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RESEARCH PAPER

Molecular Marker Study for *Hyles euphorbiae* (Lepidoptera: Sphingidae) Based on Mitochondrial DNA Genes in Erbil Province

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A B S T R A C T:

The Hawkmoths consisting more than 1,500 species over worldwide in 200 genera. The family sphingidae is splitted into two taxonomic categories which is sub-family sphinginae and macroglossinae. The mitochondrial genes sequence commonly used for taxonomic phylogeny due to maternal inheritance and less degradation. The aim of the present study was the taxon identification of *Hyles euphorbia* among other level species of this genus. Sixteen *Hyles* (eight males and eight females) were use and then DNA was extracted from insect anterior abdomen. Multiplex PCR was performed for amplification of specific targeted sequence DNA in mitochondrial cytochrome oxidase I and II genes. The *Hyles euphorbiae* was successfully identified and this corresponds to the amplification of sixteen specimens of targeted DNA fragment with using a group specific primers that covering the targeted sequence between 277 bp and 280 bp. The present study was concluded that the *Hyles euphorbiae* exists in Kurdistan region and Multiplex PCR can be done for this reason.

KEY WORDS: COI, COII, mtDNA, *Hyles euphorbiae*, Multiplex PCR.
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1. INTRODUCTION

he Hawkmoths (Lepidoptera: Sphingidae) consisting more than 1,500 species over worldwide in 200 genera and appear on every continent exclude Antarctica are one of the most obvious and widely studied insects (Kawahara and Barber, 2015, Kawahara et al., 2009, Duarte et al., 2008). Hawk moth is a successful genus that evolved in Neotropics depending on molecular data (Hundsdoerfer et al., 2019, Ernst et al., 2018).

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The sphingidae name was formulated by Samouelle in 1819. The family sphingidae is splitted into two taxonomic category, first one sub-family sphinginae that consist of 116 genera, second one sub-family macroglossinae that contain 89 genera (Messenger, 1997) and sub-family Smerinthinae (Li et al., 2018).

Sphingidae is one of the nocturnal recognizable family belong to abundant size and prevalence which is appeal to light sources (Moré et al., 2005). Mating behaviour occurred at night (Kilaso and Tigvattananont, 2018). The new island *Hyles* was collected during the day and without light (Tennent and Russell, 2015). The spurge hawk moth *Hyles euphorbiae L.* (Lepidoptera: sphingidae) are from moderate to large size and the body weight between 0.1 to 7 gram. Hawkmoth species distributed in Central/Southern Europe and Western Asia (Hundsdoerfer et al., 2005b). The mysterious biogeographic pattern in *Hyles* during speciation

stage is belong to cosmopolitan distribution, polymorphic also disapproval classification species (Mende et al., 2016, Halloway et al., 2018).

Hawkmoth has role in balance ecosystem through floral pollination, the floral member advertises and signal got to pollinator by olfactory neurons in terminal of proboscis which strongly have role in floral diversity by transferring pollen grains (Haverkamp et al., 2016, Heywood et al., 2017). The long tongued moths are adapted for pollination (Suetsugu et al., 2015).

Classification in the past was done depended on superficially phenotype body characters like pattern and of wing and abdomen in mature stage, color in larval immature stage and genital feature (Hundsdoerfer and Kitching, 2017). Species identification requires data from more sources such behavior phenotype and DNA markers so only a very little variation in molecular level is enough also quite efficient to detect unknown insect (Funk and Omland, 2003, Dayrat, 2005).

The DNA sequence of mitochondrial genes commonly used for taxonomic phylogeny due to maternal inheritance, sequence conservation, little modification, quick development and less degradation (Avise et al., 1987). The sequence data comprise about 2300 bp of the mitochondrial genes cytochrome c oxidase subunit I (COX I), cytochrome c oxidase subunit II (COX II), and the gene of the ribosomal transfer RNA for leucine (tRNA-leu). In the mitochondrial genome of *Hyles*, this ribosomal region lies between the two COX genes (Hundsdoerfer et al., 2005a). The primers were designed depending on the various marker sequence of commonly used mitochondrial genes like cytochrome oxidase I, II and 16S rRNA genes for species level classification (Folmer et al., 1994, Caterino et al., 2000). *Hyles* can be used as a model organism for biological and environmental studies like species development and role of genetic factor in ecology adaptation (Barth et al., 2018, Cock, 2018).

Deoxyribonucleic acid (DNA) barcode sequence generated for Sphingidae through which compare with global DNA insects that can find the exact geographical distribution (Haxaire et al., 2015). The various projects of insect taxonomy really realize to use specific primer not universal primers to gain accurate and success PCR (Hebert et al., 2004, Penton et al., 2004). Molecular techniques is the quick method used to develop biology fields and till now our knowledge of molecular feature evolution remains relatively limit and understanding on phylogenetic relationship depend on the analysis of molecular data (Blair and Hedges, 2005, C Regier et al., 2005). The aim of the present study was taxon identification of *Hyles euphorbia* among other level species of this genus.

2. MATERIALS AND METHODS

2.1. Sample Collection:

The larvae of *H. euphorbiae* were collected on Euphorbia macrocalda at the periods from March – April from the villages Hanara at Shaqlawa, in Iraqi Kurdistan region. The food plants restricted to the genus *Euphorbia* (Euphorbiaceae). The captured larvae were taken from field to laboratory in appropriate box. The fresh leafy spruge leaves were provided as food for developing larvae. The larvae were kept in the room temperature during the experiment. Cotton wad dipped in 10 per cent honey solution was provided as food for the moths. Sixteen *Hyles* (eight males and eight females) were collected manually using entomological net and then ethyl acetate was used as an injection solution ventrally between thorax and abdomen to kill the sphingidae. The specimens preserved in 100% ethanol or store at -20°C until DNA extraction (Hundsdoerfer et al., 2005a, Primo et al., 2013, Singh and Kaur, 2017a, Santos et al., 2015, Sondhi et al., 2017). Sex differentiation were carried out by phenotypic character of wing frenulum, male with one large and female with a brush like bristle (Primo et al., 2013).

2.2 Genomic DNA Extraction from Insect Sample

The piece of the anterior abdomen was separated from sphingidae and followed by DNA isolation from sixteen of Hyles member depend on protocol of geneaid DNA isolation kit manufacturers' instructions. Weight 10-20 mg of anterior abdomen tissue and transfer to 1.5 ml Eppendorf tube. Micropestle and mortal was used to squash the tissue, suspend in 600 µl cell lysis buffer, homogenize the sample by continue grinding (Mende and Hundsdoerfer, 2013).

Digest the tissue by adding 12 µl of Proteinase K to the tube and mix by vortex then incubate at 60ºC for 30-60 minutes, during incubation, invert the tube periodically. Add 200 µl of Protein Removal Buffer to the sample lysate then vortex immediately for 10 seconds. Centrifuge at 14-16,000 x g for 3 minutes to form a tight pellet. Transfer the supernatant to a clean 1.5 ml microcentrifuge tube then add 600 µl of isopropanol and mix well by gently inverting 20 times. Centrifuge at 14-16,000 x g for 5 minutes then carefully discard the supernatant and add 600 µl of 70% ethanol to wash the pellet. Centrifuge at 14-16,000 x g for 3 minutes then carefully discard the supernatant and air-dry the pellet for 10 minutes. Add 100 µl of DNA Hydration Buffer then gently vortex for 10 seconds. Incubate at 60ºC for 30-60 minutes to dissolve the DNA pellet. During incubation, tap the bottom of the tube to promote DNA rehydration. Then the concentration and purity of genomic DNA extracts from each insect samples were determined using Nano-Drop nd-1000 spectrophotometer by recording the concentration from (110.1 - 177.89 ng/µl) and purity (1.15 – 1.88) for each sample.

2.3. Molecular Technique Analysis

The mitogenome study can be used for phylogenetic of Lepidoptera including Bombycidae, Saturniidae and Sphingidae. Molecular technique was performed for amplification of specific targeted sequence DNA in mitochondrial cytochrome oxidase I, II genes and t-RNA gene for leucine (Hundsdoerfer et al., 2009, Kim et al., 2016, Gu et al., 2016). Multiplex PCR was carried out to amplify three fragments covering 794 bp in total (B: 277 bp, H: 280 bp and L: 237 bp), the primers that used in the study were designed specifically for *Hyles* or *Hylex euphorbiae* complex (HEC) (Mende and Hundsdoerfer, 2013).

Amplification was conducted in polymerase chain reactions in total volume 25 µl containing 3.4 µl of nuclease free water, 0.6 µl for each primer *Hyles* COIca100f:5-TAAGATTAYTAATTCGAGCAG-3, MLepR1": 5-CCTGTTCCAGCTCCATTTTC-3, HEC-COIca1110f: 5-ATGATACATATTATGTTGTAGC-3, HEC-COIca1350r: 5-GAGATATATGACCCTAATGATGA-3, HylesMCOIIf: 5-GATACTGAAGATATGAATATTC-3, HylesCOIIca2125r: 5-TTGTTTGGTTTAAACGTCCAGG-3) respectively (10 pmol/µl) (Simon et al., 1994) and 15µl of master mix (Promega, USA). Three microliters genomic DNA as a template was used. The program file condition consisted of an initial 5 min denaturation at 95º C followed by 35 cycles of 95º C for 30 s, 57º C for 1 min 30 s and 72°C for 45 s and final elongation at 60°C for 30 min and was performed on a thermal Cycler (Techne, UK). After PCR amplification,10µl PCR product was loaded on to 2% agarose then the separated bands were stained with ethidium bromide and visualized under UV light (Brown, 2016).

3. RESULTS AND DISCUSSION

3.1. Extraction Yield

The grouping and identifying of Lepidoptera is applicable depending on phenotypic characters while insect speciation in this genus cannot be used therefore PCR target amplifications of COX I, COX II and t-RNA were conducted and used as standard tool for molecular taxonomy of *Hyles* classification (Hundsdoerfer et al., 2005a, Singh and Kaur, 2017b).

The *Hyles* can be classify according to Cytochrome C oxidase *in vitro* replication (Hundsdoerfer et al., 2009, Mende and Hundsdoerfer, 2013, Mende and Hundsdoerfer, 2014). Species communities can be rapidly identify via a group of specific primers (Sint et al., 2014). The set of taxon specific primers of Multiplex PCR were created specifically that bind accurately only with complementary DNA target sequence to classify Lepidoptera (Mende and Hundsdoerfer, 2013, Sint et al., 2014).

In the present study the *Hyles euphorbiae* was successfully identified amplified two just targeted fragments 277 and 280 bp in length of the CO I/II genes. This corresponds to the amplification of sixteen specimens of targeted DNA fragment with using a group specific primers in multiplex PCR that covering targeted sequence between 277 bp and 280 bp in length. The findings of the present study are in agreement with previous investigation of Mende and Hundsdoerfer, who reported that successfully PCR

amplified targeted fragments 277 and 280 bp in length of the CO I/II genes from 143 specimens by using these specific primers (Mende and Hundsdoerfer, 2013). Among the total specimens, ten haplotypes male and female of *Hyles euphorbiae* were detected successfully as elucidated in (Figure 1). Additionally, our work was confirmed by using the same primer in Uniplex PCR and they indicated the same results (Figure 2). While the DNA amplicons 237 bp had not offered and obtained from sixteen specimens of targeted DNA fragment with using a specific primer in Uniplex PCR might be due to the lack of suitable targeted and priming sites of PCR primer in the insect's genomic DNA to amplify of the gene of the ribosomal transfer RNA of *H. euphorbiae* collected in Kurdistan Region as illustrated in (Figure 3). Hundsdoerfer et al. was found that the there is a close relation between mtDNA of *H. euphorbiae* and *H. tithymali* lineage it means the remaining specimen have different DNA loci may belong *H. tithymali* (Hundsdoerfer et al., 2005a).

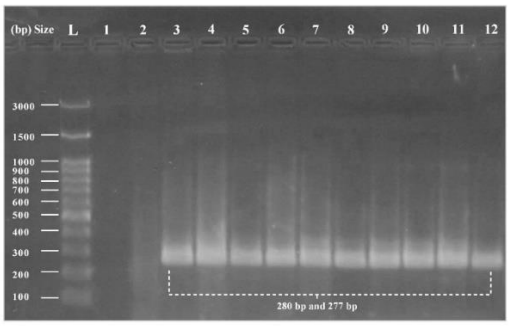

Figure 1. Agarose gel electrophoresis (2%) showing 277bp and 280 bp Multiplex PCR fragments corresponding to amplification of mitochondrial genes. Lane L: 100 bp DNA ladder. Lane 1: Negative control (H_2O was used as a template). Lane 2: *Hyles euphorbiae* not amplified with the specific primers. Lane 3-12: *Hyles euphorbiae* target DNA sequence amplified (B and H fragments) with the specific primers.

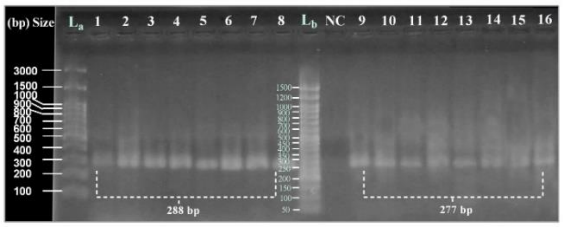

Figure 2: Agarose gel electrophoresis (2%) representing 277 bp and 280 bp Uniplex PCR products corresponding to amplification of mitochondrial genes. Lane La: 100 bp DNA ladder. Lane 1-8: 288 bp PCR DNA amplicons (H fragments) with the specific primer correspond to *Hyles euphorbiae* species. Lane Lb: 50 bp DNA ladder. Lane NC: Negative control (H2O was used as a template). Lane 9-16: 288 bp PCR DNA product sizes (B fragments) with the specific primer correspond to *Hyles euphorbiae* species.

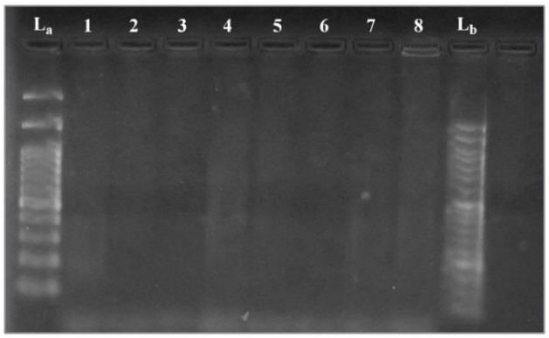

Figure 3: Illustrates failure of PCR amplification results obtained with specific primer corresponding to amplification (L fragments) of mitochondrial genes by (2%) agarose gel electrophoresis. Lane La: 100 bp DNA ladder. Lane 1-8: No PCR DNA amplicons with the specific primer correspond to *Hyles euphorbiae* species. Lane Lb: 50 bp DNA ladder.

4. CONCLUSIONS

The present study concluded that *Hyles euphorbiae* exists in Kurdistan region and Multiplex PCR can be used for this purpose. With regard to our findings of the present study, future DNA sequence analyses by recent molecular taxonomical study should examine the mitochondrial genes sequences in more detail to revealed the geographical distribution of mitochondrial lineages for *Hyles euphorbia* species with other hawkmoths species.

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