

DNA barcoding, life history and taxonomy of lablab pod borer, *Adisura atkinsoni* Moore (Lepidoptera, Noctuidae)

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ABSTRACT: DNA barcoding and morphological characters together with thorough bioecological investigations to diagnose field bean pod borer, *Adisura atkinsoni* Moore, 1881 an important pest was carried out. *A. atkinsoni* has a limited host range. The incidence of *Adisura* was higher on local varieties (photo sensitive) compared to HA4 hybrid (photo-insensitive). The field bean pod borer appeared during middle of October and continue to March. The undefined taxa were identified using both female and male genitalia. But since they could be differentiated from their morphologically closest relative, they were marked as sensu lato. While doing BLAST analysis, the mitochondrial COI Sequence of *Adisura* specimens collected from Bengaluru showed similarity to *A. bella*. In the phylogenetic tree, these two samples Sp1 and Sp2 separate new sub-clade which stands separately from the rest of the *Adisura* species. It was identified that the lepidopteran samples collected from Bengaluru are *Adisura* and the DNA sequences did not match with any of the existing species of *Adisura*. The shift in this pest's prevalence has been discussed. © 2024 Association for Advancement of Entomology

KEY WORDS: Field bean pod borer, molecular phylogenetics, bioecology, prevalence

INTRODUCTION

The field bean pod borer, *Adisura atkinsoni* Moore, is a noctuid moth first described in 1881. *Adisura leucanioides* Moore, 1881; *A. pallida* Moore, 1881 and *A. atkinsoni* Hampson, 1903 are the synonyms (Savela, 2023). Hampson (1903) and Lefroy (1909) recorded *A. atkinsoni* with notes on their distribution and larval plants from India. Kishida (2011) mentioned brief morphological description, distribution, developmental period, food

plants and sporadic outbreaks of *A. atkinsoni* in Japan. Also, there are several mentions of *A. atkinsoni* (Moore, 1881) in checklists and online databases (India Biodiversity Portal, 2016; ICAR-NBAIR, 2016; Encyclopedia of Life, 2018). *A. atkinsoni* is a specific, locally adapted, economically important pod borer on *Lablab* beans (*Lablab purpureus*). In Karnataka, the life cycle of the pod borer appears to have co-evolved with the life cycle of the local cultivars. *A. atkinsoni* is attracted to the specific odor that emanates from

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the local cultivar and its parts. Local cultivar is highly susceptible to this pod borer (Krishnamurthy and Appanna, 1948). In some *Lablab* varieties, which secrete fragrant oil on the surface of pods is preferred for consumption by humans and insects as well. The pod exudate consists of homologous fatty acids and their methyl esters-42 in all from C-11 through C-24, including odd carbon chain compounds. Apart from trans-2-dodecenoic and trans-2-tetradecenoic acids, which constitute the major percentage of the oil, other homologous Δ^2 -enoic acids and saturated acids and esters are also found (Fernandes and Nagendrappa, 1979). Local cultivar is photo-sensitive and blooms under restricted photoperiod range. The seeds of local cultivar have specific odor and taste which local people relish. *A. atkinsoni* is the dominant pod borer on local cultivar and persists on the crop from the beginning to the end (July-August to January-February) in Karnataka (Mallikarjunappa, 1989). This species is distributed across Asia and Africa (Hampson, 1894; Hampson, 1903; India Biodiversity Portal, 2016; ICAR-NBAIR, 2016 and CABI Datasheet, 2019). In present study, species identification using morphology and DNA barcoding along with bioecology of *A. atkinsoni* were undertaken. Also carried out phylogenetic relationship of *A. atkinsoni* with three other *Adisura* species and *Helicoverpa armigera* (Hubner) as outgroup.

MATERIALS AND METHODS

Field experiments were carried out at Zonal Agricultural Research Station (ZARS) of UAS, Bengaluru and ICAR-Indian Institute of Horticultural Research (IIHR), Bengaluru, Karnataka, India during 2016-2020. Geographically, ZARS, Bengaluru is located at 12°58' N; 77°35' E and an altitude of 930m above sea level. The annual average rainfall ranges from 679.1 to 888.9mm. The local photo-sensitive variety (local avare) was grown under field conditions for maintenance of laboratory culture, bioecological and life table studies. The recommended agronomic practices were followed to raise a healthy crop, except crop protection chemicals.

The initial culture of *Adisura* was obtained by collecting larvae from farmer's fields in and around areas of Doddaballapur, Bengaluru Rural district (13.29° N; 77.54° E) and maintained in laboratory at ICAR-Indian Institute of Horticulture Research during 2018-2020. The larvae were reared in the glass jars (41cm height and 30cm diameter). Fresh buds of local lablab bean were provided daily as food for larvae till pupation. Freshly formed pupae were kept individually in plastic jar along with sand, the top of which was covered with muslin cloth and secured firmly with rubber bands. On emergence of moths, they were fed with diluted (5%) honey solution. The emerged adults were paired and allowed to mate to get gravid females and were used for further studies. The moths were maintained in the laboratory (at $27 \pm 2^\circ\text{C}$ and 40% relative humidity).

Host Range: To test the host range of *A. atkinsoni* in laboratory, fresh blooms containing bud, flowers and tender pods of *L. niger* (Field bean), *Cajanus cajan* (Pigeon pea), *Vigna unguiculata* (Cow pea), *Cicer arietinum* (Chickpea) were offered to ovipositing moths in a wooden-wire-mesh cage (0.3 m³) in the laboratory under no-choice conditions. Two pairs of moths were released in to each cage and the experiment was repeated twice. To test the larval feeding response, fresh tender pods of *L. niger*, *C. cajan*, *V. unguiculata* and *C. arietinum* were offered to third instar larvae in petri dishes (9cm diameter), separately. Host acceptability was based on the orientation behavior and palpation and initiation of feeding. The experiment was repeated twice.

Species description: Different life stages were collected from the laboratory culture and stored in ethanol (10%) at the constant climatic parameters as stated above. Adults were dried, stretched, pinned and maintained in wooden insect boxes. A digital camera lucida was used for measurement. The adult moths were separated into males and females after identification of the specimens under laboratory conditions. All stages of *A. atkinsoni* were examined under an AO microscope i.e, Stereo Zoom microscope (Olympus) and mounted for species identification. All measurements like adult

and larval body length and width in adults, paired wings; length and width of thorax, abdomen and size of pupae, etc. were measured in mm and photographs were taken using Leica DFC 425 mounted on a Leica M205C.

For genitalia preparation, the abdomen of male and female moths were separated using a pair of micro scissors and placed in a test tube containing was KOH (10%) solution for overnight. Genitalia then transferred to a cavity block containing water and washed repeatedly to remove excess KOH. Then, genitalia was placed in glycerol on a slide for dissection and examination. Genitalia were taken out from the abdomen under a stereo binocular microscope. After examination, the parts of the specimens were transferred to a micro vial (10ml) containing glycerol (98% pure) and the vial was pinned below the specimen. The terminologies of Klots (1965) were adopted to describe the genitalia. Hampson (1894), Kristensen (2003) and Keegan *et al.* (2021) terms were used as the basis for identification of specimens.

DNA Barcoding: Sample collection and DNA Extraction: Third instar *Adisura* larvae were collected on local Lablab cultivar pods and stored in ethanol (95%). A total of six samples were collected on *Lablab* host plant species from three different locations of Karnataka (India) and preserved immediately in ethanol (95%) and stored at 4°C for future use. From all six samples, *Adisura* larval specimens were collected each for the marker analysis. Total genomic DNA was extracted from individual larvae. The specimens were washed briefly in sterile distilled water to remove alcohol prior to homogenization. Genomic DNA was extracted using MN- Genomic DNA from tissue kit (Macherey-Nagel, Germany) and then stored the DNA at -20°C for future use.

Mitochondrial COI gene amplification: A portion of mitochondrial COXI gene (approx 658 bp) fragment was amplified in a 25- μ l reaction mix, containing 1X PCR buffer (10 mM Tris-HCl pH 8.0), 2.5 mM MgCl₂, 0.2 mM dNTPs, 10 p mole each of Forward and reverse primers [LCO-1490 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-

3'; HCO-2198 5'- TAACTTCAGGGTGA CCAAAAATCA-3'], 1.25 U*Taq* DNA polymerase (Genei, Bangalore) and 200 ng of DNA template. PCR was performed in Veriti 96 well thermo-cycler (Life technologies-AB, USA) according to the following cycling condition, initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec; annealing temperature 54°C for 40 sec; extension at 72°C for 50 sec, and final extension at 72°C for 10 min to extend the incomplete fragment.

PCR products were visualized in agarose gel (1.2%) and the band was eluted using PCR clean-up gel extraction kit (Macherey-Nagel, Germany). Purified PCR products were cloned using pTZ57-T/A plasmid vector system and the vector was transformed into *Escherichia coli* DH5 α according to the manufacture protocol (Fermentas Life science, USA). The plasmid DNA was isolated from three randomly selected clones selected from each samples using Gene JET Plasmid mini prep kit (Fermentas Life Science, USA). Presence of the insert was checked by colony PCR using gene specific primer and visualized in agarose gel (1.2%). The sequencing was performed using M13 universal primers at Eurofins MWG Operon, India. The sequence homology was determined using BLASTn (<http://www.ncbi.nlm.nih.gov>), and the selected sequences were edited by manual using the sequence alignment editor 'BioEdit' version 7.0.

Phylogenetic analyses: Phylogenetic analyses were carried out for mtCOI marker. The sequences were aligned separately in CLUSTAL W program. Independent alignment was carried out for each taxon sample, resulting in marker dataset. Neighbor-Joining method was used to construct phylogenetic tree with kimura-2-parameter model with South American pin worm, *Tuta absoluta* as out-group. In addition, the number of substitutions, Transition (Ti)/Transversion (Tv) ratio, and nucleotide compositions for mitochondrial COI were also determined using MEGA version 7.0.

Biology: Laboratory studies on *A. atkinsoni* were carried out at the Department of Entomology, University of Agricultural Sciences, G.K.V.K.,

Bengaluru. The insect culture in laboratory was maintained at $25 \pm 2^\circ\text{C}$, 70 ± 5 RH, and photoperiod of 14:10 (L:D) at constant regimes of climatic parameters. Dates of pre-oviposition, oviposition and post-oviposition were recorded. All the plant parts were examined for eggs and counted. The total number of eggs laid by each female during life-span was counted. Such ten females were observed for the eggs laid. Observations on incubation, larval and pupal periods were recorded as per the rearing procedure in Govindan (1974) and Chakravarthy (1977).

Seasonal incidence: Field observations were recorded at the Zonal Agricultural Research Station, UAS, during September 2019 to February 2020. Seeds of local variety and HA-4 hybrid of *lablab* bean were sown during third week August 2019 in 800 m² area to record the seasonal incidence of *Adisura*. The study area was divided into four quadrates. Observations on the number of larvae per 10 plants at each quadrate were made at weekly intervals commencing from 50 per cent flowering (50 days after sowing) to pod maturity stage. ANOVA was carried out by treating quadrates as replications and dates of observations as treatments to know the differences among dates of observations.

RESULTS AND DISCUSSION

Host range: *A. atkinsoni* has a limited host range. Fletcher (1919) recorded on *Blumea* sp at Pusa; *Cajanus cajan* (L.) and *Dolichos lablab* (L.) at Coimbatore and on *Cicer arietinum* L.; *Lens esculenta* Moench at Indore, Rattan Dhâr, Ujjain, Hoshangabad, Betur and Bilaspur districts of Madhya Pradesh (Bhatia, 1962). According to Mujtaba (1918) the caterpillar was found feeding on the leaves and buds of *Blumea* sp. *Adisura* larvae have been recorded on *C. cajan* in Delhi (Issac, 1946). However, none of the above workers reared *A. atkinsoni* on plants, they reported and these plants may serve as larval plants only, not host plants. Therefore, *Lablab* is the only established host plant for *A. atkinsoni*. According to Gardner (1946), the caterpillar of this insect was found feeding on the *Hibiscus mutabilis* at

Dehradun (Uttarkand). *Adisura* has been reported to be boring into the pods of *Dolichos* in Mysore state (Krishnamurthy and Appanna, 1948). Bhatia (1962) reported that this species was found in abundance on chickpea, pigeon pea and lentil. *Blumea* sp. belong to Asteraceae and *Hibiscus* sp. *mutabilis* belong to Malvaceae. All other plants belong to Leguminaceae. Interestingly, Thontadarya *et al.* (1982) recorded *A. marginalis* (Walker) feeding on pods of redgram from the campus of the University of Agricultural Sciences, Bengaluru for the first time from south India. Preliminary experiments in laboratory revealed that while larvae of *A. atkinsoni* made directed movements towards *Lablab* bean pods, they randomly nibbled on the pods of other plants offered in the laboratory. Moths of *A. atkinsoni* did not oviposit on blooms of other legumes in laboratory, but preferred only the blooms of field beans. Thus, *A. atkinsoni* is the only pod borer of field beans which is monophagous, while other pod borers are oligo or polyphagous in Karnataka.

Biology: The moths emerged in mid-October from the pupae of previous season, when the local cultivar was in the initial reproductive phase. The pupae were formed inside the soil. During the season, insect completed three generations and fourth generation caterpillars pupated in the soil and remained till the next season of the crop. The total lifecycle depended on the total and average rainfall and accompanying temperature and their distribution in a year.

Egg: Iridescent white spherical eggs were laid singly either on the pods or on the flower buds. The incubation period on an average lasted for three to four days (3.84 ± 0.24). Tender leaves were preferred for oviposition only when flower buds and pods were not offered in laboratory. Both in field and lab, gravid *A. atkinsoni* moths did not oviposit on pigeon pea, chick-pea and cowpea leaves, flower buds, and pods observed. Ramachandra Rao (1918) and Krishnamurthy and Appanna (1948) earlier recorded similar observations.

Larva: *Adisura* underwent five larval instars, required I instar 3.20 ± 0.30 , II instar 1.94 ± 0.48 ,

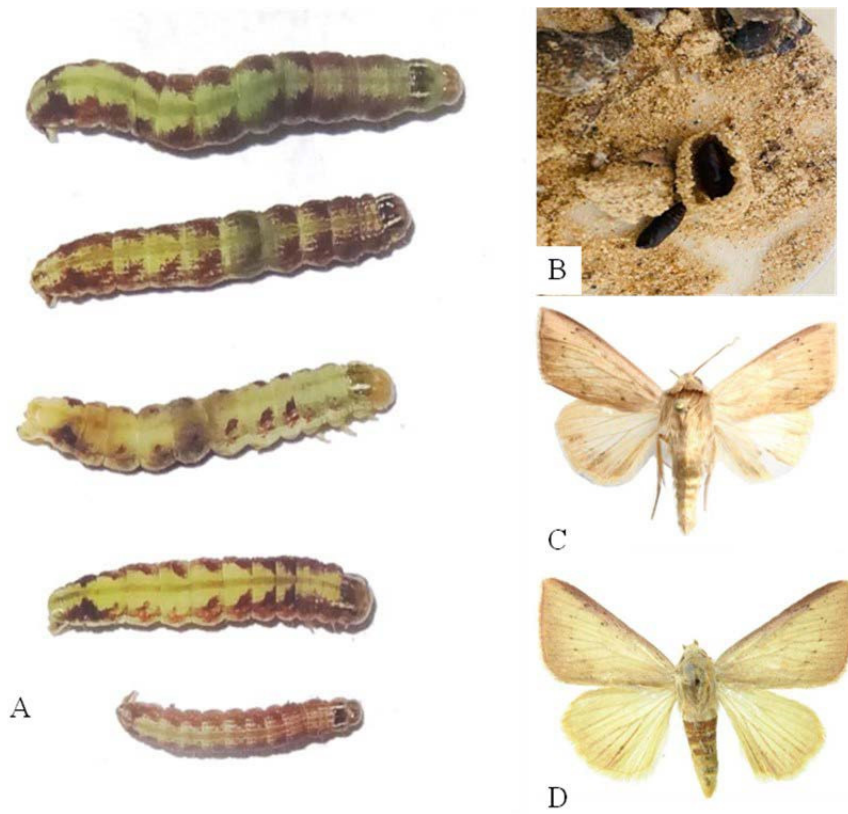


Fig.1 A - Morphological variations in *Adisura atkinsoni* larvae; B - Earthen pupal cell of *Adisura atkinsoni*; C - Adult Female and D - Male of *Adisura atkinsoni*

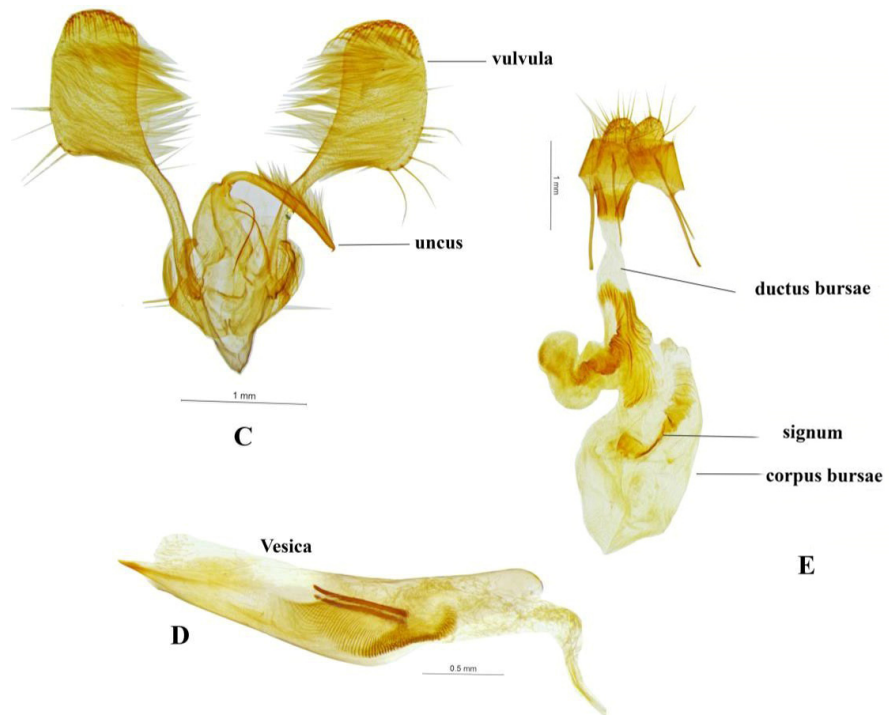


Fig. 2 C - Male genitalia, D - Aedeagus, E - Female genitalia

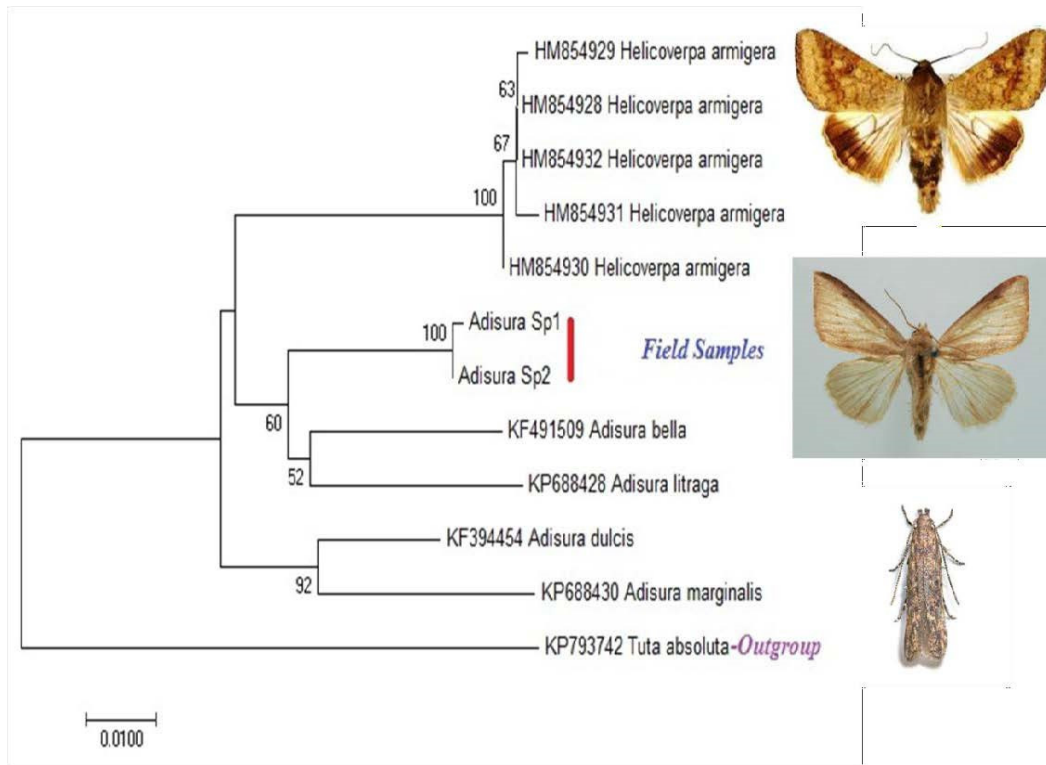


Fig. 3 Phylogenetic tree constructed for *Adisura* sp. using mitochondrial *COI* gene sequence

III instar 3.04 ± 0.32 , IV instar 3.48 ± 0.28 and V instar 6.15 ± 0.57 days respectively ($n=28$) to complete the life-stages. A fully grown 5th instar caterpillar measured 27-28mm long. A great variation was recorded in the colour of the later stages of the larvae, especially in the third generation (Fig.1A). Larvae showed cannibalistic tendency when reared in cages. These observations are in agreement with the observations of Ramchandra Rao (1918) and Krishnamurthy and Appanna (1948). According to Steven Passoa (2007) larvae of *A. atkinsoni* can be distinguished from other species of Heliiothinae by spinner spatulate and crochets bifurcate at their tip.

Pupa: The matured caterpillar entered the soil, formed an oval earthen chamber underground (Fig.1B) and pupated. However, even in the absence of soil, the larvae pupated in individual receptacles. The pupae measured 18mm long and appeared thick and red-brown. The pupal period varied from 15 to 18 days on an average of 17.08 ± 2.26 days in the first two generations to over nine months in the third generation. Govindan (1974) and

Chakravarthy (1977) too recorded observations in concurrence with the above results.

The moth completed the life cycle on an average in 38.73 ± 3.25 days ($n=3$). Longevity of male is 13.5 ± 1.78 and female is 10.8 ± 1.12 days. The fecundity of *Adisura* moths varied from 150 to 180 eggs, on an average ($n=20$). Each female moth oviposited for two to nine days in the laboratory. Mujtaba (1918), Krishnamurthy and Appanna (1951), Chakravarthy (1977), Chakravarthy (1983), Chakravarthy and Lingappa (1984, 1988), Chakravarthy (1988), Thontadarya *et al.* (1982), and Mallikarjunappa (1989) and Chakravarthy and Rajendra Prasad (2016) recorded bioecology of *A. atkinsoni* from different locations in different periods in Karnataka, south India. There were slight variations in the life history depending on weather conditions but broadly conformed to the results obtained in this study.

Seasonal incidence: Field observations revealed that the pod borer, *A. atkinsoni* was the dominant pest of field bean occurring from October to March

under field conditions. Its appearance in the field coincided with the initiation of flowering and pod formation stages of the local variety of *lablab* beans and the borer population disappeared with the local cultivar attaining senescence stage. It was found specifically feeding on flowers and pods of *Lablab*. The field bean pod borer appeared during middle of October and the per cent infestation on an average (n=100 blooms) varied from 24.50 to 60.00. The incidence of *Adisura* was higher on local varieties (photo sensitive) compared to HA4 hybrid (photo-insensitive). The pod infestation started from October through March and ranged up to 39.2 per cent on local cultivar. The peak *Adisura* borer infestation was recorded during November-January on local cultivar. The incidence on HA 4 hybrid was as low as 1.2 to 4.0 per cent. So, *A. atkinsoni* was the only dominant borer whose life-cycles coincided and completed with the local *Lablab* cultivar.

Adult: *A. atkinsoni* head and thorax were with a vestibule consisting of greyish brown hair - like scales. Abdomen covered with straw coloured, less dense scales. Palpi dark brownish with dense brownish scales on the outer side than the mesal area and proboscis well developed. Antennae setaceous; forewing greyish brown with black irregularly distributed scales; costal margin tinged with deep coppery brown; two or more less black circular spots, present on basal area of median space; an oblique series of black points present just below the sub-terminal area. Hind wing is light brownish with black suffusion on the terminal area narrowing from inner margin to the apex; fringe whitish throughout. The orbicular spot towards the base of the forewing is prominent. The male and female forewings measured on an average (n=20) about 30.73mm and 30.92mm, respectively (Figs.1C, D). According to Mathews (1991), *Adisura* has unique form of coiling of the female appendix bursae and the male vesical and dopa decarboxylase (DDC) sequences permitted *Adisura* to be treated as sister group.

Male genitalia: Uncus evenly elongated, pointed tip; tegumen small, subtriangular; valva narrow band; cucullus is well separated from other part of

valva by more or less narrow neck; succulus strongly sclerotized, broader base narrowing towards apex; vinculum U-shaped. Aedeagus long nearly uniform width, sinuate medially with apically pointed (Figs. 2C, D, E).

Female genitalia: Papillae analis sclerotized, setose plate, two times as long as wide; posterior apophysis as long as anterior apophysis; ostium sclerotized, cup shaped; ductus bursae membranous sac like, almost equal length to corpus bursae and sclerotized distally; corpus bursae membranous with a sclerotized irregular band signum.

Ecology: The pupae of *A. atkinsoni* of the previous year were maintained under 70 ± 2.0 per cent relative humidity with light: dark (13:11 h/day) in the laboratory under ordinary light and regime periods 24 ± 2.0 for eight and half months. Diapause of *A. atkinsoni* could not be broken under above conditions. Workers have observed that under Bangalore conditions, a combination of high humidity of the north-east monsoon season and heavy rainfall or heavy mist in the morning and bright sunshine during the day appeared favourable for normal appearance, multiplication and optimum activity of the *A. atkinsoni* moths and the caterpillars. It was observed that failure of the monsoon and even a poor monsoon acted adversely on the emergence of the moths early in the season and a large number of pupae of the previous season got desiccated in the soil and a number of moths even after eclosion from many pupae failed to travel upwards to the soil surface and emerged successfully. These adverse weather conditions affected growth and development of local cultivar under field conditions (Ramachandra Rao, 1918; Govindan, 1974; Chakravarthy and Rajendra Prasad, 2016).

Phylogenetic studies: The species included many of those in the catalog by Hampson (1894) and also some undefined morpho- species. These undefined taxa were identified using both female and male genitalia. But since they could be differentiated from their morphologically closest relative, they were marked as *sensu lato*; further studies will be necessary to name and validate these taxa. While

doing BLAST analysis, the mitochondrial COI Sequence of *Adisura* specimens collected from Bengaluru showed 95 per cent similarity to *A. bella* and 94 per cent to *A. dulcis*, while *A. litraga* and *A. marginalis* showed less similarity. So, *A. bella* was most closely associated with *A. atkinsoni*. In the phylogenetic tree, these two samples Sp1 and Sp2 separate new sub-clade which stands separately from the rest of the *Adisura* species. According to the above-mentioned results and the results from classical taxonomic studies, it was identified that these lepidopteran samples collected from Bengaluru are *Adisura* and the DNA sequences did not match with any of the existing species of *Adisura* (Table 1). Therefore, it was confirmed that the samples belonged to *A. atkinsoni* (Fig. 3). Cho *et al.* (1995) and (2008); Mitchell and Gopurenko (2016) conducted studies on DNA barcoding and molecular phylogenetics of heliothine moths including species of *Adisura* without *A. atkinsoni*. The above workers showed phylogenetic relationship of *A. litraga*, *A. bella*, *A. marginalis* and *A. purgata* with 26 other heliothine moth species. Cho *et al.* (2008) confirmed monophyletic nature of *Adisura* having all genes allied to the Heliothis group.

Table 1. Database of nucleotide sequence of *Adisura* spp. in BOLD system

Species	Specimens	Sequences	Barcodes > 500bp
<i>Adisura aerugo</i>	2	2	2
<i>A. bella</i>	2	2	2
<i>A. callima</i>	1	1	1
<i>A. dulcis</i>	12	11	11
<i>A. litarga</i>	9	9	5
<i>A. marginalis</i>	21	20	20
<i>A. parva</i>	2	2	2
<i>A. purgata</i>	1	1	1
<i>Adisura</i> sp.	28	28	16

<http://www.boldsystems.org/index.php/Taxonpage/SpeciesSummary?taxid=53549>

Hardwick (1965) suggested Heliothinae is the correct spelling to replace Heliothidinae or Heliothinae. Some of the world's most destructive pests belong to the noctuid subfamily Heliothinae. The subfamily Heliothinae is well-defined, comprising about 400 species of small to medium-sized noctuid moths: antennae in both sexes are filiform; palpi short, pressed; proboscis well developed; fronsconvex, sometimes with sclerotized comb; in most genera tibia of all legs armed with spines. The monophyly of Heliothinae is supported by two apomorphies. First, the larval integument is covered in conical granules each bearing a minute apical spine. Spinose skin also occurs in Herminiinae, Cuculliinae and Plusiinae (Kitching, 1984) but these conditions are non-homologous. For instance, the spinules in Plusiinae are fine and hair-like (Lafontaine and Poole, 1991). Secondly, in most noctuid larvae, seta L1 on the prothorax is vertically above seta L2, as it is in early instars of Heliothinae. But in mature heliothinae larvae L2 is positioned directly posterior to L1. The COI barcode region is for species identification, and has been used to not only corroborate morphologically defined species but also to define the species in the *Adisura* complex.

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