The puzzling mitochondrial phylogeography of the black soldier fly (*Hermetia illucens*), the commercially most important insect protein species

Gunilla Ståhls (gunilla.stahls@helsinki.fi)
Luonnontieteellinen keskusmuseo https://orcid.org/0000-0003-0505-0691
Rudolf Meier
National University of Singapore
Christoph Sandrock
Research Institute of Organic Agriculture
Martin Hauser
California Department of Food and Agriculture
Ljiljana Šašić Zorić
Biosense Institute University of Novi Sad
Elina Laiho
Finnish Museum of Natural History
Andrea Aracil
University of Alicante
Jovana Doderović
University of Novi Sad
Rozane Badenhorst
Agriprotein
Phira Unadirekkul
National University of Singapore
Nur Arina Binte Mohd Adom
National University of Singapore
Leo Wein
Protenga
Cameron Richards
Agriprotein
Jeffery K. Tomberlin
Texas A&M University College Station
Santos Rojo
University of Alicante
Sanja Veselić

University of Novi Sad

Tuure Parviainen

Teknologian tutkimuskeskus VTT Oy

Research article

Keywords: Black soldier fly, Hermetia illucens, mtDNA COI haplotypes, nuclear rDNA

Posted Date: January 3rd, 2020

DOI: https://doi.org/10.21203/rs.2.19982/v1

Version of Record: A version of this preprint was published at BMC Evolutionary Biology on May 24th, 2020. See the published version at https://doi.org/10.1186/s12862-020-01627-2.

Abstract

Background

The black soldier fly (Diptera: Stratiomyidae, *Hermetia illucens*) is renowned for bioconversion ability of organic waste, and is the worldwide most widely used source of insect protein. Despite varying extensively in morphology, it is widely assumed that all black soldier flies belong to the same species, *Hermetia illucens*. We here use genetic data of 424 rearing culture and wild caught fly samples from 38 countries and six biogeographic regions to test this assumption based on data for three genes (mitochondrial COI, nuclear ITS2 & 28S rDNA).

Results

Our study reveals a surprisingly high level of intraspecific genetic diversity for the mitochondrial COI gene (divergences up to 4.9%). This level of variability is often associated with the presence of multiple species, but tested nuclear ITS2 and 28S rDNA markers are invariant and fly strain hybridization experiments under laboratory conditions revealed reproductive compatibility. COI haplotype diversity is not only very high in all biogeographic regions (54 distinct haplotypes in total), but also in breeding facilities and research centers from six continents (10 haplotypes: divergences up to 4.3%). The high genetic diversity in fly-breeding facilities is mostly likely explained by many independent acquisitions of cultures via sharing and/or establishing new colonies from wild-caught flies. However, explaining the observed high diversity in most biogeographic regions is difficult. The origin of the species is considered to be New World (32 distinct haplotypes) and one would expect severely reduced genetic diversity in the putatively non-native populations in the remaining biogeographic regions. But distinct, private haplotypes are also known from the Australasian (N=2), Oriental (N=4), and the Palearctic (N=5) populations. We review museum specimen records and conclude that the evidence for introductions is strong for the Western Palearctic and the Afrotropical regions which lack distinct, private haplotypes.

Conclusions

Based on the results of this paper, we urge the black soldier fly community to apply molecular characterization (genotyping) of the fly strains used in artificial fly-breeding and to share this data in research publications and when sharing cultures. In addition, fast-evolving nuclear markers should be used to reconstruct the recent invasion history of the species.

Background

Black Soldier Flies (BSF), Hermetia illucens (Linnaeus, 1758) (Diptera, Stratiomyidae) are large flies with brownish wings and a pair of characteristic translucent pale patches (windows) on the abdomen [1]. The larvae of this synanthropic species are very effective decomposers of an exceptionally wide range of decaying organic matter including manure, food waste, agricultural by-products, organic leachates and cadavers (including human) [2, 3, 4, 5]. Not surprisingly, the BSF have thus emerged as the dominant

source of insect protein and are now utilized on large scale commercially worldwide due to its ability to convert organic matter into oil- and protein-rich feed (for a recent review see [6]). In nature, adult flies rarely feed but may occasionally imbibe water or honeydew. Therefore, the larvae need to accumulate enough protein and fat during the six larval stages in order to provision the females with sufficient resources for producing 500–1000 eggs [7] and males with sufficient energy for sperm production and mating on the wing. Commercial producers in China, Europe, South East Asia, Africa, South America and North America presently rear large numbers of larvae that are used as feed in aquaculture and poultry farms [8, 9]. Many cultures were started using 'Phoenix Worms' (https://phoenixworm.com/), which have been commercially available for 20 years [10], but the origin of many additional cultures is unknown. This is problematic because it is common for cultures to be shared without prior genotyping.

The native range of BSF is considered to be New World with the northern-most native populations being from the southwestern parts of the US (at least up to 40º N) and northern South America [11]. However, the species is now cosmopolitan and occurs in all other tropical, many subtropical, and some temperate regions (between 46°N and 40°S) [11, 12, 13]. All the Old World populations are considered introduced. Indeed, numerous historical records from the late 19th and early 20th century document the presence of the species in the New World [13] while the earliest records for the other regions are considerably younger. For the Afrotropical region, it is a South African record from 1915 [13] and several subsequent ones from Madagascar (1930s) [14]). The first record for the Palaearctic region is from Malta in 1926: [15] but by the 1960s the species was known from Spain, France and Italy [12, 16]. In Asia the first confirmed records are from the 1940s (e.g., Malaysia) no confirmed records from China until 1960 [13]. With regard to the Australasian region, reports of H. illucens specimens from Australia collected in 1915 [16, 17] have now been revealed to be erroneous because they belong to a related species (H. pallidipes Hill, 1919) [18]. The oldest known specimen for this conspicuous species is thus from 1948 (in Australian National Insect Collection). According to [13] many Pacific islands were colonized by H. illucens by the1940s. This includes Hawaii, the Solomon Islands, New Caledonia, Mariana Islands, Palau, and Guam. Overall, it is striking that many of the earliest known localities outside of the New World are on islands or close to the coast. This may indicate that worldwide trade played a major role in the spread of BSF [12]. Introduction via shipping is likely responsible for the fast spread of the species throughout the Pacific region in the 1940s (e.g., Guam and Palau) given that the species' arrival coincided with troop and supply movements during WWII.

Upon closer study, species with exceptionally large distributions are frequently revealed to consist of species complexes, or, to comprise several evolutionary significant units that deserve recognition in order to protect their genetic integrity [19, 20, 21]. Fragmentary evidence has been emerging that BSF may be such a species. For example, a regional study of mitochondrial cytochrome c oxidase I (COI) barcode variability in South Korea [22] revealed 10 highly diverged haplotypes. If confirmed for the populations and cultures worldwide, such high genetic diversity would have considerable implications for scientific research and commercial use of the species. For example, it took decades until genotyping revealed the presence of several species in widely used experimental cultures of leeches in the genus Helobdella. By the time of discovery, the lack of timely genotyping had done serious harm to the scientific literature

because results had been filed under incorrect scientific names [23]. One year later, similar problems were discovered for a “medical device”, the medicinal leech Hirudo medicinalis. Many leeches used for medical purposes were revealed to belong to species that were not approved for commercial use [24]. Yet, BSF captive cultures used for scientific or commercial purposes still use flies that have not been genotyped and very little is known about the genetic diversity of the species worldwide. We here carry out a first systematic survey across the species’ current range. We compare the genetic diversity of wild-caught flies with samples obtained from cultures maintained for academic or commercial purposes. In addition to mitochondrial data, we assess the genetic diversity for two fast-evolving nuclear markers (ITS2 and a loop region of 28S rDNA; [25, 26]. Lastly, we test whether two cultures with high genetic divergence for COI are reproductively compatible.

Results

COI barcode dataset and analyses. All COI barcodes were trimmed to equal lengths of 658 nt before a gap-free alignment was obtained. The A + T content was 61.7%, and 79/658 (12%) nucleotide sites were variable that coded for amino-acid changes in 5 of the 219 residues (2.3%). Our study generated 266 full-length barcodes for colony and 107 barcodes for wild-caught samples, which were complemented with 51 publically available DNA barcodes from sequence databases which represented additional geographic localities (Supplementary data Table S1). All sequences were translatable and our repeated sequencing of the same specimens yielded the same sequences for the same specimens. For subsequent analyses, identical COI barcodes from colony samples and non-colony samples from a given geographic location were merged to retain only single representative barcodes of each haplotype (HT). The final COI dataset thus had 135 barcodes representing 54 unique haplotypes, with a total of 40 new and the 14 registered haplotypes in our samples and public databases, respectively. The maximum uncorrected p-distance across the full dataset was 4.9%. The Median-Joining haplotype network for the dataset of all 135 COI barcodes resolved 54 distinct haplotypes (Fig. 1). The NJ-network tree of all COI barcodes was broadly congruent with the haplotype network and barcodes were resolved in four larger sequence clusters, each comprising both cultured and wild flies, while Neotropical samples were placed in two separate sequence clusters (Supplementary data Fig. S1).

COI haplotypes: Non-cultured flies. We produced barcodes for 107 wild-caught flies and added 51 barcodes from public databases. Wild-caught flies from the Neotropics show the highest number of distinct, private haplotypes (32 out of a total of 54) mainly represented by single individuals with sequence divergences up to 3.7% among them. Several unique haplotypes was recorded for the Australasian (2 haplotypes), Oriental (4) and Palaearctic (5) samples (Fig. 1). For single samples from the Afrotropical region (Benin and Ghana) we recorded a haplotype nested among the New World haplotypes (Fig. 1). Samples from La Reunion, Madagascar, and Zambia (Afrotropical) shared haplotypes with samples from the Australasian, Neotropical, Oriental and Palaearctic and regions (Fig. 1: II; Table 1). A haplotype recorded for the Australasian and Oriental regions was also found in samples from the Nearctic and Palaearctic (Fig. 1: III).

COI haplotypes: Cultured flies. We sequenced 266 samples and recorded ten COI haplotypes from the cultures (Fig. 2). A shared, distinct haplotype was recorded for samples traced back to the commercial provider of ‘Phoenix worms’. The samples came from China, France, Kenya, South Africa, Spain, Switzerland, and the USA (Fig. 1: I; Fig. 2). The same haplotype was also detected in wild (presumably escaped) BSF in most of these countries (Table 1). In contrast, some commercial BSF providers in Australia rear ‘local’ flies with private, distinct haplotypes. A private haplotype was also recorded for samples from several rearing facilities in Europe (Fig. 1; Fig. 2), and one European rearing culture (Switzerland) had two distinct haplotypes. The most divergent rearing culture samples differed by p-distances of up to 4.1%.

Nuclear ribosomal DNA. We obtained 453 nt of the ITS2 region for 196 samples, and only one nucleotide site was variable (some individuals were heterozygous), while all 28S loop sequences were invariant.

Hybridization experiments. Mating trials under laboratory conditions revealed a lack of reproductive isolation between the populations, as hybrid offspring were produced and successfully back-crossed with the parental population. The hybrid populations were also fertile, having been maintained for > 10 generations. Backcrosses were not attempted for these.

Discussion

High COI barcode diversity

Our data reveal many unexpected results for this widely used workhorse of insect protein production. They highlight that there is currently a surprising lack of background information for a species that is globally extensively used and shared for scientific research and organic waste recycling.

Our study revealed very high COI barcode variability, but this variability is not mirrored by the two invariant nuclear markers that were included in our study. This high COI diversity does not translate into reproductive incompatibility although the divergence levels (close to 5%) well exceeds what is typical for flies [37]. This implies that the lack of genotyping of cultures may not have done as much scientific and commercial harm in BSF as it did for leeches belonging to Helobdella and Hirudo. The second result is that this high COI diversity is structured in both expected and unexpected ways. The species is considered native to the New World; i.e., the high genetic diversity we recorded for the New World samples is according to expectations, while the remaining regions should mostly have subsets of the New World genetic diversity. This is indeed the case for the Afrotropical and the Eastern Palearctic populations, which contain a limited number of haplotypes. It appears likely that these populations were indeed introduced. However, it was surprising to find many distinct, highly diverged haplotypes in Australasian, Oriental and Palaearctic populations. This diversity remains unexplained if the species indeed originated in the New World and only recently dispersed to these regions. Possible explanations include still insufficient sampling of the Neotropical diversity or a broader geographic origin of the species.

BSF has not only unusually high levels of genetic diversity for COI. It is also morphologically very variable with regard to body size, wing and abdominal coloration, and the shape of translucent abdominal windows. Wing coloration varies and ranges from almost black with a blueish-metallic shine over a clear brownish-opaque insignia in the inner part of the wing to almost pale-translucent. The amount of white versus black coloration on the head shows great variation, while the black and white pattern on the legs seem to be more consistent. There is a tendency for males to have a reddish to red abdomen, while females rarely display red coloration on the abdomen. Much of this variation can be found within the same laboratory cultures and is thus likely due to polyphenism, but more systematic study across cultures representing different haplotype groups is needed.

Global trade and the distribution of BSF

We documented that the distinct haplotype of the commercial US breeding strain (Fig. 1: I commercial brand 'Phoenix Worms') can be found in flies from natural and urban environments in Africa (South Africa, Kenya), Asia (Bhutan, China, Indonesia) and Europe (European part of Russia, Spain). Similarly, haplotypes of flies recorded in commercial and/or research cultures in Asia and Australasia are found in wild-caught flies in these and other geographical regions (Fig. 1: II & III; Table 1). Barcodes of single flies sampled in Benin and Ghana were most similar to BSF barcodes from the Neotropical region. These patterns are compatible with human-mediated introduction across biogeographic regions, but it will require population-level nuclear markers to confirm whether the introductions were sufficiently recent to be due to global trade.

Arguably, the most convincing evidence for human-mediated introductions come from specimens collected at latitudes that are unlikely to support breeding populations [13, 38, 39, 40]. The lower threshold for BSF larval survival was observed to be 15 °C [41], and between 16 and 19 °C [42] and some Canadian BSF localities are unlikely to meet this requirement. Marshall et al. [13] thus hypothesized that the annual occurrence of BSF in Ontario (Canada) is due to the disposal of unused fishing baits (larvae) by local fishermen or the accidental release of flies into nature by owners of reptile pets. However, the evidence is not entirely conclusive because BSF flies, prepupae, and pupae are resistant to cold and can survive low temperatures (5 °C) for several weeks [43]. This may explain why there is an established BSF population in Northwestern Switzerland that has been regularly encountered for one decade. In addition, wild BSF are occasionally caught in Western and Central European countries such as Germany [38] and the Czech Republic [40]. Overall, more research is needed on the thermal tolerance of BSF and its diapausing capabilities. This and all other research needs to be based on genotyped cultures because it is likely that populations from different parts of the (native) range will differ with regard to thermal tolerance. One way or another, it appears likely that BSF will be able to increase its current range as global warming takes hold. We would suggest that urban populations will most likely spearhead the range expansion because they benefit from higher temperatures [41] and readily available organic waste. Range expansion may also be facilitated through population homogenization as more cultures are shared over

long distances and escaped flies cross-breed with native or previously established non-native populations.

Puzzling phylogeography

This study screened a comprehensive number of both cultured and wild caught BSF samples from six continents and 38 countries, including numerous from the putatively native New World and continents where BSF is an alien species. Our findings clearly highlight the need for additional sampling of New World populations as well as the Australasian and Oriental regions. In particular, the discovery of additional haplotypes currently only known from Australasian and Oriental population would strengthen the hypothesis of a New World origin of the species.

If a divergence rate of 2.3% per million years for insect mitochondrial DNA [44] were used as a guide, it could be concluded that the most distant samples of the BSF haplotype network (Fig. 1) have been separated for 0.74–1.35 million years. This would imply that the Oriental and Australasian wild samples (Fig. 1: II & III) had already diverged 1.04 MY ago. These findings could either challenge the assumption of a New World origin or the spread of BSF was not human-mediated. Arguably, the strongest argument against the latter is that black soldier flies are so conspicuous that they should have been collected by early naturalists in Asia and Australasia if they had been present. Instead, the earliest records are from the 1940s.

Conclusions

A high genetic diversity would have considerable implications for scientific research and commercial use of the species, if confirmed for populations and cultures worldwide. Assessing the genetic diversity would seem a necessary fly culture management tool, considering the possible advantages of a high genetic diversity of the BSF strains coupled with different dominant characteristics that a breeding program could capitalize on. We furthermore urge all commercial users and researchers to generate barcodes for their cultures as this can be done at a low cost, and preserve specimens for genotyping or future screening of additional nuclear markers. The available genomic studies on the BSF are presently limited [45, 46], but are expected to rapidly increase in near future. Finally, in order to unravel a more detailed dispersal history of the BSF, comprehensive population genomic analyses should be carried out.

Methods

Our study included both samples from cultures and the wild. For cultures, we screened multiple individuals (adult flies or larvae) from rearing facilities in Europe, Africa, Asia, Australia and United States. Non-colony (“wild”) fly samples were collected in both urban and pristine habitats from Africa, Asia, Australasia, Europe, and the Americas, but it is often difficult to rule out that they are escaped flies from cultures. Our data were complemented with publicly available COI barcodes retrieved from GenBank (ncbi.nih.gov) and BOLD (boldsystems.org) (for data on BSF samples, GenBank accession codes and repositories see Supplementary Table S1).

Laboratory procedures

Helsinki lab. Colony samples: Genomic DNA was extracted from about half of the thorax of an individual ethanol preserved fly, or from a small piece (2 × 2 mm) of larval tissue. The Nucleospin Tissue DNA extraction kit (Macherey-Nagel, Düren, Germany) was used for DNA extraction. Ready-to-Go PCR beads (GE Healthcare, Little Chalfont, UK) were used for amplification of the COI barcode using the universally conserved primer pairs LCO-1490 and HCO-2198 [27] of 266 samples from four countries. Non-colony samples: Genomic DNA was extracted using 1–2 legs of each pinned fly specimen obtained from collections, as a least-invasive approach. The Phire™ Tissue Direct PCR Master Mix #F-170S kit (Thermo Scientific Baltics UAB, Vilnius, Lithuania) was used for DNA extraction and PCR amplification. The DNA isolation followed the Dilution & Storage protocol of the kit with slight modifications, and PCR amplifications of the COI barcode [28] using the mentioned primers followed the recommended PCR cycle conditions. We sequenced 51 samples from 23 countries. We additionally amplified and sequenced the fast evolving second Internal Transcribed Spacer (ITS2) of the nuclear ribosomal gene cluster of a high number of both colony and non-colony samples using the primer pair ITS2a and ITS2b [29].

PCR products were enzymatically purified using Illustra ExoStar (GE Healthcare) and the same primers were employed for subsequent bidirectional Sanger sequencing reactions. The laboratory work was done at FMNH Luomus DNA Lab (Helsinki, Finland), and sequencing at Sequencing Service Laboratory (FIMM Genomics, UH, Helsinki, Finland). The raw sequences were edited for base-calling errors and assembled using Sequencher vs 5.0 (GeneCodes, Ann Arbor, USA).

Singapore lab. We screened 259 specimens from 13 countries representing all six continents by amplifying a 313-bp fragment of COI using either direct PCR (dPCR)[30] or DNA extraction with QuickExtract [31]. The use of tagged primers allowed for sequencing these fragments on HiSeq 2500 using the pipeline described in [31, 32]. Once unique haplotypes were identified based on the 313 bp piece of COI, we carried out formal DNA extractions for specimens representing the different haplotypes. DNA was extracted using BioEr's GenePure Plus Nucleic Acid Extractor following the protocol of the MagaBio Insect Genomic DNA Purification Kit. Extracts were subjected to PCR to amplify the 658-bp fragment of COI using LCO-HCO and a fast-evolving 270-bp fragment of 28S ribosomal RNA using the following primer pair: 28S_P03_F: 5'- TTYRGGAYACCTTYDGGAC-3' and 28S_P03_R: 5'-GGTTTCCCCTGACTTCDACCTGATCA-3'. Sequences for the full-length DNA barcode were obtained with Sanger sequencing while the data for the 28S rDNA fragment were obtained via tagged amplicon sequencing following the same pipeline as the 313 bp COI fragment (see above).

Sequence analyses

Median-Joining haplotype networks can be useful for both conveying population structures and estimating the genealogical relationships among various haplotypes. We generated Median-Joining haplotype networks [33] using POPART [34] for the complete dataset of all BSF barcodes, and separately for BSF samples from rearing cultures. Additionally, we used MEGA version 7 [35] for computing

uncorrected p-distance values and other DNA sequence details among the COI barcodes, and for generating a Neighbor-Joining (NJ) network tree [36] for all haplotypes (Supplementary data Fig. S1).

Hybridization experiments

To examine the reproductive compatibility between populations of BSF with high mitochondrial gene divergence, we carried out two batches of hybridization experiments involving virgin males and females from different cultures. The first experiment involved 10 virgin females from a culture established based on wild caught flies in Singapore with 10 virgin males obtained from Spain of US commercial origin (uncorrected COI p-distances between Spanish and Singapore flies range between 3.7-4.0%). Virgin hybrid flies of both sexes were separated and then back-crossed to virgin flies from their parental cultures. The following reciprocal pairings were carried out: 20 Spanish ♂♂× 20 Hybrid ♀♀; 20 Hybrid ♂♂×20 Singapore ♀♀. In order to ascertain whether flies from failed back-crossing trials were fertile, we mated them with flies from their own respective populations (20 Hybrid ♂♂ x 20 Hybrid ♀♀). A second batch of experiments involved 311 New World ♂♂ with 310 ♀♀ from Singapore and 408 New World ♂♂ with 407 Singapore ♀♀.

Abbreviations

BSF
Black Soldier Fly
COI
Cytochrome c Oxidase subunit I
HT
Haplotype
ITS2
Second Internal Transcribed Spacer
MY
Million Years
rDNA
Ribosomal rRNA genes
WWII
World War II

Declarations

Consent for Publication

Not applicable

Availability of data and materials

The data supporting the conclusions of this article are available in the TreeBase repository (http://www.treebase.org) or included in the additional files of the article.

Competing interests

All authors declare we have no competing interests.

Funding

This work was funded by the H2020 Research and Innovation Staff Exchange Programme of the European Commission (RISE), project 645636: 'Insect-plant relationships: insights into biodiversity and new applications' (FlyHigh), and the grants R-154-000-B46-114 and R-154-000-A94-592 to RM, as well as a grant of the Swiss Federal Office for Agriculture to CS ('Efficiency and sustainability of the production and feeding of insect based feedstuff to fish and poultry' project number 50084).

Author contributions

R.B, S.R., C.S. and M.H provided specimens. E.L., A.A., L.S.Z., J.D., S.V., R.B. and T.P. acquired and interpreted the data. G.S., R.M., C.S., M.H and S.R. conceived the study, G.S., L.S.Z. and R.M. analysed the data. G.S., R.M., C.S. and M.H. wrote the manuscript. All authors contributed to the revising of the final version of this manuscript, approved of and agreed to be held accountable for the content therein.

Acknowledgements

We are indebted to Elvira Rättel for technical lab assistance.

References

1. Hauser M, Woodley N, Fachin DA. Stratiomyidae. In: Kirk-Spriggs AH & Sinclair BJ (eds.) Manual of Afrotropical Diptera. Volume 2. Nematocerous Diptera and lower Brachycera. Suricata 2017;5:919-979.
2. Lord WD, Goff ML, Adkins TR & Haskell NH**.** The black soldier fly *Hermetia illucens* (Diptera: Stratiomyidae) as a potential measure of human postmortem interval: observations and case histories. J Forensic Sci. 1994;39:215-222.
3. Sheppard DC, Tomberlin JK, Joyce JA, Kiser BC, Sumner SM. Rearing methods for the black soldier fly (Diptera: Stratiomyidae). J. Med. Entomol. 2002;39:695-698.
4. Tomberlin JK, Sheppard DC, Joyce JA. Black soldier flies (Diptera: Stratiomyidae) colonization of pig carrion in South Georgia. J. Forensic Sci. 2005;50:152-153.
5. Popa R, Green TR. Using Black Soldier Fly Larvae for Processing Organic Leachates. J. Econ. Entomol. 2012;105:374-378. http://dx.doi.org/10.1603/EC11192
6. Barragan-Fonseca KB, Dickel M, van Loon JJA. Nutritional value of the black soldier fly (*Hermetia illucens* L.) and its suitability as animal feed – a review. J Ins. as Food and Feed. 2017; 3:105-120.
7. Sheppard DC, Newton GL, Thompson SA, Savage S. A Value Added Manure Management System Using the Black Soldier Fly. Biores. Techn. 1994;50:275-279. https://doi.org/10.1016/0960-

8524(94)90102-3.

8. FAO - Food and Agriculture Organization of the United Nations. 2013. Edible insects. Future prospects for food and feed security. In: Huis A. van, Itterbeeck J van, Klunder H, Mertens E, Halloran A, Muir G, Vantomme P, eds. Available from: http://www.fao.org/docrep/018/i3253e/ i3253e00.htm
9. Belghit I, Liland N, Gjesdal P, Biancarosa I, Menchetti E, Li Y, Waagbø R, Krogdahl A, Lock E-J. Black soldier fly larvae meal can replace fish meal in diets of sea-water phase Atlantic salmon (*Salmo salar*). Aquaculture. 2019;503:609-619. https://doi.org/10.1016/j.aquaculture.2018.12.032
10. Zhou F, Tomberlin JK, Zheng LY, Yu ZN, Zhang JB. Developmental and waste reduction plasticity of three black soldier fly strains (Diptera: Stratiomyidae) raised on different livestock manures. J. Med. Entomol. 2013;50:1224–1230.
11. Rozkosný R. A biosystematic study of the European Stratiomyidae (Diptera). In: Spencer, K.A. (Ed.), Clitellariinae, Hermetiinae, Pachygasterinae and Bibliography. Series Entomol. 1983;2:172-176.
12. Leclercq M. A propos de *Hermetia illucens* (Linnaeus, 1758) (“soldier fly”) (Diptera: Stratiomyidae: Hermetiinae). Bull. ann. Soc. Royale d’Entomol. de Belgique. 1997;133:275–282.
13. Marshall SA, Woodley NE, Hauser M. The historical spread of the Black Soldier Fly, *Hermetia illucens* (L.) (Diptera, Stratiomyidae, Hermetiinae), and its establishment in Canada. J. ent. Soc. Ont. 2015;146:51–54.
14. Lindner E. Stratiomyiden von Madagaskar. Stuttg. Beitr. zur Naturkunde. 1966;156:1–26.
15. Lindner E. Stratiomyiidae. In: Lindner, E. Die Fliegen der Palaearktischen region. Stuttgart; 1936; 218 pp.
16. Leclercq M. Dispersion et transport des insectes nuisibles: *Hermetia illucens* L. (Diptera Stratiomyidae). Bull. rech. agron. Gembloux, n. s. 1969;4:139–143.
17. Callan EM. *Hermetia illucens* (L.) (Dipt., *Stratiomyidae*), a cosmopolitan American species long established in *Australia* and New Zealand. Entomol. Monthly Mag. 1974; 109:232-234.
18. Lessard BD, Yeates DK, Woodley NE. Revision of the Hermetiinae of Australia (Diptera: Stratiomyidae). Austral Entomol. 2019;58:122-136.
19. Moritz C. Defining ‘evolutionarily significant units’ for conservation. Trends ecol. evol. 1994;9:373-375.
20. Bickford D, Lohman DJ, Sodhi NS, Ng PKL, Meier R, Winker K, Ingram KK, Das I. Cryptic species as a window on diversity and conservation. Trends Ecol. Evol. 2007:22:148–155. doi:10.1016/j.tree.2006.11.004
21. Dincă V, Lee KM, Vila R, Mutanen M. The conundrum of species delimitation: a genomic perspective on a mitogenetically super-variable butterfly. Proc. R. Soc. B. 2019; 286.20191311. http://dx.doi.org/10.1098/rspb.2019.1311
22. Park S, Choi H, Choi J, Jeong G. Population Structure of the Exotic Black Soldier Fly, *Hermetia illucens* (Diptera: Stratiomyidae) in Korea. **Korean J Env. Ecol. 2017;6:520-528.** https://doi.org/10.13047/KJEE.2017.31.6.520

23. Bely AE, Weisblat DA. Lessons from leeches: a call for DNA barcoding in the lab. Evol. & dev. 2006;8:491-501.
24. Siddall ME, Trontelj P, Utevsky SY, Nkamany M, Macdonald III KS. Diverse molecular data demonstrate that commercially available medicinal leeches are not *Hirudo medicinalis*. Proc. Royal Soc. B Biol. Sci. 2007;274:1481-1487.
25. Kehlmaier C, Assmann T. Molecular analysis meets morphology-based systematics - A synthetic approach for Chalarinae (Insecta: Diptera: Pipunculidae). Syst. Entomol. 2010;35:181 - 195. doi:10.1111/j.1365-3113.2009.00500.x.
26. Nedeljković Z, Ačanski J, Vujić A, Obreht D, Djan M, Ståhls G, Radenković S. Taxonomy of *Chrysotoxum festivum* Linnaeus, 1758 (Diptera: Syrphidae) - an integrative approach. Zool. J Linn. Soc. 2013;169:84–102.
27. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA Primers for Amplification of Mitochondrial Cytochrome c Oxidase Subunit I from Diverse Metazoan Invertebrates. Mol. Marine Biol. Biotechn. 1994;3:294-299.
28. Hebert, P.D.N., Ratnasingham, S., deWaard, J.R. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society. B, 2003;270 (Suppl 1):S96-S99.
29. Beebe NW, Saul A. Discrimination of all members of the *Anopheles punctulatus* complex by polymerase chain reaction- restriction fragment length polymorphism analysis. Am. J. Trop. Med. Hyg. 1995;53:478–481.
30. Wong WH, Tay YC, Puniamoorthy J, Balke M, Cranston PS, Meier R. 'Direct PCR' optimization yields a rapid, cost-effective, nondestructive and efficient method for obtaining DNA barcodes without DNA extraction. Mol. Ecol. Res. 2014;14:1271-1280.
31. Wang WY, Srivathsan A, Foo M, Yamane SK, Meier R. Sorting specimen-rich invertebrate samples with cost-effective NGS barcodes: Validating a reverse workflow for specimen processing. Mol. Ecol. Res. 2018;18:490-501.
32. Meier R, Wong W, Srivathsan A, Foo M. 2016. $1 DNA barcodes for reconstructing complex phenomes and finding rare species in specimen-rich samples. Cladistics. 2016; 32:100-110.
33. Bandelt HJ, Forster P, Rohl A. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 1999;16:37-48.
34. Leigh J, Bryant D. POPART: full-feature software for haplotype network construction. Meth. Ecol. Evol. 2015;6:1110–6. doi: 10.1111/2041-210X.12410.
35. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 2016;33:1870-1874.
36. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 1987;4:406–425. https://doi.org/10.1093/oxfordjournals.molbev.a04045437.
37. Srivathsan A, Meier R. On the inappropriate use of Kimura-2-Parameter (K2P) divergences in the DNA – barcoding literature. Cladistics. 2011;28:190-194. 10.1111/j.1096-0031.2011.00370.x.

38. Ssymank A, Doczkal D. *Hermetia illucens* (Linnaeus, 1758) (Stratiomyidae), a soldierfly new to the German fauna. Studia dipterol. 2010;16:84–86.
39. De Groot M, Veenvliet P. *Hermetia illucens* L. (Diptera, Stratiomyidae), a new alien invasive species in Slovenia. Acta Entomol. Slovenica. 2011;19:195–198.
40. Roháček J, Hora M. A northernmost European record of the alien black soldier fly *Hermetia illucens* (Linnaeus, 1758) (Diptera: Stratiomyidae). Čas. Slezské zemské muz. 2013; 62: 101–106.
41. Chia SY, Tanga CM, Khamis FM, Mohamed SA, Salifu D, Sevgan S, Fiaboe KKM, Niassy S, van Loon JJA, Dicke M, Ekesi S. Threshold temperatures and thermal requirements of black soldier fly Hermetia illucens: Implications for mass production. PLoS One. 2018;13: e0206097. https://doi.org/10.1371/journal.pone.0206097
42. Holmes LA, Van Laerhoven SL, Tomberlin JK. Lower temperature threshold of black soldier fly (Diptera: Stratiomyidae) development. J Insects as Food and Feed. 2016: 2:255-262.
43. Spranghers T, Noyez A, Schildermans K, De Clercq, P. Cold Hardiness of the Black Soldier Fly (Diptera: Stratiomyidae). J Econ. Entomol. 2017; 110:1501–1507.
44. Brower AVZ. Rapid morphological radiation and convergence among races of the butterfly Heliconius erato inferred from patterns of mitochondrial DNA evolution. Proc. Natl. Acad. Sci. 1994;91:6491-6495.
45. Qi Y, Xu J, Tian X, Bai Y, Gu X. The complete mitochondrial genome of *Hermetia illucens* (Diptera: Stratiomyidae), Mitochondrial DNA Part B. 2017; 2:189-190, DOI: 10.1080/23802359.2017.1307708.
46. Zhan S, Fang G, Cai M. *et al.* Genomic landscape and genetic manipulation of the black soldier fly *Hermetia illucens*, a natural waste recycler. Cell Res. 2019. doi:10.1038/s41422-019-0252-6.

Supplementary Information

Supplementary Fig. S1. Neighbor-Joining network (uncorrected p-distances) of all included haplotypes (labelled with country and sample code). The biogeographical region of the haplotypes is color-coded (yellow squares=Afrotropical, pink squares and circles=Australasian and Oriental, pale blue squares=Neotropical, green circles=Nearctic, blue circles=Palaearctic). All rearing culture samples are indicated with a *.

Supplementary Table S1. GenBank accession numbers for each distinct haplotype and locality/culture. Specimen repository indicated for pinned fly specimens (CSCA=California State Collection of Arthropods, USA).

Table

Table 1. Occurrences of the here observed most abundant haplotypes shared between rearing cultures and wild-caught flies.

In culture: Country of origin of the rearing facilities (different facilities in the same country indicated with letters A-C) of the studied BSF cultures. The total number of sequenced samples for culture samples in each country indicated in brackets. **In nature**: Origin of samples obtained from pristine habitats or natural areas close to rearing facilities with haplotypes identical with the samples obtained from rearing cultures.

	In culture	In nature
I	China: A & B (2); France: A, B & C (3); Kenya (17); South Africa: A, B & C (57); Spain (55); Switzerland (24); USA: A & B (9); Vietnam (1).	AFROTROPICAL: Kenya, South Africa; NEARCTIC: USA; ORIENTAL: Bhutan, China, Indonesia; PALAEARCTIC: Russia, South Korea; EASTERN PALAEARCTIC: China.
II	Australia A, B & C (65).	AFROTROPICAL: La Reunion, Madagascar, Zambia; AUSTRALASIA: Australia; ORIENTAL: French Polynesia, India, Malaysia, Papua New Guinea, Singapore, Vietnam, Bangladesh; PALAEARCTIC: France, South Korea, Switzerland; NEOTROPICAL: Bolivia.
III	Singapore (4).	AUSTRALASIA: Australia; PALEARCTIC: Pakistan, South Korea; ORIENTAL: Singapore, Thailand, American Samoa, Bhutan; NEARCTIC: Canada.

Figures

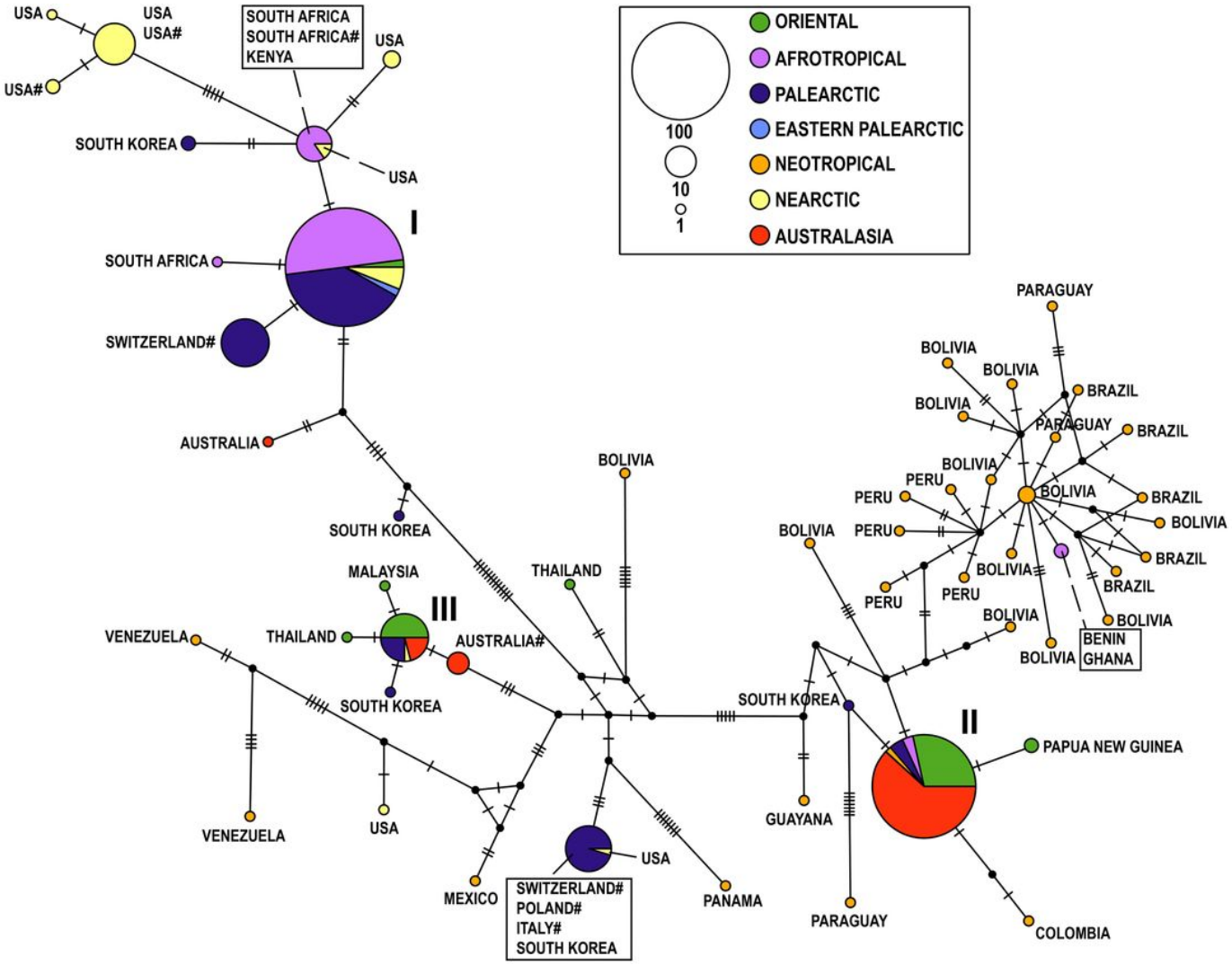

Figure 1

Median-Joining haplotype network of all 54 COI haplotypes. The biogeographical region of the haplotypes is color-coded, and the size of circles is proportional to the number of individuals per haplotype. The black circles represent putative un-sampled haplotypes. The branch lengths are largely proportional to the numbers of mutational steps separating the haplotypes, and the number(s) of mutational steps are indicated with hashmarks on the branches. All rearing culture samples are indicated with a #. The most abundant COI haplotypes are indicated with Roman numerals I-III with the geographical sources are listed in Table 1.

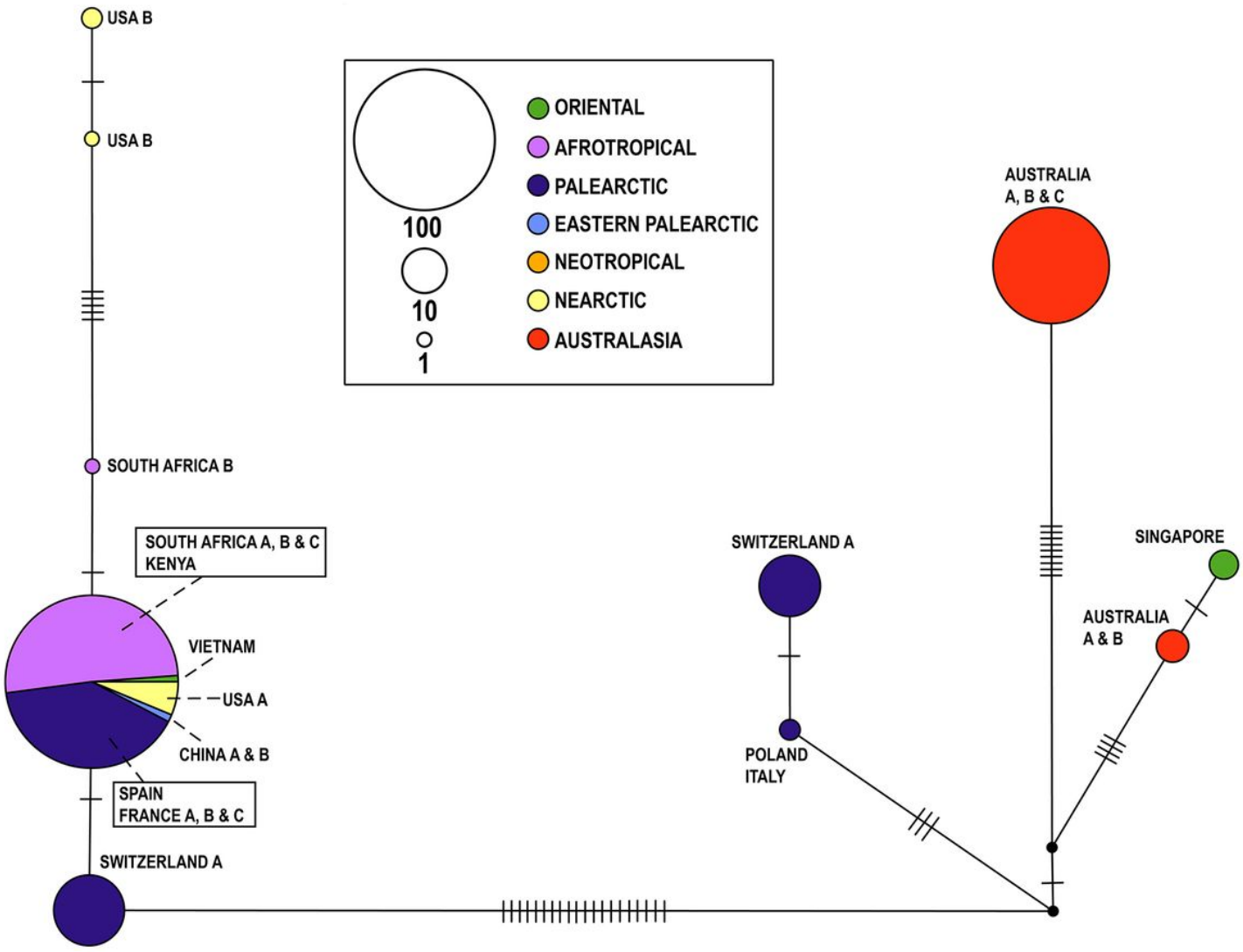

Figure 2

Median-Joining network of the ten observed rearing culture COI haplotypes. Haplotype description as in Fig. 1.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplFig.S1NeighborJoiningtreeCOIbarcodes.jpg
- SupplementaryfileS1.BSFGBaccessionnumbers.xlsx