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Assessing the nucleotide sequence diversity of COI, COII, CYTB, ND5 in several silkworm strains raised in Vietnam

TRAN THI BINH NGUYEN[1,♥], NGUYEN THI NHAI[2], LE THI XUAN[2], HO VIET DUC[1], NGUYEN DUC DUY[1], TRAN THI HONG HANH[1], NGUYEN THI NHIEN[1]

[1]Faculty of Biotechnology, Vietnam National University of Agriculture. Trau Quy Ward, Gia Lam District, Ha Noi 131000, Vietnam. Tel./fax.: +84-024-626-17586. ♥email: ttbnguyen@vnua.edu.vn

[2]Department of Silkworm Breeding, Vietnam Sericulture Research Centre. Ngoc Thuy Ward, Long Bien District, Ha Noi 131000, Vietnam

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Abstract. *Nguyen TTB, Nhai NT, Xuan LT, Duc HV, Duy ND, Hanh TTH, Nhien NT. 2025. Assessing the nucleotide sequence diversity of COI, COII, CYTB, ND5 in several silkworm strains raised in Vietnam. Biodiversitas 26: 1061-1068.* The selection of silkworm breeds is currently a subject of great interest in Vietnam, where assessing genetic diversity plays a crucial role. The main objective of this study was to evaluate the genetic diversity characteristics of the COI, COII, Cytb, and ND5 loci of mtDNA in 15 parental *Bombyx mori* (Linnaeus, 1758) lines raised in Vietnam. This includes 10 high-yielding white cocoon strains (bivoltine) and 5 low-yielding yellow cocoon strains (multivoltine), which are well adapted to the local climate conditions in Vietnam. The research used the PCR technique with specific primer pairs, performed sequencing using the Sanger method, and processed the data with BioEdit, DnaSP, and MEGA 11 software. The nucleotide sequencing results revealed a high A+T content in all four loci, particularly at the ND5 locus, where the total A+T content reached 84.94%. The study indicated that COI, COII, and ND5 markers alone are insufficient for differentiating between strains. However, the nucleotide sequences of the COII and ND5 loci can be used to identify the 5 multivoltine yellow cocoon native strains of Vietnam. Additionally, the nucleotide sequence of the Cytb locus can be used to distinguish these 5 native silkworm strains. These results will support breeders in identifying and differentiating native Vietnamese silkworm breeds.

Keywords: *Bombyx mori*, COI, COII, Cytb, ND5 sequences

Abbreviations: COI: Cytochrome c Oxidase subunit I; COII: Cytochrome c Oxidase subunit II; Cytb: *Cytochrome b* genes; ND5: the mitochondrially encoded NADH dehydrogenase 5

INTRODUCTION

Mulberry silkworms are used to produce silk for fashion clothing, shoes, handbags, wallpapers, and other textile products (Oduor et al. 2021; Gjurašić and Đurović 2023), creating additional human-use products such as pupae, biomass, and excreta, thereby enhancing their economic value (Hăbeanu et al. 2023). The domestication and selective breeding to produce high silk-yielding silkworm strains have increased inbreeding, leading to reduced resilience against changing weather conditions and decreased disease resistance. Maintaining genetic diversity is a crucial element in the long-term strategy to improve the genetics of *Bombyx mori* (Linnaeus, 1758) (Buhroo et al. 2018). Along side the conservation of genetic resources through selection and breeding by morphological characteristics, assessing genetic diversity using molecular markers can serve as an initial guide to identifying unique and valuable genes for accurately conserving valuable genetic resources.

The nucleotide sequences of mitochondrial DNA (mtDNA) have been widely used in studies evaluating genetic diversity among and within species (Kim et al. 2019; Alcudia-Catalma et al. 2021; Kim et al. 2021, 2022) due to mtDNA's distinctive characteristics such as a faster evolutionary rate compared to nuclear DNA (Allio et al. 2017). This makes it a useful tool in studies of genetic barcoding and phylogenesis (Cameron 2014). The mitochondrial DNA of insects is circular and double-stranded, with a length of 15-18kb (Cameron 2014), contains 37 genes, including 13 protein-coding regions (ND1, ND2, ND3, ND4, ND4L, ND5, ND6, COI, COII, COIII, ATP6, ATP8, and Cytochrome b) (Boore 1999; Cameron 2014). Several mtDNA sequences commonly used to assess genetic diversity in animals include the COI region (Fang et al. 2022; Fernández et al. 2022; Zhang and Bu 2022; French et al. 2023; Nguyen et al. 2023); COII region (Ostroverkhova et al. 2015; Debrah et al. 2023); Cytb region (Rahmatullaili et al. 2019; Farag et al. 2020; Davidović et al. 2022; Herrero et al. 2023) and ND5 region (Yamauchi et al. 2018; Khan 2021; Park et al. 2022).

The nucleotide sequence of the COI gene is one of the commonly used genetic sequences to assess genetic diversity among silkworm varieties worldwide (Yukuhiro et al. 2011; Fassina et al. 2014; Vimala et al. 2020). Specifically, Alcudia-Catalma et al. (2021) used the COI gene to describe the genetic characteristics of *B. mori* reared in the Philippines and to determine the phylogenetic relationships between these strains. Using 577 nucleotides of the COI gene sequence, Pan et al. (2020) identified genetic polymorphisms in all four silkworm strains. Besides the COI gene, the COII gene has also been used to evaluate genetic diversity in Korean silkworm strains (Kim et al. 2022; Park et al. 2022). Similarly,

the gene encoding the NADH-ubiquinone oxidoreductase chain 5 enzyme (ND5 gene) on mitochondrial DNA transfers electrons from NADH to the respiratory chain. This gene has also been used to assess genetic diversity (Yamauchi et al. 2018; Khan 2021; Park et al. 2022), and notably, it has been identified as distinguishing three endemic silkworm varieties in Korea. The nucleotide sequence of the Cytb gene has also been widely used in assessing genetic diversity in animals, both between species and within species (Li et al. 2005; Peterson et al. 2016; Kim et al. 2020; Chen et al. 2023; Herrero et al. 2023). Ray et al. (2024) confirmed the effectiveness of the Cytb gene as a potential DNA marker for identifying wild strains of Tasar silkworm and presented the first national report on the DNA barcoding of strain *Antheraea mylitta* (Drury, 1773).

The mitochondrial DNA nucleotide sequences and mitochondrial genes (COI, COII, Cytb, ND) have been studied to assess the genetic diversity among strains and lines of silkworms worldwide. However, in Vietnam, there are currently limited studies utilizing mitochondrial DNA sequences to evaluate genetic diversity in silkworm strains. Therefore, the goal of this research is to assess genetic diversity using the COI, COII, Cytb, and ND5 gene sequences in 15 silkworm strains cultivated in Vietnam. The information from this study provides an initial basis for developing measures and strategies to conserve, exploit, and develop silkworm strains in Vietnam.

MATERIALS AND METHODS

Sampling

The research used 15 silkworm strains including 10 bivoltine white cocoon silkworm varieties from India, South Korea, China, and Vietnam (Bivoltine *Bombyx mori* eggs require a winter dormancy period before hatching, which limits them to producing two generations of larvae per year). Five multivoltine yellow cocoon silkworm varieties native to Vietnam (these are multivoltine silkworms found exclusively in tropical regions. Their eggs typically hatch within 9 to 12 days, enabling up to eight generations of larvae per year). The characteristics of the silkworms and cocoons, as well as their origins, are described in Table 1. The silkworm samples were collected at the Vietnam Sericulture Research Centre.

Genomic DNA extraction

For each silkworm strain, two pupae were used (1 male, 1 female), and total DNA was extracted from the pupae samples using the Ausubel et al. (1996) method. The basic steps are as follows: grind 100mg of silkworm pupae; add 700 µL cell lysis solution and 20 µL proteinase K; incubate overnight at 56°C; add 4.5 µL RNase (20 mg/mL) and incubate at 37°C for 2-3 hours; add 700 µL phenol : chloroform : isoamyl alcohol (25 : 24 : 1); precipitate DNA with 100% ethanol at 4°C for 24 hours, wash with 70% ethanol, and store in TE buffer (5 mM Tris-HCl, pH 8.0, 0.5 mM EDTA). The extracted total DNA was analyzed by electrophoresis on 1% agarose gel and spectrophotometrically measured at A260/A280 nm wavelengths using the NanoDrop One (Thermo, USA) to evaluate concentration and purity.

PCR amplification and sequencing

The information on the primers used in the amplification and sequencing of mitochondrial COI, COII, Cytb and ND5 genes were shown in Table 2. The annealing temperature was calculated based on the average annealing temperature (Ta) of primers F and R, followed by applying a temperature gradient of ±5°C around the average Ta.

PCR was performed in a 50 µL volume reaction containing 25 µL PCR Master Mix (Thermo), 2.5 µL primer (5 µM), 1 µL total DNA (100 ng/µL), and 21.5 µL deionized water. The PCR thermal cycle included: initial denaturation at 95°C for three minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at T_a°C (Table 2) for 30 seconds, elongation at 72°C for 30 seconds, and a final extension at 72°C for five minutes. PCR products of the expected sizes were observed on the 1% agarose gel. For DNA sequencing the PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's protocol. After purification, the PCR products were sequenced using the ABI-3100 Avant Genetic Analyzer automatic sequencer from the 1st BASE company (Malaysia) according to the Sanger method. Sequencing was performed directly using primers F and R. BioEdit was used to assemble the sequences to create the gene sequence.

Table 1. Some biological characteristics of the silkworm strains studied

Silkworm race	Symbol	Egg	Larva	Cocoon color/shape	Origin
Bivoltine silkworm races					
In 01	I1	Black pink	Marked	White/peanut	India
In 02	I2	Black pink	Plain	White/oval	India
In 03	I3	Black pink	Plain	White/oval	India
B42	VN2	Black green	Plain	White/oval	Vietnam
QĐ7	VN7	Black pink	Plain	White/peanut	Vietnam
A1	TQ1	Black	Plain	White/peanut	China
526	TQ9	Black pink	Marked	White/peanut	China
75 xin	TQ10	Black pink	Sex -limited	White/oval	China
Keumok	HQ2	Black pink	Plain	White/oval	Korea
KoC	HQ4	Black pink	Plain	White/oval	Korea
Multivoltine silkworm races					
Hoang Lien Son	HLS	Yellow	Plain	Yellow/ spindle	Vietnam
Re vang Ha Tinh	RVHT	Yellow	Plain	Yellow/ spindle	Vietnam
Do Son Khoang	ĐSK	Yellow	Marked	Yellow/ spindle	Vietnam
Re vang Thai Binh	RVTB	Yellow	Marked	Yellow/ spindle	Vietnam
Vang Bao Loc	VBL	Yellow	Marked	Yellow/ spindle	Vietnam

Table 2. Information on primers used in the amplification of mitochondrial gene regions

Genes	Primer sequences (5' - 3')	T_a (°C)	Size (bp)	References
COI	F: ATTCAACCAATCATAAAGATATTGG	58	658	Vimala et al. (2020)
	R: TAAACTTCTGGATGTCCAAAAAACA			
Cytb	F: TGGTACTTTACCTCGTTATCG	60	591	Li et al. (2005)
	R: TATGGACCATTACGATCATCAA			
COII	F: ATTGCTTTACCATCATTACG	50	404	Park et al. (2022)
	R: ACAATAGGTATAAAACTATGATTAGC			
ND5	F: CCTAAAATTGAACCTAAAATACT	50	342	Park et al. (2022)
	R: ATCTGGATTTTATTCTAAAGA			

Phylogenetic analysis of mitochondrial gene regions:

The nucleotide sequences of the COI, COII, Cytb, and ND5 genes were processed using the BioEdit 5.0 software program (Hall 1999). Genetic diversity criteria (polymorphic sites, DNA polymorphism) were identified and analyzed using DnaSP V5 software (Librado and Rozas 2009). A phylogenetic tree was constructed using the Neighbor-Joining method with MEGA 11 software, incorporating 1,000 bootstrap replicates (Tamura et al. 2021). Nodes with bootstrap values below 80% were excluded. The genealogical tree analysis was based on approximately 100 reference genes of *B. mori* sequences from the GenBank database (The information on GenBank accession numbers is specifically presented in Table S1).

RESULTS AND DISCUSSION

Polymorphic site and haplotype of mtDNA regions

We sequenced 658 bp of COI, 405 bp of COII, 600 bp of Cytb, and 342 bp of ND5. The sequences of COI, COII, Cytb, and ND5 were submitted to GenBank (Accession numbers COI: PP734319-PP734333; COII: PP738482-PP738496; Cytb: PP738497-PP738511; and ND5: PP738512-PP738526). The nucleotide composition at all four loci, averaged across the 15 studied silkworm strains, shows a higher A+T nucleotide ratio compared to C+G. Notably, at the ND5 locus, the A+T ratio reached 84.49%. The high A+T content of the control region appears to be a phylogenetic characteristic of Insecta, and mutational pressure toward A+T may have influenced this region early in the evolutionary diversification of insects (Zhang and Hewitt 1997). The total nucleotide content of the COI region in 15 silkworm strains studied is 33.12% A, 37.08% T, 15.35% C, and 14.45% G. These results are quite similar to previous studies on the COI gene nucleotide sequencing: the A+T nucleotide ratio was 70.2%, while C+G reached 29.8% in 17 Philippines silkworm strains (Vimala et al. 2020), and the A+T ratio was 70.3%, while C+G was 29.7% in Brazilian silkworm strains (Fassina et al. 2014). The COII region consists of 37.15% A, 35.56% T, 15.56% C, and 11.74% G. The Cytb region contains 33.9% A, 40.27% T, 14.26% C, and 11.57% G, which is consistent with the study by Li et al. (2005), where Cytb gene sequencing of 14 Chinese *Bombyx mori* strains and 2 *B. mandarina* (Moore, 1872) strains showed an A+T nucleotide ratio of 71.07%, while C+G reached 28.93%. The high A+T ratio (58.3%) was also observed when sequencing the Cytb gene of the Tropical Tasar Silkworm *Antheraea mylitta Drury* (Ray et al. 2024). The nucleotide ratio of the ND5 region is 54.23% A, 30.71% T, 11.27% C, and 3.39% G. Previous studies on the complete mitochondrial DNA genome of silkworm strains specifically, and insects in general, have consistently shown a high A+T ratio (Vimala et al. 2020; Alcudia-Catalma et al. 2021; Kim et al. 2021; Perkin et al. 2021).

Using the BLAST software to search for nucleotide similarity of the COI DNA sequence for all 15 studied strains with the gene sequences published on GenBank. The results showed that the nucleotide similarity of the COI gene of the 15 studied strains reached 100% compared to the COI sequence of *B. mori* available on GenBank. This confirms the identity of the amplified gene as the COI gene and the specimens as *B. mori.* Additionally, using the BioEdit software to identify the nucleotide variation positions of the COI gene among the 15 silkworm strains. The results indicated that the COI gene sequence is highly conserved among the studied strains, with 657 out of 658 positions conserved (99.85%) (Figure 1.A). In this study, when comparing 89 *B. mori* COI nucleotide sequences in Genbank with those of the 15 researched silkworm strains, only 7 nucleotide substitution positions were observed. All sequences were divided into 7 haplotypes, with haplotype 1 being the most prevalent, containing 78/89 strains in Genbank and 14/15 of the strains raised in Vietnam (Figure 1.A). This high degree of conservation of the COI gene is also observed in *B. mori* strains worldwide, such us, the study by Fassina et al. (2014) found no significant differences among silkworm varieties from China, India, Japan, and hybrids when sequencing the COI gene. Similarly, Alcudia-Catalma et al. (2021) demonstrated that the BaiyuN strain from China showed no significant variations in the COI gene even after many years of adaptation to the conditions in the Philippines.

The BLAST results showed that the nucleotide similarity of the COII gene of the 15 studied strains reached 99.75-100% compared to the COII sequence of *B. mori* available on GenBank. High conservation is also exhibited at the COII locus, specifically with only one position (283) where a G to A substitution occurs in the COII gene sequence of the 15 studied strains, notably, this substitution occurs in all 5 indigenous Vietnamese (Figure 1.C). When comparing the COII gene nucleotide sequences of the 15 studied silkworm strains with those of 90 *B. mori* strains in the Genbank, 12 nucleotide substitution positions were observed, distributed across 13 haplotypes.

Table S1. Detailed information on the reference GenBank accession numbers for the genes

Gene	Reference GenBank accession numbers of the genes
COI	***Bombyx mori*:** MW158385.1; MW158384.1; MW158382.1; MW158380.1; MW158379.1; MW158375.1; MW158374.1; MW158373.1; MW158372.1; MW158369.1; ON310877.1; ON310876.1; ON310875.1; ON310873.1; ON310869.1; MK295810.1; MN103530.1; MN027269.1; MG797555.1; KP313778.1; KM279431.1; NC002355.1; OK358664.1; OK358660.1; OK358656.1; OK358655.1; OK358653.1; OK358652.1; OK358650.1; OK358644.1; OK358641.1; OK358639.1; OK358637.1; OK358636.1; OK358634.1; OK358633.1; OK358630.1; MW158381.1; MW158376.1; AF149768.1; AB083339.1; MW158378.1; MW158377.1; MK295808.1; KP192478.1; KP244370.1; KP192479.1; KP729110.1; OK323379.1; MZ047130.1; MZ047147.1; MZ047152.1; MZ047155.1; MZ047157.1; MZ047158.1; KJ704318.1; EF514893.1; EU141360.1 MK033728.1; MK033732.1; MK033733.1; MK033735.1; MN634343.1; MN634353.1; MN634354.1; MN626567.1; MN535969.1; MN535971.1; MN535976.1; MN535975.1; MN535977.1; MN535981.1; EU660888.1; JF700139.1; AF248711.1; AF248713.1; AF248715.1; GQ423220.1; GQ423222.1; GQ423230.1; GQ423231.1; JF700137.1; JF700138.1; AF167260.1; AF167279.1; AF167282.1; AF167283.1; AB649189.1; AB649183.1; ***Bombyx mandarina*:** AB737920.1; AB737913; AB737925.1; AB737922.1
COII	***Bombyx mori:*** MW158386.1; MW158385.1; MW158384.1; MW158382.1; MW158375.1; MW158373.1; ON310877.1; ON310876.1; ON310875.1; ON310874.1; ON310873.1; ON310872.1; ON310871.1; ON310870.1; ON310868.1; MK295813.1; MK295812.1; MK295809.1; MK295807.1; MK251837.1; MK246425.1; MW551562.1; MN103530.1; MK613835.1; MG797555.1; AY048187.1; KM279431.1; NC 002355.1; PQ329533.1; PQ329532.1; PQ329531.1; OK358665.1; OK358661.1; OK358659.1; OK358657.1; OK358656.1; OK358655.1; OK358654.1; OK358653.1; OK358652.1; OK358651.1; OK358650.1; OK358649.1; OK358648.1; OK358647.1; OK358646.1; OK358645.1; OK358644.1; OK358643.1; OK358642.1; OK358641.1; OK358640.1; OK358639.1; OK358638.1; OK358637.1; OK358635.1; OK358634.1; MW158381.1; MK295814.1; AF149768.1; GU966626.1; GU966625.1; GU966624.1; GU966623.1; GU966622.1; GU966620.1; GU966618.1; GU966617.1; KM347743.1; AB070264.1; MW551562.1; MW158376.1; OK358658.1; KP729110.1; KP313778.1; MW158378.1; MW158377.1; MK295808.1; KM875545.1; KP192479.1; KP244370.1; MW158380.1; MN027269.1; OK323379.1; MW158374.1; MW158369.1; MK295811.1; MK295810.1; OK358660.1; ***Bombyx mandarina:*** MW960640.1; MK246423.1; Ok589865.1; KT589976.1
Cytb	***Bombyx mori:*** AY343547.1; MW158386.1; MW158385.1; MW158384.1; MW158382.1 ; MW158375.1; MW158374.1; MW158373.1; MW158372.1; MW158370.1; MW158369.1 ON310877.1; ON310876.1; ON310875.1; ON310872.1; ON310870.1; ON310869.1; MK295813.1; MK295809.1; MK295807.1; MK251837.1; MK246425.1; MW551562.1; MN103530.1; MK613835.1; MG797555.1; KM279431.1; NC002355.1; OK358665.1; OK358664.1; OK358661.1; OK358660.1; OK358659.1; OK358657.1; OK358656.1; OK358653.1; OK358652.1; OK358651.1; OK358650.1; OK358649.1; OK358647.1; OK358646.1; OK358644.1; OK358641.1; OK358639.1; OK358636.1; OK358635.1; OK358634.1; OK358633.1; OK358631.1; OK358630.1; OK358629.1; MW158381.1; MW158376.1; MW158371.1; AF149768.1; GU966630.1; GU966628.1; PQ329521.1; PQ329522.1; PQ329533.1; PQ329532.1; PQ329531.1; PQ329530.1; PQ329529.1; GU966626.1; GU966625.1; GU966623.1; GU966622.1; GU966620.1; GU966618.1; GU966617.1; GU966615.1; GU966614.1; GU966611.1; GU966610.1; GU966609.1; GU966608.1; GU966607.1; GU966604.1; GU966603.1; GU966602.1; GU966601.1; GU966600.1; GU966599.1; GU966596.1; MW158380.1; MW158379.1; MN027269.1; OK358632.1; GU966624.1; GU966613.1;GU966612.1; GU966605.1; MW158378.1; MW158377.1; KM875545.1; MK295808.1; KP192479.1; OK358663.1; OK358662.1; OK358658.1; KP192478.1; KP729110.1; KP313778.1; OK323379.1; KP244370.1; AY048187.1; ***Bombyx mandarina:*** Ok589865.1 MG604734.1 MW96960642 KT589976.1
ND5	***Bombyx mori:*** MW158382.1; MW158386.1; MW158385.1; MW158384.1; MW158382.1; MW158380.1; MW158379.1; MW158378.1; MW158377.1; MW158375.1; MW158374.1; MW158373.1; MW158372.1; MW158370.1; ON310877.1; ON310876.1; ON310875.1; ON310874.1; ON310873.1; ON310872.1; ON310870.1; ON310868.1; MK295813.1; MK295812.1; MK295811.1; MK295810.1; MK295809.1; MK295808.1; MK295807.1; MK251837.1; PQ329531.1; PQ329530.1; PQ329518.1; PQ329519.1; PQ329520.1; MK246425.1; MW551562.1; MN103530.1; MN027269.1; MK613835.1; MG797555.1; AY048187.1; KP192479.1; KP244370.1; KM279431.1; NC 002355.1; OK323379.1; OK358665.1; OK358664.1; OK358663.1; OK358662.1; OK358660.1; OK358659.1; OK358658.1; OK358657.1; OK358656.1; OK358655.1; OK358654.1; OK358653.1; OK358652.1; OK358651.1; OK358650.1; OK358649.1; OK358648.1; OK358647.1; OK358646.1; OK358644.1; OK358643.1; OK358642.1; OK358641.1; OK358640.1; OK358639.1; OK358638.1; OK358637.1;OK358636.1; OK358645.1; OK358635.1; OK358634.1; OK358633.1; OK358632.1; OK358630.1; OK358629.1; MW158381.1; MW158376.1; MW158371.1; MK295814.1; AF149768.1; GU966630.1; GU966628.1; GU966626.1; GU966624.1; GU966623.1; GU966622.1; GU966620.1; GU966618.1; GU966617.1; GU966615.1; GU966614.1; GU966613.1; OK358631.1; KP729110.1; KM875545.1; KP313778.1 ***Bombyx mandarina:*** MZ982840.1; MK251840; MK251838.1; Mk251839

Of these, haplotype 12 is the most common, containing 71/90 of *B. mori* strains in the world and 10/15 of the white cocoon strains raised in Vietnam (Figure 1.C). Haplotype 13 contains only the 5 indigenous yellow cocoon Vietnamese silkworm strains (Figure 1.C). In their research on native Korean silkworm strains, Park et al. (2022) also demonstrated that SNPs in the COII gene can be used to distinguish the Sun7ho silkworm strain.

Similar to the COI gene, the lowest nucleotide polymorphism occurs at the ND5 locus. When comparing the ND5 nucleotide sequence between the 15 studied *B. mori* strains and 100 *B. mori* strains in the Genbank, only 3 substitution positions are observed (Figure 1.B). However, notably, all 5 indigenous Vietnamese silkworm strains have a nucleotide substitution at position 103 (C to T) (Figure 1.B). This particular feature was also noted by Park et al. (2022), who used the T to C substitution to distinguish the domestic silkworm strain endemic to Korea (Sammyeonhonghoeback). Therefore, the ND5 gene nucleotide sequence can be used to identify native silkworm strains in Vietnam.

High polymorphism was detected at the Cytb locus. The nucleotide similarity of the Cytb locus in the 15 studied silkworm strains compared to the silkworm strains published on GenBank ranged from 99,17-100%. When comparing the Cytb nucleotide sequences of the 15 silkworm strains in Vietnam, 7 nucleotide substitution sites were found (Figure 1.D). Furthermore, when comparing the Cytb gene nucleotide sequences of these 15 silkworm strains with 108 strains in the GenBank, 24 nucleotide substitution sites were identified, and no indel positions were observed (Figure 1.D). This result is consistent with the study by Ray et al. (2024), which showed 50 variable bp and 11 singleton sites in the Cytb gene sequences of the Tropical Tasar Silkworm *Antheraea mylitta* Drury strains.

Phylogenetic analysis based on COI, COII, ND5 and Cytb sequences

Phylogenetic trees were constructed using Maximum Likelihood methods based on the sequence data of COI, COII, Cytb, and ND5 from 15 strains of silkworms raised in Vietnam. These were compared with approximately 100 nucleotide sequences of *B. mori* strains (nucleotide sequences that are identical have been classified into the same haplotype). Additionally, the wild silkmoth, *B. mandarina*, is believed to be the ancestor of *B. mori* because these two species can interbreed and produce fertile hybrid offspring.

	0	1	1	2	3	5	5
	7	1	6	7	3	7	8
	4	7	6	7	5	9	7
Haplotype 1 (n=76); RVTB; DSK; HSL; VBL; RVHT; IN02; IN03; VN02; VN07; TQ01; TQ09; TQ10; HQ02; HQ04	G	G	A	T	C	A	G	T	C	C	C
Haplotype 2 (n=7); IN01	.	.	.	.	.	G	.	.	.	.	.
Haplotype 3 (n=2)	.	.	.	.	.	.	.	.	.	T	.
Haplotype 4 (n=1)	.	.	.	.	.	.	A	.	.	.	.
Haplotype 5 (n=1)	.	.	.	.	.	.	.	.	T	.	.
Haplotype 6 (n=1)	.	.	.	.	T	.	.	.	.	.	.
Haplotype 7 (n=1)	.	.	.	.	.	.	.	C	.	.	T

A

	1	2	3
	0	4	3
	3	2	1
Haplotype 1 (n=96); HQ2; HQ4; In01; In02; In03; TQ1; TQ9; TQ10; VN02; VN07	C	C	T
Haplotype 2 (n=1)	.	T	.
Haplotype 3 (n=3)	.	.	C
Haplotype 4 ĐSK; HLS; RVHT; RVTB; VBL	T	.	.

B

	0	0	0	0	0	1	1	1	2	2	2	3
	5	5	5	6	7	5	7	7	1	2	8	9
	1	2	5	2	5	0	4	8	6	7	3	7
Haplotype 12 (n=71); IN01; IN02; IN03;VN02; VN07 HQ02; HQ04; TQ01; TQ09; TQ10	C	C	C	C	C	C	C	C	C	G	G	C
Haplotype 1 (n=1)	T	.	.	.	.	.	T	.	T	.	.	.
Haplotype 2 (n=1)	T	.	.	.	.	.	.	.	T	.	.	.
Haplotype 3 (n=3)	.	T	T	.	.	.	.	.	.	.	.	.
Haplotype 4 (n=1)	.	.	T	.	.	.	.	.	.	.	.	T
Haplotype 5 (n=1)	.	.	T	T	.	.	.	.	.	.	.	T
Haplotype 6 (n=6)	.	.	.	.	T	.	.	.	.	.	A	.
Haplotype 7 (n=1)	.	.	.	.	.	T	.	.	T	.	.	.
Haplotype 8 (n=1)	.	.	.	.	.	.	.	T	.	.	.	.
Haplotype 9 (n=3)	.	.	.	.	.	.	.	.	T	.	.	.
Haplotype 10 (n=1)	.	.	.	.	.	.	.	.	T	A	.	.
Haplotype 11 (n=1)	.	.	T	.	.	.	.	.	.	.	.	T
VBL; RVHT; RVTB; HLS; DSK	.	.	.	.	.	.	.	.	.	.	A	.

C

	0	0	0	1	1	1	1	2	2	2	3	3	3	4	4	4	4	5	5	5	5	5	5	5
	3	5	5	1	1	4	5	9	0	0	2	3	3	7	0	2	4	8	0	1	3	4	4	6	8
	3	1	6	8	0	6	3	0	6	8	0	1	9	2	9	0	4	1	7	4	3	9	6	7
Haplotype pe10 (n=82); In03; TQ01; TQ09; TQ10; HQ02; HQ04; VN02; VN04	A	C	C	G	T	T	T	G	A	C	C	T	G	C	G	T	T	A	G	C	C	T	T	T
Haplotype pe1 (n=1)	.	.	.	.	C	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
Haplotype pe2 (n=1)	.	.	.	.	.	C	.	.	T	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
Haplotype pe3 (n=1)	.	.	.	.	.	C	.	.	T	.	C	.	.	.	.	.	.	G	.	.	.	.	.	.
Haplotype pe4 (n=2)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Haplotype pe5 (n=1)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
Haplotype pe6 (n=2)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.
Haplotype pe7 (n=2)	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	T	.	.	.	.
Haplotype pe8 (n=3)	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
Haplotype pe9 (n=8)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
Haplotype pe 11 VBL	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	.	.	.
Haplotype pe 12 DSK	.	.	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	A	T	T	.	.	.
Haplotype pe 13 HLS; RVHT	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	.	.	.
Haplotype pe 14 RVTB; In01	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Haplotype pe 15 In02	.	.	.	C	.	.	A	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
Haplotype pe 16 (n=1)	.	T	.	.	C	.	.	.	T	.	.	.	.	.	G	.	.	.	.	T	.	.	T	.	A
Haplotype pe 17 (n=1)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	G	.	A	.	T	.	C	.	.
Haplotype pe 18 (n=1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.
Haplotype pe 19 (n=1)	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Haplotype pe 20 (n=1)	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

D

Figure 1. The first column signifies the symbols of the studied samples and the haplotypes representing silkworm strains worldwide (n: is the number of silkworm strains in GenBank that grouped into that haplotype). Vertically oriented numbers indicate the variable site's position. Dot (.) signifies the identity of the reference haplotype, while different base letters represent nucleotide substitutions. Figure A. COI; B. ND5; C. COII; and D. Cytb. n: is the number of *Bombyx mori* strains with identical nucleotide sequences (information about these reference GenBank accession numbers is presented in Table 1 in the appendix)

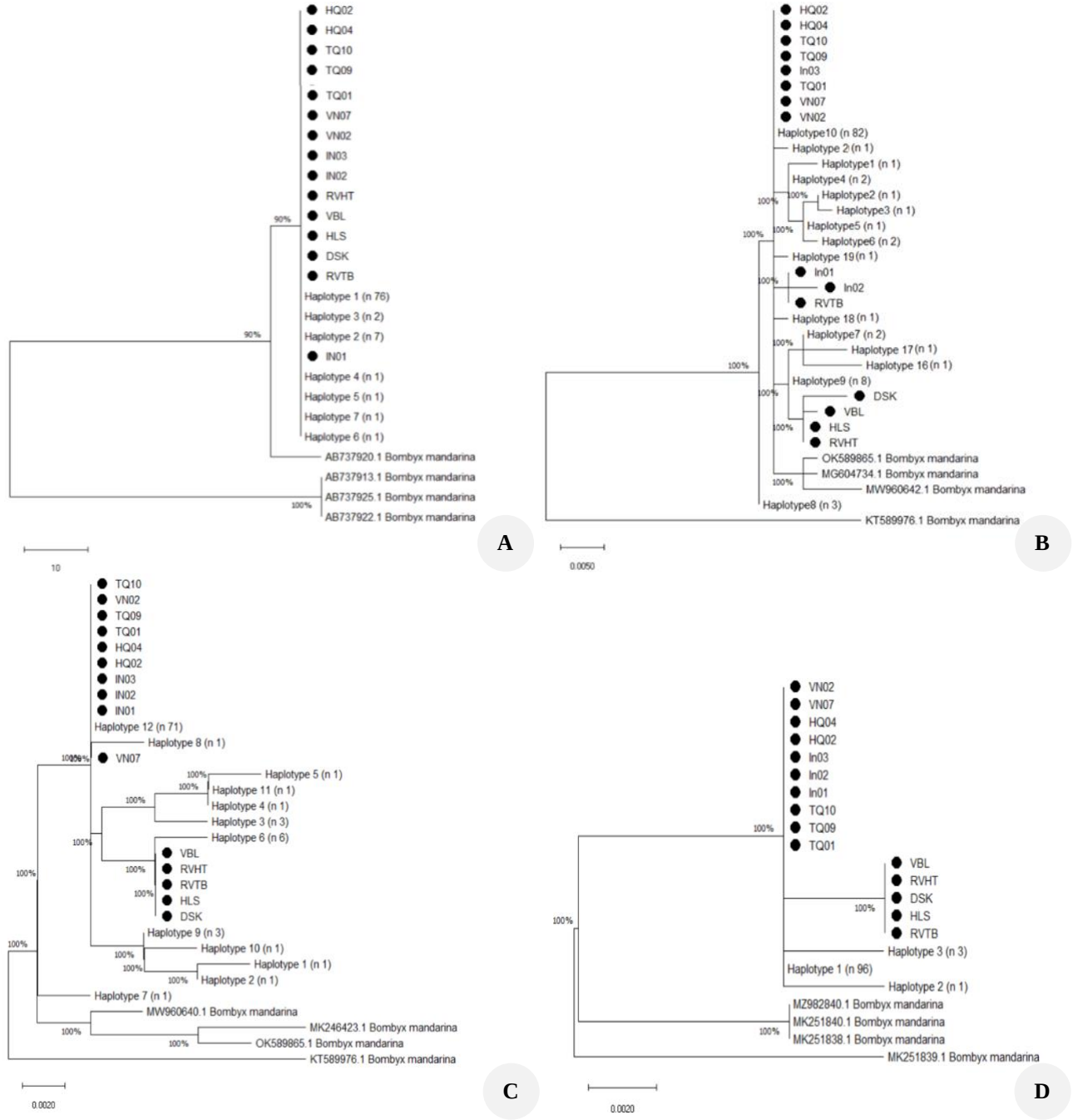

Figure 2. The phylogenetics relationship between the 15 strains of silkworms raised in Vietnam and other GenBank accessions of *B. mori* and *B. mandarina*. It was generated by the neighbor-joining method using MEGA 11 (Tamura et al. 2021) with bootstrap values for interior clades after 1,000 replications. Figure A. COI; B. Cytb; C. COII; and D. ND5. n: is the number of silkworm strains in GenBank that grouped into that haplotype (information about these reference GenBank accession numbers is presented in Table 1 in the appendix)

Bombyx mandarina exhibits various variations within the species (Yukuhiro et al. 2002). *B. mandarina* inhabiting China (Chinese mandarina) has 28 pairs of chromosomes, similar to *B. mori*. Several studies have suggested that *B. mandarina* might be a close relative of *B. mori* (Hwang et al. 1999; Yukuhiro et al. 2002). Therefore, the nucleotide sequence of *B. mandarina* was used as an outgroup (COI: AB737920.1; AB737913; AB737925.1; AB737922.1; COII: MW960640.1; MK246423.1; Ok589865.1; KT589976.1; Cytb: Ok589865.1 MG604734.1 MW96960642 KT589976.1; ND5: MZ982840.1; MK251840; MK251838.1; Mk251839).

Figure 2.A illustrates the phylogenetic tree based on the COI gene nucleotide sequences of the 15 studied silkworm strains, along with 89 *B. mori* strains and 4 *B. mandarina* strains from GenBank. The results show that 14 out of the 15 silkworm strains in Vietnam are classified into the same group, which is common group of silkworm strains worldwide (78/89 reference strains), IN01 appearing more

distantly related than the others. This result aligns with previous studies that demonstrated low polymorphism at the COI locus in *B. mori* strains (Fassina et al. 2014; Vimala et al. 2020). The results in Figure 2.A also show that the 15 studied silkworm strains and the 89 *B. mori* strains are all grouped into a single large clade, which is distinct from *B. mandarina*. Therefore, the COI gene nucleotide sequence can be used to identify *B. mori* from other species.

The Figure 2.B shows a phylogenetic tree based on the nucleotide sequence of the Cytb gene of 15 silkworm strains in Vietnam and 108 *B. mori* strains, 4 *B. mandarina* strains from GenBank. The results indicate that the 15 silkworm strains in Vietnam are divided into three groups. The first group includes 8 bivoltine white cocoon strains, which are grouped into haplotype 10, the most common haplotype (82/108 reference strains). The second group includes two bivoltine white cocoon strains (IN01, IN02) and one native multivoltine yellow cocoon strain from Vietnam (RVTB). The third group includes four native multivoltine yellow cocoon strains from Vietnam. These results indicate that the nucleotide sequence of the Cytb locus can distinguish multivoltine yellow cocoon native Vietnamese strains, although it does not yet differentiate bivoltine white cocoon strains.

Figures 2.C and 2.D show phylogenetic trees based on the nucleotide sequences of the COII gene (Figure 2.C) and ND5 gene (Figure 2.D) of 15 silkworm strains raised in Vietnam and nearly 100 *B. mori* strains, 4 *B. mandarina* strains from GenBank. The results show that all bivoltine white cocoon strains are grouped into the same cluster, a common classification of silkworms worldwide, while all 5 native multivoltine yellow cocoon strains from Vietnam are grouped into a different cluster. These findings are consistent with the study by Park et al. (2022), indicating that SNPs at the COII and ND5 loci can be used to identify certain Korean native silkworm strain.

In conclusion, The *B. mori* silkworm strains are maintained at the Department of Silkworm Breeding, Vietnam Sericulture Research Center, and have been studied for several biological characteristics. To provide genetic information about these *B. mori* strains, the research sequenced the COI, COII, ND5, and Cytb genes, which are part of the mitochondrial DNA (mtDNA). COI and Cytb are standard genes commonly used to assess species diversity and can distinguish between *B. mori* and B. *mandarina*. Therefore, this study aimed to investigate whether these genes could differentiate various *B. mori* strains in Vietnam. The COII and ND5 genes were also used because they are employed in molecular phylogenetics of closely related species and serve as SNP markers to identify certain native silkworm strains. The COI, COII, and ND5 genes showed minimal sequence variation in the 15 *B. mori* strains, and the nucleotide variations at these positions were insufficient to distinguish between the silkworm strains. However, the COII and ND5 gene sequences could be used to differentiate 5 low-yielding yellow cocoon strains (multivoltine) with 10 high-yielding white cocoon strains (bivoltine). The Cytb gene sequence exhibited higher diversity compared to the other three genes across all 15 silkworm varieties in this study and 108 strains with published sequences on GenBank.

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