

Complete Mitochondrial Genomes of the Leptocircini Species *Iphiclides podalirius* and *I. podalirinus* (Lepidoptera: Papilionidae)

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Abstract: The complete mitochondrial genomes of two *Iphiclides* species, namely *I. podalirius* and *I. podalirinus*, were sequenced, assembled, and reported in this article. Both genomes comprise 37 genes, with 13 protein-coding genes, 22 transfer RNA (tRNA) genes, and two ribosomal RNA (rRNA) genes. The gene orders and alignments agree with the reported mitogenomes of Leptocircini butterflies, while the start codon for the COX1 gene in *I. podalirinus* is CGA instead of the commonly seen ATN type. Codon preference shows that methionine and tryptophan are the poorest, while arginine, leucine, and serine are the richest. Phylogenetic analysis using Bayesian Inference shows both *Iphiclides* species are sister to the genus *Lamproptera* and are basal to all remaining Leptocircini species. The Kimura 2-parameter (K2P) distances of *I. podalirinus* from *I. podalirius* exceed 5%, demonstrating its solid species status. The K2P distance between the North African *feisthamelii* and *podalirius* exceeds 2%, indicating the reasonable elevation of *I. feisthamelii* to the full specific level as its type locality is Algeria. Future research is required to tackle the relationship between the Iberian *feisthamelii* and *podalirius* using more evidence.

Keywords: scarce swallowtail; Eurasia distribution; Hengduan Mountains; North Africa; Iberia; codon usage; DNA barcoding



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1. Introduction

The genus *Iphiclides* Hübner [1819] is strictly Palearctic, with two generally recognised species, *I. podalirius* (Linnaeus, 1758), found across the majority of Eurasia, and *I. podalirinus* (Oberthür, 1890), restricted on the eastern margin of the Qinghai–Tibetan Plateau of West China (Figure 1) [1]. *Iphiclides* is one of the genera of the tribe Leptocircini, alongside the genus *Lamproptera* Grey, 1832 [2]. All members of the genus *Lamproptera* are Oriental [3], with only *L. paracurius* (Hu, Zhang, and Cotton, 2014) found near the boundary between the Palearctic and Oriental regions [4].

The western Mediterranean race of *I. podalirius*, namely *feisthamelii* (Duponchel, 1832), is of disputed rank (Figure 1). Regardless of its constant different wing morphology, the genetic profile of *feisthamelii* does not always agree with classifications. Wiemers and Gottsberger [5] reported the discordant mitochondrial and nuclear DNA pattern between *podalirius* and *feisthamelii* around the Mediterranean area, finding that only the African *feisthamelii* (Morocco) could be differentiated from *podalirius* when using mitochondrial DNA marker (COX1), while the Iberian (Spain) *feisthamelii* could also be differentiated when using nuclear DNA marker (*EF-1α*). Later, Coutsis and Oorschot [6] reported the differences in male and female genitalia between the two races and supported the elevation

of *feisthamelii*. Based on these, Nakae [7] and Racheli and Bozano [8] listed *feisthamelii* as a distinct species in their recent publications.

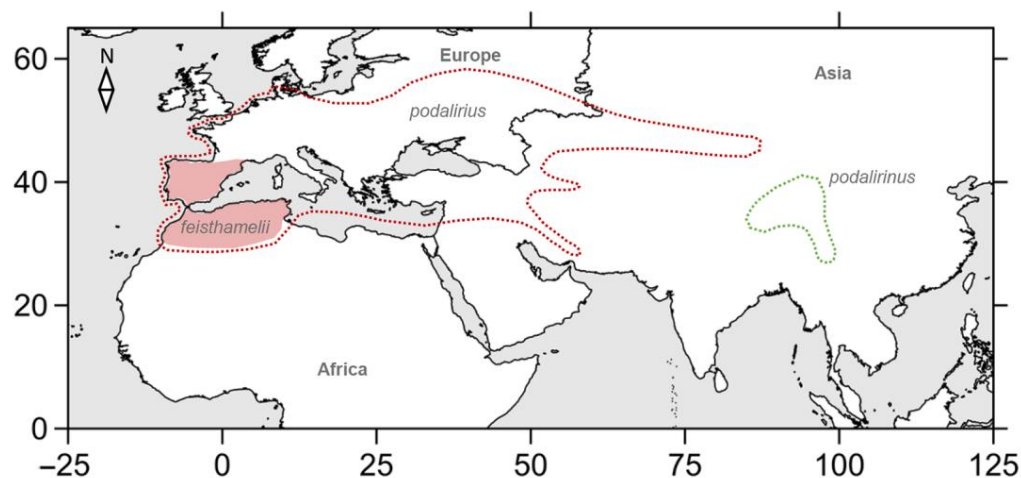


Figure 1. Distribution ranges of *Iphiclides podalirius* (red dotted line) and *I. podalirinus* (green dotted line), compiled from Racheli and Cotton [1], Wiemers and Gottsberger [5], and Racheli and Bozano [8], with red patches indicating the range of *I. feisthamelii*.

Iphiclides podalirinus is distinguished from its sibling species by wing colouration; apart from the much darker ground colour and broader black bands, the red instead of yellow-orange markings on the hindwing are prominent (Figure 2). Furthermore, the distribution range of *I. podalirinus* is completely detached from that of *I. podalirius*, making it an unmistakable species (Figure 1).



Figure 2. The female *Iphiclides podalirius* (A) and male *I. podalirinus* (B) specimens used in this study, upperside on the left side and half of the underside on the right side; scale bar = 10 mm.

In recent years, many efforts have been made to better understand the genetic diversity of *I. podalirius* and *I. feisthamelii* across Eurasia, including the whole genome data of *I. podalirius* published by Mackintosh, et al. [9]. However, being endemic to West China, the genetic study of *I. podalirinus* is still unavailable due to limited access to samples. Even though IUCN has not evaluated the threatened status of *Iphiclides* species yet [10], and CITES does not list them on any Appendices either [11], all species are still under much anthropogenic stress, including habitat loss, climate change, and commercialised collecting. In some European countries, *I. podalirius* is listed as a protected species [12]. In China, *I. podalirinus* is also protected by the regulation “List of Terrestrial Wildlife under State Protection for Ecological, Economic and Scientific Values” [13]. Enhancing genetic research on such species is, thus, crucial and would eventually benefit conservation [14].

The present study reports the complete mitochondrial genome of *I. podalirinus*, with a new genome added to *I. podalirius*. Our goal is to enrich the fundamental genetic data of this distinct and stylish swallowtail group in the hope of bringing new insights to its future conservation.

2. Materials and Methods

2.1. Taxon Sampling

One individual of each species, *Iphiclides podalirius* and *I. podalirinus*, was obtained by the corresponding author. *Iphiclides podalirius* (female) was collected from Prague, Czech Republic, in 2023, and *I. podalirinus* (male) was collected from western Sichuan, China, in 2019 (Figure 2). Specimens were killed immediately upon capture by pinching the thorax.

Three legs from the same side of a specimen were then removed, preserved in 95% ethanol, and stored at -20°C until use. Specimens were spread for morphological comparison and stored in the Lepidoptera collection of the Institute of International Rivers and Eco-security, Yunnan University. Species identification of the specimens was performed primarily by morphological comparison against Racheli and Cotton [1], as the characters are very distinct for both species.

For phylogenetic reconstruction, 54 representative species of Papilionidae with complete mitochondrial genomes were selected and downloaded from the NCBI GenBank (Table 1). The 54 species cover all taxonomic groups (subfamilies and tribes) of Papilionidae. Two Pieridae species, *Aporia hippia* (Bremer, 1861) (OP526834) and *A. bieti* (Oberthür, 1884) (KX495165), were also obtained from GenBank as outgroups.

Table 1. Mitochondrial genomes of Papilionidae representative species used in the present study.

Subfamily	Tribe	Species	GenBank Accession
Baroniinae	Baroniini	<i>Baronia brevicornis</i>	OR063968
Parnassiinae	Sericinini	<i>Allancastris cerisy</i>	LS974636
		<i>Bhutaniitis mansfieldi</i>	OP936023
		<i>Sericinus montela</i>	MN013020
		<i>Luehdorfia chinensis</i>	OR058734
	Parnassiini	<i>Parnassius epaphus</i>	KM373898
		<i>Parnassius stubbendorfi</i>	OP709281
		<i>Parnassius hardwickii</i>	OP963809
		<i>Parnassius stoliczkanus</i>	OP963808
		<i>Parnassius imperator</i>	KM507326
		<i>Lamproptera meges</i>	LT999978
Papilioninae	Leptocircini	<i>Lamproptera curius</i>	KJ141168
		<i>Graphium parus</i>	MT198821
		<i>Graphium mullah</i>	KJ472924
		<i>Graphium eurous asakurae</i>	MW549198
		<i>Graphium confucius</i>	OK136253
		<i>Graphium antiphates</i>	ON620258
		<i>Graphium agetes</i>	OQ935417
		<i>Graphium leechi</i>	KX011066
		<i>Graphium chironides</i>	KP159289
		<i>Graphium doson</i>	MK144328
		<i>Graphium agamemnon</i>	OQ852718
		<i>Graphium sarpedon</i>	ON675575
		<i>Graphium cloanthus</i>	OQ852719
	Teinopalpini	<i>Teinopalpus imperialis</i>	KR018842
		<i>Teinopalpus aureus</i>	HM563681
	Troidini	<i>Cressida cressida</i>	MK507889
		<i>Euryades corethrus</i>	LT999972
		<i>Losaria neptunus</i>	LT999979
		<i>Pachliopta kotzebuea</i>	LS975120
		<i>Pachliopta aristolochiae</i>	KU950357

Table 1. Cont.

Subfamily	Tribe	Species	GenBank Accession
		<i>Atrophaneura dixonii</i>	LT999977
		<i>Byasa polyeuctes</i>	OQ673106
		<i>Byasa alcinous</i>	KJ540880
		<i>Trogonoptera brookiana</i>	LT999986
		<i>Troides aeacus</i>	EU625344
		<i>Ornithoptera priamus</i>	LT999981
		<i>Ornithoptera alexandrae</i>	OQ590009
	Papilionini	<i>Papilio machaon</i>	HM243594
		<i>Papilio xuthus</i>	KT922004
		<i>Papilio rex</i>	KX033354
		<i>Papilio dardanus</i>	KX033351
		<i>Papilio demoleus</i>	KR024009
		<i>Papilio paris</i>	OQ927990
		<i>Papilio bianor</i>	JN019809
		<i>Papilio syfanius</i>	KJ396621
		<i>Papilio maackii</i>	KC433408
		<i>Papilio polytes</i>	KM215138
		<i>Papilio helenus</i>	KP247522
		<i>Papilio protenor</i>	KY272622
		<i>Papilio macilentus</i>	OP324646
		<i>Papilio agenor</i> ¹	MH981597
		<i>Papilio janaka</i> ²	OQ955740
		<i>Papilio alcmenor</i>	ON964516

¹ *Papilio memnon* in the GenBank record. ² *Papilio bootes janaka* in the GenBank record. Based on the specimen information in GenBank records and the latest taxonomic research on the genus *Papilio* [15,16], the species names *Papilio agenor* and *Papilio janaka* were adopted herein.

To compare the genetic differences between all *Iphiclid* taxa, 184 full-length (658 bp) DNA barcodes (COX1) of *I. podalirius* and *I. feisthamelii* were downloaded from the Barcode of Life Database (BOLD System v4; <https://www.boldsystems.org/>) (accessed on 1 April 2024) (Table 2) and analysed with the data obtained in the present study.

Table 2. The 184 DNA barcode (COX1) sequences of *I. podalirius* and *I. feisthamelii* downloaded from BOLD for this study.

Taxon	Country	BOLD Accession
<i>I. podalirius</i>	Portugal	BDE369-19, EULEP5983-18, EULEP5984-18
	Spain	EULEP5951-18, EULEP5952-18, EULEP5980-18, EULEP5994-18, WMB3432-14, BIBSA922-15, BIBSA2258-20, BIBSA2271-20, EULEP4099-16, EULEP5979-18, EULEP5986-18, EULEP5987-18, EULEP5988-18, EULEP5989-18, EULEP5992-18, EZSPM448-09, OXB1065-15, OXB1259-15, OXB1366-15, OXB1388-15, WMB354-11, WMB1173-13, WMB1730-13, WMB1800-13, WMB3465-14, WMB3893-14, WMB3912-14, WMB3916-14, WMB3945-14, WMB3971-14, WMB4637-14, WMB5327-14
	France	EULEP2164-15, EULEP4098-16
	Switzerland	GWORA2430-09
	Germany	ABOLC133-16, ABOLD084-16, LEASS703-17, LEASS856-17
	Austria	BIBSA349-15, BIBSA580-15, BIBSA701-15, BIBSA970-15, BIBSA1031-15, BIBSA1140-15, BIBSA1171-15, BIBSA1329-15, BIBSA1423-15, BIBSA1600-16, BIBSA1613-16, BIBSA1618-16, BIBSA1667-16, BIBSA1766-16, EULEP5662-17, EULEP5953-18, EULEP5961-18, EULEP5965-18, EULEP5982-18, EULEP5991-18, EULEP5999-18, EULEP6000-18, LEATD016-13, LEATG462-14, LEASZ969-22, OXB587-15, OXB888-15, OXB910-15, OXB974-15, OXB1028-15, WMB127-11, WMB259-11, WMB641-11, WMB1895-13, WMB2120-13, WMB2216-13, WMB2360-13, WMB2361-13, WMB2442-13, WMB3834-14, WMB3853-14, WMB4131-14, WMB4271-14, WMB4288-14, WMB4342-14, WMB4765-14, WMB4804-14, WMB4855-14, WMB4968-14, WMB5040-14, WMB5084-14, WMB5497-14, WMB6563-18
	Italy	

Table 2. Cont.

Taxon	Country	BOLD Accession
<i>I. feisthamelii</i>	Slovakia	EULEP5022-16
	Ukraine	EULEP4835-16
	Romania	EULEP001-14, EULEP5973-18, EZRMN332-08, EZROM219-08, EZROM220-08, EZROM351-08, EZROM1028-08, EZROM1029-08, EZROM1030-08
	Bulgaria	EULEP897-15, EULEP1197-15
	Greece	EULEP701-15, EULEP1306-15, EULEP1367-15, EULEP1442-15, EULEP1600-15, EULEP1767-15, EULEP5962-18, EULEP5971-18, EULEP5972-18, EULEP5974-18, EULEP5975-18, EULEP5978-18
	Turkey	EULEP5950-18
	Iran	IRANB315-08
	Kazakhstan	EULEP5956-18, EULEP5957-18, EULEP5958-18, EULEP5959-18, EULEP5960-18
	Russia	LEFIL147-10
	Morocco	EULEP5954-18, EULEP5955-18, EULEP5990-18, EULEP5996-18, EULEP5997-18, WMB245-11, WMB373-11, WMB399-11, WMB421-11, WMB3604-14, BDE293-19, BDE294-19
	Algeria	EULEP5964-18, EULEP5981-18, EULEP6001-18, WMB1472-13, WMB1473-13, WMB1476-13
	Tunisia	EULEP5970-18, WMB1405-13, WMB1406-13, WMB6562-18
	Portugal	WMB4400-14, BDE553-19
	Spain	EULEP5949-18, EULEP5966-18, EULEP5967-18, EULEP5968-18, EULEP5969-18, EULEP5993-18, EULEP5995-18, EULEP5998-18, EULEP6002-18, EZSPC449-09, EZSPC450-09, EZSPC451-09, EZSPM099-09, EZSPM275-09, EZSPM362-09, EZSPM419-09, EZSPM450-09, EZSPM611-12, EZSPM629-12, EZSPN330-09, EZSPN403-09, EZSPN477-09, EZROM725-08, WMB3126-14, WMB3315-14, WMB3481-14, WMB3777-14, WMB3995-14, WMB4046-14
	France	EULEP002-14, EULEP003-14, EULEP5985-18

2.2. Molecular Work

Genomic DNA was extracted from all three legs using the Rapid Animal Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China), then the concentration was determined on a Qubit® Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and it was stored in the refrigerator at −20 °C. The quality of the resultant DNA was examined by 1% agarose gel electrophoresis under a Gel Image Analysis System (FR-980A, Furi Science & Technology Co., Ltd., Shanghai, China).

The extracted DNA was then sequenced on an Illumina NovaSeq 6000 System (Illumina Inc., San Diego, CA, USA). The chromatograms were analysed and translated with base calling to generate raw reads in the FASTQ file. The quality of raw reads was evaluated and processed using fastp 0.36 [17] to obtain valid, high-quality, clean data.

Due to the high redundancy in high-throughput sequencing data, the de novo assembly strategy was used to align the reads and target sequences bidirectionally using SPAdes 3.15 [18]. Sequencing errors were corrected first on the input sequences, and then the sequence was spliced using multiple Kmer values. Once the splicing was completed, BLASTn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (accessed on 23 September 2023) was applied to compare scaffolds with the NCBI nucleotide library to assess similarity and extract the sequencing depth and coverage information of each scaffold. After filling gaps between all contigs using GapFiller 1.11 [19], the PrInSeS-G method [20] was applied to correct clipping errors and indels of small fragments during splicing. Further, built-in scripts were employed to process and evaluate contigs to obtain a complete circular genome.

2.3. Genomic Analysis

The mitochondrial genomes of Leptocircini are circular and approximately 14–16 kb in length, containing 37 genes (13 protein-coding genes (PCGs), 22 transfer RNA genes, and two ribosomal RNA genes) [21–26]. tBLASTn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (accessed on 23 September 2023) and GeneWise (<https://www.ebi.ac.uk/jdispatcher/psa/>)

genewise) (accessed on 23 September 2023) were used to perform reverse comparison with reference genomes of *Graphium parus* (MT198821) [24], *G. eurous asakurae* (MW549198) [25], and *G. confucius* (OK136253) [26] to obtain PCG boundaries. The tRNA regions were annotated using MiTFi [27], and CMsearch [28] was employed with the Rfam database [29] for non-coding rRNA identification. Based on the annotation and the aforementioned results, the schematics of mitochondrial genomes were mapped using Circos [30].

Codon preference plays an important role in evolution and, thus, was analysed in the present study. Relative synonymous codon usage (RSCU) was employed to measure the use frequency of codons, which shows the ratio of actual utilisation value to theoretical utilisation value. When $RSCU < 1$, the utilising frequency of the focal codon is lower than that of other synonymous ones; when $RSCU > 1$, the explanation is vice versa; and when $RSCU = 1$, the focal codon shows no preference [31]. The resultant RSCU plots were generated using the “ggplot2” package [32] in R 4.3.3 (<http://www.r-project.org> (accessed on 10 February 2024)).

2.4. Phylogeny, Genetic Distances, and BOLD BINs

To test the phylogenetic position of the obtained *Iphiclides* genomes against other taxonomic and systematic theories, a phylogenetic tree was reconstructed using PhyloSuite 1.2.3 [33,34] using the *Iphiclides* genomes with the downloaded genomes (Table 1).

ModelFinder [35] was used to estimate the best substitution model for the dataset, and Bayesian Inference (BI) was performed by MrBayes 3.2 [36] with the selected model with 10 million generation Markov Chain Monte Carlo (MCMC) (sampled every 1000th generation with a 25% burn-in) to calculate the clade posterior probabilities (PPs). The average standard deviation of split frequencies (ASDFs), the potential scale reduction factor (PSRF), and the effective sample size (ESS) were adopted to evaluate the robustness of the BI analysis. Good robustness was considered when ASDF approaches 0, PSRF approaches 1, and $ESS > 200$ [36,37]. The resultant tree file was annotated using FigTree 1.4.4 [38] and Adobe Illustrator CS6 (Adobe Systems Inc., San Jose, CA, USA; licensed serial number 9229-8586-7036-7176).

To estimate the degree of genetic differentiation of all *Iphiclides* taxa, the DNA barcode fragments (COX1) of the *Iphiclides* genomes were extracted and compiled with those listed in Table 2. The dataset was split into four groups in MEGA X [39] under the current taxonomic system plus geographic ranges (Figure 1), that is, *I. podalirius*, *I. feisthamelii* North Africa, *I. feisthamelii* Iberia, and *I. podalirinus* [1,7]. The Kimura 2-parameter (K2P) genetic distance [40] between groups was calculated and shown in percentage. The Barcode Index Numbers (BINs) of both *I. podalirius* and *I. feisthamelii* were also detected automatically using the web-based tool in the BOLD System (https://www.boldsystems.org/index.php/Public_BarcodeIndexNumber_Home; accessed on 2 July 2024) with the keyword “Iphiclides”.

3. Results

3.1. Sequencing Quality and Genomic Components

A total of 22.748 million reads covering 3.404 billion bases with a 150 bp average read length were obtained from the sample of *I. podalirius* from two reads; the ratio of Q20 reads was 98.48% and 96.50%, and that of Q30 reads was 95.28% and 90.32% in two reads, respectively. A total of 20.803 million reads covering 2.969 billion bases with a 143 bp average read length were obtained from the sample of *I. podalirinus* from two reads; the ratio of Q20 reads was 98.66% and 97.09%, and that of Q30 reads was 95.89% and 93.71% in two reads, respectively.

The complete mitochondrial genome of *I. podalirius* is 15,166 bp in length (PP566972), and that of *I. podalirinus* is 15,229 bp in length (PP566973). Both genomes are circular and contain 37 genes, including 13 protein-coding genes (PCGs), 22 transfer RNA genes, and 2 ribosomal RNA genes (Figure 3). Among the 13 PCGs, *ND2*, *COX1*, *COX2*, *ATP8*, *ATP6*, *COX3*, *ND3*, *ND6*, and *CYTB* are coded by plus strands, while *ND5*, *ND4*, *ND4L*, and *ND1* are coded by minus strands (Table 3). The CG content of the mitogenome of *I. podalirius* is 18.1%, and that of the mitogenome of *I. podalirinus* is 19.1%.

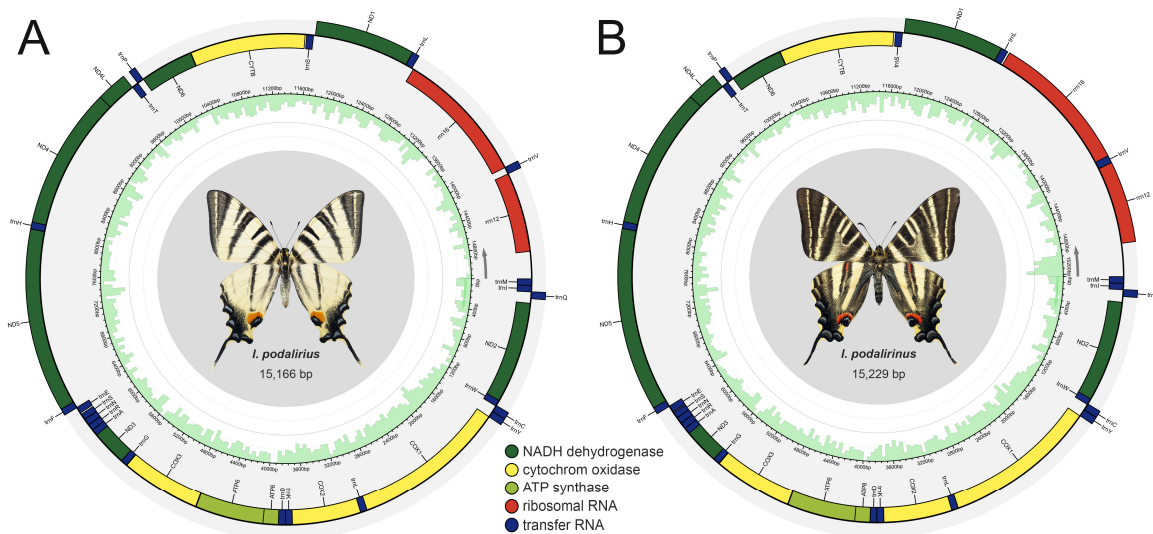


Figure 3. Mitochondrial genomic schematics of *Iphiclidus podalirius* (A) and *I. podalirinus* (B) assembled in this study.

Table 3. The basic components, strand properties of each gene, anti-codon of tRNA, and start and stop codons of 13 protein-coding genes of the mitochondrial genomes of *Iphiclidus podalirius* (A) and *I. podalirinus* (B).

Gene	Strand	Span		Anti-Codon	Start Codon		Stop Codon	
		A	B		A	B	A	B
<i>tRNA-Met</i>	+	1–66	1–67	CAU	—	—	—	—
<i>tRNA-Ile</i>	+	67–130	68–131	GAU	—	—	—	—
<i>tRNA-Gln</i>	—	128–196	129–197	UUG	—	—	—	—
<i>ND2</i>	+	243–1256	256–1296	—	ATT	ATT	TAA	TAA
<i>tRNA-Trp</i>	+	1255–1319	1268–1332	UCA	—	—	—	—
<i>tRNA-Cys</i>	—	1312–1379	1325–1391	GCA	—	—	—	—
<i>tRNA-Tyr</i>	—	1380–1444	1394–1464	GUA	—	—	—	—
<i>COX1</i>	+	1445–2978	1468–2998	—	ATG	CGA ¹	T-tRNA	T-tRNA
<i>tRNA-Leu</i>	+	2979–3045	2999–3065	UAA	—	—	—	—
<i>COX2</i>	+	3046–3727	3066–3747	—	ATG	ATG	T-tRNA	T-tRNA
<i>tRNA-Lys</i>	+	3728–3797	3748–3817	CUU	—	—	—	—
<i>tRNA-Asp</i>	+	3798–3865	3818–3884	GUC	—	—	—	—
<i>ATP8</i>	+	3866–4024	3885–4043	—	ATC	ATA	TAA	TAA
<i>ATP6</i>	+	4018–4695	4037–4714	—	ATG	ATG	TAA	TAA
<i>COX3</i>	+	4701–5492	4720–5508	—	ATA	ATA	TAA	TAA
<i>tRNA-Gly</i>	+	5496–5561	5512–5577	UCC	—	—	—	—
<i>ND3</i>	+	5562–5915	5578–5931	—	ATT	ATA	TAG	TAA
<i>tRNA-Ala</i>	+	5914–5976	5931–5993	UGC	—	—	—	—
<i>tRNA-Arg</i>	+	5977–6039	5994–6057	UCG	—	—	—	—
<i>tRNA-Asn</i>	+	6041–6105	6059–6123	GUU	—	—	—	—
<i>tRNA-Ser</i>	+	6107–6167	6125–6187	UCU	—	—	—	—
<i>tRNA-Glu</i>	+	6183–6247	6197–6263	UUC	—	—	—	—
<i>tRNA-Phe</i>	—	6246–6310	6262–6326	GAA	—	—	—	—
<i>ND5</i>	—	6311–8050	6327–8066	—	TTG	TTG	TAA	TAA
<i>tRNA-His</i>	—	8051–8116	8067–8132	GUG	—	—	—	—
<i>ND4</i>	—	8116–9455	8132–9471	—	ATG	ATG	T-tRNA	T-tRNA
<i>ND4L</i>	—	9456–9746	9472–9762	—	ATG	ATG	TAA	TAA
<i>tRNA-Thr</i>	+	9749–9813	9765–9828	UGU	—	—	—	—
<i>tRNA-Pro</i>	—	9814–9878	9829–9893	UGG	—	—	—	—
<i>ND6</i>	+	9881–10,411	9896–10,426	—	ATT	ATT	TAA	TAA

Table 3. Cont.

Gene	Strand	Span		Anti-Codon	Start Codon		Stop Codon	
		A	B		A	B	A	B
CYTB	+	10,416–11,564	10,431–11,579	—	ATG	ATG	TAA	TAA
tRNA-Ser	+	11,580–11,647	11,597–11,665	UGA	—	—	—	—
ND1	—	11,665–12,603	11,683–12,621	—	ATG	ATG	TAG	TAG
tRNA-Leu	—	12,605–12,672	12,623–12,689	UAG	—	—	—	—
16S-rRNA	+	12,691–14,029	12,709–14,056	—	—	—	—	—
tRNA-Val	—	14,028–14,097	14,055–14,126	UAC	—	—	—	—
12S-rRNA	+	14,098–14,895	14,127–14,900	—	—	—	—	—

¹ Start codon not determined.

3.2. Codon Usage, Preference, and Substitution

Both species share the same set of anti-codons for all 22 transfer RNAs. For *I. podalirius*, 12 out of 13 start codons are the ATN type, while only one of the *ND5* gene is a specialised TTG; 8 out of 13 stop codons are the typical TAA; and two (*ND3* and *ND1*) are TAG. The stop codons of *COX1*, *COX2*, and *ND4* are incomplete and inferred to be compensated by the 3'-adenine residuals to the mRNA. For *I. podalirinus*, the usage of both start and stop codons is very similar to those of *I. podalirius* in general, while the start codon of *COX1* is CGA and the stop codon of *ND3* is TAA instead of TAG (Table 3).

The codon preference analysis showed that both methionine and tryptophan possess only one codon each, being the poorest, while arginine, leucine, and serine possess five codons each, being the richest. Among the remaining amino acids, nine are coded by two codons, one is coded by three codes, and five are coded by four codes. The codon usage and preferences of *I. podalirius* and *I. podalirinus* are shown in Figure 4.

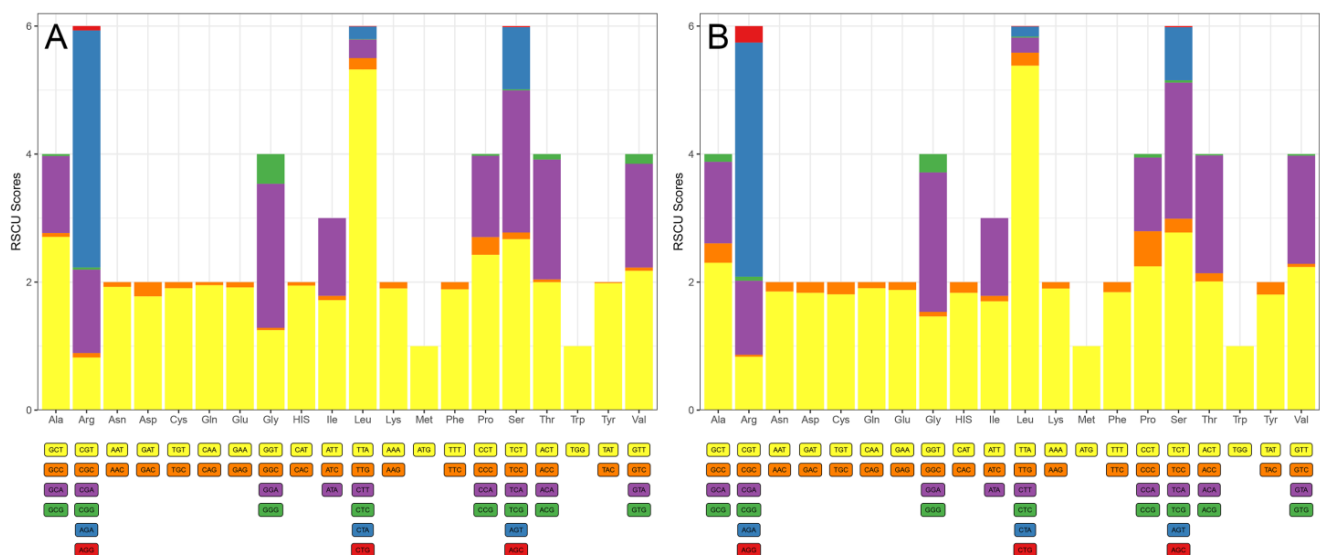


Figure 4. RSCU scores of codon usage in the mitochondrial genomes of *Iphiclides podalirius* (A) and *I. podalirinus* (B).

3.3. Phylogenetic Validation, Genetic Distance, and BINs

The BI analysis resulted in an ASDf = 0.000207, an average PSRF = 1.000 (maximal 1.001), and an average ESS = 6645.74 for all parameters, indicating the runs have reached excellent robustness.

The phylogenetic tree reconstructed by Bayesian Inference produced a robust topology and clear division of all subfamilies and tribes in Papilionidae, as reported by Condamine, et al. [2], with most node values (PPs) in the backbone of subfamilies and tribes exceeding 0.90, if not maximal. Within each tribal clade, most nodes were supported

by maximal PP values, with only that between genera *Iphiclides* and *Lamproptera* being the lowest (0.85) (Figure 5 and Figure S1 in Supplementary Materials).

The two *Iphiclides* species are sister to each other and were altogether placed sister to genus *Lamproptera* at the basal part of tribe Leptocircini (Figure 5), reflecting the taxonomic position of genus *Iphiclides* based on morphological characters.

The K2P distances of DNA barcode (COX1) between *I. podalirius* and the remaining taxa reached 5.280–5.530%, being the largest, and that between *I. podalirius* and the Iberian *I. feisthamelii* was only 0.134%, being the smallest (Table 4). The K2P distances between the North African *I. feisthamelii* and both *I. podalirius* and the Iberian *I. feisthamelii* are intermediate, being 2.184–2.234% (Table 4). The BIN search for “*Iphiclides*” from BOLD data collection yielded two BINs, with North African *I. feisthamelii* being one distinct BIN (25 sequences), while the typical *I. podalirius* and Iberian *I. feisthamelii* together being another BIN (198 sequences) (accessed on 2 July 2024) (Table 5).

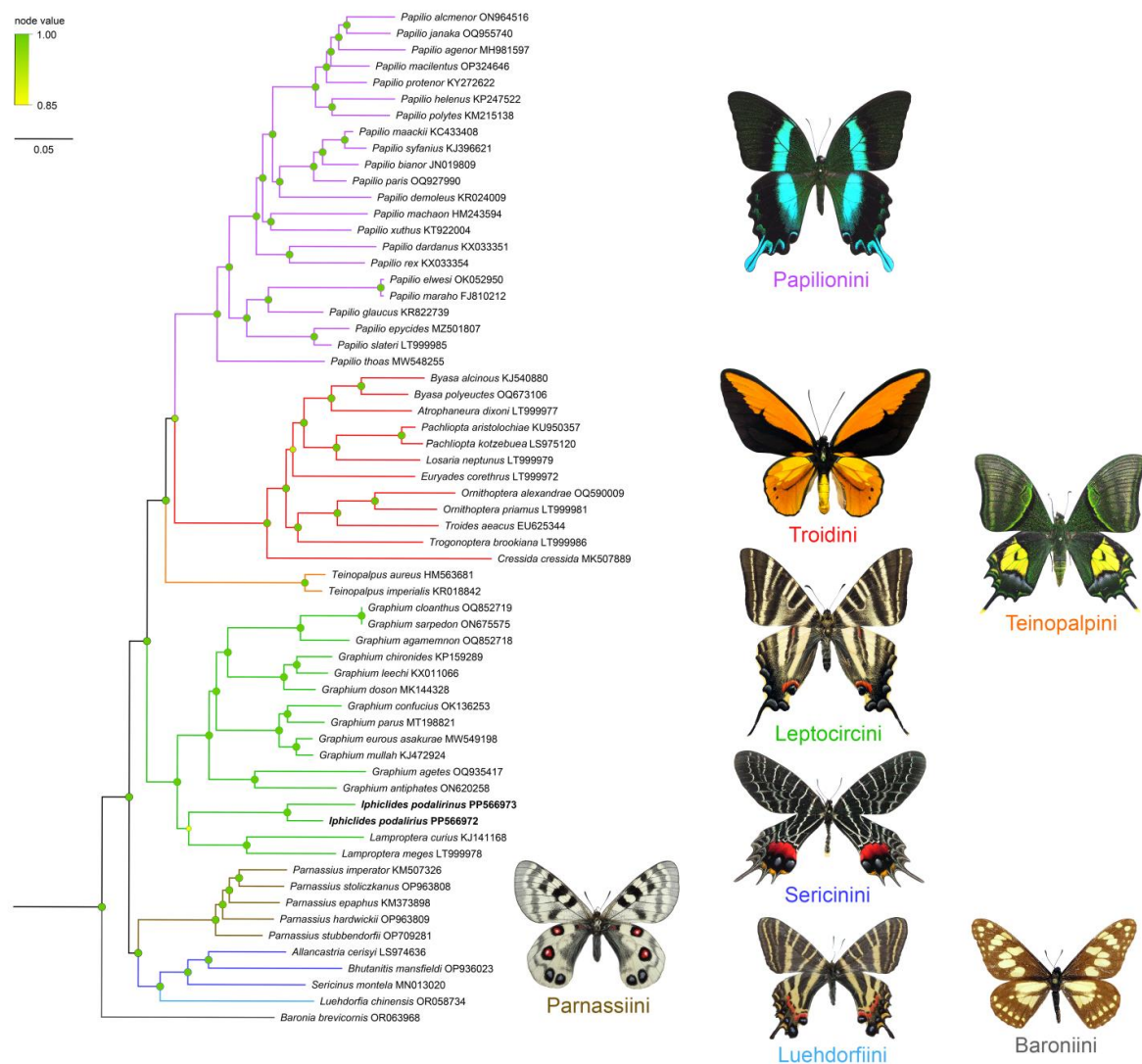


Figure 5. The phylogenetic tree of representatives of all subfamilies and tribes in Papilionidae using Bayesian Inference (BI); outgroups are not shown; circles at the nodes represent node values (PPs); and the tip names of the focal *Iphiclides* species are in bold font. Pictures of representative species for each tribe were photographed and edited by Shao-Ji Hu and Adam M. Cotton. Pictures are not proportional to actual butterfly sizes.

Table 4. The Kimura 2-parameter (K2P) genetic distances between *I. podalirius*, *I. feisthamelii* (two geographic groups), and *I. podalirinus* in percentage.

	1	2a	2b	3
1. <i>I. podalirius</i>				
2a. <i>I. feisthamelii</i> North Africa	2.234			
2b. <i>I. feisthamelii</i> Iberia	0.134	2.184		
3. <i>I. podalirinus</i>	5.309	5.530	5.280	

Table 5. The Barcode Index Numbers (BINs) search result using “Iphiclides” in the BOLD System; the numbers in parentheses after country/region names indicate the number of sequences contained.

BIN Number	No. of Sequences	Species Included	Country/Region
BOLD:AAB2272	198	<i>I. podalirius</i> <i>I. feisthamelii</i>	Italy (58); Spain (40); France (34); Greece (16); Romania (10); Portugal (7); Austria (6); Switzerland (5); Kazakhstan (5); Czech Republic (3); Iran (3); Russia (2); Slovakia (2); Bulgaria (2); Turkey (2); Germany (1); Ukraine (1); Unknown (1)
BOLD:AEA3098	25	<i>I. feisthamelii</i>	Morocco (13); Algeria (7); Tunisia (4); Unknown (1)

4. Discussion

The two *Iphiclides* species share the same mitochondrial genomic configurations with each other, as do most other Leptocircini butterflies [21,23–26]. The most frequently used codons are TTA and AGA, and codons in the third position in A or T reflect the AT bias of the insect mitochondrial genome. It is noticeable that in the mitogenome of *I. podalirinus*, the start codon for the COX1 gene is CGA instead of the regular ATN type (Table 3). Kim, et al. [41] argued that the CGA start codon (arginine) could be a synapomorphic character of arthropods, and Papilionidae species used in this study, such as *Parnassius stubbendorffii*, *Sericinus montela*, *Graphium sarpedon*, *Papilio xuthus*, and *P. elwesi*, all possess this start codon for the COX1 gene. Future studies are required to tackle the origin of the uncertainty of the start codon for the COX1 gene in Lepidoptera and integrate the start codons of more species.

Molecular studies have been instrumental in supporting and revising taxonomies based on morphology and geographical distribution. Our analysis shows that the K2P genetic distances between *I. podalirinus* and the remaining taxa of this genus are all over 5% (Table 3), demonstrating its solid species status, as represented by morphological differences on the wings. However, the genetic differences between *I. feisthamelii* and *I. podalirius* are interesting. For the North African *feisthamelii*, the genetic distance between it and *I. podalirius* reached 2.234% (Table 4), indicating its distinct species status when one takes 2% as the threshold for species delimitation [42]. On the contrary, the Iberian *feisthamelii* is much closer to *I. podalirius* genetically (Table 4). BOLD BIN search also agreed with this delimitation (Table 5). Similar discordance has been reported in the Iberian *Melitaea* species when nuclear gene markers performed better in species delimitation than using mitochondrial gene markers alone [43].

Given that the type locality of *feisthamelii* is Algeria in North Africa [44], it is reasonable to treat it as a distinct species with reported morphological and genitalic differences [6], as well as the genetic differentiations reported for either mitochondrial or nuclear genes. However, the status of Iberian *feisthamelii* has long been obscured due to the paradox in its genetic relationship with *I. podalirius* using different genetic markers [5] until Gaunet, et al. [45] discovered such discordance is the result of genetic sweeping and mitochondrial introgression due to repeated *Wolbachia* infections.

Wolbachia is a type of inheritable Rickettsia-like endosymbiont that can infect insects with certain taxonomic specialisations [46]. Populations infected by *Wolbachia* either exhibit a modified mitochondrial genetic profile, which could affect subsequent analysis of popu-

lation genetics, demographic history, and even phylogenetic relationships [45,47]. Hence, future taxonomies involving molecular evidence must either evaluate the *Wolbachia* infection among samples or include sufficient nuclear genes to avoid obscure results hindering our recognition of distinctive taxa [48,49].

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/d16070392/s1>, Figure S1: The Bayesian Inference phylogenetic tree with numeric node values and outgroups.

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