitud confirma la ubicación de estos taxones indios dentro del clado Theclini, mostrando bajas distancias genéticas

intergenéricas (5,0-6,5 %) que se alinean con las hipótesis genómicas recientes que sugieren una posible sinonimia dentro del grupo *Hypaurotis* Scudder, 1876. Estos hallazgos proporcionan bases morfológicas y moleculares esenciales para los Lycaenidae raros de gran altitud y subrayan la importancia de integrar la morfología genital con herramientas moleculares para resolver la compleja sistemática de la tribu.
Palabras clave: Lepidoptera, Lycaenidae, Theclini, *Chrysozephyrus*, código de barras de ADN, COI, genitalia, taxonomía integrativa, India.

Introduction

The ecological diversity and functional roles of Papilionoidea serve as crucial indicators of biodiversity change (Lomov et al. 2006). However, documenting this diversity is often hindered by identification challenges arising from complex life cycles, polymorphism, and regional phenotypic variations. The tribe Theclini Swainson, 1831, is predominantly distributed across East Asia, comprising nearly 200 species situated within the ecotonal regions of the Eastern Palaearctic and Northern Oriental realms (Fujioka, 1993; Koiwaya, 1999). In India, the tribe is represented by 33 species across 13 genera (Varshney & Smetacek, 2015). Among these, the genus *Chrysozephyrus* Shirôzu & Yamamoto, 1956, exhibits the highest species richness, with over 50 formally described taxa (Koiwaya, 2007).

Species within *Chrysozephyrus* show remarkable interspecific morphological similarity in external wing patterns, necessitating the examination of genital structures for accurate identification (Huang, 2021). Historically, Howarth (1957) utilized external forewing characteristics to categorize the *Chrysozephyrus birupa* group, including *C. syla* (Kollar, [1844]) and *C. birupa* (Moore, 1877). While both species share overlapping distributions across the Himalayas from Himachal Pradesh to Uttarakhand and exhibit similar metallic violet-green reflections in males, their internal diagnostic features remained under-documented, particularly for female taxa.

The taxonomic placement of these species has undergone significant revision. Varshney and Smetacek (2015) initially assigned *C. birupa* and *C. syla* to the genera *Shirozuozephyrus* Koiwaya, 2008 and *Inomataozephyrus* Koiwaya, 2007, respectively. These were later synonymized under *Chrysozephyrus* by Saito & Hasegawa (2016). More recently, Zhang et al. (2020) proposed a broader synonymization of several genera, including *Chrysozephyrus*, under *Hypaurotis* Scudder, 1876, based on genomic evidence. Additionally, Garlani (2024) documented the presence of these two species within Himachal Pradesh.

To address these taxonomic complexities, integrative approaches combining morpho taxonomy with DNA barcoding have become essential (Wahlberg et al. 2005; Prieto et al. 2018). The mitochondrial cytochrome oxidase I (COI) gene remains the standard molecular marker for species delimitation due to its conserved nature and efficacy in resolving taxonomically challenging insect groups (Hebert et al. 2003).

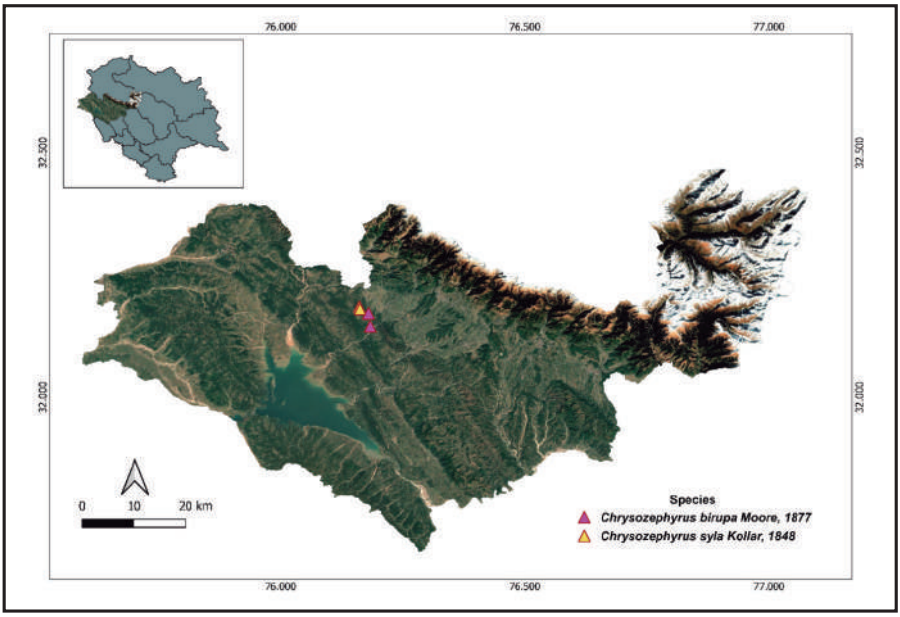
In the present study, we address existing knowledge gaps by providing the first formal description of the female genitalia of *C. birupa* and comprehensive genital illustrations for both sexes of *C. syla*. Furthermore, we present the first COI-based DNA barcodes for these species from the Indian Himalayas. By integrating detailed morphological dissections with preliminary molecular data, this study aims to clarify the diagnostic characteristics of these high-altitude Lycaenidae and contribute essential data to the ongoing systematic re-evaluation of the tribe Theclini.

Materials and Methods

Collection and Preservation: In the present study, four samples of the genus *Chrysozephyrus* were collected by sweeping net from various locations in Kangra, Himachal Pradesh (India), from 2022-2024. Wings were detached and placed in envelopes as vouchers. The bodies of the samples were placed in glass vials containing 100% ethanol. All ethanol-preserved samples were also stored at -20ºC until further use.

Morphological and Genitalia Examination: Specimen identification was conducted based on wing morphology and colouration patterns, following the methods of Evans (1932); Wynter-Blyth (1957), Cantile (1963) and Kehimkar (2016), supplemented by data from (http://www.ifoundbutterflies.org). Genitalia dissections were prepared via Robinson (1976) protocol for Microlepidoptera, with nomenclature standardized according to Klots (1970). Imaging and documentation were performed using a Leica image processing unit. Mapping was performed via QGIS software (Figure 1).

Figure 1. Map indicating the locations within Kangra district of Himachal Pradesh.



DNA Extraction and COI Amplification: Genomic DNA was extracted from ethanol-preserved samples, specifically from the legs and abdominal musculature. Extraction was performed via Qiagen DNA extraction kit. Agarose gel electrophoresis was used to check the integrity of the extracted DNA. For COI gene amplification, universal forward and reverse primers (Folmer et al. 1994) were used (LCO-1490-5'GGTCAACAAATCATAAAGATATTGG3' and HCO-2198- 5'TAAACTTCAGGGTGACCAAAAAAT-CA3'). Amplification was performed in a total volume of 28 µl, containing 8 µl of PCR water, 14 µl of Master Mix, 1.5 µl of each primer (LCO-1490 and HCO-2198), 1 µl of BSA and 2 µl of DNA. The thermal cycling program of the PCR procedure was as follows: 1 cycle for initial denaturation at 95ºC for 5 minutes; 35 cycles for denaturation at 95ºC for 1 minute, annealing at 45ºC for 1 minute, and extension at 72ºC for 1 minute; and 1 cycle for a final extension at 72ºC for 5 minutes. All the PCR products were visualized via 1% agarose gel electrophoresis with ethidium bromide (EtBr) staining under UV light via a gel documentation system to confirm successful DNA amplification. The amplification of the COI gene was performed. Amplified products were sent to the Research Lab Biology, New Delhi, for sequencing via the Sanger dideoxy sequencing method. The obtained sequences were approximately 600-680 bp in length and were submitted to the NCBI database, and accession numbers were obtained. The NCBI BLAST search engine was employed to identify conspecific sequences by analysing genetic similarities. Four COI (cytochrome oxidase I) gene sequences, representing two species, were submitted to the NCBI GenBank database. Unique accession numbers were assigned to these sequences, making them accessible for future research and comparative analysis.

Sequence alignment, accession numbers, genetic distance analysis and phylogenetic analysis: Corresponding COI sequences of conspecific samples deposited by other workers were downloaded from GenBank via a BLAST search. The raw sequences were aligned, edited and trimmed manually via ClustalW in MEGA XII software. Interspecific genetic distances and conserved, variable and parsimony informative sites of the COI gene fragments of all the species were recovered via MEGA XII. A phylogenetic tree was constructed via the maximum likelihood method and the Tamura (1992) model for all the species and *Thecla betulae* (Linnaeus, 1758) of the subfamily Theclinae designated as the outgroup (Table 1)**.**

Table 1. Accession numbers, voucher numbers, sequence lengths, localities and references of the species used in the phylogenetic analysis.

Reference	Species	Sequence Length (bp)	Voucher Number	Accession number	Locality
Odagiri, 2004	*Chrysozephyrus smaragdinus* Shirôzu & Yamamoto, 1956	507	98ZE32	AB195511	Japan
Odagiri, 2004	*Favonius cognata* (Staudinger, 1892)	507	98ZE45	AB195541	Japan
Odagiri, 2004	*Favonius jezoensis* (Matsumura, 1915)	507	98ZE05	AB195538	Japan
Odagiri, 2004	*Favonius korshunovi* Dubatolov & Sergeev, 1982	579	98ZE39	AB195536	Japan
Odagiri, 2004	*Favonius leechina* Lamas, 2008	579	98ZE51	AB195531	Japan
Odagiri, 2004	*Favonius orientalis* (Murray, 1875)	579	98ZE13	AB195530	Japan
Odagiri, 2004	*Favonius saphirina* (Staudinger, 1887)	579	98ZE40	AB195522	Japan
Odagiri, 2004	*Favonius taxila* (Bremer, 1861)	579	98ZE03	AB195526	Japan
Odagiri, 2004	*Favonius ultramarine* (Fixsen, 1887)	579	98ZE18	AB195544	Japan
Odagiri, 2004	*Favonius yuasai* Shirôzu, 1948	579	98ZE36	AB195534	Japan
Odagiri, 2004	*Hypaurotis crysalus* (Edwards, 1873)	507	98ZE31	AB195512	Japan
Odagiri, 2004	*Neozephyrus japonica* (Murray, 1875)	507	97ZE20	AB195513	Japan
Odagiri, 2004	*Sibataniozephyrus fujisanus* (Matsumura, 1910)	507	98ZE33	AB195514	Japan
Kim et al. 2010	*Neozephyrus koreanus* (Riley, 1939)	603	NA	GU372574	Korea
Hausmann et al. 2011	*Quercusia quercus* (Linnaeus, 1758)	579	BC ZSM Lep 30684	HM393195	Germany
Dinca et al. 2021	*Hypaurotis quercus* (Linnaeus, 1758)	579	RVcoll1 5F391	MW503561	Portugal
Nakabayashi & Ohshima, 2024	*Hypaurotis orientalis* (Murray, 1875)	507	YT-048	OP443378	Japan
Lukhtanov et al. 2009	*Thecla betulae* (Linnaeus, 1758)	534	2005-LOWA-736	FJ664048	Russia
Current study	*Chrysozephyrus syla* (Kollar, [1844])	603	DT46	PQ850024	India
Current study	*Chrysozephyrus syla* (Kollar, [1844])	603	DT43	PQ850022	India
Current study	*Chrysozephyrus birupa* (Moore, 1877)	603	DT12	PQ849159	India
Current study	*Chrysozephyrus birupa* (Moore, 1877)	603	DT33	PQ677695	India

Results and Discussion

Taxonomic Descriptions

Genus *Chrysozephyrus* Shirozu & Yamamoto, 1956
Chrysozephyrus Shirôzu & Yamamoto, 1956; *Sieboldia, 1*(4), 381
Hypaurotis; Zhang, Cong, Shen, Opler & Grishin, 2020, *Taxonomic Rep., 8*(7), 13
Type species: *Thecla smaragdina* Bremer, 1861

Diagnostic characters: Eyes hairy, labial palpi projected forward, projecting beyond head, with a hairy second segment; forewing with 11 veins; R_3+R_5 stalked, M_1 arising freely beyond the end of the cell; male genitalia with uncus bifid, short and flattened; tegumen with prominent lateral processes, brachia serrate at base, smoothly curved medially, distally straight, vesica furnished with multiple cornute; female genitalia with prominent genital plate, eye-shaped signa.

Key to species of the genus *Chrysozephyrus* Shirozu & Yamamoto, 1956

1. Forewing dorsally in male with border thicker at the apex; ventrally brownish white; male genitalia with base strongly dentate and spinose; saccus broader and smaller; aedeagus shorter, broader; female genitalia with ductus bursae shorter, semi-sclerotized tube……. *birupa* Moore
- Forewing dorsally in male with uniform border; ventrally silvery-white; male genitalia with the base of the brachia not dentate; saccus exceptionally long; aedeagus longer, narrower; female genitalia with the ductus bursae narrow, elongated, membranous and tubular…….. *syla* Kollar

Chrysozephyrus birupa (Moore, 1877) (Figure 2: (A-G))
Moore, 1877, *Ann. Mag. nat. Hist.* (4)*20*(115), 51 (*Dispas*); Howarth, 1957, *Bull. Br. Mus. nat. Hist., 5*(6), 266 (*Neozephyrus*); Shirôzu, 1962, *Tyô to Ga, 12*(4), 147 (*Chrysozephyrus*); Zhang, Cong, Shen, Opler and Grishin, 2020, *Taxonomic Rep., 8*(7), 13 (*Hypaurotis*).

Material examined: 1 ♀, 19-V-22, Dharamkot, Kangra; 1 ♂, 18-VI-24, Kareri Village, Kangra, Himachal Pradesh.

Adult (Male): Forewing dorsally metallic green, with black scales interspersed, bordered by a smooth, continuous black margin, ventrally brownish white, with an end-cell spot, a submarginal band, a discal band extending from the costa; hindwing crenulate, metallic green dorsally with powdery black scales bordered black, tailed ventrally brownish white, an obscure terminal cell spot, an elongated discal band, an indistinct submarginal band, a tornal region with black spot, crowned orange, with an additional black spot, encircled orange, all markings brown, white edged.

Adult (Female): Forewing dorsally dark brown, with two bluish-white subapical spots and slight blue suffusion at the base, otherwise like male.

Male genitalia: Uncus bilobed, each lobe filamentous, densely pilose; elongated, basal region produced into a strongly dentate, elongated elbow-like structure, with abundant spines, curved arms, tegumen very broad, lateral windows deeply clefted; lower half with narrow vinculum, saccus elongated; valvae rectangular, costa narrow, harpe with rounded apex, ampulla extends into an elongated finger-like process, pilose; juxta V shaped, arms broad; aedeagus long, subzone larger than suprazonal portion, bulbus ejaculatorius entering dorsally, rounded coecum, vesica with numerous spines.

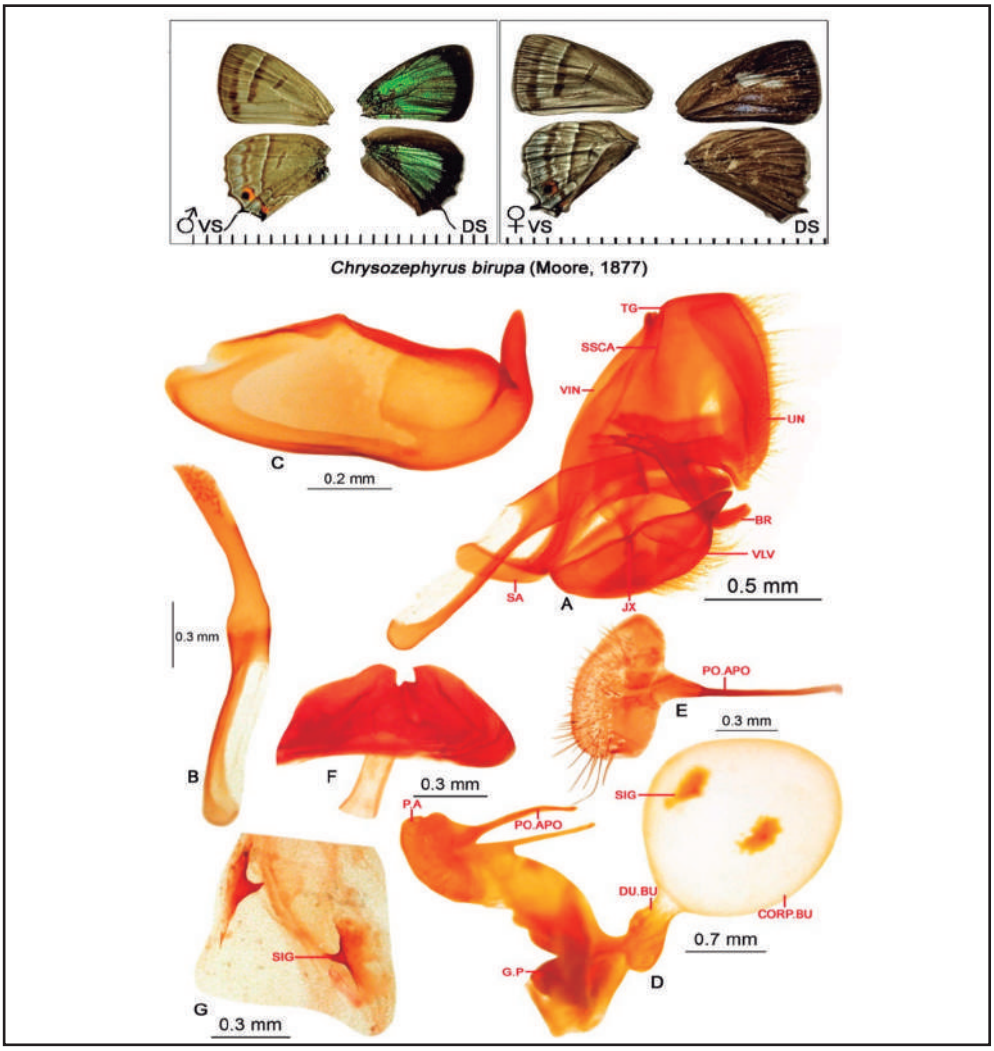
Female genitalia: Lodix rounded; genital plate broadly sclerotized and bilaterally symmetrical; ductus bursae small, semi-sclerotized tube, connects ostium bursae to the corpus bursae; corpus bursae rounded, membranous with pair of well-developed signae; signae sclerotized and pointed, apophyses anterioris sharp; posterior apophyses short and broad near base; papilla analis oval, pilose.

Wing expanse: Half: Male: 19.0 mm, Female: 19.5 mm. Range: 1400-2700 m ASL.

Old distribution: India: Shimla to Kumaon, Kangra, Mussoorie, Doon Valley. Other Countries: East to Central Nepal. Larval host plant: *Rhododendron arboreum* Sm.

Remarks: During the survey period (2022-2024), *C. birupa* was recorded at altitudes ranging from 1900-2100 m ASL. Specific collection sites included the vicinity of Dal Lake and Kareri village (Kangra district), where specimens were observed in riparian zones and temperate forested habitats. The ventral wing surfaces exhibit silver-brown cryptic maculation; similar phenotypes in Theclini have been noted for their resemblance to dried leaf litter (Howarth, 1957). Adults demonstrated high vagility and rapid escape flight when disturbed, a behavioural trait common to high-altitude Theclinae. The spatial association of adults with *Rhododendron arboreum* suggests a potential host-plant relationship or specific habitat correlation, although further larval rearing is required for confirmation. This investigation provides the first formal documentation and illustration of the female genitalia for this species, significantly expanding the known morphological record for the *C. birupa* group in the Indian Himalayas.

Figure 2. A. Male genitalia (lateral view). **B.** Aedeagus (lateral view). **C.** Valva (inner view). **D.** Female genitalia (lateral view). **E.** Papilla analis. **F.** Genital plate. **G.** Signum.



Chrysozephyrus syla (Kollar, [1844]) (Figure 3: (A-G))
Kollar, [1844] in Hügel, *Kaschmir und das Reich der Siek, 4*, 414 (*Thecla*); Moore, [1858] in Horsfield & Moore, *Cat. lep. Ins. Mus. East India Coy, 1*, 30 (*Dispas*); Howarth, 1957, *Bull. Br. Mus. nat. Hist., 5*(6), 267, (*Neozephyrus*); Shirôzu, 1962, *Tyô to Ga, 12*(4), 148 (*Chrysozephyrus*); Zhang, Cong, Shen, Opler and Grishin, 2020, *Taxonomic Rep., 8*(7), 13 (*Hypaurotis*).

Material examined: 1 ♀, 1 ♂, 18-VI-24, Kareri Trek, Kangra, Himachal Pradesh.

Adult (Male): Forewing dorsally metallic green iridescence, with a narrow black margin, ventrally silvery white with an end-cell spot, a brown curved discal band from the costa to the inner margin, a faint

submarginal band, white bordered outwardly; hindwing dorsally metallic green, broad black border, tailed, ventrally a postdiscal band, bordered white outwardly, a distinct tornus adorned with prominent tornal spots in black, bordered by orange.

Adult (Female): Forewing dorsally iridescent violet-bluish with white spot at the cell end, black dark border, apically broad; otherwise, like males.

Male genitalia: Uncus bilobed with each lobe long, pilose; brachia long, with broad base, arms slender, curved; tegumen broad, lateral window deeply cleft, viniculum broad in upper half, terminates into exceptionally long tubular saccus; valva large, triangular, well divided into ampulla and harpe, ampulla broad at base produced into an elongated narrow curved apical process, pilose; harpe with elongated curved apex, pilose; juxta V-shaped; exceptionally long narrow, slightly curved aedeagus, suprazonal portion much longer than subzonal; bulbus ejaculatorius enters dorsally, coecum small rounded; vesica with numerous, minute cornuti.

Female genitalia: Lodix rectangular; genital plate broad, flattened, triangular in shape with sclerotized edges; ductus bursae narrow, elongated and tubular, dilated near base; corpus bursae rounded, bulbous and membranous with two distinctly large, pointed, spine-like well sclerotized signae; anterior apophysis sharp; posterior apophysis long, broad and curved near base; papilla analis subovate, weakly sclerotized, pilose.

Wing expanse: Half: Male: 18.0-21.5 mm, Female: 18.5-21.0 mm. Range: 1800-2700 m ASL.

Old distribution: India: Northwest India to Sikkim and Manipur, Jammu and Kashmir to Kumaon (including Safed Koh, Chitral-Kumaon). Other Countries: Afghanistan, Pakistan, Nepal, Bhutan.

Remarks: Specimens of *C. syla* were recorded at an elevation of approximately 2450 m ASL during surveys along the Kareri Lake trek (altitudinal range: 2100-2934 m). The species was observed in close spatial association with oak-dominated forests (*Quercus* spp.), which is consistent with the known ecological niche and host-plant associations for this genus in the Western Himalayas (Wynter-Blyth, 1957). The ventral wing surfaces exhibit cryptic coloration; a morphological trait frequently associated with predator-avoidance in sub-canopy environments. Field observations indicated a bimodal diel activity pattern, with peak flight occurring during the early morning and late afternoon. This temporal partitioning may be related to environmental thermoregulation, as activity was significantly reduced during periods of high solar radiation at midday. Notably, the male and female genitalia of this species are described and illustrated here for the first time, providing a critical morphological baseline for Indian populations. In accordance with national conservation priorities, this species is legally protected under Schedule II of the Wildlife (Protection) Act, 1972, as amended in 2022.

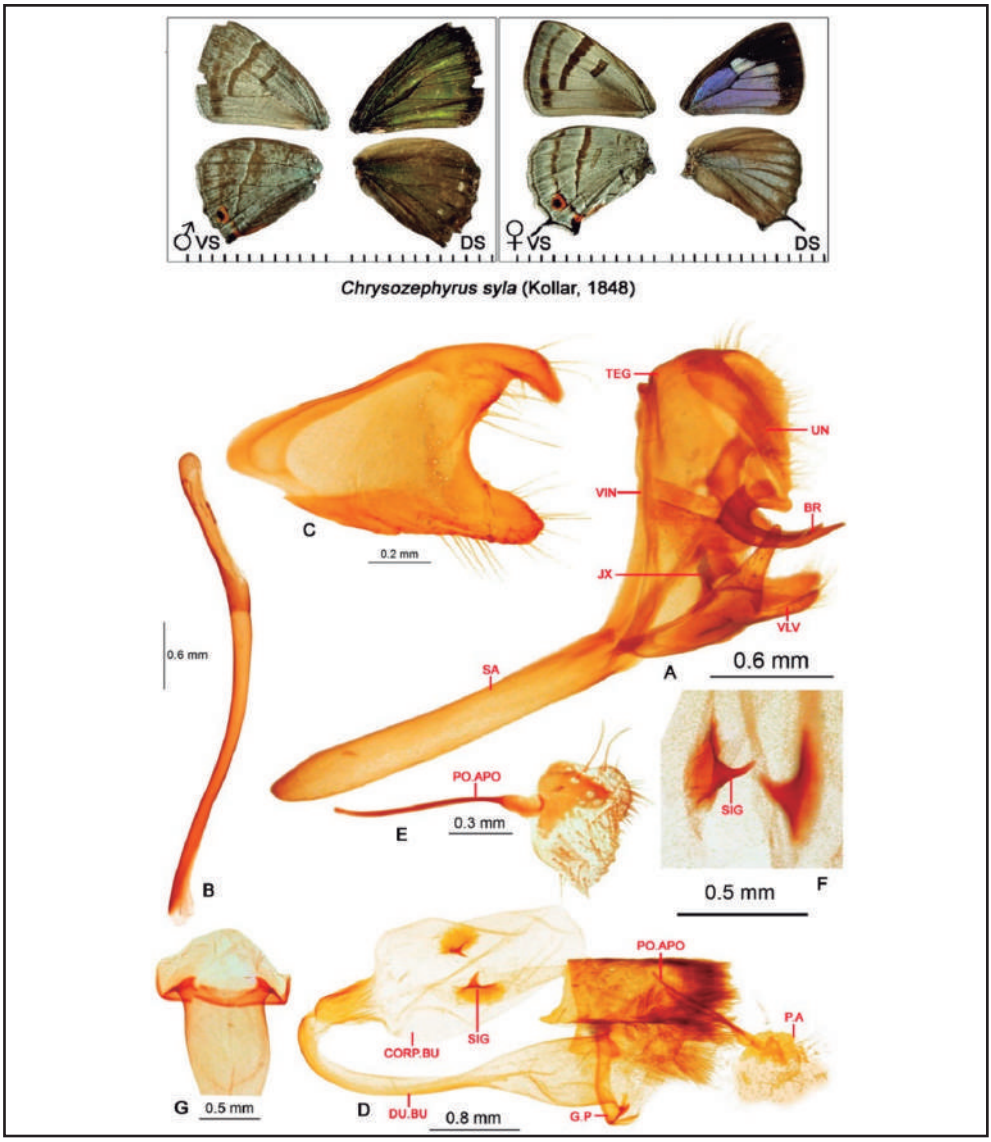
Phylogenetic Insights

DNA Barcoding: In the present study, sequences representing two species of the genus *Chrysozephyrus* (*C. syla* and *C. birupa*) were successfully barcoded and deposited in the GenBank database. This is the first global molecular barcoding effort for these species within the subfamily Theclinae, providing a significant contribution to the molecular repository and advancing phylogenetic and taxonomic studies in this group.

Nucleotide Composition, Conserved, Variable, Parsimony Informative Sites and Singleton Sites*:* Across the final alignment of COI sequences of 603 bp, there are 475 conserved sites, 128 variable sites, 71 parsimony informative sites and 57 singleton sites in the dataset, which confirms that the COI gene is highly conserved. Figure 4 shows the nucleotide composition percentages for all the species. The average nucleotide frequencies are 31.37% (A), 38.92% (T), 15.09% (C) and 14.62% (G) (Figure 4). The nucleotide composition across codon positions in insects follows a distinct and consistent pattern, with a strong A + T bias, particularly in the first and third codon positions, whereas the second codon position has a more balanced composition. Yang et al. (2021) reported that in the mitochondrial genomes of Tortricidae, adenine (A) is the most frequent nucleotide at the first codon position, followed by thymine (T), guanine (G), and cytosine (C). This aligns with the observed values (A-1: 34.3 > T-1: 30.3 > G-1: 24.2 > C-1: 11.2), suggesting that the first codon position is relatively conserved, with a preference for purines (A, G) over pyrimidines (T, C).

At the second codon position, thymine (T) is dominant (44.4%), followed by cytosine (C) (26.4%), adenine (A) (15.2%), and guanine (G) (14.0%), which is consistent with the findings of Kokate et al. (2021) in *Drosophila* Fallén, 1823. The second codon position plays a crucial role in determining amino acid properties, as it directly affects protein function, which may explain the greater presence of T and C, as these bases are often associated with hydrophobic or structurally stable amino acids.

Figure 3. A. Male genitalia (lateral view), B. Aedeagus (lateral view), C. Valva (inner view), D. Female genitalia (lateral view), E. Papilla analis, F. Signum G. Genital plate



The third codon position exhibited the greatest variability, with adenine (50.6%) and thymine (41.6%) being dominant, whereas cytosine (7.3%) and guanine (0.6%) were significantly underrepresented. This finding supports the findings of Näsvall et al. (2023), who highlighted a strong G/C to A/T substitution bias in butterflies. This suggests that natural selection and mutational pressures favour A + T richness at the third codon position, which is known to be the most synonymous and variable site in codon evolution. These findings indicate that the A + T content is highest in the third codon position, followed by the first, whereas the second position is relatively balanced. This pattern is consistent across different insect taxa, reflecting the influence of mutation pressure, translational efficiency, and natural selection in shaping codon usage bias (Figure 5).

Figure 4. Bar graph showing the nucleotide compositions of all the species in percentage.

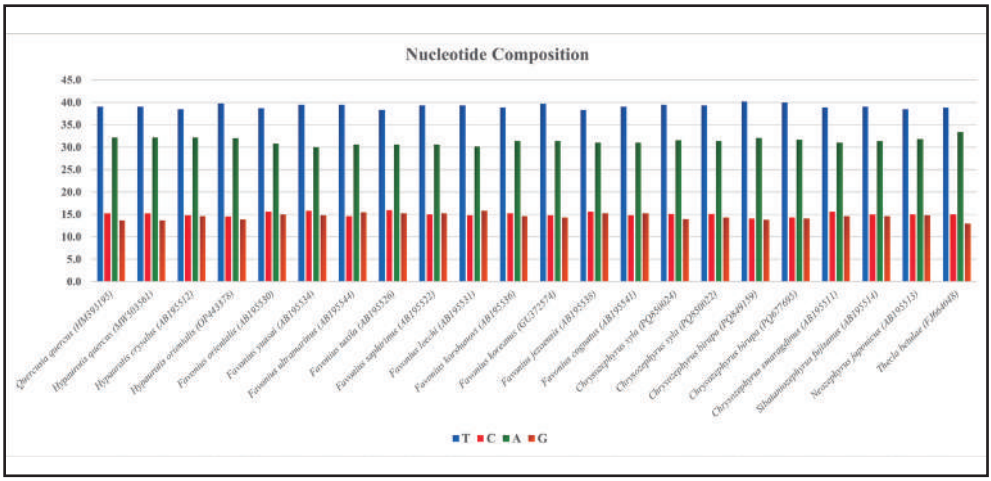
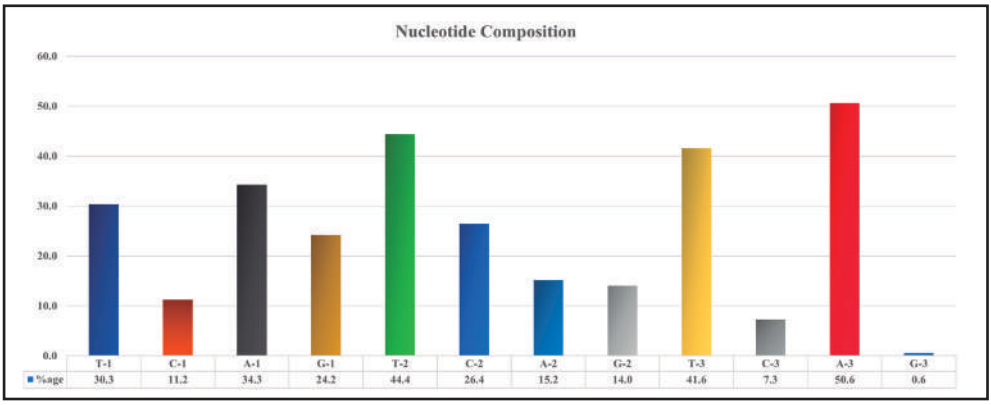


Figure 5. Nucleotide composition at the 1st, 2nd, and 3rd codon positions.



The transition/transversion rate ratios are $k_1 = 2.49$ (purines) and $k_2 = 7.098$ (pyrimidines). The overall transition/transversion bias is $R = 2.138$, where $R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]$. The analytical procedure encompassed 22 coding nucleotide sequences at the 1st, 2nd, 3rd, and noncoding positions. The pairwise deletion option was applied to all ambiguous positions for each sequence pair, resulting in a final dataset comprising 603 positions. Evolutionary analyses were conducted in MEGA12 (Table 2).

Table 2. Each entry shows the probability of substitution (r) from one base (row) to another base (column). For simplicity, the sum of the r values is equal to 100. The rates of different transitional substitutions are shown in **bold**, and those of transversionsal substitutions are shown in *italics*.

	A	T	C	G
A	-	*5.58*	*2.16*	**5.22**
T	*4.5*	-	**15.35**	*2.09*
C	*4.5*	**39.58**	-	*2.09*
G	**11.19**	*5.58*	*2.16*	-

Genetic distance (Table 3): The interspecific divergence ranged from 0.2% to 7.9%, excluding the outgroup. The low interspecific genetic divergence (0.2-0.8%) observed among *Favonius cognatus* (Staudinger, 1892)*, F. koreanus* (Riley, 1939), and *F. ultramarinus* (Fixsen, 1887) suggests recent evolutionary divergence, potentially influenced by ongoing gene flow or shared ecological adaptations. This finding is consistent with that of Lavina et al. (2017), who reported that some Papilionoidea species exhibit minimal interspecific divergence, sometimes below 1%, indicating recent speciation events or incomplete lineage sorting. Similarly, Dinca et al. (2021) reported low genetic distances among European butterflies, reinforcing the notion that closely related species may retain genetic similarities despite being taxonomically distinct. These parallels suggest that while greater genetic divergence is typical among species, low divergence values, as observed in *Favonius*, could reflect either recent divergence or the need for taxonomic re-evaluation. Therefore, comprehensive morphological, ecological, and genomic investigations are crucial to better understand their evolutionary relationships and validate their species status. The high genetic divergence (7.9%) between *Chrysozephyrus birupa* and *Favonius yuasai* Shirôzu, 1948 is due to the primarily to long-term evolutionary separation, geographic isolation, distinct ecological adaptations and the accumulation of genetic differences over time. *C. birupa* shows greater divergence from *C. smaragdinus* (4.5%), indicating that it is the most genetically distinct within this group. *C. syla* is closer to *C. smaragdinus* (3.7%), suggesting that they may share more recent common ancestry. *C. syla* and *C. birupa* also significantly diverged (4.3-4.8%).

The intergeneric distance values among *Hypaurotis* Scudder*,* 1876, *Favonius* Sibatani & Ito, 1942*, Chrysozephyrus* Shirôzu & Yamamoto, 1956*, Sibataniozephyrus* Inomata, 1986 and *Neozephyrus* Sibatani & Ito, 1942 range from 5.0-6.5, with the minimum distance of 5.0 observed between *Hypaurotis* and *Favonius*, suggesting a close evolutionary relationship, whereas the maximum distance of 6.5 between *Favonius* and *Chrysozephyrus* indicates greater divergence. These distances, although relatively low, suggest a close genetic and evolutionary affinity among these genera. Wiemers & Fiedler (2007) reported that the mean congeneric interspecific sequence divergence in Lycaenidae was 5.1% for *Agrodiaetus* Hübner, [1822] and 5.0% for other genera. Hebert et al. (2003) established an intergeneric divergence threshold of 10-16% in Lepidoptera, whereas Ratnasingham & Hebert (2013) reported that insect genera rarely exhibit genetic divergence below 10%. Given these findings, the observed distances in this analysis fall well below the expected intergeneric divergence range, reinforcing the hypothesis that these genera may not be evolutionarily distinct at the genus level. This aligns with the findings of Zhang et al. (2020), who suggested that, on the basis of COI barcode analysis, *Sibataniozephyrus, Neozephyrus, Chrysozephyrus,* and *Favonius* should be considered junior subjective synonyms of *Hypaurotis* rather than distinct genera. This taxonomic interpretation is further supported by the morphological similarities among these species, as noted by Eliot (1973). The low intergeneric distances observed in the present study provide additional evidence supporting the synonymization of these taxa, suggesting that they represent a single evolutionary lineage rather than separate genera (Table 4).

Table 4. Intergeneric distance percentages.

Genera	*Hypaurotis*	*Favonius*	*Chrysozephyrus*	*Sibataniozephyrus*	*Neozephyrus*
Hypaurotis					
Favonius	5.0				
Chrysozephyrus	5.3	6.5			
Sibataniozephyrus	5.9	6.2	5.9		
Neozephyrus	5.7	5.3	5.9	5.8	

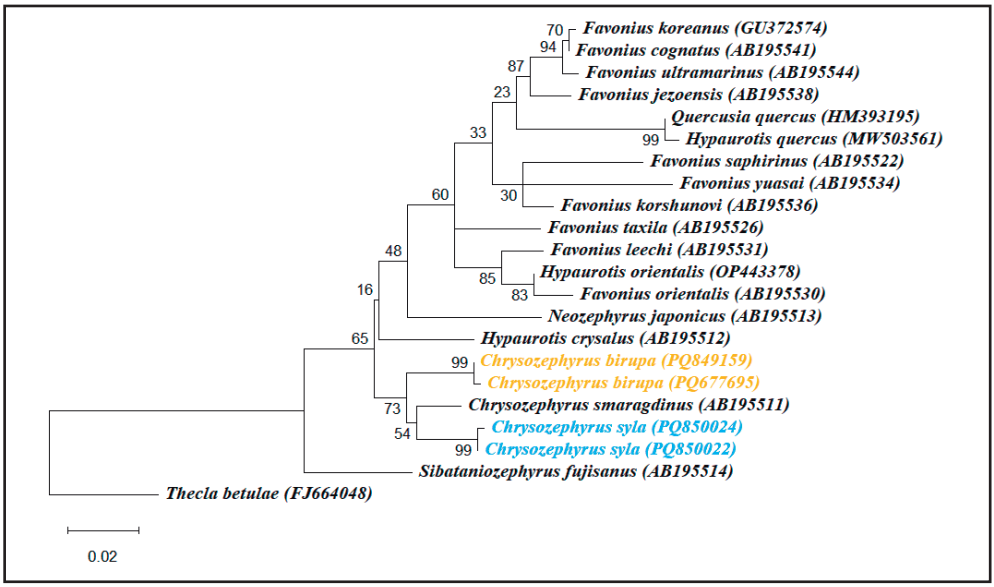
Thecla betulae, used as an outgroup, exhibited genetic divergence ranging from 7.7% to 10.5% among the analysed species, with the lowest divergence (7.7%) observed with *Hypaurotis crysalus* (Edwards, 1873), whereas the highest (10.5%) was recorded with *Favonius yuasai*. In the present study, the genetic divergence rate between *Hypaurotis crysalus* and *Favonius orientalis* was found to be 6%, whereas the divergence rate between *H. crysalus* and *Thecla betulae* was 7.7%. These findings align with those of Zhang et al. (2020), who reported a 6% divergence between *H. crysalus* and *F. orientalis*, indicating a relatively close genetic relationship, whereas

the divergence between *H. crysalus* and *T. betulae* was slightly greater (8.2%). The minor discrepancy in the *H. crysalus*-*T. betulae* divergence values between the two studies (7.7% vs. 8.2%) may result from differences in sample populations, sequencing methodologies, or alignment techniques.

Phylogenetic analysis: A phylogenetic tree was reconstructed on the basis of the mitochondrial gene COI gene sequences of 19 species along with the outgroup (*Thecla betulae*). Tree was generated via the maximum likelihood method and the Tamura (1992) model (Figure 6). Five major clades ((*Favonius koreanus* + *Favonius cognatus* + *Favonius ultramarinus*) + *Favonius jezoensis* + (*Quercusia quercus* +*Hypaurotis quercus*) + (*Favonius saphirinus* + *Favonius yuasai* +*Favonius korshunovi*) + *Favonius taxila* + (*Favonius leechi*, + *Hypaurotis orientalis* + *Favonius orientalis*)) + (*Neozephyrus japonicus*) + (*Hypaurotis crysalus*) + (*Chrysozephyrus birupa* + *Chrysozephyrus smaragdinus* + *Chrysozephyrus syla*) + (*Sibataniozephyrus fujisanus*).

The Indian specimens of *Chrysozephyrus birupa* and *C. syla* clustered into distinct monophyletic clades with maximum bootstrap support (99%), confirming their molecular identity at the species level. The topology reveals that *C. syla* (India) shares a more recent common ancestor with the Japanese *C. smaragdinus* (73% support) than it does with *C. birupa*. This confirms that *C. smaragdinus* and *C. syla* have undergone less evolutionary divergence from each other, potentially reflecting a closer evolutionary link between Western Himalayan and East Asian populations within this genus. While species-level clades are robustly supported, several deeper intergeneric nodes exhibit low bootstrap values, such as the nodes with 16%, 23% and 33% support. These values indicate that while the COI gene fragment is highly effective for species-level barcoding, it lacks sufficient signal to fully resolve the deep-time ancestral relationships within the Theclini tribe. Acknowledging these limitations directly addresses known biases associated with exclusive reliance on mitochondrial markers for genus-level taxonomy. The tree illustrates a high degree of paraphyly among the genera *Favonius*, *Hypaurotis* and *Chrysozephyrus*. Notably, the clustering of *Quercusia quercus* and *Hypaurotis quercus* with 99% support, combined with the low intergeneric genetic distances observed across the tree, provides molecular evidence that aligns with the recent proposal for the synonymization of these taxa under *Hypaurotis* Scudder, 1876.

Figure 6. Evolutionary analysis via the maximum likelihood method and the Tamura (1992) model of nucleotide substitutions and the tree with the highest log likelihood (-1,863.70) are shown. The percentage of replicate trees in which the associated taxa clustered together (1,000 replicates) is shown next to the branches. The evolutionary rate differences among sites were modelled via a discrete gamma distribution across 5 categories (+*G*, parameter = 0.6489), with 62.19% of sites deemed evolutionarily invariant (+*I*). The analytical procedure included 22 nucleotide sequences with 603 positions in the final dataset.



Conclusion

The present study provides an integrative molecular and morphological analysis of the tribe Theclini (Lepidoptera: Lycaenidae, Theclinae) from the Western Himalayas, specifically focusing on *Chrysozephyrus syla* and *C. birupa*. Through this investigation, the female of *C. birupa* and both sexes of *C. syla* have been studied and illustrated for the first time, filling critical gaps in the morphological record of these high-altitude taxa. The integration of DNA barcoding and phylogenetic reconstruction recovered five major clades with high bootstrap support for species-level monophyly. Nucleotide composition analysis confirmed a strong A + T bias (average 70.3%), particularly at the third codon position, which is consistent with the mitochondrial genome evolution of insects. The identification of conserved, variable, and parsimony-informative sites further clarifies the genetic architecture of these species within the Indian Himalayas. Most notably, the low intergeneric distances (5.0-6.5%) and the paraphyletic nature of the tree topology provide robust molecular evidence supporting the synonymization of several taxa, including *Chrysozephyrus*, under *Hypaurotis*. These findings challenge older morphological classifications and underscore the necessity of integrative taxonomic approaches that combine genital morphology with molecular data. By refining species delineations and providing the first barcodes for these protected Himalayan species, this study not only enhances our understanding of Papilionoidea phylogenetics but also contributes essential data to future conservation strategies within the tribe Theclini.

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Conflict of Interest

The authors declare that there is no known financial interest or personal relationship that could have influenced the work presented in this article.

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Table 3. Estimates of evolutionary divergence between sequences.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Quercusia quercus (HM393195)																						
Hypaurotis quercus (MW503561)	0.3																					
Hypaurotis crysalus (AB195512)	5.4	5.6																				
Hypaurotis orientalis (OP443378)	5.0	5.0	5.4																			
Favonius orientalis (AB195530)	6.1	6.1	6.0	1.0																		
Favonius yuasai (AB195534)	5.4	5.4	6.4	4.5	4.9																	
Favonius ultramarinus (AB195544)	5.0	5.0	5.6	4.5	5.1	5.3																
Favonius taxila (AB195526)	5.0	5.0	5.3	4.1	3.9	5.8	5.3															
Favonius saphirinus (AB195522)	5.6	5.6	5.7	5.2	5.5	5.1	4.1	4.9														
Favonius leechi (AB195531)	6.6	6.6	6.0	1.9	2.6	4.5	4.9	4.1	5.3													
Favonius korshunovi (AB195536)	4.7	5.2	4.5	4.1	4.5	3.6	3.4	4.3	3.7	4.1												
Favonius koreanus (GU372574)	4.3	4.3	6.0	4.1	5.1	5.3	0.8	5.4	4.1	4.9	3.5											
Favonius jezoensis (AB195538)	5.0	5.4	5.8	4.7	5.4	5.8	2.2	5.2	4.5	4.9	3.3	2.2										
Favonius cognatus (AB195541)	4.7	4.7	5.8	4.3	4.9	5.1	0.6	5.1	3.9	4.7	3.2	0.2	2.0									
Chrysozephyrus syla (PQ850024)	5.8	6.0	4.1	5.0	6.2	7.3	5.8	6.0	6.6	6.4	5.3	5.4	6.2	5.6								
Chrysozephyrus syla (PQ850022)	5.6	6.0	4.9	4.8	6.6	7.7	6.2	6.6	7.1	6.9	5.8	5.7	6.7	6.0	0.7							
Chrysozephrus birupa (PQ849159)	4.8	4.8	6.0	4.7	6.9	7.9	6.9	7.3	7.7	7.1	6.9	6.4	7.8	7.1	4.7	4.3						
Chrysozephyrus birupa (PQ677695)	5.0	5.0	5.3	4.8	6.2	7.3	6.2	6.6	7.1	6.4	6.2	5.9	7.1	6.4	4.5	4.8	1.0					
Chrysozephyrus smaragdinus (AB195511)	6.5	7.0	4.1	5.4	5.8	6.8	5.8	6.2	6.6	6.0	5.3	6.2	6.2	6.0	3.2	3.7	4.5	3.9				
Sibataniozephyrus fujisanus (AB195514)	6.3	6.3	5.5	5.6	6.6	7.1	6.0	6.9	6.2	5.8	6.0	6.0	6.0	5.8	5.5	6.0	6.6	6.0	5.5			
Neozephyrus japonicus (AB195513)	6.5	6.5	5.1	4.5	5.1	6.4	4.5	5.5	6.2	4.9	5.1	4.9	5.6	4.7	6.0	6.4	6.6	5.5	5.1	5.8		
Thecla betulae (FJ664048)	9.2	9.2	7.7	10.0	9.7	10.5	10.3	9.0	10.3	9.8	9.8	10.0	10.3	10.0	8.9	8.9	8.7	8.9	8.7	8.2	9.2	