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A Comparative Account of External Sculpture of the Eggs of two Tiger-Moths (*Erebidae: Arctiinae: Lepidoptera*) from India

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Abstract

This study aimed to update the taxonomic data of two tiger moths which were earlier based upon the taxonomic characteristics of adult specimens only like male and female genitalic features. This study is an attempt to prove that the ultrastructural characteristics of the egg chorion of these tiger moth species are also important and they can be used in early taxonomic identifications of these species based on egg characteristics as they are also found to be species-specific. A scanning electron microscope was used to inspect, characterize and depict eggs of two species referable to Spilarctia Butler and Cladarctia Koda of the Erebidae family. The descriptions and comparative morphological analyses of the eggshells of Spilarctia multiguttata (Walker) and Cladarctia quadriramosa (Kollar) were compiled to present the structural complexity of these tiny eggs and depict the distinct patterns of structural features including the central micropylar pit, micropylar rosette, number of micropyles and aeropyles. The SEM analysis revealed their unique architecture which allows for unrestricted exchange of oxygen and carbon dioxide while limiting water loss because of the presence of minute air pores all over the chorion. From the results, it is evident that the ultrastructural egg chorion characters are of great taxonomic value at specific as well as generic levels and these types of investigations must be amplified to improve and escalate the morphological personation of tiger moths.

Keywords: *Chorion, Micropyle, Aeropyles, Erebidae, Scanning Electron Microscopy*

1. Introduction

Eggs are a well-established mode of reproduction among invertebrates including insects, spiders, mollusks, and crustaceans. Like most other insects, the lepidopterans are oviparous or "egg-bearers" (Gullan and Cranston, 2004). Many species lay their eggs singly in a widely spread manner, in tiny clumps or masses, while others lay them in masses that are further coated by a hardened fluid from the female's abdomen glands (Holland, 1898, 1903).

In Lepidoptera, the eggs are usually elliptical, spherical, hemispherical, flattened, slightly cuboid, and asymmetrical in shape. The chorion may have a simple and basic structure or crinkled surface, consisting of divisions, with transverse or longitudinal ridges. The eggshell generally possesses microscopic minute grooves or holes (airspaces, air-pores, or aeropyles) which can only be seen underneath the high magnification and resolution power of an electron microscope. These airpores permit the respiratory interchange of oxygen (O_2) and carbon dioxide (CO_2) between the environment and developing larvae with a fairly very small amount of water loss. The upper side of the egg is somewhat depressed and creates a small medial chamber with a minute aperture known as a micropyle (Evans, 1932). It is an exceptional aperture near the anterior top of the egg which provides a portal for the introduction of male gamete during fertilization in most insects.

This micropylar region is located on the upper anterior portion of eggs which are globular, conical, or cylindrical in shape, and on the outer perimeter or rim in flattened or lenticular-shaped eggs (Holland, 1898, 1903). This region is guarded with a distinct micropylar rosette. The number of micropyles varies from 1-20 (Hinton, 1981).

There are different techniques to analyze the taxonomic characters viz., morpho-taxonomy, molecular taxonomy, behavioral taxonomy and ecological taxonomy, etc. The present work is a precise benefaction, in regards to improving and escalating the morphological personation of tiger moths. These SEM inspections of eggs and eggshells of moths will prove to be an advanced approach and greatly helpful regarding the recognition and discrimination of different taxa at the levels of family, genus, and species even for resolving species complexes.

2. Materials And Methods

Collection and Preservation:

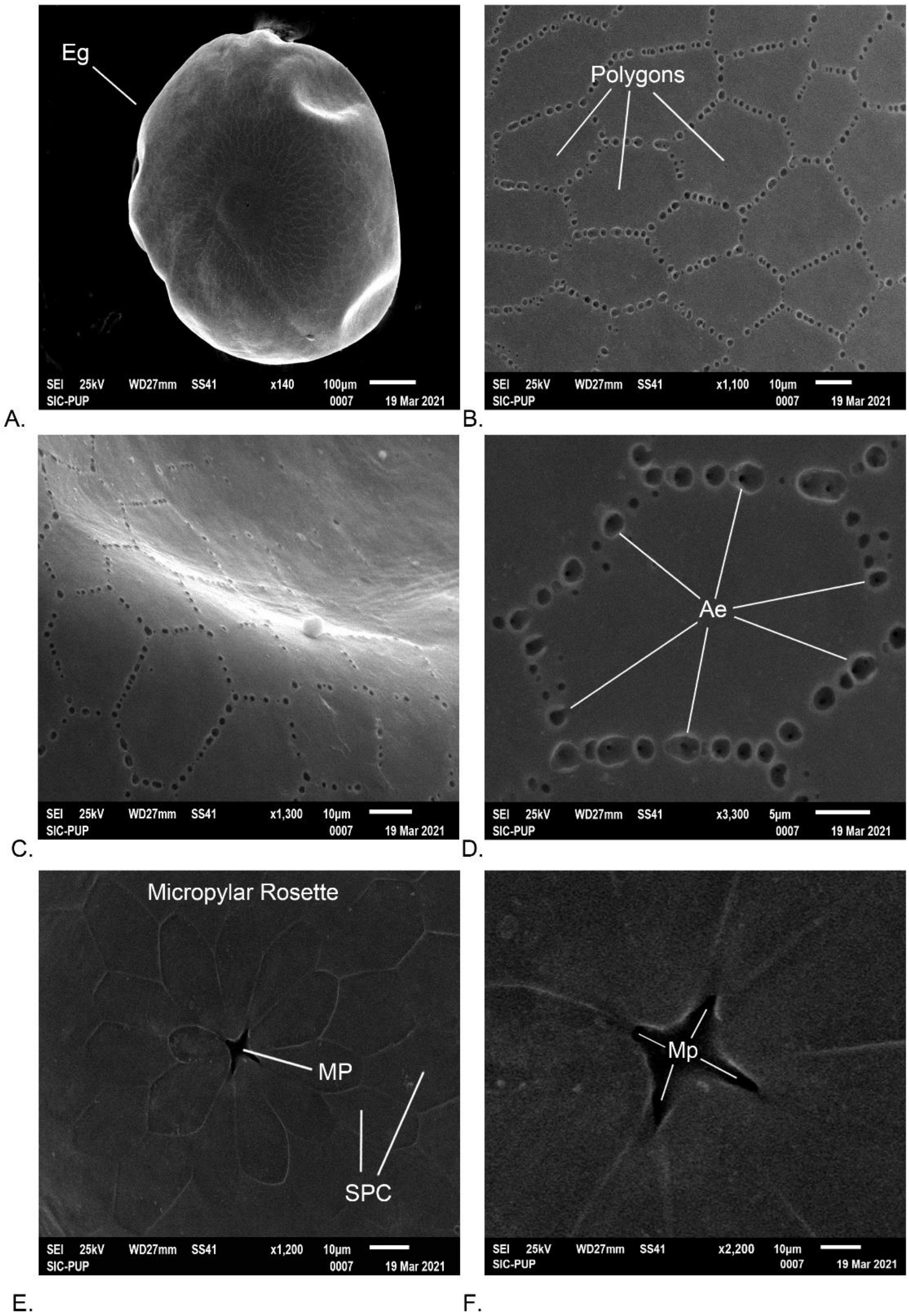
The tours were conducted in different localities of district Kullu of Himachal Pradesh to collect the eggs of moths in September 2020. The freshly laid eggs by the female moths were located and hand-picked from the underside of leaves and other parts of the plants with the help of fine forceps and brushes. The collected eggs were preserved in glass vials containing 70% alcohol and glycerol in a ratio of 8:2. The vials were labeled with proper reference numbers and field data such as the name of locality, collector, and date of collection. The adult specimens were also collected, stretched and preserved in entomological boxes for identification. The identification of adult representatives was carried on with the help of relevant literature i.e., Hampson (1894) and comparison with the reference collections present in our department. The relevant literature was retrieved from University Library and through web surfing.

Preparation of samples and procedure for SEM studies:

To prepare samples for scanning electron microscopic studies, the sample substance i.e., the eggs were fixed in 2.5% glutaraldehyde for a minimum duration of one hour. The material was shifted to phosphate buffer solution (PBS) having pH 7.4 and rinsed repeatedly for a minimum period of 15 minutes. Then, it was dehydrated by maneuvering a succession of graded ethyl alcohol. After proper dehydration, the sample material was mounted on aluminum stubs with double-sided adhesive carbon tape and sputter coated with a mixture of gold and platinum. The sputtered sample of the eggs was observed and studied through Scanning Electron Microscope (JEOL) JSM-6510LV available in the Sophisticated Instrumentation Centre of Punjabi University, Patiala. The sample material was photographed to assess the chorion, micropylar region, arrangement of micropylar rosettes, aeropyles and other external ultra-structures present on the eggshell. The eggs were classified according to the nomenclature systems proposed by Doring (1955) and Peterson (1964). The terminology used by Zolotuhin and Kurshakov (2009) and Dolinskaya (2019) has been followed in the present study.

3. Results and Discussion

The descriptions and comparative morphological analyses of the eggshells of *Spilarctia multiguttata* (Walker) and *Cladarctia quadriramosa* (Kollar) were compiled to elucidate the structural complexity of these tiny eggs.



1. (A-F) Egg of ***Spilarctia multiguttata*** (Walker 1855)
A: Dorsal view (Egg); B-C: Egg Chorion; D: Aeropyles;
E-F: Micropylar Region

Spilarctia multiguttata **(**Walker, 1855**)**
Figs. 1(A-F)
Type Species: *Phalaena lutea* Hufnagel, 1766
Distribution: India, Bhutan, Cambodia, China, Japan, Korea, Malaysia and Nepal.

Remarks: This genus is represented by more than 150 species from different parts of the world. Out of which, 21 species are known from India. Kirti and Kaleka (1994) studied and described the genitalic features of total of four species of this genus from India viz., *Spilarctia comma* (Walker), *Spilarctia obliqua* (Walker), *Spilarctia casigneta* (Rothschild) and *Spilarctia multiguttata* (Walker). Later, Kaleka (2005a) sorted out four new species viz., *himachalensis*, *multicornutiata*, *nirmalae* and *valvata* from this species complex. In the present study, scanning electron microscopic studies have been performed on the eggs of *Spilarctia multiguttata* (Walker) for the first time to explore the ultrastructure of egg chorion.

Egg Shape:
The egg is hemispherical in shape from a dorsal view and depressed and concave at the base to form a point of attachment for the egg. The outer external chorion of the egg is very thin and delicate.

Egg Chorion:
The general surface area, except the micropylar region, is very smooth without any depression, cavities, groves, or ridges. The egg chorion is highly sculptured with numerous pentagonal, hexagonal and heptagonal cells all over the surface area up to the middle of the outer circumference boundary from where the base of the egg starts.

These polygonal cells are with many circular aeropyles along with their margins which help to demarcate the actual shape of the polygonal cells. As such, these polygonal cells have no clear, thick, thin or raised boundaries. The internal surface of these cells is also plain and smooth without any sculptures or bumps on the egg chorion.

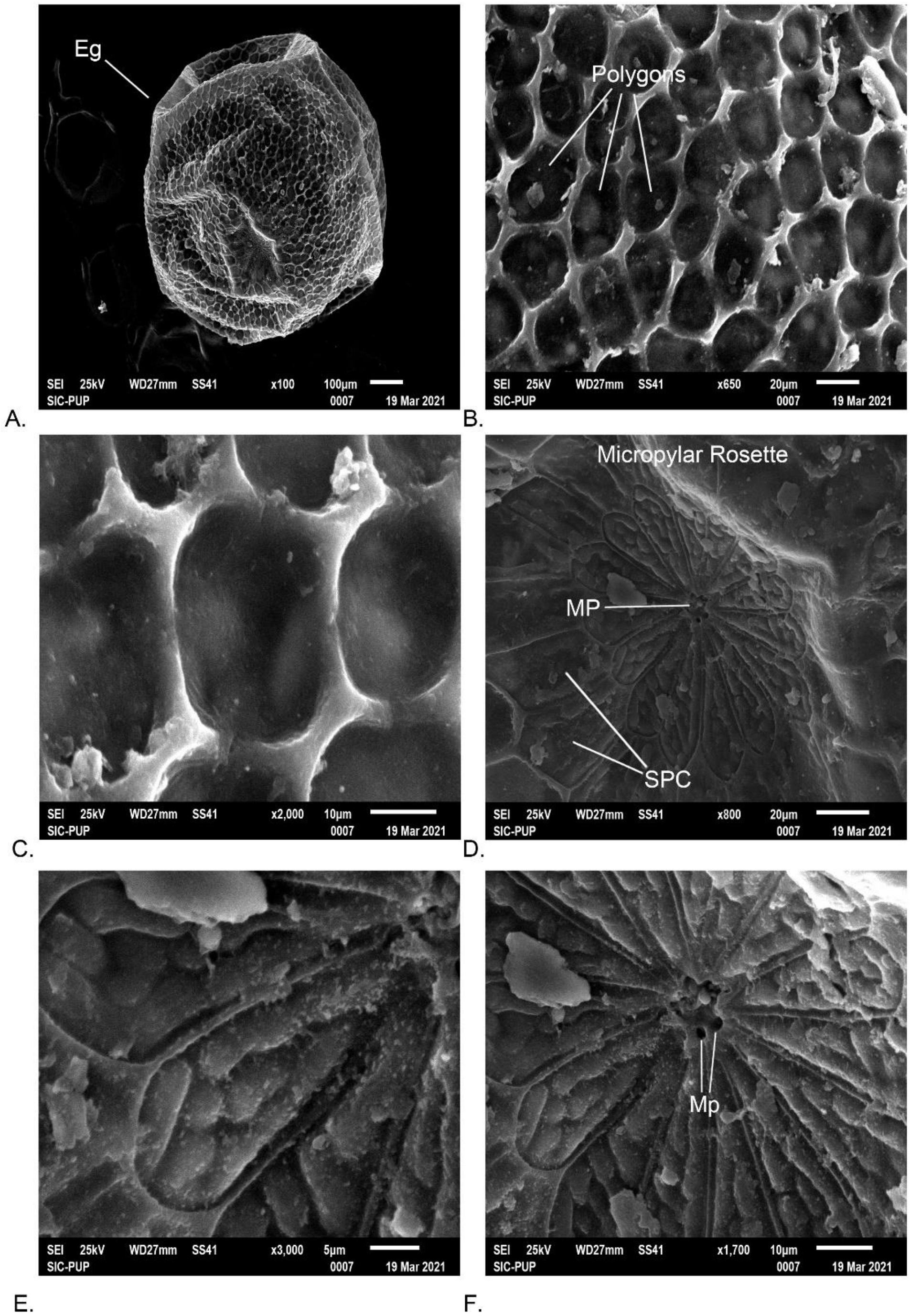
Micropylar Region:
The micropylar region in the eggs of this species is composed of a micropylar opening and micropylar rosette having primary and secondary petaloid cells. The micropylar region is present at the center of the top anterior end of the egg and surrounded by different polygonal cells all over the exo-chorion.

Micropylar Rosette:
The micropylar rosette is composed of 10 primary petal-shaped cells around micropyles. One petaloid cell is much smaller in size as compared to the remaining 9 cells and forms a similarity i.e., 9+1 arrangement in the rosette. The cells are attached with each other in the micropylar pit up to ¼th of their length from where the curves of the cells start. These primary cells are further surrounded by a series of secondary petal-shaped cells. Their boundaries merge or fade into a general chorionic surface forming a transition zone to demarcate the end of the micropylar region.

Micropyles:

The micropylar openings are at the center of the micropylar region in a tetragonal micropylar pit with clearly pointed endings and four micropyles are present at each pointed corner of the micropylar pit.

Aeropyles:
The aeropyles or air cavities are observed throughout the general chorionic surface of the egg. The aeropyles are present on the boundaries of each polygonal cell as small circular minute holes bordering each polygonal cell. The number of these aeropylar openings is different according to the shape and size of the polygonal cell ranging from 43-46 per cell. No depressions or raised boundaries are present around the aeropyles.



2. (A-F) Egg of ***Cladarctia quadriramosa*** (Kollar [1844])
A: Dorsal view (Egg); B-C: Egg Chorion;
D-F: Micropylar Region

Cladarctia quadriramosa **(**Kollar, 1844**)**
Figs. 2(A-F)
Type species: *Euprepia quadriramosa* Kollar, 1844
Distribution: India: Kashmir and North-Western Himalayas; China, Japan, Nepal, Tibet.

Remarks: Koda (1988) erected the present genus for proper placement of its type species i.e., *quadriramosa* (Kollar) which was earlier discussed under genus *Alphaea* Walker by Hampson (1894). Later, he synonymized this genus under genus *Diacrisia* Hübner but transferred the species *quadriramosa* (Kollar) under genus *Estigmene* Hübner (Hampson, 1901). Based on the distinct genitalic features, Koda (loc. cit.) justified its placement under genus *Cladarctia* Koda as its type species. Kaleka (1999, 2005b) and Fang (2000) agreed with this placement as it is genetically distinct from the North American genus *Estigmene* Hübner. During present study, the eggs of *Cladarctia quadriramosa* (Kollar) have been scanned scanning electron microscope for the first time to explore the ultrastructural details.

Egg Shape:
In this species, the egg is hemispherical in shape with wrinkles and large depressions on the egg surface due to the presence of a very thin and delicate exo-chorion over the egg and concave base.

Egg chorion:
It has been observed that the chorionic surface of the egg is highly sculptured all over the egg surface except the micropylar region present at the anterior pole of the egg. The chorion is completely covered with irregularly shaped polygonal cells up to $3/4^{th}$ surface of the egg till the base of the egg starts. These polygonal cells are of different shapes and sizes, some of these cells being simple squares, pentagonal and hexagonal. These polygonal cells have raised ridges or boundaries to form somewhat deep craters and depressions all over the chorionic surface in each cell. The internal area of these polygonal cells is almost smooth and without any markings or bumps. The boundaries or ridges of the polygonal cells are observed to be thin and uniform in between the corners and somewhat thick at the corners of the polygons. There are no aeropyles or air cavities present on the ridges and boundaries of these cells as observed in the eggs of other studied species.

Micropylar Region:
In this egg, the micropylar region is present in the center at the top anterior pole of the egg. The micropylar region in this species is composed of important parts such as micropylar openings and micropylar rosette with petaloid cells.

Micropylar Rosette:
The micropylar rosette is present in the center of micropylar region with highly sculptured primary petaloid cells. These cells are 15 in number and present around the micropylar pit. These petaloid cells are of the same shape, size and length. These cells are present distinctly from each other i.e., each with separate boundaries and appears as double-walled structure. The internal area of these petal-shaped cells is also highly sculptured with an appearance of cracked ground. These primary cells are further surrounded by a row of secondary petaloid cells to form a transitional zone.

Micropyles:
The micropyles are observed in the center of the micropylar rosette in a simple star-shaped micropylar pit. In total 5 micropylar openings are present at each corner of the star as a deep circular tiny hole. The central area of the star-shaped micropylar pit is completely smooth and flat.

Aeropyles:
In this egg, no aeropylar openings are seen i.e., complete absence of aeropyles both on exo-chorion as well as in the micropylar region. This may be due to the thin and delicate exo-chorion layer because of its ability for general air exchange from the environment directly.

During the present study, the egg morphology, ultrastructure of egg chorion and their different patterns in two species of tiger moths namely *Spilarctia multiguttata* (Walker) and *Cladarctia quadriramosa* (Kollar) have been investigated and studied with scanning electron microscope. The external morphology or ultrastructure of an egg's chorion is distinct in both of the studied species of two distinct genera with respect to their chorion sculpturing, number of primary cells forming the micropylar region, number and shape of micropylar and aeropylar openings.

In *Spilarctia multiguttata* (Walker), the egg is hemispherical in shape with a concavely depressed basal posterior end for its attachment to the substratum. The exo-chorion is very thin and delicate forming some folds as seen under a scanning electron microscope. The chorion of the egg is highly patterned with pentagonal, hexagonal and heptagonal cells all over up to the base of the egg except the anterior pole. The micropylar region is present at the anterior pole and has 4 micropyles in the tetragonal micropylar pit. The micropylar rosette is composed of 10 petaloid cells. Out of which, 9 are of the same size and 1 is much shorter. This asymmetric arrangement of primary cells is followed by a series of secondary polygonal cells. These polygonal cells possess very fine thin boundaries. The aeropyles are present on the sides of these polygonal cells as smooth flat tiny holes. Their number varies from 43-46 according to the shape and size of the polygon on the chorion.

In *Cladarctia quadriramosa* (Kollar), the shape of the egg is found to be hemispherical with a concave base for its attachment with some folds and wrinkles due to very thin and delicate exo-chorion. The chorionic surface is highly patterned with irregularly shaped polygonal cells in the form of simple squares, pentagonal or hexagonal cells. These cells are with raised boundaries or ridges and have deep craters on the surface of the chorion. The micropylar region of the egg is present at the anterior pole of the egg and has a distinct micropylar rosette. The rosette is composed of 15 primary petaloid cells which have separate boundaries and appear as double-walled structures. The micropylar pit is star-shaped and 5 micropyles are present at each corner of the star. The primary cells are further surrounded by a series of secondary petaloid cells with very fine boundary lines which form a transitional zone. The chorionic surface is completely devoid of aeropyles or air cavities.

The eggs of two studied species of tiger moths revealed the general pattern of egg chorion as found in other species of these moths by Doring (1955) and Peterson (1963) by means of light microscopy. The egg shapes of these moths are observed as round and hemispherical when seen from above completely resembles the eggs of other species referable to Erebidae family.

As far as the information about aeropylar openings present on the egg chorion in lepidopteran eggs is concerned, quite limited studies are there. Fehrenbach *et al.* (1987) attempted to count the number of aeropyles present on egg chorion in two Noctuid species namely *Heliothis virescens* (Fabricius) and *Spodoptera littoralis* (Boisduval). They recorded 50 aeropyles per egg in *Heliothis virescens* (Fabricius) and 400 aeropyles per egg in *Spodoptera littoralis* (Boisduval). In the present study, an attempt has been made to examine the number as well as shape of aeropylar openings in the studied species. The number of aeropyles per polygonal cell present on the egg chorion has been recorded and revealed a great variance in their number in these two studied species of tiger moths. The highest number of aeropyles per polygonal cell has been observed in the eggs of *Spilarctia multiguttata* (Walker) as 43-46 whereas, no such openings were found on the egg chorion in *Cladarctia quadriramosa* (Kollar). The ultrastructural details of the eggs of these two studied species have been summarized in Table 1.

Table 1: Comparative account of ultrastructural characters in the studied species

Egg Features	**Studied Species**	
	Spilarctia multiguttata (Walker)	*Cladarctia quadriramosa* (Kollar)
Egg Shape	Hemispherical (Concave base)	Hemispherical (Concave base)
Chorion Texture	Highly Sculptured	Highly Sculptured
Shape of Polygons	Pentagonal, Hexagonal & Heptagonal	Square, Pentagonal & Hexagonal
Number of Aeropyles (Per polygonal cell)	43-46	-
Number of Micropyles	4	5
Primary Petaloid Cells (PPC)	9+1 Petaloid	15 Petaloid
Secondary Petaloid Cells (SPC)	Present	Present
Shape of Micropylar Pit	Plus-Shaped	Star-Shaped
Ridges And Carinae	Absent	Present

The superficial morphological attributes such as general coloration, ornamentation of the head, thorax and abdomen, wing maculation, wing venation and more particularly the male and female genitalic features are conventionally employed for identification and differentiation of various taxa and in the formulation of identification keys. Based on the present work, it can be easily concluded that the ultrastructural features also provide remarkable features and these can further authenticate and strengthen the morpho-taxonomy. It is quite difficult to differentiate the closely related species based on observation of the morphological features such as shape, size, color and texture of the eggs with the naked eye. So, more well-grounded taxonomic attributes need to be incorporated for proper differentiation. For this, the ultrastructure investigations on different aspects such as shape, chorion texture and pattern and other structural details of eggs of these two species of ditrysian moths have been performed and these studies provided ample features. Further inspection of such features present on the eggs of more species of various moths needs to be conducted. These investigations will contribute significant facts and details to further strengthen the taxonomy and phylogenetic studies of the moths and prove appreciable for the delimitation of closely linked taxa. Such attempts can engross these egg structures in recognition of tiger-moth species. It is justified to mention here again that these SEM inspections of the eggs and eggshells of these moth species will prove to be an advanced approach, help in the discrimination of different taxa at family, generic or specific levels and even for resolving out species complexes. Hence, this research will set a foundation and contribute a suitable model for carrying out such investigations on other species of Indian moths.

4. Conclusion

This study was conducted to update the taxonomic data of two tiger moths based upon the ultrastructural characters present on their egg chorions because when it comes to identification and differentiation of discrete taxa, only superficial morphological attributes particularly, male and female genitalic features are traditionally used. In *Spilarctia multiguttata* (Walker, 1855), the egg was hemispherical with a concave base having a highly patterned chorion layer with pentagonal, hexagonal and heptagonal polygons. The micropylar rosette at the anterior top of the egg was formed of a 9+1 petaloid arrangement with 4 micropyles present in the center. Approximately 43-46 tiny aeropyles were seen present encircling the polygonal cells. In *Cladarctia quadriramosa* (Kollar, [1844]), the egg was hemispherical shape in with a concave base having a highly patterned chorion layer with square, pentagonal and hexagonal cells that form deep craters and ridges all over the chorionic surface. The micropylar region was present at the anterior top and had 15 petaloid cells forming a rosette and 5 micropyles in the center. In this egg, no air-pores or aeropyles were observed. It is difficult to distinguish between closely related species based on morphological features such as the shape, size, color, and texture of the eggs. But sadly this research area has been not getting enough attention when it comes to escalating the taxonomic data of the ditrysian moths for their identification and we are still lacking behind even after technology like SEM has become a popular tool for redefining this type of research on ultrastructural characters. So, for proper differentiation, more well-founded taxonomic attributes must be included such as the egg chorion characters investigated in the present work. Based on current and relevant previous important works, it is easy to conclude that the ultrastructural features are also noteworthy and can authenticate and strengthen the morpho-taxonomy of these moths. These investigations will contribute significant facts and details to further strengthen the taxonomy and phylogenetic studies of the moths and will prove useful for the delimitation of closely related taxa. Additionally, these types of investigations can be carried out in regards to pest management programs and crop protection from potential pest species which are harmful agricultural pests and they can be eradicated in their early immature life stages after identification through pet pest management programs.

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Conflict of interest:
The authors declare no conflict of interest.

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