The Natural History of the Black Soldier Fly, *Hermetia illucens*: Insights From Complete Mitochondrial Genome Sequences

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Abstract

Background

The Black Soldier Fly (BSF) *Hermetia illucens* is a cosmopolitan fly heavily used by industrial companies to reduce biowaste and produce protein and fat for poultry and aquaculture feed. However, the natural history and the genetic diversity of the BSF are poorly known.

Results

In this study, we analyzed 677 CO1 sequences derived from samples found all over the five continents, leading us to discover 52 haplotypes, including ten major haplotypes. We refined the definition of these haplotypes by sequencing 59 mitochondrial genomes. We could derive an estimate of the separation events of the different haplotypes at more than two million years for the oldest branches. This worldwide cryptic genetic and genomic diversity is mirrored at a local scale in France, in which we found five major haplotypes sometimes in sympatry.

Conclusions

Our data resolve the phylogenetic relationships between the major lineages and give insights into the dispersal and the numbers of BSF neo-introduction at global and local scales. Our results indicate that commercial BSF stock's genetic and genomic diversity is very low. In addition, this broodstock participates in disseminating the BSF in the wild. Taken together, these results call for a better understanding of the genomic diversity of the BSF to unravel possible specific adaptations of the different lineages for industrial needs and to initiate the selection process.

Background

The black soldier fly (BSF) *Hermetia illucens* (Linnaeus, 1758) is a Diptera of the Stratiomyidae family. It probably originated from Mexico, then spread to the south of the USA and Latin America over the last thousand years ¹. These geographical areas are now considered their natural area ^{1,2}. The BSF is now a cosmopolitan fly found in tropical, subtropical, and tempered regions, from the 40th parallel North and the 45th South ^{2,3}, where it is considered an exotic, non-invasive species ^{4,5}. This fly likely spread through shipping routes ⁶. While studies based on naturalistic observations show that it was first discovered in Malta in 1926 ⁷, it has been suggested that it was introduced in Italy about 500 years ago ⁸. The BSF appeared in the early 1950s in the South-East of France ⁹ before spreading through the South-West ¹⁰ along the Rhône valley ¹¹ and finally along the Atlantic coast ¹². Currently, the BSF can be found on the whole European territory, with few exceptions. It is reasonable to assume that the rise of international traffic and the industrial use of the BSF may have led to a steady flow of introductions in France and worldwide ^{13,14}.

The BSF was initially used in forensic entomology to date the post-mortem time ¹⁵. Industrial companies heavily use it to reduce biowastes ^{16,17}, to produce protein ^{17,18,19}, and fat ^{17,18,20}. These products are used to make animal food ²¹ or biodiesel ^{22,23}.

BSF industries face similar issues as other farms: knowing how to master their model's biological and genetic aspects to get the best out of it. To this end, it is crucial to know the genetic diversity of BSF existing internationally, nationally, and locally. Many articles exist about the nutritional aspect of the BSF ^{20,24,25,26,27} or its microbiota ^{28,29,30}, few have focused on the genetic diversity of the BSF. We have some evidence on the extent of BSF genetic diversity from previous studies. Ten haplotypes based on the CO1 gene were found in South Korea with 245 individuals ³¹. Still, on the CO1 gene and a worldwide sampling, 56 haplotypes were found with a divergence rate up to 4.9%, and it has been shown that individuals belonging to divergent lineages are still interfertile ³². Finally, based on 15 microsatellite markers, 16 genetic clusters were found with hot spots in South America ³³. Other works focused on more fundamental aspects of BSF and assembled a reference mitochondrial genome and a chromosomal-scale nuclear genome ^{34,35,36}.

Within the framework of the BSF industry, phylogeography can allow on the one side to account for genetic diversity at different scales (global, national, and local) and, on the other side, to have keys on the geographical origin of the species present in a territory or used by companies. This study may suggest local adaptations or some phenotypic plasticity due to habituation to different biomes.

In this study, we quantified this diversity at different scales: worldwide by studying the variation within the CO1 gene, and more locally at the level of France. We also analyzed the role of livestock BSF farming in the diversity present on the French territory. Our report is based on data obtained by sequencing 113 individuals for the CO1 gene and mining 564 CO1 sequences. We also sequenced and assembled 59 mitochondrial genomes. We also show the great and hidden genetic diversity within *Hermetia illucens* at all observation scales, focusing specifically on France (90 French individuals were analyzed, allowing us to find nine different haplotypes). In addition, we are also providing here date of the separation events of the different haplotypes found. This separation turns out to be more than two million years old for the most distant haplotypes.

Materials And Methods

Sampling

We decided to conduct a global sampling followed by a specific focus on France. A detailed table of samples and associated metadata is provided in (Table S01 - supplementary). The origin of all CO1 sequences used in our study is summarized in Table 1, and a summary of sample numbers by continent is provided in Table 2. Samples were collected during summer 2020 and stored in 100% ethanol at -20°C prior to DNA extraction. In France, the collection was done with the help of the compost citizen network ³⁷. The larvae came exclusively from compost and the adults collected were close to the composting area.

Between one and ten individuals (larvae or adults) were collected per area. In some French localities, collections in the wild have been made near BSF breeding areas (about five kilometers). For the other parts of the world, the individuals came either from compost or breeding farms.

In SRA, we gathered all the CO1 sequences on NCBI and Bold and whole-genome sequencing data and reconstructed the mitochondrial genome. In France, sampling was carried out during the summer of 2020 in shared and personal compost sets. We obtained larvae in the areas of Blois, Bordeaux, Toulouse, Montpellier, Lyon, Paris. In Africa, individuals from compost or livestock farms were collected during 2019. In Latin America, larvae from French Guiana compost were collected during summer 2019. An adult was found in 2016 in Mexico. In Asia, individuals collected from Taiwan either from a breeding farm or compost. These were mainly L5 larvae and pupae; some adults were also recovered. We were surprised in the Bayonne area to have BSF-like larvae, which turned out to be *Exaireta spinigera* larvae.

DNA extraction

We extracted DNA from larvae, pupae, and imago under the same conditions by grinding individuals in liquid nitrogen using a mortar and pestle. The ground samples were then processed according to the protocol of the Macherey-Nagel AXG 100³⁸ kit for the isolation of genomic DNA from tissue with some modification: we added a second purification step with 3.5 ml of freshly prepared ethanol and centrifugation at 15,000 rpm at 4 ° C for 15 min just before the final re-suspension. The DNA pellet was finally re-suspended in 100 µL of Tris 10 mM EDTA 1 mM pH 8.0. The purity was evaluated by spectrophotometry using a Nanodrop³⁹, and the concentration was quantified using the Qubit kit DNA Broad range⁴⁰. DNA integrity was monitored using routine agarose gel electrophoresis.

CO1 Sequencing

We designed specific CO1 primers from the reference mitochondrial DNA³⁴ (Table S02 - supplementary), then a mix was realized using NEB 5X standard buffer One Taq. The mix is then amplified in ONETAQ HOT 45 PCR. We purified the PCR products using the Illustra Exostar 1-step GE Healthcare kit before Sanger cycle sequencing. Cycle sequencing reactions were performed using the BigDye 3.1 chemistry, purified by precipitation, and sequenced using an ABI 3130 sequencer (Applied Biosystems). Base-calling was performed using the sequencing analysis software version 5.3 (Applied Biosystems). We assembled forward and reverse reads using the Geneious V R11⁴¹ assembler with the sensitivity set at medium/fast. The regions containing the primers were excised, and we kept only the sequences having more than 75% of high-quality base calls.

Multiple sequence alignment and haplotype network reconstruction

We aligned CO1 sequences obtained in the laboratory and those recovered from the databases using the MAFFT⁴² aligner included as a plug-in in the Geneious software⁴¹. We used the algorithm G-INS-i with a scoring matrix 200 PAM / k=2, a gap open penalty at 1.53, and an offset value at 0.123. We exported the multiple sequence alignment in nexus format⁴³ for haplotype network reconstruction and visualization using POPART⁴⁴. We modified the output file to add geographical origins to the sequences.

Shotgun sequencing and mitochondrial DNA assembly

A total of 56 BSF individuals were used for whole-genome shotgun sequencing using an Illumina method by Novogene UK^{45,46,47,48,49}. The technique used is pair-end with a coverage rate of 4,68X (SD 0,50). The resulting sequences were checked for quality and counted for read parity. We take advantage of the fact that mtDNA is highly repeated in insect DNA. In insects, mtDNA represents on average 0.42% of the total DNA in the genome sequence project⁵⁰. As stipulated, we used 5000 kb of data/sample (4-5x coverage) for the mitochondrial assembly, which on average contains (5000*0,42)/100 = 21000kb of mtDNA. As the mitochondrial genome is about 15 kb, we obtain coverage of 1400x for this genome which is largely sufficient to obtain a complete genome in one contig. The sequences were assembled de novo with the MEGAhit software⁵¹ (based options); the mitochondrial genomes were thus recovered from the assemblies by blast⁵² using the reference BSF mitochondrial genome sequence (NC_035232). THE MITOCHONDRIAL DNAS WERE ANNOTATED using MITOS online software⁵³, and the D-Loop zone was removed. We added four complete mitochondrial sequences from databases or reconstructed via reading sequences available on the SRA⁵⁴ using the same assembly procedure and then aligned under the same conditions as previously mentioned.

CO1 and whole mitochondrial genome phylogeny

We use the MEGAX⁵⁵ software to determine the best model and compute an ML Tree with 500 Bootstraps using a complete deletion parameter. We visualized the data with the online tool ITOL⁵⁶, and we rooted the tree with the Stratiomyidae *Exaireta spinigera*. We chose *Exaireta spinigera* because it was the closest species for which we have a complete mitochondrial genome (the blastn result of the complete mitochondrial genome of *Hermetia illucens* vs. *Exaireta spinigera* gives us 81.28% of similarity). To simplify the interpretation of the phylogeny, we have kept only a single identical sequence per haplotype.

Time tree

We used the BEAST software⁵⁷ to estimate the divergence time of the different BSF groups. The analysis was performed with an expansion growth model and an uncorrelated lognormal relaxed clock with the proposed insect molecular clock. Ten million iterations were performed, and then with the TreeAnnotator⁵⁸, the obtained trees were clustered with a burn of 25% of the trees. The visualization of the final tree was done with the online software ITOL⁵⁶.

Results

Haplotype Network

We reconstructed a haplotype network from the analysis of 677 sequences spanning 658 nucleotides of the CO1 mitochondrial gene. A total of 52 distinct haplotypes were delineated from the analysis of this CO1 fragment of one to 230 fly samples (Figure 1).

We found 73 segregating sites with a Tajima-D of 45.1413, suggesting a lack of rare alleles, which could come from a contraction of the population.

There were 31 haplotypes made of a single sequence. We observed 11 haplotypes made of between two and four sequences derived from the same continent; that total of 42 haplotypes will be referred to as minor haplotypes. We observed ten haplotypes made of between five and 230 sequences, including six multi-continental haplotypes that we will refer to as major haplotypes. In conclusion, we found an important diversity both within and between continents using the CO1 phylogenetic mitochondrial gene. Minor haplotypes represent 8.5% of all sequences (58 sequences) and 80.8% of haplotypes. Major haplotypes represent 91.5% of the sequences (619 sequences) and 19.2% of the haplotypes.

In Europe, we found ten distinct haplotypes from 118 sequences, nine for Asia from 277 sequences, five for Oceania from 106 sequences, 30 for South America from 55 sequences, four in Africa from 78 sequences, and seven in North America from 43 sequences. Some major haplotypes are represented in a more restricted area. For example, haplotype B is mainly present in Asia (figure 1). We observed a high haplotype diversity in South America with 28 minor haplotypes that contain between one and four sequences. The haplotype C contains all the sequences derived from farmed strains of the industry working on BSF.

Global distribution analysis

We visualized the actual distribution of the different haplotypes found previously on a distribution map (figure 2).

Each haplotype from A to J is now represented with a color; haplotypes that are numbered in the haplotype network (figure 1) are now represented in grey.

We observed great variability in South America highlighted on the map by the grey pie charts representing mostly minor haplotypes. We observe 22 singletons in South America out of the 52 haplotypes found, more than 40% of the total diversity. On the other hand, we found six out of ten major haplotypes in Europe.

Haplotype A and C were the most represented haplotypes in our sampling: Haplotype A was present on all continents and represented 34% of all sequences, and haplotype C was only absent from South America and represented 18% of all sequences. Some haplotypes seemed to be restricted to some world areas, such as haplotype G, which was present in all the localities in West Africa and surprisingly in a Portuguese sample, or the haplotype H in South America. Haplotype F was present in Europe, Uganda, and South Korea. Haplotype E was more present in Asia and Oceania as well as in North America. Finally, some haplotypes such as B, I, and J present rare sequences found in more restricted areas such as B in Korea or H and J in France (figure 2).

CO1 Phylogeny

During our CO1 gene analyses, some BSF-like larvae were identified as *Exaireta spinigera* larvae. The sequences were amplified and sequenced with the same primers as for BSF.

By taking only one sequence for each haplotype, we have reconstructed a phylogeny of the CO1 gene sequence. The CO1 phylogeny was rooted with the *Exaireta spinigera* (figure 3). A sample from Cameroon was revealed to belong to a closely unidentified species of BSF and was termed *Hermetia* sp. Cameroon in our subsequent analysis (Blastn score between *Hermetia* sp. from Cameroon and BSF gives us 85.01% identity with an E Value of 2e-174).

The CO1 gene phylogeny supports the analyses performed with the haplotype network (Figure 1), showing remarkable sequence diversity among the CO1 gene. Interestingly, all the commercial strains are found in the C haplotype (a detailed phylogenetic tree is given as a supplementary with an asterisk placed on individuals from farms (Figure S03 - Supplementary).

A group containing haplotypes 35 to 42 and haplotypes B and C forms a divergent group from the others, with strong statistical support (bootstrap value of 0.99). Some individuals captured in the wild have been found near BSF commercial breeding sites. For example, we have captured C haplotype individuals in Avignon close by companies working with the BSF.

Although the global resolution of the deep branches of the tree is low, it is also possible to better understand the relationship between the haplotypes: haplotype B and C are close to each other and well separated from other haplotypes. The other haplotypes are not very well resolved with the CO1 sequences. Finally, the samples coming from Latin America are scattered all over the tree, showing their great diversity and supporting the hypothesis of the geographical origin of the BSF.

The global BSF diversity is mirrored at the local level in France

Among the ten major haplotypes, our sampling effort in France showed that five are present in France in addition to a unique haplotype only found in France (Figure 4) in La Rochelle. Haplotype A remains the dominant haplotype in France (71.1% of all sequences) in seven of the eight localities (excluding Beaufort en Vallée, where the flies come from a company). Regarding the haplotype C, BSF belonging to this group have been found in four locations in the regions of Poitiers, Bordeaux, Beaufort-En-Vallée and Montpellier. Except for Beaufort-En-Vallée, where the flies come from a company, the other BSF come from natural environments. BSF industries exist in all these localities. More surprisingly, different haplotypes coexist in some identical localities. For example, we found three different haplotypes in the region of Paris and four in the region of Montpellier. Finally, we found a singular haplotype in Colomiers (J) and La Rochelle (1).

Complete mitochondrial genome analyses

To better understand BSF phylogeny and estimate the timing of haplotype divergence, we reconstructed and analyzed the mitochondrial genomes of 60 individuals, including 3 *Exaireta spinigera* (used as an outgroup). The 60 reconstructed mitochondrial genomes have the typical structure of insects mtDNA: 13 protein-coding genes, 22 tRNA, two rRNA-coding genes, and non-coding regions and control regions (an example is given as a supplementary (Figure S04 - Supplementary). The genetic differences are homogeneously distributed overall mtDNA and are not localized on specific genes (Figure 5A). A great genetic diversity at the mitochondrial genome level is observed. Two very homogeneous groups, A and C, are well represented, and sequences of the C haplotype are wildly divergent all along the genomes from the others. The two sequences with the most differences are 55-F-Angouleme and 21-Ghana, with a similarity percentage of 96.373% (all results are in supplementary (Figure S05 - Supplementary).

We also wanted to know if the mutations are equally (or not) distributed between the different genes. Therefore, we compared the level of similarity of the 13 mitochondrial genes of the 57 BSFs (fig 5B) to those of *Exaireta spinigera*. The results are presented as percent sequence similarity.

We grouped the individuals into three different categories:

-Haplotype A (Figure 5 B1), all genes show the same differences with those of *Exaireta spinigera*; only the NAD6 gene shows slight differences.

Haplotype C (Figure 5 B2) shows that most of the mitochondrial genes are identical except for the ATP8 gene, which is different for four individuals (11 Angouleme; 14 Kenya; 19 Kenya, and 15 Kenya). Slight differences are also present in the NAD3 and NAD6 genes.

-Haplotype D-F-G-H-I (Figure 5 B3), the profiles are slightly different, with a more substantial disparity for the ATP8, NAD1, and NAD5 genes, especially the Mexican one (57). The profiles show the same pattern except for the ATP8 gene of the individual from Mexico (57), which is more conserved.

Complete mitochondrial genome phylogeny

The complete mitochondrial genome phylogeny demonstrates the very high genetic divergence among BSF populations found with the CO1 gene analysis (Fig 6A). The increased sensitivity of the analysis, due to the size of the sequences (13.96 kb, 13 genes), robustly resolves the relationships between different haplotypes identified previously (nodes >95%). In addition, it allows us to distinguish subgroups within the previously determined haplotypes. Two major divisions emerge within the tree: group C (which includes all commercial strains) and others. Two subgroups emerge within the C group (11 – F – Angouleme; 14 Kenya; 19 Kenya; 15 Kenya and the others).

The other large group contains the sequences of the A D I G and H haplotypes. The I G and H haplotypes are related and close to each other. The very cosmopolitan A haplotype is well conserved with limited mitochondrial sequence divergences.

Moreover, although phylogenetically close, the I G and H haplotypes are found in very disparate areas (the G haplotype is mainly in Africa, the I in Europe, and the H in Latin America), supporting multiple worldwide introductions.

Strikingly the high level of the conservation of the ATP8 sequence described above in the Mexican sample (haplotype D) does not explain the basal position of the sequence in the phylogeny; indeed, we tested the phylogeny by removing the ATP8 gene, and no change was found (Figure S06 - Supplementary).

The ATP8 gene alone is not sufficient to explain the appearance of the observed subgroups. However, unlike the CO1 gene, which varies only slightly within this haplotype, the NAD2, NAD3, NAD5, and NAD6 genes are more variable.

The average percentage of identity between haplotypes A and C is about 96.39%. Between haplotype C and D 96.54%. Between haplotype C and H 96.40% and 96.43% between C and I. Between A and D 97.82% (all results are in Figure S03 - Supplementary).

The divergence time of the different groups

The time tree (fig 6B) gives us information about the divergence time of the different mitochondrial genomes. We obtained identical Bayesian and ML trees. We noted a relatively long divergence time between haplotype C and the others (2.2 million years). The others have diverged more recently (about 1.2 million years). Within the C haplotype, a divergence has been about 300.000 years, while in the other groups, the divergence times are much lower, about 20.000 years for the A haplotype.

The time of divergence with *Exaireta spinigera* has been estimated at 110 million years, confirmed by the literature that gives us 112 million years with the Timetree tool ⁵⁹.

Discussion

Hermetia illucens is now considered a species of major interest for the food industry ⁶⁰. Thus, it became urgent to study the genetic diversity within the different populations at different scales. Analyzing the different haplotypes in the same geographical area is a powerful tool to study the recent history of BSF. An in-depth study of the genetic variations between the different haplotypes may allow us to resolve better the scenario of the arrival of BSF in a territory.

In this study, we uncovered a high genetic variability of mitogenomes within the species *Hermetia illucens*. We found that haplotype C is the most divergent. The dating of the separation events with the other haplotypes goes back more than 2 million years. We found a well-resolved separation within haplotype C (>95%); the other haplotypes are also well resolved (>95%).

Our study confirms the genetic diversity found in previous papers^{31,32,33}. We found a comparable number of genetic clusters, although we added many more sequences of diverse origin. For example, Ståhls et al.³² found 56 distinct haplotypes with 418 CO1 sequences, and we found a comparable number of haplotypes based on the analysis of 677 CO1 sequences. These results suggest that we have captured a major part of the extant BSF worldwide diversity. However, in South America, we found 30 haplotypes among the 55 sequences sampled that open up the possibility of a still unknown genetic diversity in this part of the world.

With the most remarkable genetic diversity being found in South America, the hypothesis of the geographical origin of BSF in Latin America is further confirmed. Apart from this continent of origin, there seem to be many neo-introductions on all continents with divergent mitochondrial lineages. The analysis of complete mitogenomes supports the hypothesis of several distinct introductions in Europe and other continents. Our results contradict the scenario of Kaya et al.³³ based on microsatellites for a single BSF introduction in Asia and Australia. Indeed, our data support the presence of highly divergent major haplotypes A, C, and E in Australia and major haplotypes A, B, C in Asia, in addition to several orphan haplotypes. As the divergence times between these major haplotypes is estimated at around two million years, this pattern is incompatible with a single introduction by man in the recent period but supports multiple and independent introductions.

In the same way, Kaya et al.³³ propose at least three distinct introductions in Europe. Our data considerably increases this number: at least seven different haplotypes are found (A, I, J, G, F, C, and E), supporting at least seven distinct introductions. Similarly, at least four introductions in Africa arose. By increasing the sample size and using more resolutive markers, we obtained a more precise description of BSF genetic diversity and better understood its natural history. However, due to the frequent, multiple, and probably ongoing introductions worldwide of the BSF, understanding the detailed introduction history might be challenging.

Even at a national scale, the BSF genetic diversity is vast. By taking the example of France, we were able to show that the diversity observed at the global scale is also found at the national scale (five of the ten major haplotypes found worldwide). Moreover, we have haplotype mixtures at the local level, confirming the observations on microsatellite data³³. For example, in the region of Paris, we have a mix of three haplotypes (A, F, and I), and in the region of Montpellier, a mix of four haplotypes (A, B, C, and E) – Figure 4.

France contains on its territory a great diversity of haplotypes, whose origin can be explained either by the historical introduction of the year 1950⁹ in addition to more recent neo-introductions. The haplotype A, which is by far the most common haplotype in wild BSF found in France (71.1%) and in Europe, may correspond to the initial introduction. However, it is difficult to understand the exact scenario of the introduction of BSF in France. For example, the divergence times within French flies belonging to haplotype A (20.000 years) tend to prove the recent but multiple introductions in France of flies belonging to this group rather than a single introduction by men followed by diversification on the territory. Our data support a complex scenario involving multiple and repetitive introductions of the BSF from closely related (same phylogenetic groups) and distantly related (divergent phylogenetic groups) individuals in France. A possible scenario involves introducing the haplotype A in the 1950s⁹, which spread gradually on the territory. More recent introductions arose explaining the occurrence of the haplotypes J, G, and I. Finally, introductions related to the industrial activity of the BSF of the haplotype C arose.

Jefferey Tomberlin, a pioneer in the field, also indicates in a recent article on the structure and demography of the BSF based on microsatellite data³³ that the captive populations used for breeding in Europe and North America derive from a common, genetically related strain. Our analysis based on CO1 and complete mitochondrial genome sequences confirms this result: the haplotype that we have named C (for commercial) contains all the sequences of industrial origin with very few genetic diversity. Interestingly, this commercial group does not have any South American relatives, but Kaya et al. report wild North American populations related to the haplotype C (but not similar). This result suggests a possible North American origin for the BSF commercial stock. Our data support a possible alternative scenario because both network haplotypes and CO1 phylogeny indicate that the C haplotype is closely related to the B haplotypes and eight other haplotypes distributed in Asia and Europe. However, the exact origin of the commercial stocks might be difficult to decipher due to the naturalization of the C haplotypes in many locations.

Indeed, specific individuals collected in the wild and belonging to this group were collected in the vicinity (around 5 km) of industries breeding BSF. For example, if we focus on the commercial haplotype analysis in France (the C haplotype), the wild BSF belonging to the haplotype C may have escaped from industries in the regions of Poitiers, Bordeaux, Avignon, and Montpellier. This last point raises the question of the biosecurity of BSF farms around the world. Indeed, suppose the industries carrying outbreeding of BSF do not opt for optimal safety. In that case, the escape of BSF of industrial origin can lead either to establishing the BSF in territories where it was not yet present or to a replacement-hybridization of the local populations by the industrial strain. This would lead inexorably to a loss of the local genetic diversity, thus potentially erasing some local adaptation to specific environmental conditions.

However, it is interesting to note that in Ståhls et al. 2020³², in contrast to Kaya et al. 2021³³ and our study, some genetic diversity in the breeding strains in Europe and North America have been evidenced (10 haplotypes). This may be due to a broader sampling effort of commercial strains. Indeed, Ståhls³² had the opportunity to sample many small farms leading to 292 BSF sequences from rearing cultures. This allowed them to access a broader genetic diversity not found in our study and Kaya et al.³³ It is also possible that smaller farms did not obtain BSF from the dealers who constituted most of the more prominent industries but directly from *in Natura* acquisitions. Nevertheless, our results confirm that the industrial actors work with the same and unique haplotype.

We also evidence that the mitochondrial genome is perfectly suited to differentiate the genetic diversity and resolve phylogenetic relationships. It allows us to date the natural history of the BSF, bringing a sum of details that were impossible to distinguish based only on the CO1. For example, we report puzzling variations in the level of conservation of the ATP8 genes for some samples (haplotypes C and D). Interestingly, these variations have already been found in other insect species (for example, in Coleoptera, see Zhang et al. 2019⁶⁰, and in grasshoppers, see Li et al. 2018⁶¹). The ATP8 gene is small (160 bp on average in insects) and encodes for a subunit of the complex V of the ATP synthase. In *Hermetia illucens*, the initiator codon of the ATP8 gene is ATT, is 167

bp long, and it overlaps with the ATP6 gene. It has been assumed in other species that the modification of the sequence of the ATP8 gene can affect the structure of mitochondrial complex V and thus its function.

Furthermore, it has been shown that changes within ATP synthase can be explained by adaptation to different ecological environments^{62,63,64}. This indicates that some local adaptation driven by the ATP8 genes may exist in BSF, as it has already been shown in other insects. This can lead to an adaptation to survive in high-altitude environments, i.e., at a lower oxygen level and low temperature⁶⁵. Even if these variations did not change the phylogeny of the BSF, it could be a clue to some local adaptation signal.

Complete mitochondrial genome data allows us to make a robust and well-resolved time tree of the species. The divergence times between the different haplotypes are surprisingly long (more than two million years for the most divergent lineages), and the differences observed between the sequences prove that there is still unsuspected and hidden genomic diversity for the species. Comparing it to *Drosophila*, two million years correspond to many speciation events leading to different reproductively isolated species⁶⁶. By contrast, Stahls et al³². indicated that BSF belonging to phylogenetically divergent haplotypes can still cross and generate offspring, indicating no apparent reproductive isolation in laboratory conditions. Interestingly, our data evidence that the cohabitation of different haplotypes at the same locality could lead to crosses between haplotypes. Further investigations are thus needed to understand the possible genetic barriers between highly divergent BSF groups but also the possible patterns of introgression in natural BSF populations. This phenomenon might considerably confuse the elucidation of the population structure and the natural history of these species.

Finally, it is interesting to recall that, if indeed, most industrial actors use the same haplotype for the industry, the other haplotypes present in France are wildly divergent from this one. Strikingly, we found in one compost bin in the region of Paris flies belonging to two different haplotypes (A and F). Thus, crossbreeding between these two individuals belonging to these two haplotypes would bring a solid genetic mix that could benefit breeding. Associated with this significant genetic diversity, BSF may also have some significant phenotypic diversity that might be useful for specific industrial usage and selection processes to increase performances or ameliorate peculiar traits or behaviors⁶⁷.

Conclusion

Through the analysis of 60 mitochondrial genomes during this study, we proposed a robust and well-resolved time tree of the BSF that confirms the vast and hidden genetic diversity within *Hermetia illucens*. Our result elucidates the phylogenetic relationship and time divergence between the different BSF lineages and provides a plausible scenario for BSF expansion in the world via multiple and independent introductions. With 677 COI gene sequences coming from a broad geographical sample, we highlighted the genetic diversity at the level of the different continents evidencing recurrent and multiple introductions outside the geographical area of origin in Latin America. However, the exact scenario of introductions, even at a national scale in France, is still challenging to understand due to probable multiple and independent introductions and possible interbreeding between different populations that coexist in the same geographical area. We have also shown that many BSF industrial companies work with flies that derive from a unique population. We would also like to remind that the apparent biosecurity deficiency within some BSF industrial companies probably led to the escapement and possible local introduction/replacement of natural populations of BSF. Finally, our data indicated that it becomes extremely interesting to take a closer look at the nuclear genomes' evolution of the BSF to understand better the natural history of the BSF as well as specific adaptations of the different lineages that might be useful for industrial needs and to initiate the selection process for peculiar traits.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not Applicable

Availability of data and materials

The dataset generated during the study has been deposited in the European Nucleotide Archive database under Accession Numbers PRJEB48031 Bioprojet. Alignment and raw data can be downloaded following this link <http://gofile.me/2ppPR/QWjM33T7y> the supplementary can be downloaded following this link: <http://gofile.me/2ppPR/rwaGSXDQU>

Competing interests

The private company Cycle Farms partially granted this project, but at any moment, the company has influenced the design and the analysis of the results nor the writing of the manuscript and its conclusions. No commercial products have been developed based on the outputs of this article. None of the authors have any competing interests.

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Author's Contributions

JG carried out the sampling, performed data curation, and wrote the original draft. NP performed data curation and reviewed the manuscript. GB helped with the data curation and reviewed the manuscript. JF conceptualized and supervised the study, performed data curation, reviewed and finalized the manuscript.

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Tables

Table 1 : Origin of CO1 sequences and mitochondrial genomes

Origin	Number of individuals (CO1)	Number of individuals (Mitochondria)
Collection from the wild	91	43
Industrial origin	23	13
Mined from databases (GenBank & Bold)	560	1
Assembled from SRA (raw sequences)	3	3
Total	677	60

Table 2: Geographic origin of CO1 sequences and mitochondrial genomes

Geographic origin	Number of Individuals – CO1	Number of Individuals – mitochondria
South America	55	4
North America	43	2
Asia	277	4
Oceania	106	0
Africa	78	11
Europe (including France)	118 (90)	39 (38)
Total	677	60

Figures

Figure 1

CO1 haplotype network of *Hermetia illucens*

The haplotype network generated by PopArt after multiple alignments are based on the CO1 gene of *Hermetia illucens*. Analysis of the differences between the CO1 gene sequences of 677 individuals. The pie charts represent the same haplotype, and their relative size indicates the number of individuals sharing this haplotype. The number of bars between each segment indicates the differences between the two sequences. Within a pie chart, the colors represent the geographical origin of the individuals. The major haplotypes are more than five sequences (A B C E F G H 30 38 & 41). The minor haplotypes are the others. The list of sequences and their exact origin is available as an additional document.

Figure 2

World CO1 haplotype distribution

Global distribution of the different *Hermetia illucens* haplotypes based on the CO1 gene. The pie charts represent the proportion of each haplotype within a country. The country's name is followed by the number of individuals analyzed. A colored band representing the continents is next to each pie chart. The colors represent the haplotypes determined in figure 1. The grey haplotypes are represented with a number in figure 1. Separation is made in the pie chart when the haplotypes change for the same country. For example, Venezuela contains three unique haplotypes, meaning that these three individuals are different. A summary table can be found in supplementary table 3.

Figure 3

CO1-based phylogenetic tree

ML phylogenetic tree. Rooted phylogeny with *Exaireta spinigera* (Stratiomyidae). Bootstrap values are materialized with colored circles: green for values > 0.85 and red for values between 0.7 and 0.85. We only kept one sequence per haplotype to visualize the relationship between them. Next to each haplotype, colored circles represent the geographical origin of the species present in this haplotype. The order indicates the relative abundance of the individuals of a container in each haplotype. The statistical support was estimated with 500 Bootstraps with MegaX software using the Tamura 92 G+I model.

Figure 4

France CO1 haplotype distribution

Distribution of the different haplotypes of *Hermetia illucens* in France. The dots represent the specific area of collection. The pie charts represent the distribution of haplotypes in the corresponding area. The names of the main cities in each area are indicated above and the number of sequenced individuals.

Paris includes Vincennes and Nogent Sur Marnes. Blois includes Valloire Sur Cisse. Lyon includes Saint Marcellin En Forez, Corbas, Champagne Au Mont d'Or and Dardilly. Montpellier includes Avignon, Trescléoux and Dieulefit. Bordeaux includes Lormont La Force and Périgueux. Poitiers includes Saint Georges Lès Baillargeaux.

Figure 5

A: Mitochondrial DNA alignment

Multiple alignments of mitochondrial DNA sequences from *Hermetia illucens*. Alignment was performed with the MAFFT aligner. The colors of the names come from their haplotypes determined with the CO1 sequences. Each black mark represents a difference with the reference (1 – China). Above the alignment, there is a representation of the mitochondrial genes.

B: Sequence conformance between *Hermetia illucens* and *Exaireta spinigera*

Sequence conformity of mitochondrial genes of *Hermetia illucens* to *Exaireta spinigera*. The sequences are grouped according to haplotypes. The A haplotype (B1), the C haplotype (B2), and the other haplotypes (B3). The sequences inside each graph are represented in the alignment (5A). Sequences from 23 to 46 plus sequences 51, 52, and 55 for haplotype A (5-B1); from 1 to 22 for haplotype C (5-B2); and from 47 to 57 (except sequences 51, 52, and 55) for the other haplotypes (5-B3).

Figure 6

6A: Mitochondrial DNA phylogenetic tree

ML phylogenetic tree, of 57 mitochondrial DNA sequences of *Hermetia illucens*, deprived of D-Loop regions. MAFFT was used to align the sequences, 500 Bootstrap replicas were performed. The outgroup used is *Exaireta spinigera* (Stratiomyidae). The colors of the names correspond to the haplotypes determined with the CO1 gene sequences (Figure 1). Bootstrap values are indicated by a green circle when they are > 0.95. Commercial individuals are marked with an asterisk.

B Mitochondrial DNA time tree

Bayesian time tree obtained with BEAST of 60 mitochondrial genomes of *Hermetia illucens* rooted with *Exaireta spinigera*. The scale is in millions of years. A break in scale is made at the root level. The colors of the names correspond to the haplotypes determined with the CO1 sequences (figure 1). Commercial individuals are marked with an asterisk.

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