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Life history studsies of *Anopheles (Cellia) subpictus* Grassi (Anophelinae: Culicidae) with the aid of scanning electron microscopy (SEM) from Punjab

Jagbir Singh Kirti and Shipali

Abstract
Anopheles (Cellia) subpictus Grassi exists in a complex of four sibling species i.e. A, B, C and D. The identification of the species becomes difficult due to the presence of sibling species. Scanning Electron Microscopic (SEM) studies have been conducted for the first time on egg, larva, pupa, cibarium, male and female genitalia to discover new/additional taxonomic attributes which will be incorporated for updating the status of present taxa.

Keywords: *Anopheles (Cellia) subpictus*, cibarium, egg, larva, male female genitalia, pupa, scanning electron microscopy.

1. Introduction
Anopheles (Cellia) subpictus was described by Italian scientist Grassi in 1899. This species is widely spread in abundance in the Oriental region. It is found in Sri Lanka in South; China in North of India, west of India in Afghanistan, Pakistan and Iran and to the east in New Guinea and in Marina's Islands. In India, it is found throughout the mainland (Rao, 1984) [1]. During the recent collection-cum-survey tours a large number of adult representatives of the present species were captured from various localities of Malwa, Doaba and Majha region of Punjab. Various workers have conducted taxonomic studies on the adult and immature stages of the present species. Some of the eminent workers (Stephens & Christophers, 1902a & 1902b) [2, 3]; (Christophers & Barraud, 1931) [4]; (Christophers, 1933) [5]; (Walch & Walch-Sorgdrager, 1934 & 1935) [6, 7]; (Wu, 1936) [8]; (Saliternik, 1942) [9]; (D'Abrera, 1944) [10]; (Leeson & Buxton, 1949) [11]; (Weyer, 1954) [12]; (Reid, 1968) [13]; (Reinert, 2010) [14] worked on egg of this species. Other workers (Chaudhary *et al*., 2013)[15]; (Surendren *et al*., 2010)[16]; (Elango *et al*., 2011)[17]; (Tikar *et al*., 2011)[18]; (Suguna *et al*., 1994)[19]; (Singh *et al*., 2014)[20]; (Reuben and Suguna, 1983)[21]; (Kumari *et al*., 2009)[22] and (Nagpal and Sharma, 1995)[23] studied other aspects of this species. However, none of these workers has tried to explore ultra structures present in immature and adult stages of *An. subpictus.*

An. subpictus has resemblance with other species i.e. *An. stephensi* Liston, *An. sundaicus* Rodenwaldt and *An. vagus* Doenitz. *An. stephensi* resembles to this species because of its often fawn color; *An. sundaicus* resembles for speckling of femora and tibiae and *An. vagus* confused to this because of apical band on maxillary palpi. Because of the complex behavior and resemblance to other species, SEM studies have been conducted for the first time on various immature stages and adult representatives of the species.

2. Material and Methods
About 1000 representatives of *An. subpictus* were captured from various localities of Punjab state during July 2011- September 2013. The blood fed females were reared in laboratory and shifted to test tube containing some fresh water and after 2-3 days eggs were laid by female. Some eggs were preserved in 70% ethanol whereas, some were reared. For SEM studies, protocol given by (Chaudhary and Gupta, 2004)[24] was followed. Eggs were dehydrated in graded series of alcohol and mounted on SEM specimen stubs using only a small strip of double-sided adhesive tape (Kirti & Kaur, 2011)[25]. The samples were then sputter coated with

gold and scanned under JSM- 6100 scanning electron microscope at Indian Institute of Technologies (IIT) Ropar. Micrographs of about 5-10 eggs were examined from all desired directions.

After hatching of eggs, some larvae were preserved in 70% ethanol (Larvae were boiled in water for some time to kill them before preservation) while others were reared for developing into adults. For SEM studies, same method was followed as for eggs but larvae were passed through Critical Dry Point before mounting on SEM stubs.

After the emergence of adult from pupa, the pupal exuviae were preserved for SEM studies in 70% ethyl alcohol. Pupal exuviae were not passed through critical dry point to avoid rolling or breakage.

For SEM studies of cibarial armature, the method given by (Lee and Craig, 1983)[26] has been adopted. The head of adult female mosquitoes was snipped off from body and boiled in 10% KOH solution till clearance. Dissected material was washed several times with water. The head was placed on a slide with a drop of water and dissection was completed with needles under the binocular microscope. Compound eyes were slowly pulled apart in order to expose cibarium that is located immediately behind the clypeus. Dissected material was washed several times with water and dehydrated by passing through ascending grades of alcohol. The specimens were placed on stubs in dorsal position after air drying on filter paper and then coated with gold.

For genitalic attributes, last three segments of both male and female specimens were dissected with the help of forceps. These were first boiled in 10% KOH for 20-25 minutes, washed with water several times, air-dried and mounted on stubs for microgaphs.

Further procedure for larva, pupa, cibarial armature and genitalia was same as explained for eggs. For all the above said stages/parts of the species under reference, taxonomic keys developed by (Puri, 1931)[27], (Ross & Roberts, 1943)[28], (Nagpal & Sharma, 1995)[23], (Amersinghe *et al.*, 2002)[29] were used for identification. The terminology given by (Hara, 1959)[30], (Harbach & Knight, 1978 & 1980)[31, 32] and (Sirivanakarn, 1978)[33] has been used for various structures.

3. Results and Discussions

3.1 EGG (Fig. 1-6):

Eggs laid singly, black in color, boat-shaped, whole egg surface covered with tubercles. On dorsal and ventral surface, tubercles irregular in shape and size and interconnected to each other. Float small, broad at middle, rounded having 30-32 float ridges. Frill stiff, continued all around the margin of upper surface except at ends. Lobed tubercles were 3 at posterior end and 5 at anterior end opposite to micropyle. Micropyle clearly visible consisting of disc and collar.

3.2. Larva (Fig. 11-13):

3.2.1. Ventral aspect of head: Some structures of larvae head were damaged during sample preparation. So, few structures which were studied are A (Antenna), LPB (Lateral palatal brush), Mx (maxilla), Lat (Lateralia), PTP (Posterior tentorial pit), HEL (hypocranial ecdysial line), HyS (Hypostomal suture), PL (paraclypeal lobe), Mn (Mandible).

Mentum consists of Ventromentun (Vm) and Dorsomentum (Dm).

3.3. Pupa (Fig. 7-10):

Pupa showing emergence of adult mosquito. Trumpet angusticorn type. Ventral aspect of terminal abdominal structure showing genital lobe, IX- sternum and VIII- sternum.

3.4. Cibarial Armature (Fig. 14-17):

As in Anopheline species, length of cibarium twice its width and Anterior Hard Palate (AHP) about one third length of cibarium. Cibarium consists of two parts: Cibarial armature and cibarial sense organs.

3.4.1. Cibarial armature consists of two rows of teeth i.e. Rods and Cones.

a. Rods: The number of rods always is species specific. In the present communication, number of rods were 13-14, long, broad at base and middle, pointed at tip, fabricated ends with lateral spines on either side.

b. Cones: The number of cones was same for rods i.e. 13.

3.4.2. Cibarial sense organs: It consists of two types of papillae namely dorsal papillae and Trichoid papillae. Only one dorsal papillae and trichoid papillae have been studied.

3.5. Male Genitalia (Fig. 18-21):

The arrangement and number of parabasal and other differentiated spines on the coxites are characteristics for different subgenera of *Anopheles*. In subgenera *Cellia,* two (occasionally one or three) large parabasal spines arising from eminences found, in subgenera *Anopheles* there are 4-5 smaller spines not on raised area and internal spine of subgenera *Anopheles* is usually absent (Reid, 1968)[13]. The shape of dististyle was represented by an oblong, conical, sickle-shaped bent formation. Study under SEM demonstrated that it was uniformly bent rather than bent apically, as it was mentioned by some authors (Mohrig, 1969)[34].

Shape of dististyle was sickle shaped with claw at tip. Basistyle were covered by several kinds of setae and with numerous long and short microtrichae. One internal spine which was very long and thin; two accessory spines, all were almost of same shape and size; parabasal spines not studied. 12-15 more spines which were shorter than accessory spines and 3 small spines were present. Clasepettes were very prominent with spoon-shape (lower stem like part was thin with rounded upper portion). Aedeagus looked like pyramid.

3.6. Female Genitalia (Fig. 22-23): Female genitalia consisted of cerci, Post Genital Lobe (PGL) and IX-tergum. Pair of cerci covered with several types of small and large setae. PGL clearly visible with pair of long and thin seta. Shape of IX-tergum arc- like; Insula slightly downwards in middle making bowl like structure; Cowl curved at end.

Egg of *Anopheles* (*Cellia*) *subpictus* Grassi

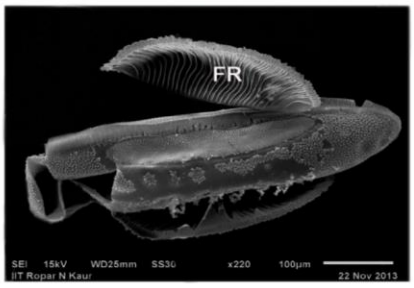

Fig. 1 Hatched egg

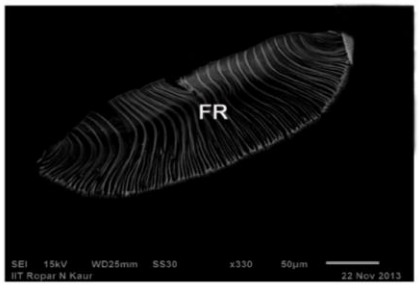

Fig. 2 Float

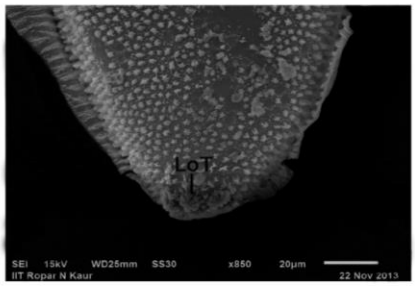

Fig. 3 Lobed tubercles

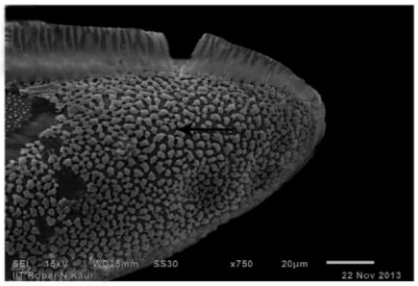

Fig. 4 Posterior end showing tubercles

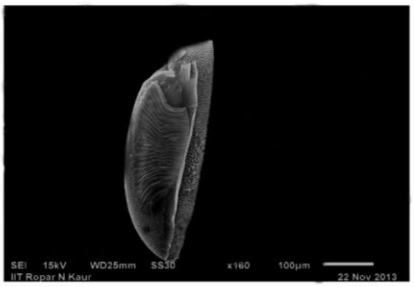

Fig. 5 Lateral view

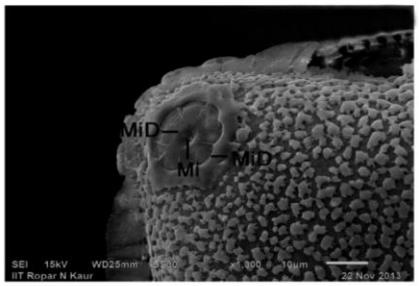

Fig. 6 Posterior end showing micropyle

Anopheles (*Cellia*) *subpictus* Grassi

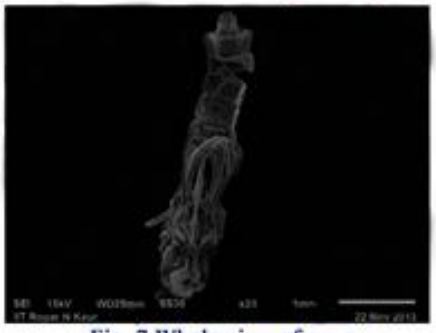

Fig. 7 Whole view of pupa

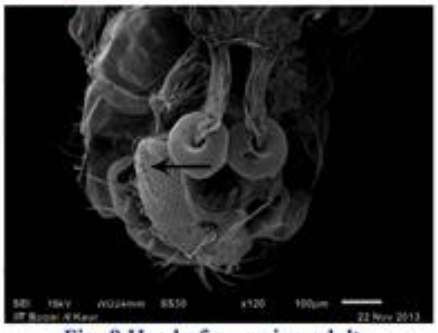

Fig. 8 Head of emerging adult

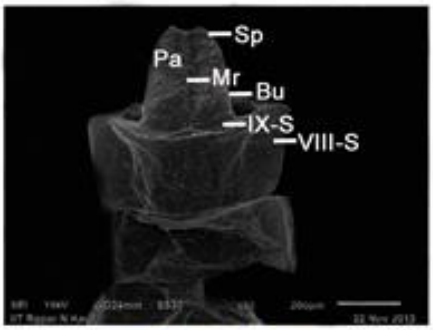

Fig. 9 Genital lobe

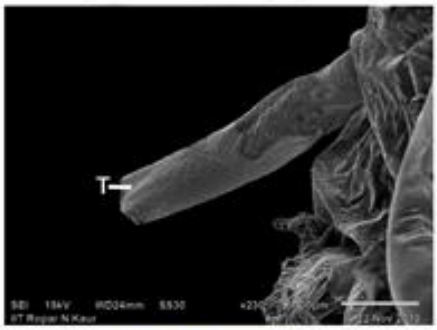

Fig. 10 Trumpet

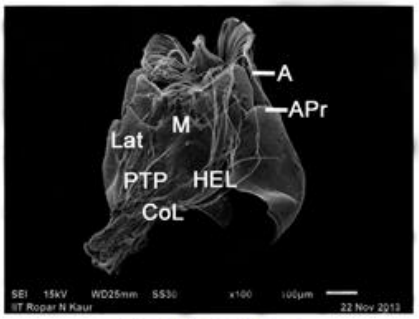

Fig. 11 Larval head

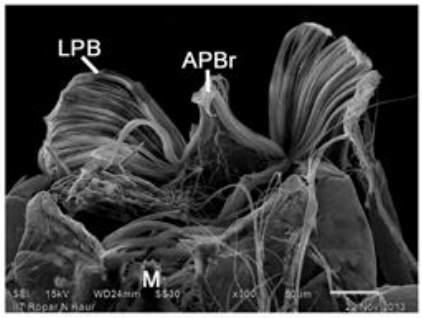

Fig. 12 Magnified view

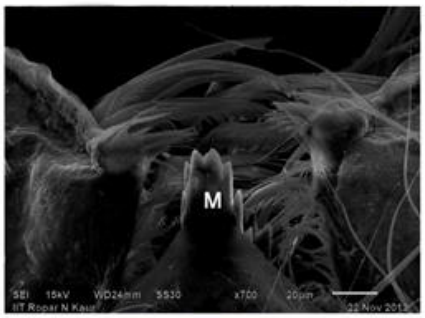

Fig. 13 Mentum

Cibarial armature of *Anopheles* (*Cellia*) *subpictus* Grassi

Fig. 14 Cibarial armature

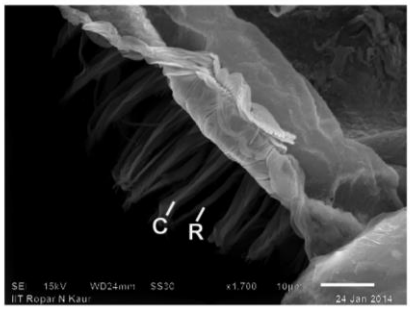

Fig. 15 Cibarial teeth (Magnified)

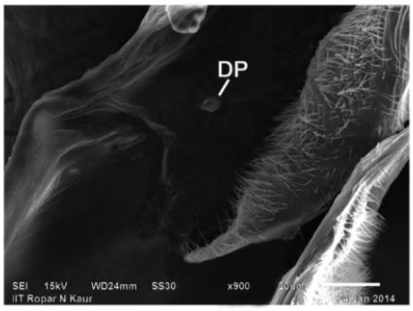

Fig. 16 Dorsal Papillae

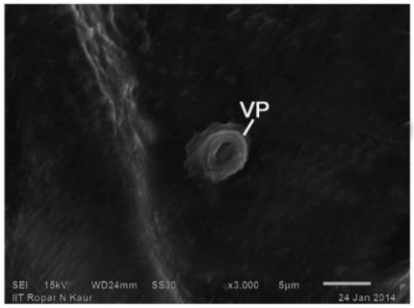

Fig. 17 Ventral papillae

Genitalia of *Anopheles* (*Cellia*) *subpictus* Grassi

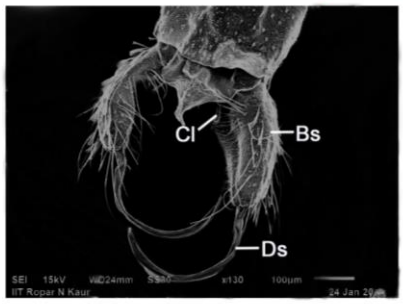

Fig. 18 Male genitalia

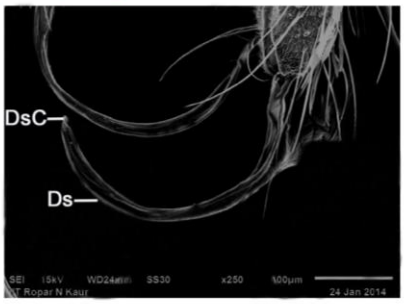

Fig. 19 Dististyle

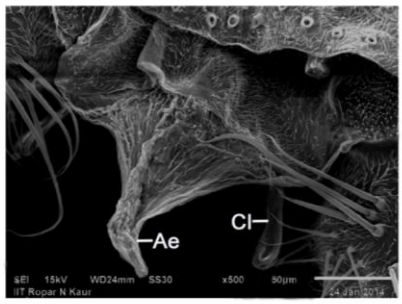

Fig. 20 Phallosome

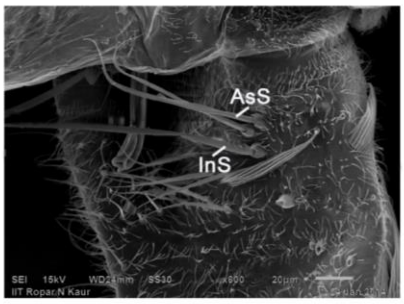

Fig. 21 Spines on basistyle

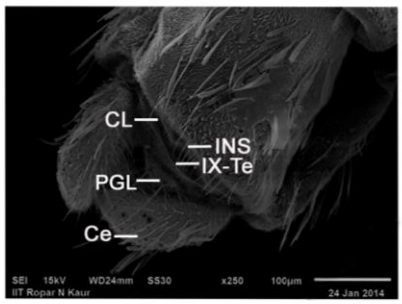

Fig. 22 Female genitalia

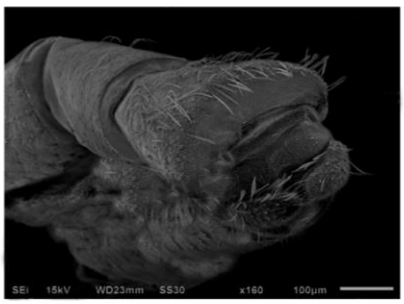

Fig. 23 Dorso-caudal view

4. Conclusion

Mosquito species in general and members of genus Anopheles Meigen are known to found in species complexes. The species *An. subpictus* also exist in a complex of four sibling species in India. Degree of interspecific divergence in these morphological characters differentiates them more than usual for sibling species (Suguna *et al.*, 1994) [39]. An effort has been made to study the ultra structures of the present species on egg, larva, pupa, cibarium, male and female genitalia. These new/additional characteristics will not only have taxonomic significance to discriminate between allied species of *An. subpictus* but will also be helpful in resolving the species complex. These attributes will certainly be added in the diagnosis of the species to update its status.

5. Abbreviations

A (Antenna), Ae (Aedeagus), AsS (Accessory spine), Bs (Basistyle), C (Cones), Ce (Cerci), Cl (Claspette), Cl (Cowl), Dm (Dorsomentum), DP (Dorsal papillae), Ds (Dististyle), DsC (Dististyle claw), FR (Fringe), HEL (Hypocranial ecdysial line), HyS (Hypostomal suture), INS (Insula), InS (Internal spine), IX-Te (IX- Tergum), Lat (Lateralia), LPB (Lateral palatal brush), Mi (Micropyle), MiC (Micropyle collar), MiD (Micropyle Disc), Mn (Mandible), Mx (maxilla), PGL (Post Genital Lobe), PL (Paraclypeal lobe), PTP (Posterior tentorial pit), R (Rods), Ventromentun (Vm), VP (Ventral papillae).

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