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Genetic diversity of black soldier flies in Vietnam based on DNA COI sequence

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Abstract. *Nguyen TN, Tran TBN, Tran HN. 2023. Genetic diversity of black soldier flies in Vietnam based on DNA COI sequence. Biodiversitas 24: 6727-6732.* The Black Soldier Fly (BSF), *Hermetia illucens* (Linnaeus, 1758), is found in temperate and tropical regions of the world. In recent years, BSF has been cultivated as fodder in some areas of Vietnam. The genetic diversity of BSF in Vietnam has not yet been assessed. This study aimed to investigate the nucleotide diversity in COI sequences of BSF in Vietnam. We examined the COI nucleotide sequences of 22 BSFs using flies or larval tissue samples for DNA extraction and subsequent sequencing. Our analysis showed that the nucleotide composition of BSF consisted of 25.36% A, 36.04% T, 21.25% C, and 17.35% G, with a significant prevalence of A+T nucleotides of 61.40% compared to 38.60% for G+C nucleotides. Among 22 individual BSFs samples collected from ten provinces and cities, spanning seven agro-ecological regions in Vietnam, we identified 9 different haplotypes, which were distributed in five clades but mainly clustered in two main clades: E and F. Clade E, prominently represented by branch E, showed a higher abundance in Asia, while Clade F has been identified in Europe, Uganda, South Korea, Oceania and North America. Our results highlight the relatively high genetic diversity of the Vietnamese BSF population. In addition, the COI nucleotide sequences of these Vietnamese BSF samples were similar to those of Asian and Australian countries, showing significant similarities to the Palearctic and Oriental regions. These results provide valuable insights to inform BSF breeding and development in Vietnam.

Keywords: Black soldier fly, DNA-COI sequence, genetic diversity

Abbreviations: BSF: Black Soldier Fly, COI: Cytochrome c Oxidase subunit I

INTRODUCTION

Black Soldier Fly (BSF), *Hermetia illucens* (Linnaeus, 1758), are large flies, as adult individuals can reach 8mm~16mm in length depends on the type of diet (Gobbi and Rojo 2013). BSF reaches maturity with a lifespan ranging from 12 to 17 days. They do not consume anything other than water, avoid approaching humans, do not bite or sting, and do not transmit diseases (Shaphan 2019). Essential factors for their environment include access to water and nutrients. Adult BSF individuals lay eggs in locations near water sources and food, ensuring a suitable environment for the hatching and development of the larvae with an adequate food supply. The BSF is known to live in the natural habitat characterized by a temperature range of 25-32°C and humidity levels between 70-80% (Tomberlin et al. 2009; Holmes et al. 2012). BSF's larvae are very efficient at breaking down a wide range of decaying organic matter, including manure, food waste, agricultural by-products, organic leachate, and plant and animal remains (Tomberlin and van Huis 2020). BSF larvae can be reared on most organic materials and can be used as a source of animal feed, and its use in agriculture and waste management is of increasing interest worldwide (Spranghers et al. 2017; Biasato et al. 2019; Heuel et al. 2021).

It has been reported that BSF probably originated in Mexico, then spread to the north and south America over the last thousand years (Guilliet et al. 2022), however the earliest specimen has been found in the southern United States by the 18th century (Marshall et al. 2015; Khamis et al. 2020; Guilliet et al. 2022). Today, the species is cultivated internationally and found in tropical and subtropical regions (Marshall et al. 2015; Rhode et al. 2020; Ståhls et al. 2020). There have been records of BSF breeding in tropical Africa, such as South Africa in 1915 and Madagascar in 1930, or near northern territories like Malta in 1926 and Spain, Italy, and France in the 1960s. In Asia, the first record was in Malaysia in 1940, but in China, the BSF was not raised until 1960. It was recorded in Australia and neighboring countries in the 1940s (Marshall et al. 2015). The marketing of BSF larvae and adults on a global scale, as well as the mixing of BSF genetic resources, made the identification of the original genetic source and its development challenging (Rhode et al. 2020; Ståhls et al. 2020; Guilliet et al. 2022).

Therefore, to date, research has mostly focused on nutritional claims (Van Huis 2013; Spranghers et al. 2017; Biasato et al. 2019; Schiavone et al. 2019; Hoc et al. 2020; Heuel et al. 2021) or the functional role of the gut microbiota in the bioconversion performance of BSF (Khamis et al. 2020; Eke et al. 2023). It is also reported that BSF has

found applications in waste management (Surendra et al. 2020) and environmental management tools (Lalander et al. 2015; Gold et al. 2018; Giannetto et al. 2020). Fewer studies have been conducted using molecular markers to examine BSF's genetic diversity. Therefore, there is a need to determine the genetic lineage and genomic variation of BSF collected locally. In particular, BSF is receiving increasing attention because it is a species with high economic value and global distribution (Rhode et al. 2020). Although BSF is still considered a biological species, it has very high intraspecific variability and contains many different types and genetic groups (Ståhls et al. 2020; Guilliet et al. 2022). A genealogical origin and traceability study using the entire mitochondrial genome nucleotide sequence of 60 strains worldwide showed that BSF has an origin of about 2.8 million years, starting from the Isthmus region of Panama (O'Dea et al. 2016) and North America about 2 million years, from there it spread globally (Guilliet et al. 2022). Up to 52 haplotypes were found, including 10 major haplotypes, indicating that BSF has a very high and complex genetic diversity in forming a global genetic network (Khamis et al. 2020; Ståhls et al. 2020; Guilliet et al. 2022).

DNA barcoding is a method of species identification involving the sequencing of a short fragment of the mitochondrial COI gene. The COI barcode region is relatively conserved at the intraspecific level and sufficiently variable to detect genetic divergence gaps that delineate interspecific boundaries helping to differentiate between divergent phylogenetic lineages within conventionally recognised morphospecies. Animal DNA barcoding focuses primarily on the mitochondrial COI gene, as COI often varies from population to higher taxonomic level (Hebert et al. 2003). Sequencing the 5'-mitochondrial COI gene has become the most widely used approach (http://www.boldsystems.org/). COI sequence data provide accurate estimates of species richness using either pre-set thresholds (cluster) or inference from the pre-set dataset (Hendrich et al. 2010). For BSF, the sequencing of the COI gene has been some research used to assess genetic diversity and phylogenetic analyses (Park et al. 2017; Qi et al. 2017; Khamis et al. 2020; Ståhls et al. 2020; Ferdousi et al. 2021; Guilliet et al. 2022). So far, registered in the Gene Bank (GenBank), there is only one nucleotide sequence of the COI gene in a standard sample (voucher) collected from the BSF cultured in Vietnam and identified as belonging to the Oriental group (Ståhls et al. 2020). Therefore, the objective of this study was to evaluate the genetic diversity of the BSF collected in Vietnam, supply breeders with information, and inform managers about the diversity of BSF.

MATERIALS AND METHODS

Sampling

We have selected BSF from 10 provinces and cities namely Son La, Hai Duong, Hai Phong, Phu Tho, Da Nang, Quang Nam, Can Tho, Dak Lak, Dong Nai, and Ho Chi Minh. These 10 locations are selected based on their representativeness to the seven agro-ecological regions in Vietnam. Detailed information on samples and GenBank accession numbers is presented in Table 1.

Samples of BSF were collected in the natural environment at seven agro-ecological regions of Vietnam, including Northwestern region (Son La), Northeastern region (Phu Tho), Red River Delta region (Hai Phong, Hai Duong), South Central region (Da Nang, Quang Nam), Central Highlands region (Dak Lak), Southeastern region (Dong Nai, Ho Chi Minh City), Southwestern region (Can Tho). BSF sampling covered seven ecological regions in Vietnam to ensure a comprehensive assessment of genetic diversity. Each region is characterized by distinct climatic and soil conditions. For detecting more genetic variations, we tried to ensure the gene pool be adequately sampled is possible. The samples were collected in October 2011 (autumn) and July 2012 (summer), because the temperature and humidity in autumn and summer in Vietnam is closest to the natural habitat of BSF. Regardless of the climat, BSF populations remained relatively low in the natural environment. Adult BSFs were trapped using nets and collected in mesh insects. During transportation to the laboratory, adult BSFs were preserved in 96% alcohol at 4°C. They were then stored at -20°C before DNA extraction for molecular characterization.

The origin of all COI sequences used in our study is summarized in Table 2.

Table 1. Sample information

Sample code	Sampling site	GenBank accession numbers assigned
Hill-SL-VN	Son La Province, Northern Vietnam	OP164686
Hill-HD-VN	Hai Duong Province, Northern Vietnam	OP164687
Hill-PT1-VN	Phu Tho Province, Northern Vietnam	OR646558
Hill-PT2-VN		OR646559
Hill-HP1-VN	Hai Phong City, Northern Vietnam	OR646551
Hill-HP1-VN		OR646552
Hill-DNN-VN	Da Nang City, Central Vietnam	OP164688
Hill-DNN2-VN		OR646555
Hill-DNN3-VN		OR646556
Hill-DNN4-VN		OR646557
Hill-QN1-VN	Quang Nam Province, Central Vietnam	OP164689
Hill-QN2-VN		OP164690
Hill-QN3-VN		OP164691
Hill-DL-VN	Dak Lak Province, Central Vietnam	OP164692
Hill-DL2-VN		OR646553
Hill-DL3-VN		OR646554
Hill-DN1-VN	Dong Nai Province, Southern Vietnam	OP164693
Hill-DN2-VN		OP164694
Hill-HCM1-VN	Ho Chi Minh City, Southern Vietnam	OR646549
Hill-HCM2-VN		OR646550
Hill-CT1-VN	Can Tho Province, Southern Vietnam	OP164696
Hill-CT2-VN		OP164697

Table 2. Reference samples

Haplotype	Country of collection	Accession number	References
Palaearctic	Voucher/ United States	KC192965	GenBank
Oriental 2	Voucher/Singapore	MT186669	Ståhls et al. 2020
East Palaearctic	Voucher/United States	MT181122	Ståhls et al. 2020
Palaearctic	Voucher/Poland	MT178512	Ståhls et al. 2020
East Palaearcti	Voucher/China	MT178511	Ståhls et al. 2020
Palaearctic	Uganda	MT520680	Khamis et al. 2020
East Palaearctic	United States	MT520672	Khamis et al. 2020
Neotropical	Nigeria	MT520662	Khamis et al. 2020
East Palaearcti	South Africa	MT181122	Khamis et al. 2020
East Palaearctic	Kenya	MT483940	Khamis et al. 2020
East Palaearctic	Australia	MT483921	Khamis et al. 2020
Oriental 1	Voucher/Malaysia	MT178479	Ståhls et al. 2020
Neotropical	Voucher/Peru	MT178472	Ståhls et al. 2020
Neotropical	Voucher/Bolivia	MT178476	Ståhls et al. 2020
Neotropical	Voucher/Kenya	MT483929	Ståhls et al. 2020
Oriental 2	Voucher/United States	LR792226.	Ståhls et al. 2020
Oriental 2	Voucher/Thailand	MT178506	Ståhls et al. 2020
A	Voucher/Vietnam	MT178480	Ståhls et al. 2020
Palaearctic	Switzerland	LR792262	GenBank
Palaearctic	South Korea	FJ794401	GenBank
Oriental 2	Australia	LR792254	GenBank
H	Paraguay	LR792241	GenBank
H	Brazil	LR792236	GenBank
H	Bolivia	LR778193	GenBank
A	Zambia	LR778156	GenBank
A	Bolivia	LR778195	GenBank
A	Madagascar	LR778154	GenBank
E	Bhutan	LR778159	GenBank
E	Australia	HM399363	GenBank
D	Mexico	LR778208	GenBank
F	Switzerland	LR792262	GenBank
F	Switzerland	LR812715	GenBank
C	Kenya	LR792260	GenBank

Genomic DNA extraction

BSF samples were collected from North, Central, and South Vietnam and 10 provinces and cities described above. The number of samples, sample locations, and GenBank accession numbers are shown in Table 1. The origin of all COI barcodes obtained from GenBank (ncbi.nih.gov) and used for analysis in our study is summarized in Table 2. Genomic DNA was extracted from 22 samples from 10 provinces and cities in Vietnam. Ethanol-preserved BSF were used for DNA extraction with the Thermo Scientific GeneJET Genomic DNA Purification Kit. The quantity and quality of genomic DNA were checked with a UV spectrophotometer and agarose gel electrophoresis. DNA samples were stored at -20°C until analysis.

PCR amplification and sequencing

The primer sequences used for COI amplification were as follows: HILC1 F: 5'TTTCAACAAATCATAAAGATA TTGG 3' HILC1 R: 5' AGATATAAACTTCTGGGTGTCC 3'. This primer pair was designed based on the insect COI-barcoded primer pair LCO1490/HC02198 (Folmer et al. 1994). PCR was performed in a 50 µL reaction containing 25 µL PCR master mix (Thermo Scientific), 10 pM primers, 100 ng genomic DNA, and 1.5 µL DMSO. The reaction mixture was placed in a DNA thermal cycler. In PCR amplification, an initial denaturation at 94°C for one minute was followed by 35 cycles of denaturation at 94°C for one minute, annealing at 48°C for one minute, extension at 72°C one minute, and a further extension of 72°C for seven minutes. PCR products were purified for DNA sequencing using a GeneJET PCR Purification Kit (Thermo Scientific). Purified PCR products were used for sequencing with ABI (3100 Genetic Analyzer, Applied Biosystems).

Phylogenetic analysis of COI sequences

The analysis was performed using the following methods. Nucleotide sequences were identified using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). Multiple nucleotide alignments were performed using BioEdit (Hall 1999). Diversity parameters, including nucleotide diversity haplotypes, the number of polymorphic sites, the number of haplotypes, and haplotype diversity of all sequences, were estimated using DnaSP v.6 software (Rozas et al. 2017). A Neighbor-Joining (NJ) phylogenetic tree with a bootstrap value of 1,000 replicates was performed using MEGA version X (Tamura et al. 2021). The phylogenetic tree analysis was constructed based on Ståhls et al. (2020) and Guilliet et al. (2022) classifications with the COI reference sequences listed in Table 2 of the GenBank database. closely related species was inferred using the Maximum Likelihood method by MEGA X (Kumar et al. 2018).

RESULTS AND DISCUSSION

Polymorphic site and haplotype of COI

Moreover, 22 COI sequences with a total length of 658 bp were submitted to GenBank (accession numbers are listed in Table 1). A comparison of the 22 nucleotide sequences of the COI gene in BSF revealed that there were 39 sites of nucleotide diversity, including 35 parsimony-informative sites, three single variable sites (Figure 2), and one parsimony-informative site (Figure 2). The nucleotide composition of all haplotypes was 25.36% A, 36.04% T, 21.25% C, 17.35% G, 61.40% A+T, and 38.60% G+C. Our results are consistent with a study conducted across 600 BSF personnel from 39 countries (Ståhls et al. 2020). Their study looked at a sequence of 658 nucleotides and found that A+T was 61.7%, while C+G comprised 38.3%. The A+T haplotype was significantly more common than the G+C haplotype. This result is consistent with another study (Wirth et al. 1999), which showed that most insect COI gene sequences are A- and T-rich. The study used BFS references in clade B (FJ794367.1) to compare BSF nucleotide sequences collected in Vietnam, as clade B is mostly found in Asia (Guilliet et al. 2022). Nucleotide variations between the BSF COI sequences and the representative sequence (FJ794367.1) are shown in Figure 2. The results show that the average percentage of polymorphic sites was 5.93% (39/658). Furthermore, for 39 sequence variations, all these nucleotide substitutions were identified.

The results of using DnaSP software to determine the haplotype on 658 nucleotides of 22 COI sequences of BSF revealed that the 22 nucleotide COI sequences from black soldier flies were classified into 9 distinct haplotypes. Among these, haplotype 1 comprised 8 samples, while haplotypes 2 and 7 each contained 3 samples. Haplotypes 5 and 2 were each represented by 2 samples, whereas haplotypes 4, 6, 8, and 9 consisted of only one sequence each. The difference in nucleotide sequence between the haplotypes of the BSF is shown in Figure 1. The Haplotype diversity index (Hd) of the mitochondrial COI region of the BSF samples collected in Vietnam had relatively high genetic diversity (Hd: 0.858).

Phylogenetic trees were constructed using Maximum Likelihood methods and were constructed using 22 COI sequences along with 33 reference sequences that corresponded to the main clades defined by previous studies (Khamis et al. 2020; Ståhls et al. 2020; Guilliet et al. 2022) (Figure 2).

Figure 2 shows that 11 BSF samples classified as clade F were collected from different provinces, including Da Nang, Phu Tho, Dak Lak, Can Tho, Quang Nam, Son La, Hai Duong, and Hai Phong. These specimens belong to the following ecological regions: Northwestern region (Son La), Northeastern region (Phu Tho), Red River Delta region (Hai Phong, Hai Duong), South Central region (Da Nang, Quang Nam), Central Highlands region (Dak Lak), Southwestern region (Can Tho) which is similar to BSF found in Europe, Uganda, and South Korea. Furthermore, another 6 samples belong to the clade E collected from Hai Phong, Da Nang, and Quang Nam also frequently observed in Asia and Australia. Additionally, three samples were assigned to clade A collected from Ho Chi Minh, Dak Lak, and Dong Nai. One BSF sample from Phu Tho Province was classified as clade C (Figure 2).

Clade A and C were the most common and were found on all continents except South America, where clade C was absent. Clade A is the most common haplotype of wild BSF in France and Europe. Only one specimen collected from Ho Chi Minh City was classified as clade B, identified in BSF from Europe and Asia (Guilliet et al. 2022). The distribution of genetic diversity of BSF samples collected in Vietnam is spread across most ecological regions (each has different climatic conditions, weather conditions, and agricultural practices). Even within the same region, there is great genetic diversity. This is shown in the same sample collection in Hai Phong, Dong Nai, and Quang Nam provinces where the sample population belongs to both clades F and E. Or more specifically, the sample population collected in Dong Nai but is distributed in branches F, E, A. This may indicate that the ecological conditions of Vietnam do not greatly affect the genetic diversity of BSF; the genetic closeness of BSF collected in Vietnam compared to Europe, Uganda, and South Korea is more reasonable. This genetic diversity implies the possibility of foreign BSF species entering Vietnam through global trading.

	0	0	0	0	0	0	0	1	1	2	2	2	2	3	3	3	3	3	3	4	4	4	4	4	4	4	5	5	5	5	5	5	6	6	6	6	6
	0	2	3	4	5	7	8	6	6	0	0	6	9	1	1	6	7	7	8	0	3	3	5	6	9	9	1	1	2	3	5	6	6	2	2	3	3	3	4
	1	2	4	0	8	9	2	3	6	5	8	3	5	3	6	4	0	3	5	6	3	6	1	6	0	6	7	8	6	8	3	2	5	5	8	1	4	7	9
FJ794367.1 class 1B	T	A	T	G	T	T	T	A	G	C	A	T	C	C	A	T	T	A	C	C	G	T	A	A	T	A	A	A	A	C	T	T	G	A	A	A	T	C	A
Haplotype 1 (1)	T	A	T	G	T	T	T	A	G	C	A	T	C	C	A	T	T	A	C	C	G	T	A	A	T	A	A	A	A	C	T	T	G	A	A	A	T	C	A
Haplotype 2 (8)	T	.	.	A	.	C	C	T	A	.	G	C	A	T	G	C	A	.	.	T	.	.	T	.	C	.	G	G	.	T	C	C	A	G	.	T	C	.	.
Haplotype 3 (2)	A	.	.	A	.	C	C	T	A	.	G	C	A	T	G	C	A	.	.	T	.	.	T	.	C	.	G	G	.	T	C	C	A	G	.	T	C	.	.
Haplotype 4 (3)	.	.	C	A	C	G	C	T	A	A	G	C	A	T	.	C	A	.	.	T	.	.	T	.	C	.	G	G	.	T	.	C	A	.	G	T	C	A	G
Haplotype 5 (1)	.	.	.	A	.	A	C	T	A	.	G	C	A	T	G	C	A	.	.	T	.	.	T	.	C	.	G	G	.	T	C	C	A	G	.	T	C	.	.
Haplotype 6 (2)	.	.	C	A	C	A	C	T	A	A	G	C	A	T	.	C	A	.	.	T	.	.	T	.	C	.	G	G	.	T	.	C	A	.	G	T	C	A	G
Haplotype 7 (3)	A	G	.	A	C	.	C	T	A	.	G	C	A	T	.	C	A	G	T	T	A	C	T	.	.	G	G	G	G	T	.	.	A	G	.	T	C	.	.
Haplotype 8 (1)	A	.	C	A	C	.	C	T	A	A	G	C	A	T	.	C	A	.	.	T	.	.	T	.	C	.	G	G	.	T	.	C	A	.	G	T	C	A	G
Haplotype 9 (1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	G	.	.	.	.	A	.	.	.	.	.

Figure 1. Nucleotide polymorphisms were observed in the COI of the BSF Vietnamese (658 bp). The first column signifies the identification number of samples and references BSF. Vertically oriented numbers indicate the variable site's position. Dots (.) indicate identity with the reference sequence (GenBank accession number FJ794367.1), while different base letters denote substitutions

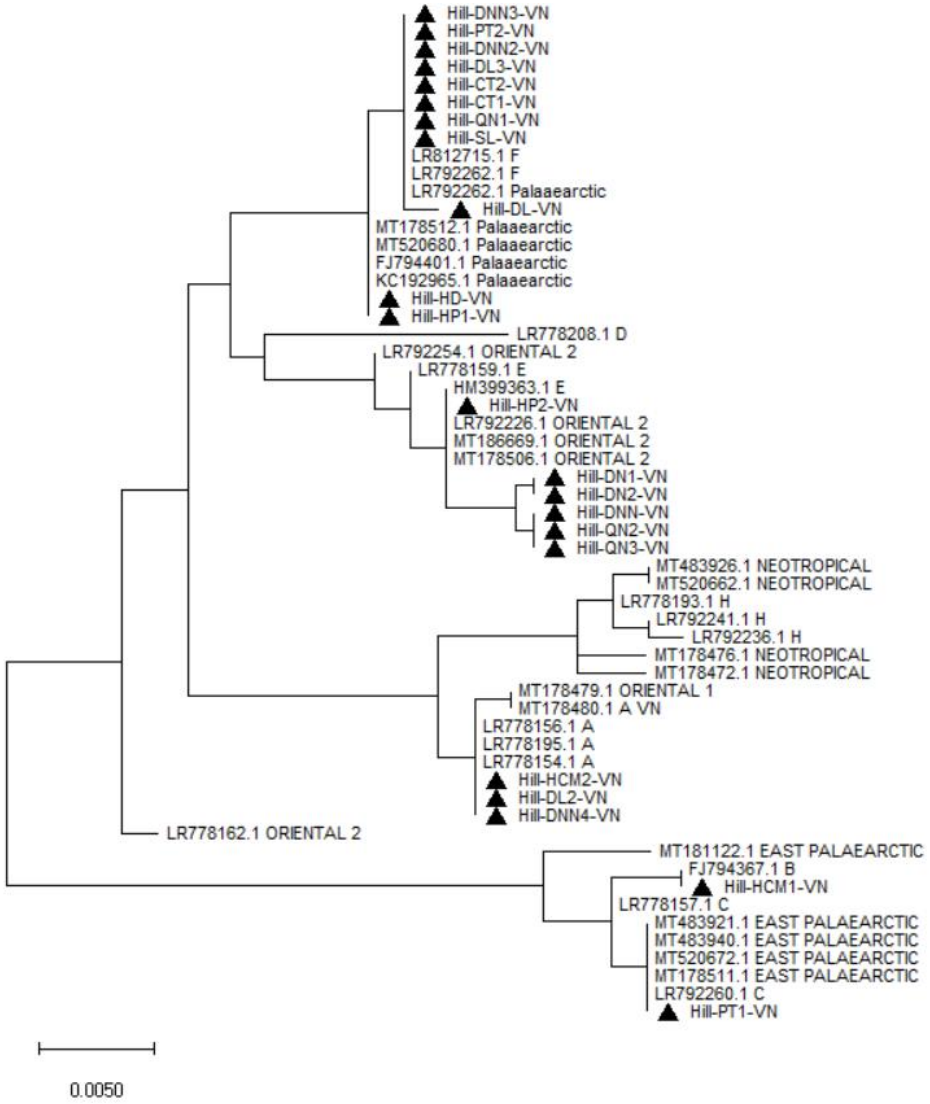

Figure 2. The phylogenetic relationship between the *Hermetia illucens* samples collected from different locations of Vietnam and other GenBank accessions of closely related species was inferred using the Maximum Likelihood method by MEGA X (Kumar et al. 2018)

Our study showed considerable genetic diversity in the COI sequence of *Hermetia illucens*. This finding is consistent with a previous study (Ståhls et al. 2020; Kaya et al. 2021). These results allow us to capture a significant part of the diversity of the Vietnamese BSF. This information is valuable for both growers and livestock regulators, facilitating more efficient BSF agriculture. In addition, population genetics studies can play a key role in ongoing efforts to identify insect lineages with improved decomposition properties. This is significant in terms of being the scientific and genetic basis for selecting and creating BSF varieties to serve in future agricultural production.

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