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MOLECULAR IDENTIFICATION USING MTDNA (COI GENE) AND SCANNING ELECTRON MICROSCOPY OF A FORENSIC CALLIPHORIDAE FLY *CHRYSOMYA RUFIFACIES* (MACQUART 1843)

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ABSTRACT:

In forensic entomology insect is used to determine a minimum post-mortem interval (PMI min). A PMI estimation is based either on species specific developmental rates or on succession patterns and species diversity on the carrion. Hence correct species identification is a very important step in forensic entomology. Unfortunately, lack of taxonomist or sufficient keys to identify the species lead to occasional errors in species identification. The eggs or larvae of many forensically important dipteran species are particularly difficult to distinguish morphologically and incorrect identification can seriously harm an investigation. To overcome the limitations of morphology-based identification, molecular identification using mtDNA (COI gene) performed by sequencing fragment of 658 bp of COI gene. This study emphasizes on identification of *Ch. rufifacies*, one of the forensically important hairy blowfly and a myiasis causing agent.Scanning electron microscopy was performed to observe distinguishing characteristics of *Ch. rufifacies* like pattern of folding in frontal field, number of anterior spiracles, posterior spiracles, number of tubercles, structure of button, spiracular hair and middle sacrum.

KEY WORDS: *Molecular, Succession, Entomology, Colonization, Microscopy, Taxonomy, COI.*

INTRODUCTION:

Insects are the most numerous and diverse organisms on the planet. For thousands of years, human is associated with carrion feeding insects. The first reference to blowflies produced more than 3600 years ago in the Har-ra-Habulla, a collection of cuneiform writings on clay. It is the oldest known book in zoology and first mentions the greenfly and the blue fly (Greenberg and Kunich, 2002). Entomology derived from the Greek word Entomol. Entomon means insect and logos meaning the study of insects (Gupta and Setia, 2004). An accurate estimation of the time since death is of fundamental importance in many forensic cases. A pathologist using different methods such as algor mortis, livor mortis, vitreous concentrations, electrical excitability of skeletal muscles and gastric content, can estimate PMI (Henssage et al., 2002). However, these methods measure accurately in the two or three days following death (Amendt et al., 2004). An additional method that may help with the estimation of the time since death is the entomological approach. The collection and analysis of insect evidence from a forensic investigation used to assist in forensic, legal or medico-legal cases (Varatharajan and Sen, 2000).

In the first 72 hours after death, the pathologist considered able to provide a reasonably accurate determination of the time of death. This based upon the condition of the body itself and such features as the fall in body temperature. Beyond this time, there is less medical information with which to correlate PMI. So another area of expertise is required to clarify the time of death. The forensic entomologist can provide a measure of the possible post mortem interval, based upon the life cycle stages of particularly fly species recovered from the corpse or from the succession of insects present on the body. This estimate can give over a period of hours, weeks or years. The start of the post mortem interval calculated to coincide with the point when the fly first laid its eggs on the body and its end to be the discovery of the body and the recognition of life stages of the oldest colonizing species infesting it. The duration of this stage, in relation to the particular stage of decay, gives an accurate measure of the probable length of time the person has been dead and maybe the best estimate that is available (Gennard, 2007).

Accurate species identification is an essential first step when applying entomological evidence to forensic practices. Furthermore, using the morphological method to identify the immature was very difficult or sometimes impossible due to the lack of a comprehensive key for any geographical region. Therefore, a scanning electron microscope (SEM) has provided ample morphological identification key to differentiate carrion flies understudy for the correct identification. The

identification of entomological species is a very critical step in forensic investigations. Morphology based identification sometimes may be difficult. To overcome the limitations of morphology-based identification, DNA based sequence data applied to identify insect species. Using molecular techniques morphologically similar species or genera can be distinguished easily. In this study, molecular identification of *Ch. rufifacies* has been done using the barcoding of the mtDNA technique. Cytochrome C oxidase subunit I (COI) gene used to confirm the morphological identification of. Sequence this sample of calliphorid species uploaded to the BOLD system to confirm the identification and analysis of the barcoding data.

MATERIAL AND METHODS:

For collection, the fresh beef liver sample purchased from the local slaughterhouse. Partially putrefied liver/meat exposed in the air and within a few minutes, the flies attracted. Similarly, maggots and adult flies collected from dead bodies of road kill cadaver from different regions of Osmanabad district, Maharashtra. Collected samples were brought to the laboratory as laboratory rearing was very important to maintain the pure culture of each species. From mixed culture eggs of each species were collected and grown separately to identify the species. Different stages of the life cycle of collected species dissected to observe the identification marks and followed by the published identification keys. Third instar larvae were dissected to observe the posterior spiracles and cephaloskeleton as these are species-specific and offers correct identification. Adult and maggot studied morphologically with the help of a stereo-zoom light microscope (Magnus trinocular microscope). Morphological characters were photographed by using 16.0 Megapixel Nikon Coolpix Optical Zoom Digital Camera. Based on larval identification both the species were grown in a separate cage under the hygienic conditions for further research. Morphology based identification is quite tedious as proper identification key is not available. Sometimes due to identical morphology, it is difficult to differentiate two species. To overcome this problem scanning electron microscopy was performed to differentiate larvae based on number of tubercles, the structure of integument, posterior spiracular button, body spines, spiracular hair, sensillae, antenomaxillary complex The body size, shape of wrinkled head, number of papillae, anterior, dorsal and posterior spines.

Cleaning Larvae and pupa for SEM: Larvae obtained from a laboratory pure culture. The intermittent shaking of the vial separated batches of the eggs. This process continued only for a minute. Care was taken not to shake the vial with its contents too much to prevent damage to the eggs. Adhered eggs separated by mechanical teasing with fine dissecting needles. Slight pressure applied to the egg mass with a small blunt object (back of a dissecting pin). The cleaning solution

rinsed from the eggs with numerous batches of distilled water until no soapiness observed. Care taken not to damage the eggs throughout the process. Larvae killed by immersing them in the hot water, which killed the larvae instantaneously and aided in straightening out the larvae. To provide adequate room for the larvae to extend fully enough wide container used. For cleaning of the larvae, the brushing of larvae performed to remove dust or any debris attached to their body surface. However, the most time effective and hassle-free method was to place the specimens in a vial of lukewarm water to which a few drops of the 1% household detergent was added and then to intermittently shake the vial for not more than a minute. The cleaning solution removed by washing the specimens in a few batches of distilled water until no soapiness observed anymore. Puparia placed in hot water to arrest their further developmental process. Puparia cleaned by placing them in a vial with warm soapy water and shaking the vial in an attempt to get rid of the debris adhering to the puparium surface. Small paintbrushes used to brush them and remove dust and debris. The cleaning solution removed by washing the specimens in a few batches of distilled water until no soapiness observed anymore. Larvae were stored in 70% ethanol (Brink L.S. 2009). Finally, the larvae subjected to critical point drying to complete the dehydration process. Following the dehydration process, the larvae attached to double-stick tape on aluminum stubs in order to coat with gold in the sputter-coating apparatus to enable viewing under a JEOL- JSM840A scanning electron microscope.

Molecular identification

For barcoding DNA abdominal part was removed from the male adult specimens and kept in an Eppendorf tube containing 3 ml of absolute Ethanol. All instruments used to remove the abdominal part cleaned in 70% ethanol to avoid infection. Molecular identification method was done according to (Wells and sperling, 2001; Hebert et al.2003a; Chen et al.,2004; Jefferies et al.,2007 Wells and Williams,2007; Park et al.,2009; Bajpai,2010; Meiklejohn et al.,2011, Singh et al .,2014).

DNA Extraction:

Cells grown in monolayer was lysed by suspending 1-3 colonies aseptically and mixed with 450 µl of “B Cube” lysis buffer in a 2ml microcentrifuge tube and lysis was done by repeated pipetting.4µl of RNAse A and 250µl of “B Cube” added for neutralization of buffer. After, vortex the content and the tubes were incubated for 30 minutes at 65ºC in the water bath. To minimize shearing the DNA molecules, DNA solutions mixed by inversion. Centrifugation of the tubes done for 15 minutes at 14,000-rpm at100C.The following centrifugation; the resulting viscous

supernatant was transferred into a fresh 2 ml microcentrifuge tube without disturbing the pellet. After adding 600 µl of "B Cube" binding buffer to the content, mixed thoroughly by pipetting, incubated the content at room temperature for 5 minutes, and transferred 600 µl of the contents to a spin column placed in 2ml collection tube. It then centrifuged for 2 minutes at 14,000 rpm and discarded flow through. The spin-column was reassembled and the collection tubes were transferred the remaining 600 µl of the lysate. Centrifugation done for 2 minutes at 14,000 rpm and discarded flow-through. The addition was 500 µl "B Cube " was done for washing buffer I to the spin column and centrifuged at 14,000 rpm for 2 minutes and discard flow-through. Reassembled the spin column, added 500-µl "B Cube" washing buffer II, centrifuged at 14,000 rpm for 2 minutes, and discarded flow-through. Then transferred the spin column to a sterile 1.5 ml microcentrifuge tube. Added 100 µl of "B Cube" Elution buffer in the middle of the spin-column without touching filter. Tubes incubated for 5 minutes at room temperature and centrifuged at 6000 rpm for one minute. The above process was repeated 14 and 15 for complete elution. The buffer in the microcentrifuge tube contains the DNA. DNA concentrations measured by running aliquots on 1% agarose gel. The DNA samples were stored at -200C until further use.

PCR Protocol:

Primer details:

LCO-1490 GGTCAACAAATCATAAAGATATTGG 25 base pairs

HCO-2198 TAAACTTCAGGGTGACCAAAA 21 base pairs

Polymerase Chain Reaction (PCR) is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a very unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. DNA polymerase adds nucleotides to the 3` end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3` end to generate an extended region of double-stranded DNA.

Added 5 µL of isolated DNA in 20 µL of PCR reaction solution (1.5 µL of Forwarding Prime and Reverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix). Performed PCR using the following thermal cycling conditions.

1. Denaturation

The DNA template is heated to 94°C for 3 minutes. This breaks the weak hydrogen bonds that hold DNA strands together in a helix, allowing the strands to separate creating single-stranded DNA.

2. Annealing

The mixture is cooled to anywhere from 94℃ for 30 sec, 50℃ for 60 sec, and 72℃ for 60 sec. This allows the primers to bind (anneal) to their complementary sequence in the template DNA.

3. Extension

The reaction is then heated to 72° C for 10 mins, the optimal temperature for DNA polymerase to act. DNA polymerase extends the primers, adding nucleotides onto the primer in a sequential manner, using the target DNA as a template.

Purification of PCR Production

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).The PCR product was sequenced using the 27F/1492R primers. Sequencing reactions were performed using an ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

Sequencing protocol: Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Bioinformatics protocol:

1. The 18s r RNA sequence was blast using the NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.

2. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as a Substitution model.

3. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering. (Dereeper et al., 2008).

Sequences were submitted to NCBI and the Gen Bank accession Number of the samples was obtained *Chrysomya rufifacies* MG816778.

RESULTS AND OBSERVATIONS:

Observations of the present study are as depicted bellow,

Morphological identification of *Ch. rufifacies* (Macquart 1843)

Ch. rufifacies is an Australian blowfly. This Calliphoridae species is one of the important hairy blowflies. Adults are shiny metallic blue-green, face and cheeks are with dense silvery hair on dark brown to a black surface (Fig 1. l, m). Anterior spiracles are pale, creamy or white. Lower squama covered with fine hair above. Rows of conspicuous tubercles are present on the body so this species identified without the aid of a microscope (Fig 1. d, e). The anterior spiracle is open and the presence of proepisternal seta (stigmatic bristle). 9 and 10 papillae observed during the present study (Fig 1. j). First instar larvae are smooth and without tubercles (Fig 1. b). In Posterior spiracles, each spiracle has two spiracular slits attached to it. Second instars show the presence of developed tubercles. Posterior spiracles show two separated spiracular slits with thick incomplete peritreme. The open area present in between the ends of peritreme is quite broad. Tubercles of second instar larvae are slender in shape with three-minute dark spines. The ventral and dorsal surface of the third instar shows well-developed tubercles. In the third instar larvae, there is the absence of hairy like structure at the base of tubercles in the caudal region; three rows of numerous crowns on the tip are present (Fig 1. i).

Scanning Electron Micrographs of *Ch. rufifacies* (Macquart 1843)

I instar: The first instar shows the 12-segmented body with muscoid-shape. A dorsal organ, terminal organs are located on the cephalic segment. A pair of mouth hooks situated mid-dorsally. The mouth hooks situated mid dorsally of the mouth region. The anterior spiracle is apparent only as a small depression and is located at the posterior margin of the prothorax. A pair of the posterior spiracular disc is located in a deep depression in the caudal segment (Fig 2. n).

II instar: The second instar possesses the same general morphology similar to that of the first instar. The dorsal and terminal organs do not possess much difference. Many distinct differences

were apparent in the second instar as compared to the first instar. A pair of large, robust mouth hooks observed. The labium is comprised of three large, round lobes. The oral groove area well developed as an array of ridges between oral grooves used for channeling liquid into the mouth opening. The number of papillae on the anterior spiracles are 10. All papillae arranged in a single row. The whole body covered with numerous dome-shaped papillae. The posterior spiracular discs observed well developed in the second instar as compared to the first instar. A button or ecdysial scar is located on the medial edge of the peritreme of each spiracular disc and appears as a circular depression (Fig 2. o). The structural pattern of an antennal sensory complex of *Ch. rufifacies* was observed in the second instar (Fig 2. t).

III instar: Overall morphological features were similar to that of the second instar. The labium is not trilobed. A very distinct ultrastructure of the surface integument is present in the third instar and covered with several net-like patches (Fig 2. p). The elongated tubercles encircling body segments are located along the body and are each slender in shape, with a tip having circular rows of spines. Each spiracular disc contains three straight slits and a relatively thick peritreme (Fig 2. v, z). 11 anterior spiracles were observed (Fig 2. w). The structural pattern of an antennal sensory complex of *Ch. rufifacies* was observed significantly in third instar (Fig 2. u).

Antennomaxillary Complex: The antenna (asc) was located in an anterior position. A dome-shaped base portion was present with a pointed distal portion. The maxillary complex (mxsc) of first instar larvae was different from that of the second instar larvae, but quite similar to third instar larvae ((Fig 2. q, r, s).

Pupa: Generally both the pupa was oblong shaped (fig f). The structure of the mouth scar was very prominent and distinct in SEM of *Ch. rufifacies* (fig x, y). Based on distinguishing characteristics at the frontal field it is categorized into unwrinkled, wrinkled and structural folds or ridges. As a diagnostic feature distinct ridges and folds with the specific pattern observed at the frontal field of *Ch. rufifacies*. The folds in the *Ch. rufifacies* were in the form of a defined loop especially in the frontal region prominent hairpin like unique structure was observed (fig y). The texture of integument of puparia in *Ch. rufifacies* was very rough and with numerous pointed spines

Molecular Identification: Species with sample ID 14 which morphologically identified as *Ch. rufifacies* , sequence identification using species database shown 100% match to *Ch. rufifacies* . The mean of nucleotide composition frequency of *Ch. rufifacies* were A= 30.83, T= 37.40%, G= 16.12 % and C= 15.65 %.

DISCUSSION:

As per the results first instar larvae were smooth without tubercles, posterior spiracles, each spiracle has two attached spiraclular slits (V shape). Second instar larvae started developed the tubercles; Posterior spiracles with two separated spiraculer slits with thin incomplete peritreme, open area between the ends of peritreme is broad. Tubercles of second instar larvae stage, slender in shape with 3-minute dark spines or crown tubercles well developed in both dorsal and ventral surface of third instar larvae (Abd-Al Galil F. M. A., 2015). The main characteristic features of the *Ch. rufifacies* are its sclerotized spines, fleshy processes and heavily sclerotized mouth hooks, which used to penetrate the bodies of other maggots for fluid extraction during predation (Baumgartner, 1993).

The adults of *Ch. rufifacies* had a stout body and were brilliant blue-green in colour. The terminal edge of the abdominal segments was dark purple to blue. The adults of this species were usually the first to arrive on carrion so they are very important in forensic entomology. The larvae of this species were readily distinguished from other larvae of the calliphoridae family by the presence of prominent fleshy protrusions along their body. The main characteristic feature of this species is if the food supply becomes depleted the larvae will consume and eliminate other species from the carcass. The larvae were also be able to burrow several inches into the soil to colonize buried remains (Baumgartner, 1986, 1993; Baumgartner and Greenberg, 1984; Gagne, 1981; Greenberg, 1971, 1988; Hall, 1948; Hall and Townsend, 1977; James, 1947; Oldroyd and Smith, 1973; Richard and Ahrens, 1983; Smith, 1986; Wells and Greenberg, 1992; Zumpt, 1965).

Sukontason *et al.,* (2006), also have differentiated the species based on the presence of tubercles along with the dorsal and lateral segments of the *Ch. rufifacies* and *Ch. villeneuvi*. Another characteristic difference between the puparia of both species was the number of globules at the bubble membrane on the dorso-lateral border of the fifth segment, and SEM could only observe this. A larger number of globules found in *Ch. villeneuvi* than in *Ch. rufifacies.*

In *Ch. rufifacies* the puparium surface was similarly adorned with processes and papillae (Sukontason *et al.,* 2006b). The micrographs presented by Sukontason *et al.,* (2006b) indicated that the small papillae and the spines covering the tips of the processes, i.e. the same characteristics used to distinguish among the third instar larvae of the two species, could be utilised for the distinction between the puparia of *Ch. albiceps* and *Ch. rufifacies*.

Dipteran pupa are of forensic importance as they shows most of the morphological characters of larval III instar (Erzinçlioglu, 1985; Greenberg, 1989), except for some collapsed parts, such as the

pseudocephalon. The arrangement of some structural parts like spines in segments and anal division are more or less conserved in the pupa. The posterior spiracles, allowing the correlation to larval morphology for identifying purposes. These structures observed by light microscopy although SEM better describes it. Moreover, pupae morphology shows exclusive structures, such as the bubble membrane and respiratory horn, which are very closely related. Bubble membrane appears in young pupae and disappears when the respiratory horn developed since both structures placed in the same location.

As per Nelson *et al.,* (2007) the mean nucleotide frequency distribution within *Chrysomya were* A=30.4%, T=38.1%, C=15.8%, G=15.6%. Study done by Bajpai *et al.,* 2013 shows the average nucleotide composition as T=39.6%. A=31.4%, C=15.4%, G=14%. The effect of the geographical distribution leads to the small non-significant variation of the nucleotide composition. According to this database species level in BOLD search, if the value 100% matches indicate the matches with a high confidence level and if the values lower than 99% match, that indicates alow level of matching.

Bharati and Singh, (2017) also have studied the DNA-based identification of forensically important blowflies (Calliphoridae) from India. In this study, mitochondrial COI gene sequenced from *Calliphorinae, Luciliinae* and *Chrysomyinae f*amilies respectively. All species included in the study displayed very low intraspecific divergence (<0.3%) whereas interspecific divergence ranged from 0.11% (for the most closely related species *C. megacephala* and *C. chani*) to 18.14% (for the most distantly related *C. vicina and C. rufifacies*). Similarly, congeneric and intergenric divergence ranged from 0.21% (*Calliphora*) to 6.11% (*Lucilia*) and 11.04% (*Lucilia- Calliphora*) to 13.46 % (*Calliphora- Chrysomya*), respectively. In between *C. megacephala* and *C. chani* there was no overlap between intraspecific (0.30%) and interspecific (1.96) genetic diversity. *C. megacephala* and *C. chani* showed low interspecific divergence (0.11), *C. pinguis* and *C. chani* showed (1.96) and *C. pinguis* and *C. megacephala* showed (2.08) divergence respectively.

CONCLUSION:

Molecular identification provides a strong basis for species identification and will prove valuable contribution to forensic entomology when applying entomological evidences to forensic practices. Furthermore, using the morphological method to identify the immature was very difficult or sometimes impossible therefore, scanning electron microscope (SEM) can provide ample morphological identification keys to differentiate carrion flies. Molecular taxonomy plays a vital role in the confirmation of identification of the species.

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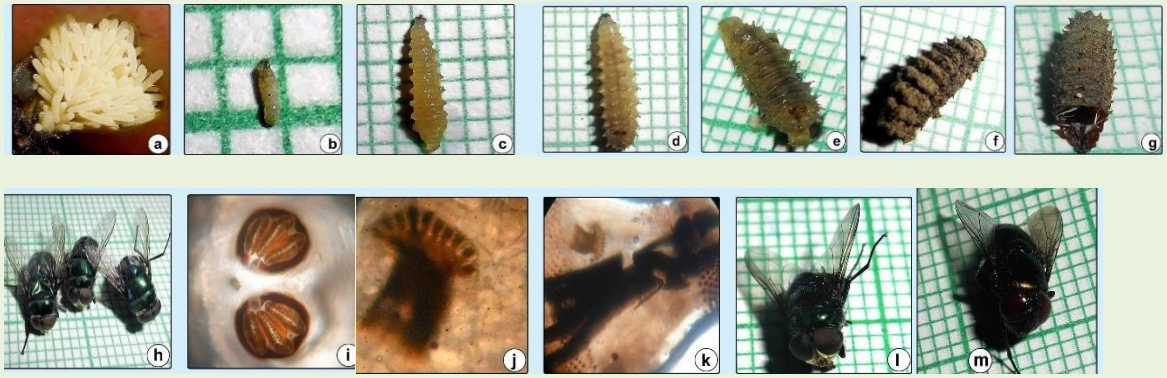

Fig. 1. Images of Morphological Features of *C. rufifacies* (Macquart 1843)

(**a**-eggs, **b**- first instar larvae, **c**-second instar larvae, **d**-third instar larvae, **e**-prepupa, **f**-pupa, **g**-empty pupa after emergence of an adult, **h**-adult flies, **i**-posterior spiracles, **j**-anterior spiracles, **k**-cephaloskeleton, **l**-male of *Ch. rufifacies* , **m**-Female of *Ch. rufifacies*)

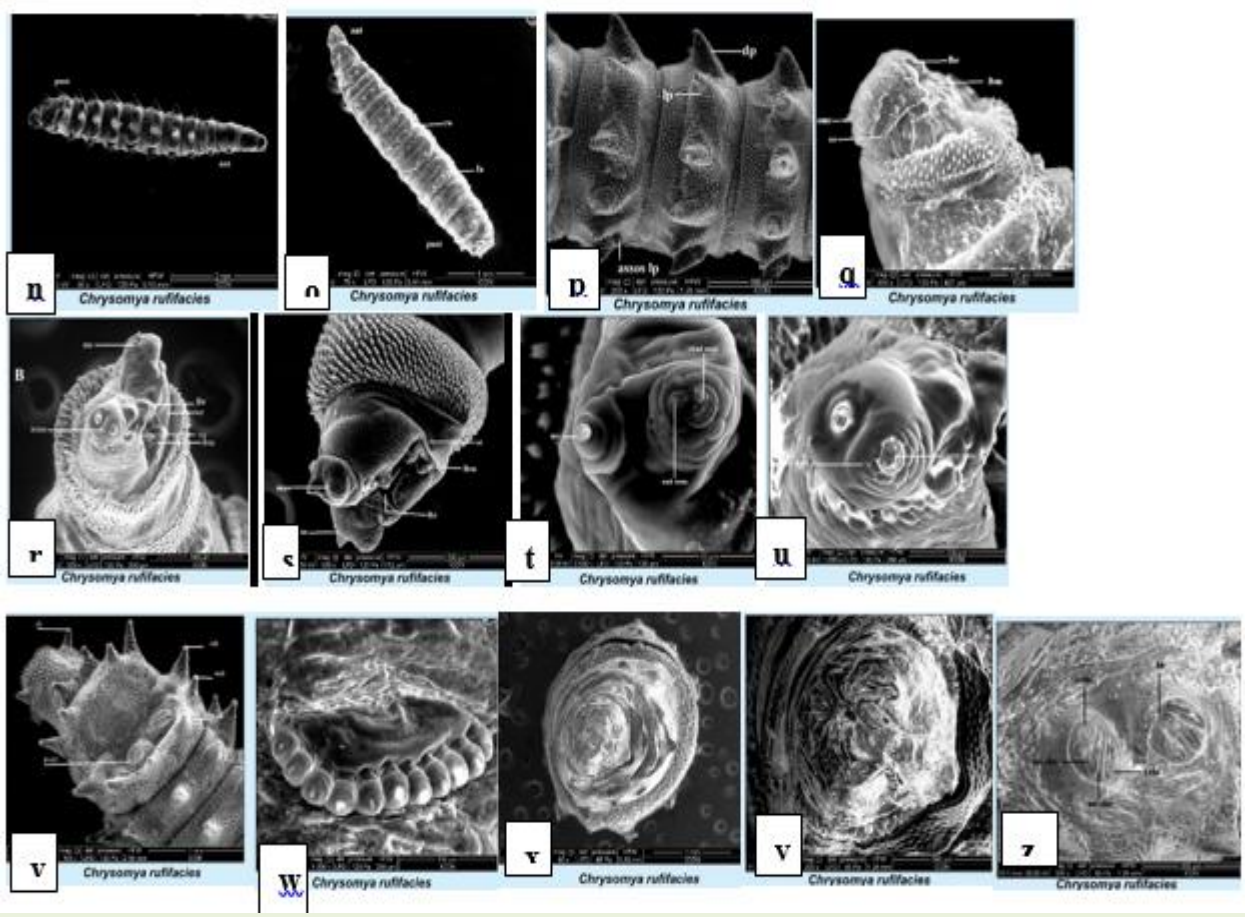

Fig. 2. Scanning electron micrographs of *C. rufifacies* (Macquart 1843)

(n- first instar, o-second instar, p-third instar, q-antennomaxillary complex of first instar larvae, r-antennomaxillary complex of second instar larvae, s antennomaxillary complex of third instar larvae, t-antennal sensory complex of second instar, u- antennal sensory complex of third instar, v-posterior spiracles of third instar, w- anterior spiracles of third instar, x-anterior end of puparia, y-frontal field of puparia, z-posterior view of puparia).

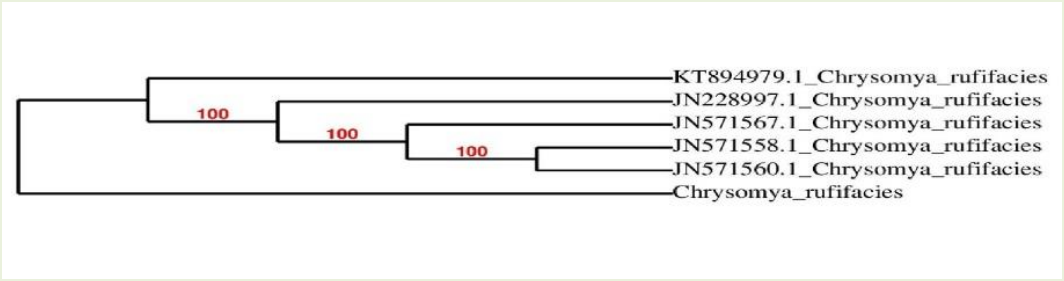

Fig. 3. Phylogenetic tree of *C. rufifacies* (Macquart 1843)