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Original Article

Complete mitogenome sequence of *Caryophyllaeus brachycollis* (Cestoda: Caryophyllidae) from China: Characterization and phylogenetic analyses of Caryophyllidea

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Abstract

Caryophyllaeus brachycollis mainly parasitizes the intestines of globally distributed freshwater fishes, and infection causes significant economic losses to the aquaculture industry. However, data on the molecular epidemiology, population genetics, and systematics of *C. brachycollis* are scarce. In this study, we sequenced the complete mitogenome of *C. brachycollis* isolated from Beijing, China. This circular mitogenome comprised 14,273 bp, which was 231 bp shorter than that of *C. brachycollis* isolated from Wuhan, China. The mitogenome contained 12 protein-coding genes, 22 transfer RNA genes, 2 ribosomal RNA genes, and 2 noncoding regions. Bayesian inference revealed that *C. brachycollis* belonged to the family Caryophyllaeidae. The taxonomic status of *C. brachycollis* is controversial when based solely on morphological features. A comparative analysis of the mitogenome sequence obtained in this study revealed novel molecular markers for the accurate ascertainment of the phylogenetic position of this parasite.

Keywords: Carp tapeworm, *Caryophyllaeus brachycollis*, mitochondrial genome, phylogenetic analyses

Introduction

Caryophyllideans, the earliest diverging group of non-segmented tapeworms, are described by morphological and biological features and typically have a monozoic (non-proglottized) body plan [1-3]. They are globally distributed and host-specific, mainly parasitizing in the intestinal tracts of Cypriniformes and Siluriformes fishes. Infection leads to weight loss, reduced fecundity, and high mortality rates, severely impacting the aquaculture industry [4-9]. The evolution of Eucestoda is unclear. The results of studies analyzing nuclear DNA genes (large and small subunits of ribosomal DNA) suggest that Spathebothriidea is the most divergent eucestode [10,11], whereas those analyzing morphological features and large fragments of mitochondrial DNA suggest that Caryophyllidea is the earliest divergent taxon [3,12]. Therefore, a better understanding of the relationship between orders Spathebothriidea and Caryophyllidea is warranted.

Although the order Caryophyllidea is regarded as a key group in Eucestoda research, the systematic classification and phylogenetic relationships of its members remain controversial. The families Balanotaeniidae, Capingentidae, Caryophyllaeidae, and Lytocestidae [1,2] are generally considered to belong to this order. Caryophyllaeidae, which frequently para-

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Conflict of interest
The authors declare no conflict of interest related to this study.

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sitize cyprinid fish in the Palearctic region, is the biggest group within Caryophyllidea, comprising 20 genera [13]. However, a recent study suggested reclassification into 10 genera [2], including *Caryophyllaeus* (Gmelin, 1790). The number of species in this genus varies from the previously described 42 nominal taxa to the recently recognized 7 valid species [14,15], of which *Caryophyllaeus brachycollis* is an important member. *C. brachycollis* consists of 2 morphotypes based on morphological and molecular (large subunit of ribosomal DNA and *cox1*) characteristics [16,17]. Although the mitogenome is a useful marker for tapeworm identification, differentiation, and systematic analyses [18-21], the complete mitogenome is only available for 1 morphotype, which was isolated from Wuhan, Hubei Province, China (CBCW). Because there is no data on the other morphotype, the molecular differentiation of *C. brachycollis* in China is unclear.

Thus, our study focused on obtaining the complete mitogenome of the other *C. brachycollis* morphotype, which was isolated from Beijing, China (CBCB). The aims were as follows: (i) sequence and annotate the complete CBCB mitogenome, (ii) determine differences between CBCB and CBCW, (iii) infer the phylogenetic relationship among members of the order Caryophyllidea, and (iv) identify the systematic and taxonomic status of the genus *Caryophyllaeus*. These findings would provide more molecular data for use in further Caryophyllidea analyses.

Materials and Methods

Ethics statement

The experimental protocol was established according to the ethical guidelines of the Institutional Animal Care and Use Committee and approved by the Ethics Committee of Hunan Agricultural University (No. 202282).

Sample collection and DNA extraction

Samples were collected from the intestines of *Cyprinus carpio* from Beijing Zoo, China. They were washed in physiological saline, and those morphologically identified as caryophyllidean were fixed in 70% (v/v) ethanol and stored at -40°C until use.

Total genomic DNA was extracted using sodium dodecyl sulfate/proteinase K treatment, followed by spin-column purification (Wizard SV Genomic DNA Purification System; Promega, Madison, WI, USA). The purified DNA was used as the template in PCR assays with the primer pair JB3/JB4.5 targeting a fragment of *cox1* for amplification [22].

Sequencing, assembling, and verification

A genomic DNA library (with 350 bp inserts) was prepared and sequenced by Novogene (Tianjin, China) using a HiSeq 2500 sequencing platform (Illumina, San Diego, CA, USA) to obtain 250 bp paired-end reads. Geneious v11.1.5 software was used to filter and clean the raw data and assemble the complete CBCB mitogenome using the amplified *cox1* sequences as initial references. The parameters were set as follows: minimum overlap identity, 99.0%; minimum overlap, 150 bp; and maximal gap size, 5 bp. The assembled mitogenome was further verified by long PCR experiments (Supplementary Fig. S1) using the primers listed in Supplementary Table S1.

Annotation, visualization, and sequence analysis

The boundaries of protein-coding genes (PCGs) were identified using the NCBI tool ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). The initiation/termination codons for each PCG were identified following alignment with the CBCW mitogenome sequence using MAFFT v7.122 software. All PCG nucleotide sequences were translated to amino acid sequences using MEGA v11.0. Twenty-two transfer RNA (tRNA) genes were detected using ARWEN [23] and tRNAscan-SE [24]. Nucleotide composition and codon usage frequencies of the PCGs were analyzed using MEGA v11.0. The annotated CBCB mitogenome was visualized using the online server Proksee (https://proksee.ca/). The CBCW sequence was used as the reference genome for comparisons with CBCB.

Phylogenetic analyses

Phylogenetic analyses were performed using 6 mitogenome sequences of tapeworms within the order Caryophyllidea (Table 1) and *Paruterina candelabraria* (GenBank accession No. NC_039533) as the outgroup. Amino acid sequences were aligned using MAFFT v7.122 and concatenated into a single dataset. Ambiguous bases or gaps were excluded using Gblocks 0.91b software with the default parameters [25]. Phylogenetic analyses were performed using Bayesian inference (BI) and maximum likelihood (ML) methods. BI analysis was run in MrBayes 3.1.1 using an approach similar to that of Ronquist and Huelsenbeck [26]. ML analysis was performed using PhyML 3.1 software. "JTT+I+G+F" was selected as the best suitable model by ProtTest v3.4.2 based on the Akaike information criterion [27] and was used for the BI and ML analyses. Phylograms were drawn using FigTree v.1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

cox1 sequence analysis

To determine the systematic relationship of the families in Caryophyllidea, BI analysis was performed using 20 *cox1* sequences of the order Caryophyllidea and *Gigantolina magna* (GenBank accession No. JQ268545) as the outgroup.

Results

Genome composition and organization

Sequencing of the CBCB mitogenome generated 4.08 Gb of raw data, from which approximately 7,577,818×2 clean reads were obtained following filtering and used for further as-

Table 1. Complete mitogenome sequences of the order Caryophyllidea used for phylogenetic analysis

Family/order	Species	Size (bp)	GenBank accession No.
Lytocestidae	*Khawia sinensis*	13,759	NC034800
	Atractolytocestys huronensis	15,130	NC035635
Caryophyllaeidae	*Caryophyllaeus brachycollis*	14,504	NC035430
	Parabreviscolex niepini	15,034	NC040117
Capingentidae	*Breviscolex orientalis*	14,011	NC035634
Cyclophyllidea	*Paruterina candelabraria*	13,634	NC039533

sembly. The complete circular CBCB mitogenome (GenBank accession No. OP359069) was 14,273 bp and comprised 36 genes, including 12 PCGs (excluding *atp8*), 22 tRNA genes, 2 ribosomal RNA genes (*rrnS* and *rrnL*), and 2 noncoding regions (NCRs; control or AT-rich regions) (Fig. 1; Table 2). All 36 genes were transcribed in the forward direction. In *C. brachycollis* mitochondrial DNA, the AT-skews were all negative (-0.481 to -0.125), and the GC-skews were all positive (0.167 to 0.465) (Table 3), suggesting a bias toward T and G bases. The lengths of the 2 NCRs in CBCB were 845 and 423 bp, respectively, while those in CBCW were 641 and 871 bp, respectively (Table 2). The large NCR (LNCR) was located between tRNA-Leu2 and *cox2*, and the small NCR (SNCR) was located between tRNA-Gly and *cox3* (Fig. 1). Alignment using BLAST revealed 100.0% nucleotide similarity with CBCW (GenBank accession No. NC_035430).

Composition of PCGs, ribosomal RNA genes, and tRNA genes

ATN was extensively used as the initiation codon, especially ATG. GTG is an uncommon start codon in tapeworms, but it was observed in *cytb*, *nad1*, and *nad4L* in this study (Table 2). Furthermore, TAA and TAG were frequently used as termination codons (TAG: *cytb*, *atp6*, *nad3*, *nad4*, and *nad4L*; TAA: *nad6*) (Table 2). Incomplete stop codons T and TA were also prevalent in termination, with *cox1*, *cox3*, and *nad2* using T and *cox2*, *nad1*, and *nad5* us-

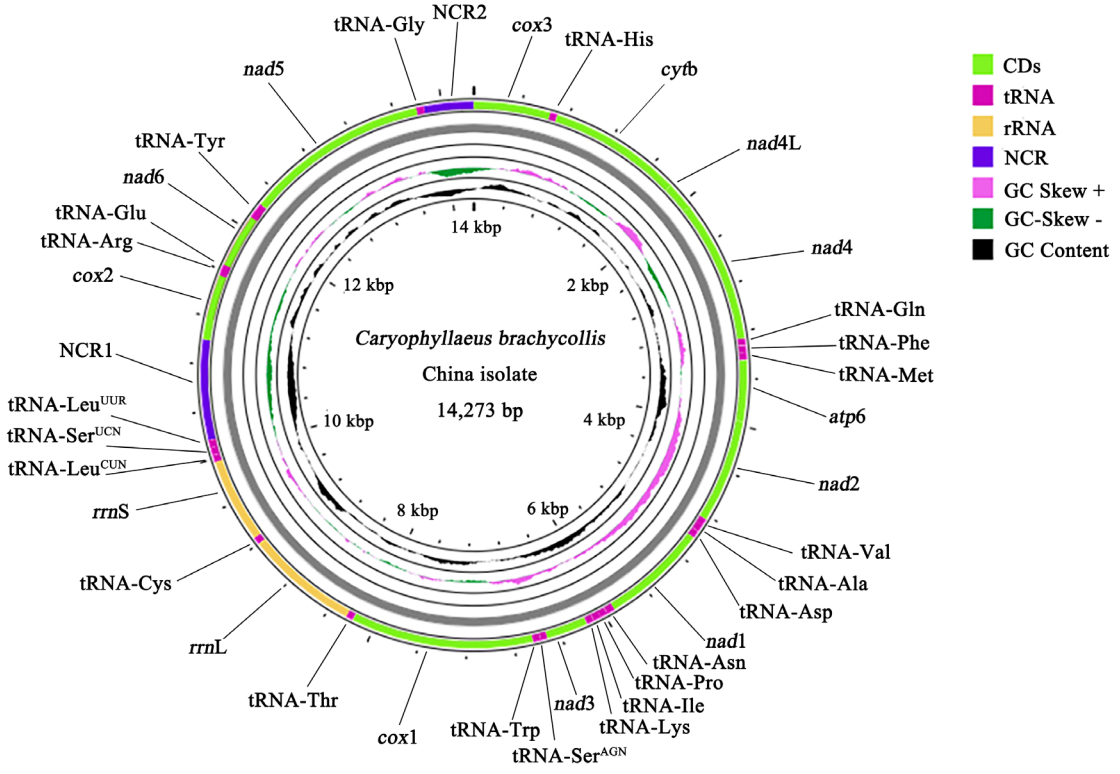

Fig. 1. Mitogenome organization of *Caryophyllaeus brachycollis* isolate from China. Gene scaling is approximate. CD, coding region; NCR1, long noncoding region; NCR2, short noncoding region; tRNA, transfer RNA; rRNA, ribosomal RNA.

Table 2. Comparative organization of the complete mitogenome of *Caryophyllaeus brachycollis* isolates from Beijing and Wuhan, China

Gene/region	Positions		Size (bp)		No. of aa		Ini/Ter codons		Anticodons
	CBCB	CBCW	CBCB	CBCW	CBCB	CBCW	CBCB	CBCW	CBCB/CBCW
cox3	1–643	1–643	643	643	214	214	ATT/T	ATT/T	
tRNA-His (H)	644–705	644–705	62	62					GTG
cytb	707–1825	707–1825	1,119	1,119	372	372	GTG/TAG	GTG/TAG	
nad4L	1828–2067	1828–2067	240	240	79	79	GTG/TAG	GTG/TAG	
nad4	2028–3260	2028–3260	1,233	1,233	410	410	ATG/TAG	ATG/TAG	
tRNA-Gln (Q)	3264–3318	3261–3322	55	63					TTG
tRNA-Phe (F)	3323–3377	3320–3382	55	63					GAA
tRNA-Met (M)	3378–3440	3378–3440	63	63					CAT
atp6	3444–3959	3444–3959	516	516	171	171	ATG/TAG	ATG/TAG	
nad2	3960–4830	3960–4830	871	871	290	290	ATG/T	ATG/T	
tRNA-Val (V)	4831–4890	4831–4890	60	60					TAC
tRNA-Ala (A)	4889–4949	4888–4949	61	62					TGC
tRNA-Asp (D)	4954–5014	4954–5014	61	61					GTC
nad1	5015–5907	5015–5908	893	894	297	297	GTG/TA	GTG/TAG	
tRNA-Asn (N)	5908–5975	5908–5975	68	68					GTT
tRNA-Pro (P)	5980–6040	5980–6040	61	61					TGG
tRNA-Ile (I)	6040–6103	6041–6102	64	62					GAT
tRNA-Lys (K)	6106–6165	6106–6165	60	60					GTT
nad3	6170–6517	6170–6517	348	348	115	115	ATG/TAG	ATG/TAG	
tRNA-Ser1 (S_1)	6520–6574	6518–6575	55	58					GCT
tRNA-Trp (W)	6575–6638	6575–6638	64	64					TCA
cox1	6642–8178	6642–8201	1,537	1,537	512	512	ATG/T	ATG/T	
tRNA-Thr (T)	8183–8242	8183–8242	60	60					TGT
rrnL	8243–9193	8238–9191	951	954					
tRNA-Cys	9194–9254	9192–9252	61	61					GCA
rrnS	9255–9966	9253–9965	712	713					
tRNA-Leu1 (L_1)	9967–10028	9966–10027	62	62					TAG
tRNA-Ser2 (S_2)	10030–10093	10028–10093	64	66					TGA
tRNA-Leu2 (L_2)	10095–10157	10094–10156	63	63					TAA
NCR1	10158–11002	10157–10797	845	641					
cox2	11003–11553	10798–11336	551	539	183	179	ATG/TA	ATG/TAG	
tRNA-Glu (E)	11587–11646	11370–11429	60	60					TTC
nad6	11647–12105	11430–11888	459	459	152	152	ATG/TAA	ATG/TAA	
tRNA-Tyr (Y)	12113–12174	11896–11957	62	62					GTA
tRNA-Arg (R)	11183–12236	11966–12021	54	56					TCG
nad5	12237–13786	12023–13570	1,550	1,547	516	515	ATG/TA	ATG/TA	
tRNA-Gly (G)	13787–13850	13570–13633	64	64					TCC
NCR2	13851–14273	13634–14504	423	871					

CBCB, *Caryophyllaeus brachycollis* isolates from Beijing; CBCW, *C. brachycollis* isolates from Wuhan.

ing TA (Table 2). The AT-skews of the 12 protein genes were all negative (-0.481 (*nad3*) to -0.181 (*cox2*)), and the GC-skews were all positive (0.167 (*cox2*) to 0.465 (*nad4L*)), suggesting a large T base bias in *nad3*, a G base tendency in *nad4L*, and a minor tendency of T+G bases in *cox2*.

The 22 tRNA genes ranged from 54 to 68 bp (Table 2). *rrnL* and *rrnS* were 951 and 712 bp, respectively, with an A+T content of 62.2% and 62.4%, respectively (Table 3).

Comparative analyses of CBCB and CBCW

Comparisons between the CBCB and CBCW mitogenome sequences revealed that CBCB

Table 3. Nucleotide composition and skews of the *Caryophyllaeus brachycollis* mitogenome

Gene	Nucleotide frequency				A+T (%)	AT-skew	GC-skew
	A (%)	G (%)	T (%)	C (%)			
atp6	16.7	25.0	42.2	16.1	58.9	-0.434	0.297
cox1	21.7	23.0	40.3	15.0	62.0	-0.230	0.212
cox2	25.4	22.1	36.7	15.8	62.1	-0.181	0.167
cox3	18.7	24.9	40.4	16.0	59.1	-0.368	0.217
cytb	22.0	23.2	39.9	14.9	61.9	-0.289	0.218
nad1	19.6	25.9	41.8	12.8	61.4	-0.361	0.339
nad2	16.6	26.5	45.8	11.0	62.4	-0.467	0.411
nad3	16.1	25.3	46.0	12.6	62.1	-0.481	0.333
nad4	20.8	22.4	41.7	15.1	62.5	-0.335	0.195
nad4L	19.6	26.2	44.6	9.6	64.2	-0.390	0.465
nad5	20.1	23.4	42.5	13.9	62.6	-0.357	0.254
nad6	20.0	22.9	42.3	14.8	62.3	-0.357	0.214
rrnS	27.0	23.9	35.4	13.8	62.4	-0.135	0.269
rrnL	27.2	22.7	35.0	15.0	62.2	-0.125	0.203
22 tRNAs	25.9	24.8	34.3	15.0	60.2	-0.139	0.246
Total	23.7	22.6	39.5	14.2	63.2	-0.250	0.227

was approximately 231 bp shorter than CBCW. In both CBCB (Fig. 1) and CBCW, the SNCR and LNCR were located in "tRNA-Gly and *cox3*" and "tRNA-Leu2 and *cox2*," respectively. The size (*cox2* and *nad5*) and the initiation/termination (*cox2*) codons of PCGs in CBCB and CBCW differed (Table 2). *cox2* was 539 bp and 551 bp in CBCW and CBCB, respectively, while *nad5* was 1,547 and 1,550 bp, respectively. The initiation/termination codons of *cox2* were ATG/TAG and ATG/TA in CBCW and CBCB, respectively.

Comparison of the nucleotide sequences of PCGs between CBCB and CBCW revealed differences of 0% (*nad4L*) to 2.2% (*cox3*) (Table 4). Differences in the amino acid sequences of CBCB and CBCW ranged from 0% (*nad4L*) to 2.7% (*cox2*) (Table 4). *nad4L* typically shows interspecies variability. However, our study revealed that it was highly conserved in *C. brachycollis*. These findings suggested that *cox2* might be a better molecular marker for identifying cryptic species within *C. brachycollis*.

Phylogenetic analyses

The phylogeny of Caryophyllidea is currently unclear due to a lack of complete mitogenome sequences. To better clarify the phylogenetic relationships among the members of this order, 6 mitogenome sequences were phylogenetically analyzed using BI and ML methods. The BI and ML tree topologies were identical (Fig. 2). There were 2 main clades: 1 clade contained Caryophyllaeidae and Lytocestidae, and the other clade contained Capingentidae, Caryophyllaeidae, and Lytocestidae, indicating that families Caryophyllaeidae and Lytocestidae were paraphyletic. The mitochondrial DNA sequences of CBCB and CBCW grouped with short topological distance (BPP = 1, BF = 100) (Fig. 2), indicating that they were closely related. Furthermore, phylogenetic analyses of *cox1* also showed that Caryophyllaeidae and Lytocestidae were paraphyletic and revealed a close evolutionary distance between CBCB and CBCW (Supplementary Fig. S2).

Table 4. Nucleotide and amino acid sequence differences between the isolates from Beijing, China and Wuhan, China mitogenomes

Gene/region	NT sequence length		NT difference (%)	No. of AA		AA difference (%)
	CBCB	CBCW	CBCB/CBCW	CBCB	CBCW	CBCB/CBCW
atp6	516	516	1.7	171	171	1.2
cox1	1,537	1,537	0.8	512	512	1.0
cox2	551	539	1.5	183	179	2.7
cox3	643	643	2.2	214	214	1.4
cytb	1,119	1,119	1.3	372	372	0.3
nad1	893	894	0.6	297	297	0.7
nad2	871	871	0.6	290	290	0.3
nad3	348	348	0.3	115	115	0.9
nad4	1,233	1,233	1.4	397	410	0.7
nad4L	240	240	0.0	79	79	0.0
nad5	1,550	1,547	1.0	516	515	0.8
nad6	459	459	1.1	152	152	1.3
rrnS	712	713	1.7	-	-	-
rrnL	951	954	0.8	-	-	-
All 22tRNAs	1,339	1,361	2.1	-	-	-

NT, nucleotide; AA, amino acid; CBCB, *Caryophyllaeus brachycollis* isolates from Beijing; CBCW, *C. brachycollis* isolates from Wuhan.

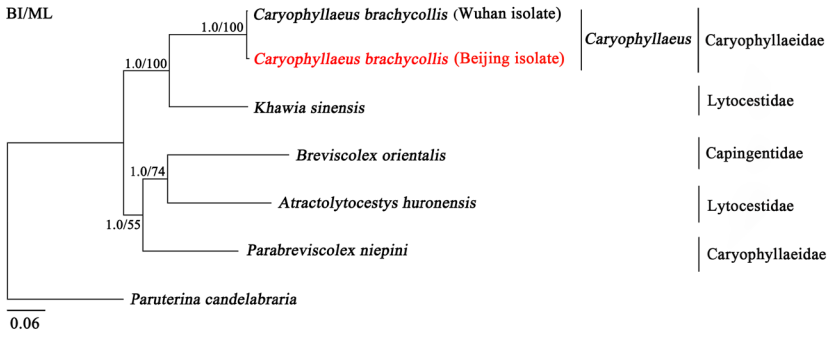

Fig. 2. Inferred phylogenetic relationships among species in the order Caryophyllidea. The concatenated amino acid sequences of 12 mitochondrial protein-coding genes were analyzed utilizing maximum likelihood (ML) and Bayesian analysis (BI) methods, with *Paruterina candelabraria* as the outgroup. The *Caryophyllaeus brachycollis* form was isolated from carp tapeworm in Beijing in this study.

Discussion

The complete mitogenome of CBCB was 14,273 bp and encoded 36 genes. The A+T base bias (73.2%) was significantly higher than the G+C base bias (26.8%). The AT-skew ([A-T]/[A+T]) and GC-skew ([G-C]/[G+C]) were useful methods to estimate compositional asymmetry [28].

Regarding the initiation codons, ATG was the most prevalent in 8 of the 12 PCGs (*atp6*, *cox1*, *cox2*, *nad2*, *nad3*, *nad4*, *nad5*, and *nad6*), and ATT was used in *cox3*. TAA and TAG

have been reported as common termination codons [29,30] and were identified as frequent termination codons in our study. There were 22 tRNA genes in the CBCB mitogenome. Similar to tapeworms *Breviscolex orientalis* and *Parabreviscolex niepini* [31,32], most tRNA genes in CBCB presented as a typical 'cloverleaf' structure, excluding tRNA-Ser^{AGN} and tRNA-Arg, which lacked DHU arms. The *rrnL* was located between tRNA-Thr and tRNA-Cys, and *rrnS* between tRNA-Cys and tRNA-Leu1, similar to the arrangement in *B. orientalis* and *Atractolytocestus huronensis* [32].

Although the mitogenome sequence of CBCW was longer than that of CBCB, the arrangements of the 36 genes were the same and consistent with the order observed in most caryophyllideans [31,32]. Considering that the differences in mitogenome sequences between closely related nematode species are 10.0%–20.0% [33,34], and species-level variations in mammals are typically 2.0%–10.0% [35], the level of differences between CBCB and CBCW indicated that they were identical.

Comparison between the CBCB and CBCW mitogenomes revealed that *cox2* is a reliable molecular marker for cryptic species identification. Furthermore, *nad4L* was highly conserved in *C. brachycollis*. This comparative mitogenomic analysis elucidated evolutionary and phylogenetic relationships among related species, enhancing the accuracy of taxonomic classification.

To summarize, our study determined and characterized the complete mitogenome sequence (14,273 bp) of CBCB, revealing that it was shorter than that of CBCW. The CBCB mitogenome provided evidence supporting a close phylogenetic relationship between *C. brachycollis* and other members of the Caryophyllidae family. Because only a limited number of specimens from a single geographic location were analyzed, future studies should include a broader sample to further assess intraspecific variations.

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Supplementary Information

Supplementary material is available with this article at https://doi.org/10.3347/PHD.25044.

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