Bangladesh J. Zool. **48**(2): 429-440, 2020 ISSN: 0304-9027
eISSN: 2408-8455

MOLECULAR IDENTIFICATION AND LIFE CYCLE OF BLACK SOLDIER FLY (*HERMETIA ILLUCENS*) IN LABORATORY

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Abstract: Molecular identification and life cycle of the Black Soldier Fly (BSF), *Hermetia illucens* were carried out from the Bangladesh bio-geographical area. The sequencing result and phylogenetic analysis of BSF showed 99-100% similarity with *H. illucens* from GenBank. The average duration of life cycle of male and female were 45.08±4.46d and 46.15± 4.12d respectively. The adult female is 16.3±0.91mm long, whereas the adult male is 14.30±0.19 mm long and smaller than female. The number of eggs per clutch was 537.37±40.21 which hatched in 4.36±0.24 days. The mean duration of the developmental stages were 16.07±2.59, 15.4±2.50, 9.95±1.48 and 10.33±1.89 d for larva, pupa, male and female respectively, when cultured at 29.40±1.77° C, RH 68.25±2.32 %, 14:10 (L: D) photoperiod. The mature larval weight (0.20±0.03 g) was highest among other developmental stages.

***Key words*:** Molecular identification, phylogeny, life cycle, black soldier fly, *Hermetia illucens*

INTRODUCTION

The Black Soldier Fly (BSF) *Hermetia illucens* (Linnaeus, 1758: Diptera: Stratiomiydae) is a potential insect that offers an effective technology for waste management (Myers *et al.* 2014, Sheppard *et al.* 1994, Oonincx *et al.* 2015, urRehman *et al.* 2017). The distribution of this species has extensively expanded in temperate and tropical regions throughout the world (James *et al.* 2015, Martínez-Sánchez *et al.* 2011, Tsagkarakis *et al.* 2015, Callan 1974). The voracious larvae of BSF are able to consume a wide variety of organic materials, ranging from animal waste to fruits, vegetables and plant material converting into fat, protein and minerals to morphing into pupae, and later, into adults (St-Hilaire *et al.* 2007; Myers *et al.* 2008, Diener *et al.* 2009, 2011, Kalová and Borkovcová 2013, Cičková *et al.* 2014). In addition, adults of *H. illucens* are not considered as pests because they have no functional mouthparts, do not bite

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nor feed, and do not vector spreading diseases (Cičková *et al.* 2014). BSF is considered as a good source of nutrients like proteins, lipids, minerals (Spranghers *et al.* 2017). Some suggests high protein enriched BSF larvae/prepupae could be utilized as diet for different species as fish, chicken and pigs (Newton *et al.* 1977, Cummins *et al.* 2017) and as a pet food (Bosch *et al.* 2014). Recently, investigations have been conducted on renewable biodiesel production from lipids of the BSF larvae (Cičková *et al.* 2015, Li *et al.* 2015), and the residue byproduct after BSF culturing can be adopted as bio-fertilizer (Zheng *et al.* 2012). Lastly, insect-based feed production technologies at low cost propose the potential to allocate employment opportunities and livelihood improvement for both farmers and urban entrepreneurs (Diener *et al.* 2015).

Although a few sporadic studies on the culture techniques and potentials of *H. illucens* have been reported in Bangladesh (Rana *et al.* 2015) no research has been reported on genetics and comprehensive life cycle of local species in Bangladesh. DNA barcoding established as an alternative to traditional taxonomic identification methods. Mitochondrial DNA is maternalistic without recombination with ancestral male mitochondrial DNA (Nelson and Cox 2005, Alberts *et al.* 2005). However, cytochrome c oxidase subunit 1 (*CO1*) gene is largely manipulated markers in the studies of population genetics and evolution (Hebert *et al.* 2003). However, the molecular biology of BSF, is inadequately researched. Khamis *et al.* 2020 had observed genetic variability and microbial diversity among BSF populations from different geographic locations in the world using the barcode region of the mitochondrial cytochrome oxidase I (mtCOI) gene and microbiome through 16 S metagenomics (Khamis *et al.* 2020). Some investigations have been conducted on genetics of BSF (Ståhls *et al.* 2020, Gao *et al.* 2019, Yingju *et al.* 2017).

The knowledge about life history traits and molecular biology of a species plays very important role to establish breeding industry and mass scale production. Additionally, previous studies have been supervised on life history traits of BSF on different substrates to utilize industrialization in temperate zone (Jucker *et al.* 2017, Shumo *et al.* 2019, Gobbi *et al.* 2013). In this study, molecular identification using the barcode region of the mitochondrial cytochrome oxidase I (mtCOI) gene and life cycle of local species, *H. illucens* from Bangladesh were investigated under laboratory condition.

MATERIAL AND METHODS

Rearing of BSF under laboratory conditions: Eggs were obtained from wild BSF from the campus area of Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka, Bangladesh. The culture was commenced by keeping domestic organic waste composed of fruits (peels of mango, water melon, pineapple, jackfruits and spoiled apple) and vegetables (spoiled gourds, spoiled carrots, peel of green papaya, bottle gourd and potato) wastes (1:1) cut into small pieces in two containers covered by lid to attract the adult flies to lay eggs. These containers carried four holes (2 inch diameter) on the upper portion to allow adult flies. A cluster of corrugated cardboards of 4 inches long tied together and attached in a bamboo splits establishing in the wastes inside the container for laying eggs of BSF (Booth and Sheppard 1984). After two days, egg clutches of BSF were collected with corrugated sheets for next studies.

Three experimental culture of BSF was conducted in the insectarium of Biological Research Division, BCSIR in June to August, 2018. The experimental diets previously described was kept in the plastic container having cemented ladder from the bottom to collection pipe at angle of 45° that facilitates the self-harvesting of mature larvae as they morph into pre-pupa. The egg clutches with corrugated sheets were directly positioned in the experimental diets. Eggs and larvae were maintained at 29.40±1.77° C, RH 68.25±2.32 %, 14:10 (L: D) photoperiod. Ten larvae from three replicate experimental containers was collected, measured length by electronic caliper and weighed on an analytical balance. After measuring, larvae were restored in their respective containers. According to Nguyen *et al.* (2015), the average time to reach prepupal stage (days ± SE) and the final mean larval weight (g ± SE) were determined when 40% of the larvae reached the prepupal stage, indicated by the transform of their creamy white color to black. Prepupae were counted daily and were kept in the wooden black box which did not allow any light but breathable. This box facilitates pupation and adult emergence. Thirty pupae from each container were weighed with the analytical balance and their length measured using an electronic caliper. Emerged adults were counted, sexed, and placed in pairs in ten sets of identical transparent plastic containers (10L) from three replicates for determining their longevity and fecundity. These setups were kept in the insectarium with artificial lighting (60W) and 29.40±1.77° C, RH 68.25±2.32 % under a 14:10 (L: D) photoperiod where they could mate. The oviposition substrates (poultry diet mixed with water) with cardboard as attractant for females (Booth and Sheppard 1984) were provided in adult rearing containers. Cardboard strips were checked every day for egg masses. Egg clutches were collected and the eggs counted under a microscope. The adult flies' longevity

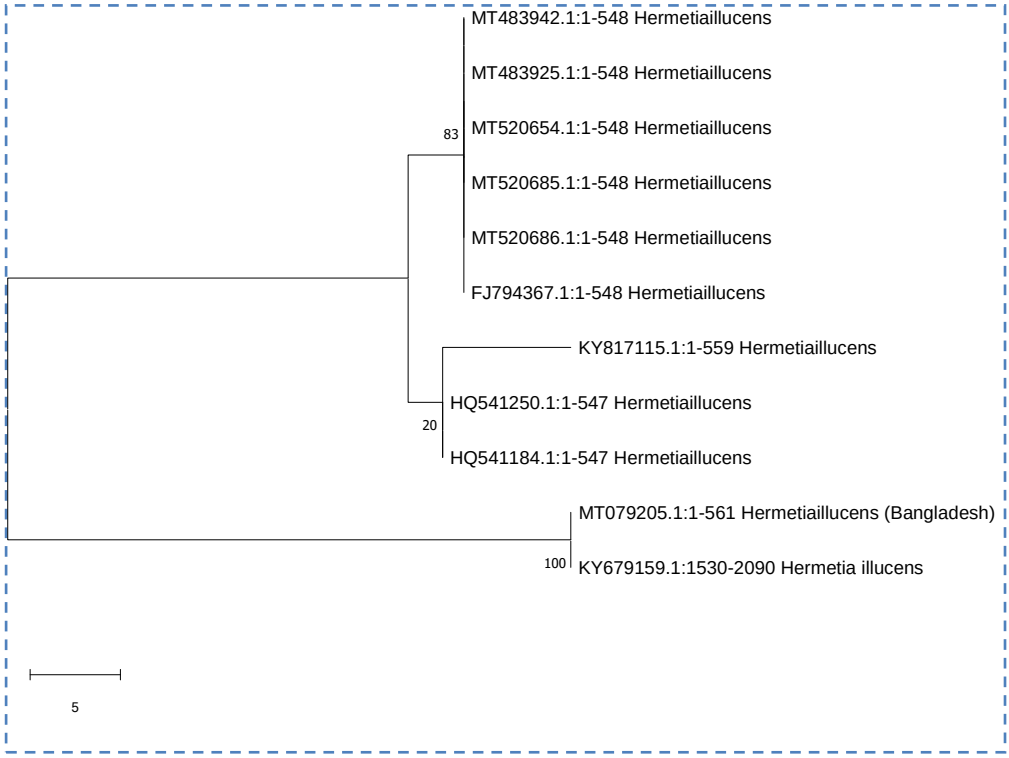

Fig. 1. Neighbor-Joining (K2P) tree of *H. illucens* from Bangladesh, based on COI gene

was noted daily until all the containers' flies were expired and comparing of adult weights, 30 adults were weighed and length measured.

DNA extraction, Polymerase Chain Reaction (PCR) and Sequencing: For genomic DNA extraction from alcohol preserved fifth instar larva was used NucleoSpine® DNA Insect Kit (Takara, Japan) according to the manufacturer's guidelines. The mitochondrial cox1 gene was amplified in PCR applying primers LCO-1490 (5´GGTCAACAAATCATAAAGATATTGG3´) and HCO- 2198 (5´-TAAACTTCAGGGTGACCAAAAAATCA-3´) (Herbert et al., 2003) followed describing conditions (Zagon *et al.* 2018). The amplified PCR product was visualized under UV light after gel electrophoresis in 1.5% agarose gel stained with ethidium bromide. The PCR product was sequenced from commercial sequencing service (Apical Scientific, Malaysia) by Sanger's method using the same primers used for DNA amplification.

Sequences analyses and phylogeny trees reconstruction: The sequences were further aligned using MEGA-X-10.2.1 software. The comparison among obtained sequence with sequences available in GenBank was employed with BLAST service available at http://www.ncbi.nlm.nih.gov:80/BLAST. Phylogenetic studies were accomplished with MEGA-X-10.2.1software using the "Maximum Likelihood method" (Tamura *et al.* 2013). The Phylogenetic trees were constructed using neighbor joining (NJ) method. Phylogenetic analyses were conducted in MEGA-X software. Bootstrap support values were obtained by 1,000 replications using method (Tamura *et. al.* 2013). The reference accession numbers of NCBI database sequences for *H. illucens* (KY679159.1, KY817115.1, MT520686.1, MT520685.1, MT520684.1, MT520654.1, MT483942.1) were used in construction of ML tree.

Data analysis: The recorded data were analyzed using MS excel. Descriptive statistics, mean and standard deviation (SD) were calculated first. Then in order to test the equality of these parameters, analysis of variance (ANOVA) was performed. A few parameters varied significantly ($p<0.05$). Finally, Duncan Multiple Rank Test (DMRT) of Post Hoc series of tests were performed.

RESULTS AND DISCUSSIONS

BSF Identification and Phylogeny: COI sequence of *H. illucens* was generated in this study that is the first COI sequence for the Bangladesh biogeographical area. The sequence was a 561bp fragment of nucleotides. Similarity and phylogenetic analyses were performed for identification of species (Table 1). The

Table 1: Similarity level of *H. illucens* based on alignment analysis on NCBI website

Bangladeshi Strain	***Hermetia* species**	**Identity**	**Accessions Numbers**
Hermetiaillucens MT079205.1	*Hermetia illucens* voucher hsm-1	100%	KY679159.1
	Hermetia illucens	100%	KY817115.1
	Hermetia illucens isolate USA-1-42	100%	MT520686.1
	Hermetia illucens isolate USA-1-41	100%	MT520685.1
	Hermetia illucens isolate USA-1-40	100%	MT520684.1
	Hermetia illucens isolate SA-4	100%	MT520654.1
	Hermetia illucens isolate Ken-36	100%	MT483942.1
	Hermetia illucens isolate Aus-54	100%	MT483925.1
	Hermetia illucens isolate Chi-60	100%	MT483917.1
	Hermetia illucens isolate Yangyang 2	99.64%	FJ794367.1
	Hermetia illucens voucher Seonghwan-falaw-pu-3	99.63%	HQ541250.1
	Hermetia illucens voucher Gurae-10	99.63%	HQ541184.1

Table 2: Life cycle duration of *H. illucens*

	Fecundity	Incubation period (days)	Time to first Preparation (days)	Time to First adult emerging from prepupae (days)	Adult male longevity (days)	Adult female longevity (days)	Developmental time egg to adult male (days)	Developmental time egg to adult female (days)
Mean±SD	537.37±40.21	4.36±0.24	16.07±2.59	15.4±2.50	9.95±1.48	10.33±1.89	45.08±4.46	46.15±4.12
Minimum	450	3.95	12	11	8.08	6.2	34.21	38.83
Maximum	625	4.75	22	20	12.63	13.58	54.34	54.13

Table 3: Lengths and weights of developmental stages of *H. illucens*

	Egg length (mm)	Length of mature larvae (mm)	Weight of mature larvae (g)	Length of Pupa (mm)	Wight of Pupa (g)	Length of adult male (mm)	Weight of adult male (g)	Length of adult female (mm)	weight of adult female (g)
Mean±SD	0.91±0.06	20.53±2.37	0.20±0.03	17.93±2.49	0.11±0.02	14.30±0.19	0.06±0.01	16.3±0.91	0.07±0.01
Minimum	0.8	16	0.136	13	13	12	0.034	15	0.049
Maximum	1	25	0.263	22	22	16	0.082	18	0.085

result displayed the highest percentage of nucleotides identity ranging from 99 to 100% of the current investigation of NCBI submitted nucleotides sequence of *H. illucens* (MT079205.1) with NCBI available KY679159.1, KY817115.1, MT520686.1, MT520685.1, MT520684.1, MT520654.1, MT483942.1, MT483925.1, MT483917.1, FJ794367.1, HQ541250.1, HQ541184.1 *Hermetia* sequences respectively (Table 1). The phylogeny tree was consisting of 2 monophyletic clades with *H. illucens* H GenBank. *H. illucens* of our study was on the second monophyletic clade. Thus the variation of BSF in Bangladesh with *H. illucens* from GenBank has based on the evolutionary relationship.

Life Cycle of H. illucens: This investigation has shown that *H. illucens* successfully accomplished life cycle (Table 2 and 3; Plate. 1) when cultured on the domestic organic waste (composed of fruits and vegetables waste). However, former investigations reveals that the diet, temperature and moisture have strongly influenced the life traits of *H. illucens* (Jucker *et al*. 2017, Cammack *et al*. 2017, Shumo *et al*. 2019). In this study, the duration of the female life cycle was longer than male life cycle (Table 2), which supports results from previous

research performed on BSF (Jucker *et al*. 2017). Sivanantharaja and Gnaneswaran, (2018) registered the duration of total lifespan from egg to adults

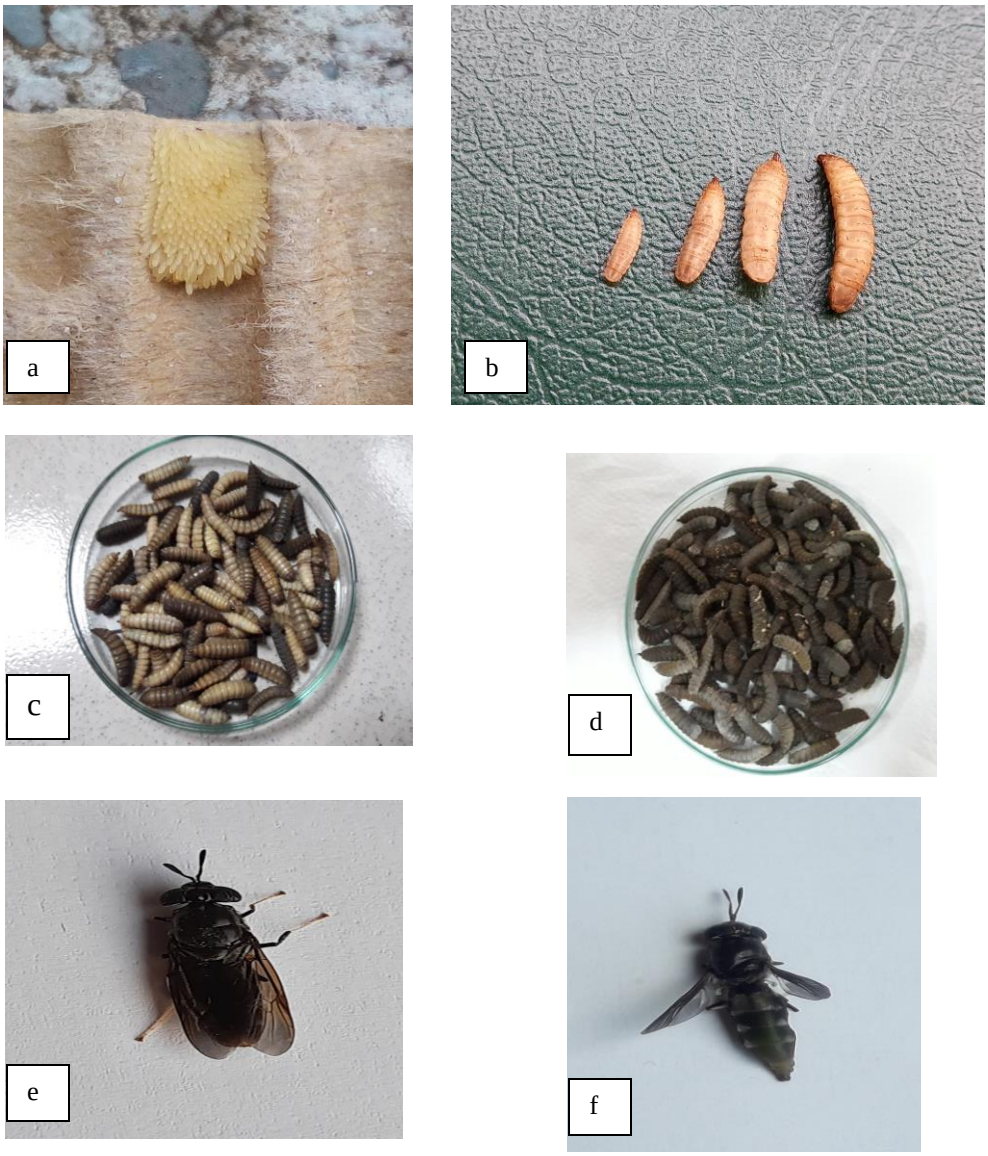

Plate 1: Different developmental stages of *H. illucens*: (a) Egg; (b) Different instar larvae (c) Prepupae (d) Pupa (e) Male (f) Female

was 57.8 days at 30.15 ± 0.26°C. As previously observed (Jucker *et al.* 2017), the longevity of *H. illucens* female was significantly higher than male longevity. This is similar to the result of male and female longevity in our research. The results of egg incubation, larval and adult emergence period (Table 2) were agreed with antecedent investigations (Shumo *et al.* 2019, Sivanantharaja and Gnaneswaran 2018). There was a significant interaction among diet (Jucker *et al.* 2017; Nguyen *et al.* 2013, Zhou *et al.* 2013), temperature (Shumo *et al.* 2019) and moisture (Cammack *et al.* 2017) content on developmental time, length and weight for larvae and prepupae.

The average length (20.53±2.37mm) and weight (0.20±0.03g) of mature larvae or prepupae (Table 2) in our study were similar to previous literature (Shumo *et al.* 2019, Sivanantharaja and Gnaneswaran, 2018). The pupal and adult length in this investigation (Table 3) were same to findings of former studies (Jucker *et al.* 2017, Sivanantharaja and Gnaneswaran, 2018) but weights (Table 3) were higher than findings of early research (Jucker *et al.* 2017). Additionally, the weights were depended with the last-instar larval weights, as holometabolous insects turn a critical weight to stimulate the hormonal cascade that leads to interruption of feeding and metamorphosis (Davidowitz *et al.* 2003, Nijhout 2003, Stern 2003). Moreover, the nutrient of diets largely stimulated endocrine events influencing the final body size and weight during different stages of life cycle of insects (Nijhout 2003). In this study, *H. illucens* females (Table 3) were always remarkably bigger than males which was observed in previous researches (Tomberlin *et al.* 2002, Jucker *et al.* 2017). The sex ratio of males to females was 2.85:1 (n = 600) which did not support the results of prior studies (Jucker *et al.* 2017, Sivanantharaja and Gnaneswaran, 2018). The number of male in this study was significantly greater than female. It was noted that, diet significantly influenced sex ratio (Jucker *et al.* 2017, Sivanantharaja and Gnaneswaran, 2018). The observed number of eggs in a single clutch and egg size were same with former investigations (Jucker *et al.* 2017). Additionally, egg production of insects depends on nutritional reserves (Chippindale *et al.* 1993, Kaspi *et al.* 2002, Tomberlin *et al.* 2002).

CONCLUSION

Using the mitochondrial cytochrome oxidase I (mtCOI) genewe were able to identify *H. illucens*. Our results mention that the duration of life cycle of BSF ranging 34.21 to 54.34 days. Hatching of all eggs lasted 4.36±0.24d and larval stage was the longest among other stages of the life cycle. Female survived significantly longer time than male. This information regarding molecular

identification and life cycle of *H. illucens* might be important for identification and rearing of BSF in Bangladesh, which addresses both the bioconversion of organic waste and production of an alternate protein source.

Acknowledgement: Authors are grateful to Bangladesh Council of Scientific and Industrial Research (BCSIR) for providing the research facilities and financing this study under grant "Research and development project". Special thanks to Ministry of Science and Technology (MOST) for funding under "Special Allocation Project- 2018-2019".

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(Manuscript received on 20 September, 2020 revised on 25 December, 2020)