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Biology, morphology and DNA barcodes of *Tessaratoma javanica* (Thunberg) (Hemiptera: Tessaratomidae)

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Abstract

Tessaratoma javanica (Thunberg) (Hemiptera: Tessaratomidae) an important sucking pest of litchi is studied for supplementing information on its biology, morphometrics of life stages and mtCOI (DNA barcodes). More details generated on the study add to the description of stages namely egg, 1st to 5th nymphal instars and adults. The evaluation of morphometrics of the life stages reveal that the progression of growth is more during 2nd to 3rd nymphal stages, and these are critical as far as the growth and development is concerned. The life cycle takes about 141.7±4.25 days; eggs last for 12.81±1.4 days with 97.14±2.86% hatchability; and duration of 1st , 2nd, 3rd, 4th and 5th nymphal instars were 11.69±0.58, 7.23±0.2, 8.63±0.55, 13.04±0.55 and 26.31±0.97 days, respectively. In addition mtCOI analyses have been done employing standard 658 bp barcode fragments facilitating molecular diagnostics of the adults and other life stages and the phylogenetic tree with available sequence in the GenBank.

Key words: Litchi, Bionomics, Life stages, mtCOI, Barcoding, India

Introduction

Tessaratoma javanica (Thunberg) (Hemiptera: Tessaratomidae) is an economically important sucking pest of *Litchi chinensis* Sonn. (Sapindaceae) from India (Kumar and Singh 2007; Choudhary *et al.* 2012). Like *T. javanica*, *T. papillosa* Drury causes damage to litchi in China (Kershaw 1907) and are commonly known as litchi bugs. Apart from litchi, it is causing damage to kusum (Glover 1933; Mehra and Kapur 1955) and also been reported feeding on *Sapindus* sp. (Distant 1902; Scahefer and Ahmad 1987), *Schleichera oleosa* (Mehra and Kapur 1955) and *Michelia champaca* (Kumar and Singh 2007). In view of economic importance, the biology of these bugs had been studied earlier by many workers (Liu 1965; Liu and Gu 2000; Mehra and Kapur 1955; Kumar and Singh 2007). The genus *Tessaratoma* has about 26 species of which only 6 species are known from India.

Litchi is a tropical and subtropical tree native to Southern China, Taiwan and South East Asia, and now cultivated in many parts of the world. India is the second largest producer of litchi in the world after China, with an area and production of 77,600 ha and 497,300 t, respectively, during 2010–11 (http.nhb.gov.in). There are many species of bugs that attack litchi, among them *T. javanica* is the most destructive. The damage is caused due to the sap sucking habit of its gregarious nymphs as well as adults on tender parts of the litchi tree, such as growing buds, leaf petioles, inflorescence, fruit stalks, and fruits. This results in drying of growing buds and tender shoots and further heavy fruit drop lead to total loss.

Recently, an outbreak of *T. javanica* was observed in the Chotanagpur plateau of Jharkhand with damage exceeding 80% (Choudhary *et al.* 2012). Despite its economic importance, *T. javanica* biology and life cycle stages are less understood and a taxonomy oriented morphological description especially of nymphal instars is lacking. Also, there is a need to develop mtCOI barcodes so that these life stages could be subjected to molecular diagnostics in a demanding situation, such as phytosanitation. The present study is thus an attempt in this direction,

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and the biology of *T. javanica* has been studied along with morphology and morphometrics of the nymphs and adults leading to its redescription. In addition, mtCOI analyses of life stages have been performed using 5' end of the mitochondrial gene cytochrome oxidase I.

Material and methods

For studying the life cycle, freshly laid egg clusters attached to leaves (n=20) were collected from litchi orchards of ICAR Research Complex for Eastern Region, Research Centre, Ranchi, and kept individually in insect rearing cages (20×40 cm) at 25±1°C, RH=60±5%, and light: dark phase, 16:8 hr. The neonates that emerge were transferred with a brush to rearing jars, supplied with fresh litchi twigs and suitably sealed. When these developed into adults, pair of males and females were placed in rearing jar for mating, provided with egg laying substrates like leaves and stems. These rearing cages and jars were disinfected periodically with Protasan DS® to enable healthy stock culture for the required experiments.

The morphology and morphometric studies were carried out with Nikon SMZ 10 and Leica MZ16A stereozoom microscopes fitted with a drawing tube. Images were taken with a Sony DSC H50 digital camera and DFC 425 digital camera attached onto a Leica 205FA stereozoom microscope. Measurements were taken in millimeter (mm) with standard deviation (SD) using a micrometer eyepiece and scale given in the figures is 1 mm.

For mtCO1 analyses, DNA was extracted from egg, single leg of nymphs/adults using DNeasy Blood and Tissue Kit (Quiagen GmbH, Germany) as per manufacturers' protocol. The leftover part of specimens were preserved as voucher material and deposited in the National Pusa Collection, Division of Entomology, Indian Agricultural Research Institute, New Delhi (Table 2). The extracted genomic DNA was visualized using 0.8% agarose gel and quantified using fluorometer using standard procedures. Depending upon the concentration, the DNA samples were diluted with molecular gradient water to get a working solution of 10–30 ng/µl. A portion of the total DNA was preserved in glycerol (10%) at -80 °C.

Polymerase Chain Reaction (PCR) was carried out in a thermal cycler (Verti; ABI-Applied Biosystem, Foster City, CA) with PCR template of 2.5µl of 10X PCR buffer, 2 µl of 25mM $MgCl_2$, 0.5µl of 10 mM dNTPs, 0.5µl each of forward and reverse primer, IU of Taq, and 17 µl of UltraPure water (Invitrogen), for a final PCR product of 25 µl. mtCOI barcoding region was amplified using the forward primer LCO-1490 in combination with the reverse primer HCO-2198 (Folmer *et al*. 1994). PCR conditions were as follows: denaturation for 5 min at 94ºC, followed by 35 cycles of denaturing for 30 sec at 94ºC, annealing for 40 sec at 54ºC and extension time of 40 sec at 72ºC, with a final extension for 5 min at 72ºC. PCR products were visualized by electrophoresis on agarose gel and purified using a QIAquick PCR purification kit (Quiagen GmbH, Germany).

The purified PCR products were sequenced directly in both directions using an automated sequencer (ABI prism® 3730 XL DNA Analyzer; Applied Biosystems, USA) at Scigenomics Lab, Cochin, India. The sequences were aligned on BioEdit 4.0 software, using ClustalW 1.8 (Thompson *et al.* 1994). mtCOI sequences identity were confirmed by BLAST. For the phylogenetic analysis, sequences available in the NCBI GenBank of *Tessaratoma* spp. (Accession nos. HQ236466 and AY252948) were obtained and aligned with data generated. The phylogenetic tree was constructed using MEGA6 (Tamura *et al*. 2013) based on Maximum Likelihood method (ML) using the sequences of *T. javanica, T. papilliosa* and an unidentified *Tessaratoma* sp. *Coridius chinensis* (Dallas) and *C. nigriventris* (Westwood) were used as outgroups.

Results

Biology. Adults start copulation within 42±17.5 days after emergence, but it takes longer (182.5±35.2 days) during hibernation period (August to February). Adults remain in coitus position for nearly 4–5 hrs and copulation is always observed end to end like other heteropteran bugs (Fig. 1). Oviposition occurred 13.24±4.92 days (n=10) after mating, eggs were usually laid in clusters of 14.3±4.2 eggs, arranged in 3–4 rows. The smaller egg cluster observed had 8 eggs, and the largest, 32. The eggs remain attached to the substrate with a sticky substance secreted by female before oviposition (Fig. 2). Eggs were preferably laid under leaf surface (75±5%) (Fig. 3), but also observed on inflorescences, fruits and over the already laid eggs. Immature stages are gregarious (Fig. 4). Female

fecundity varied between individuals. In a few cases, a female laid only 2–3 clusters of eggs and in extreme case a maximum of 16 clusters. The mean fecundity observed was 13.4 clusters (n=10). The life span of all immature stages as well as adults (male and female) has been provided in table 1.

FIGURE 1–4. 1. Mating pair. 2. Freshly laid eggs. 3. Egg clusters. 4. Nymphal stages in gregarious phase.

Morphology. Eggs are globular, 2.76±0.05 mm long and 2.5±0.04 mm in diameter. Freshly laid eggs are pale pink and change to dull pink at maturity. Eggs with 42–46 chorionic processes medially (Fig.12). A few days before hatching, a pair of small dark patch appears, indicating the development of eyes. The egg color gradually becomes darker towards eclosion. T-shaped sclerotized egg busters become visible. The chorion opened distally, allowing a cap to separate out which either remained attached or completely detached. Hatchability of 97.14 ±2.86% was observed.

First instar nymph with head brown, gradually deceasing head width anteriorly, lateral margins sinuate before eyes, eyes brown, touching anterior pronotal margin, ocelli not defined (Fig. 5); antennae brown, I antennal segment smallest and pale to orange coloured, remaining antennal segments brown to black, IV longest with white or grey setae which become dense distally, annuli white; labium red except apical segment and extending up to or beyond mesocoxae. Pronotum medially convexed, with a median ochraceous line from apex to base longitudinally; lateral margins yellow with small, globular protuberances. Legs brown, tarsi two segmented with claws and a pair of pulvill observable in light microscopy; and tibiae setose, gradually increasing towards tarsus. Abdomen oval with a median dorsal transverse spot on segments I and II; 3 pairs of dorso abdominal glands present on oval to subquadrate brown tergite; also present on segments VII and VIII, and a black semicircular tergite on each lateral margins (Fig. 13); ventrally pale orange with brown or black, semicircular spots in the middle of each segment; a spiracle each on lateral side from I–VII segments while a pair of trichobothria on segment III–VII caudal to spiracle; and a brown median oval spot from IV–VIII segments, their size keeps increasing posteriorly.

TABLE 1. Biology and morphometrics of *Tessaratoma javanica*

Stage	Measurements (mean±SD)															
	Length	Head		Pronotum		Antennae					Rostrum					Duration (days)
		Length	Width	Length	Width	I	II	III	IV	Total	I	II	III	IV	Total	
Egg Length	2.7±0.10	--	--	--	--	--	--	--	--	--	--	--	--	--	--	12.8±.1.40
width	2.5±0.10															
Instar I	6.18±0.40	0.5±0.04	1.2±0.02	0.7±0.10	2.9±0.10	0.3±0.02	0.9±0.04	0.9±0.02	1.0±0.10	3.0±0.10	0.3±0.02	0.5±0.04	0.3±0.02	0.3±0.03	1.6±0.01	11.7±0.60
Instar II	8.7±0.40	1.2±0.15	8.7±0.50	2.5±0.10	4.4±0.10	0.5±0.02	1.8±0.04	1.1±0.03	1.3±.03	4.1±0.10	0.6±0.04	0.7±0.04	0.3±.02	0.5±0.02	2.1±0.10	7.2±0.20
Instar III	17.4±0.83	1.8±0.11	2.6±0.10	2.6±0.10	8.8±0.40	0.7±0.03	1.9±0.07	1.9±0.10	2.3±0.01	6.9±0.20	0.8±0.04	1.3±0.07	0.5±.04	0.6±0.03	3.2±0.10	8.6±0.55
Instar IV	20.2±0.60	1.9±0.13	3.2±0.10	3.8±0.30	11.2±0.30	1.0±0.02	2.3±0.10	2.2±0.10	2.8±0.10	8.3±0.30	0.9±0.03	1.5±0.08	0.4±.03	0.7±0.03	3.7±0.10	13.0±0.55
Instar V	22.4±0.50	2.1±0.11	3.2±0.10	4.04±0.10	11.5±0.60	1.0±0.05	1.6±0.07	0.5±0.02	0.7±0.03	3.8±0.10	1.0±0.10	1.6±0.07	0.5±.02	0.7±0.03	3.8±0.12	26.3±0.90
Male	27.6±0.70	2.3±0.10	3.6±0.30	10.6±0.50	13.8±.30	1.1±0.02	2.5±0.10	2.3±0.20	2.8±0.43	8.6±0.60	1.0±0.01	1.6±0.10	0.5±0.04	0.7±.02	3.9±0.01	60.2±7.20
Female	29.7±1.60	2.3±0.10	3.8±0.07	9.7±0.50	14.5±.50	1.1±0.05	2.4±0.10	2.4±0.10	3.1±0.10	9.1±0.20	1.1±0.02	1.7±0.10	0.6±0.10	0.8±0.01	4.3±0.10	72.6±9.30

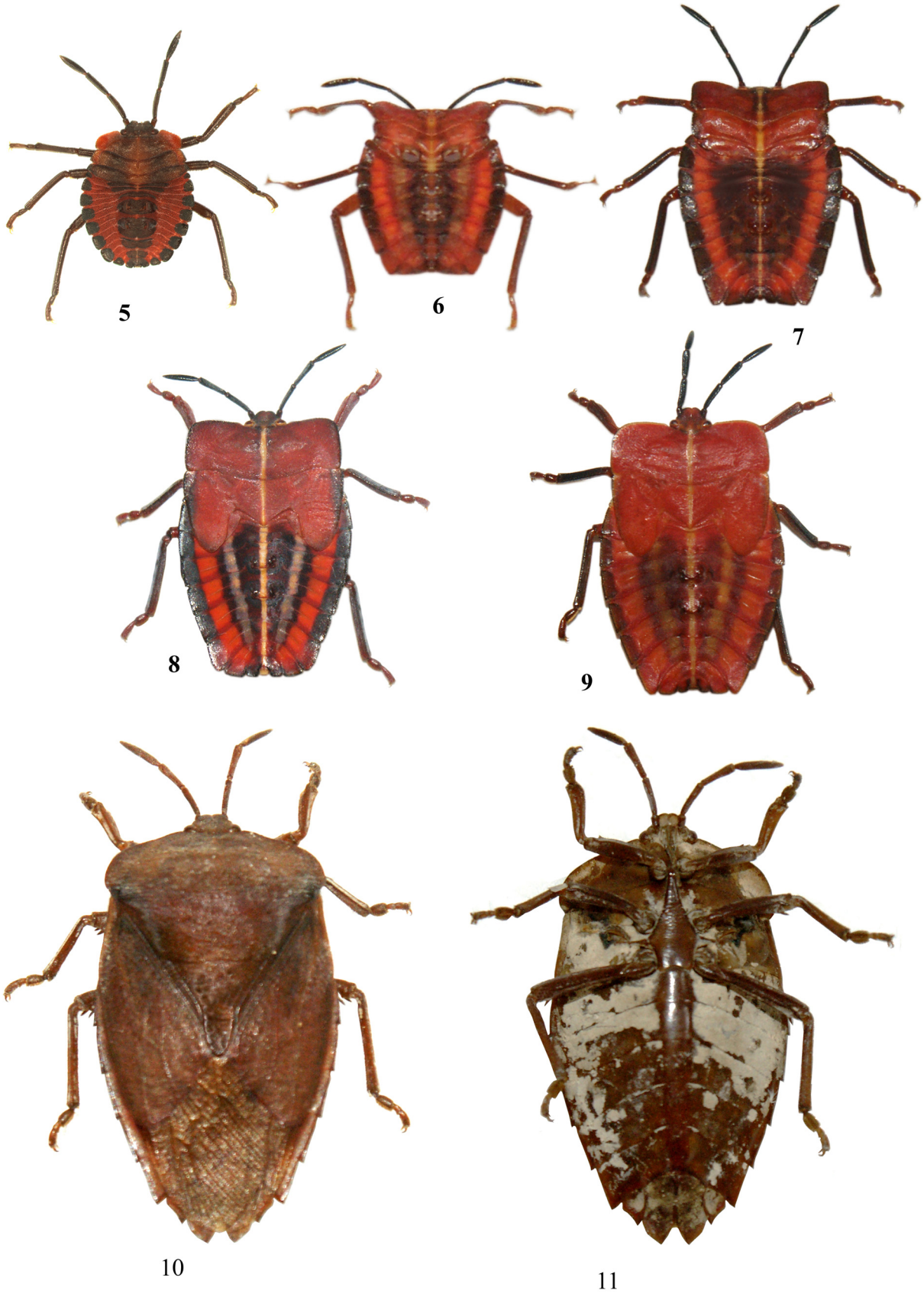

FIGURE 5–11. 5. First instar. 6. Second instar. 7. Third instar. 8. Fourth instar. 9. Fifth instar. 10. Adult habitus (dorsal). 11. Adult habitus (ventral).

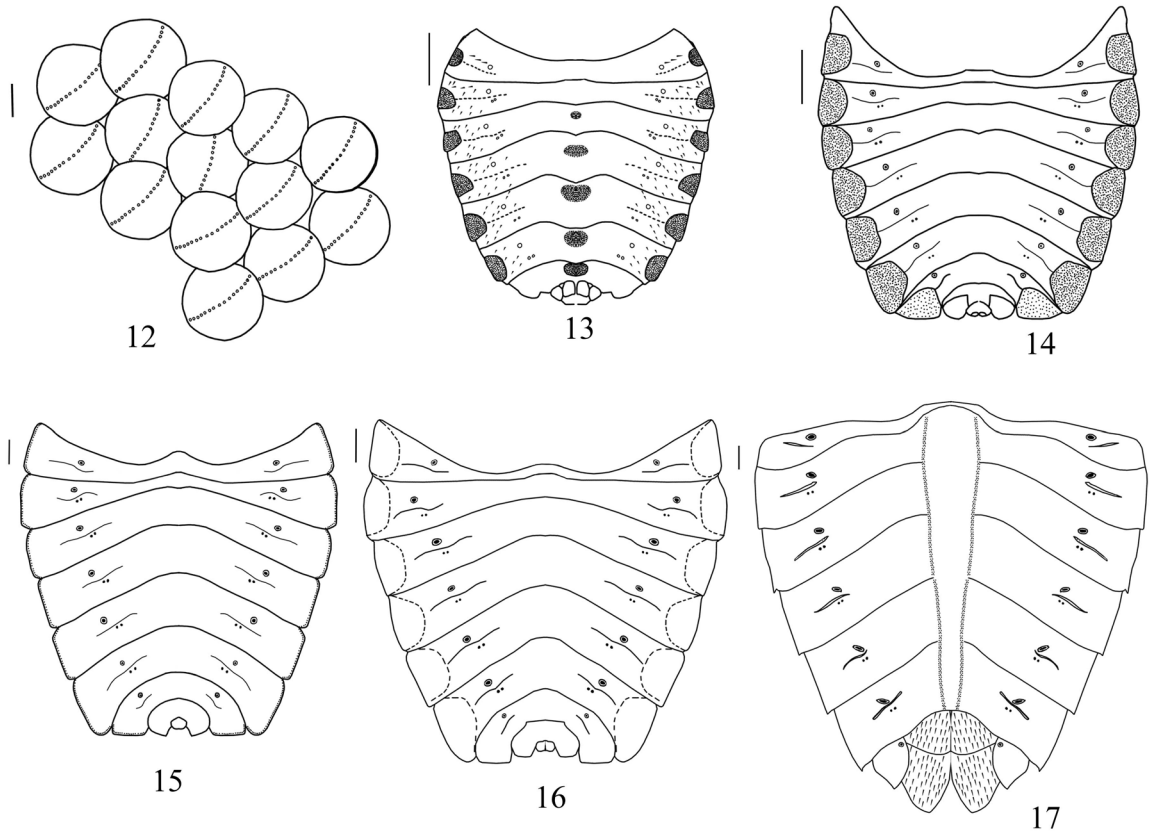

FIGURE 12–17. 12. Egg cluster. 13–17. Ventral view of abdomen for different stages. 13. First instar. 14. Second instar. 15. Third instar. 16. Fourth instar. 17. Adult female.

Second instar nymph with head brown to black, mandibular plates extends beyond clypeus, sinuation of lateral margins lesser compared to first instar, round protuberance in apical area, eyes red, touching the anterior pronotal margin, an ocharceous band present near eyes. Antennae black, I smallest, IV longest, grey coloured setae present, distally dense; labium orange except apical tip, extends upto mesocoxae. Lateral pronotal margins subquadrate, with an ochraceous line medially, and small and round protuberance all over the surface. Legs red with two segmented tarsi and a pair of apical pulvilli. Abdomen oval, dorso–lateral margins with subquadrate black spots on each segment. An ochraceous line goes medially from apex to base and similar impression on both sides runs parallel (Fig. 6), and surface with a dense protuberance. Ventrally, the lateral margins with same kind of spots and pattern, a spiracle present on I–VIII segment and a pair of trichobothria on II–VI segments caudal to spiracles present (Fig. 14).

Third instar nymph with head pale to orange, lateral margins black with slight sinuation, mandiblar plates extending beyond clypeus, eyes red, touch the anterior pronotal margin, protuberance present but dense in apical areas. First and apex of IV segment and annuli of antennae red while rest of the parts black, grey or with pale coloured small setae; labium orange except apical tip**.** Lateral pronotal margins black and subquadrate, a median, pale coloured line runs between apical pronotal margin to posterior end of abdomen. Legs red, tarsi two segmented with a pair of claws and pulvilli at apical tarsal segment, and femora with a spine at apex ventrally. Abdomen oval but subquadrate apically, lateral margins black, segment II–VI possess subquadrate brown spots dorsally, medial part of abdomen brownish–black (Fig. 7). Ventrally abdomen with similar patches on lateral margins as on dorsal surface, segment I–VII with a single spiracle on each lateral side and a pair of trichobothria on segments II–VII, caudal to spiracle (Fig. 15).

Fourth instar nymph with head pale to orange, lateral margins black and slightly sinuated, mandiblar plates extending beyond clypeus, eyes red and touch the anterior pronotal margin; antennae black except I segment and annuli, with grey coloured setae on antenna but few on I segment; labium colouration same as in 3rd instar and extends before or upto mesocoxae. Anterior pronotal margin straight, wing pads visible and extend upto II abdominal segment (Fig. 8). Legs orange–reddish, ventral surface of hind femora with a pair of spines subapically,

tarsi two segmented, with a pair of claws and pulvilli on apical segment. Metathoracic segment possess a spine on its ventral surface. Abdomen oval, subquadrate apically, other spots similar as in III instar; ventro-lateral margins black, abdominal segment possesses an impression of subquadrate spot; a spiracle on I–VII abdominal segment and a pair of trichobothria present on segment II–VI, caudal to spiracle, small, round and dense pubescence present all over the surface (Fig. 16).

Fifth instar nymph with head orange to red, lateral margins black and sinuated slightly, with a small and dense protuberance on the dorsal surface; antennae four segmented, I segment red while the rest black, small grey coloured setae present except for I segment; labium with I segment orange–red while others orange to brown and extending beyond forecoxae. Lateral pronotal margins straight and black with an additional yellow marking throughout, medial line as in third and fourth instars (Fig. 9); ventrally a metasternal spines present. Foreleg red, mid and hind tibiae brownish black while the rest of femora and tarsi red, a pair of subapical spines present on ventral surface of hind femora. Abdomen oval, subquadrate posteriorly and a subquadrate foveal impression on each abdominal segment, also with dense and fine protuberances on whole surface; ventral lateral margin with a black and yellow coloured continuous margin, with a subquadrate foveal impression on lateral side of each abdominal segment, a spiracle on I–VII segment on each lateral side and a pair of trichobothria on II–VI caudal to spiracle.

Key to the nymphal instars of *Tessaratoma javanica*

1.	Body round to oval, mandibular plates not go beyond clypeus	First instar
1'	Body quadrate, mandibular plates surpass the clypeus	2
2.	Body size never goes beyond 10mm	Second instar
2'	Body size always bigger than 15mm	3
3.	Wing pads not developed, body measures <20mm	Third instar
3'	Wing pads developed size >20mm	4
4.	Femora without apical spine, wing pads reach upto the middle of third abdominal segment	Fourth instar
4'	Femora with a pair of apical spine, wing pads goes beyond middle of third abdominal segment	Fifth instar

Adult with head triangular, lateral margins black with slight sinuation, mandiblar plates extend beyond clypeus; eyes brown touching the anterior pronotal margin, ocellus located near pronotal margin; antennae four segmented, brown reaching apex of head, with setae small and sparse; labium four segmented, orange except apical tip black. Anterior pronotal margin sinuated, lateral margins round or ovate, posterior margin straight with area near anterior and lateral margins densely punctate (Fig. 18). Scutellum triangular, anterior margin overlapped by posterior edge of pronotum, posteriorly tapered, with sparse punctations. Legs pale to orange, a pair of apical spines on ventral surface of each femora and tarsi 3 segmented. Sternum with a broad and triangular keel, occupying area between fore and hind coxa; exterior of metathoracic scent glands with ostiole round, located on metapleuron, ostiolar groove distally wider, peritremal lobes reaching 0.33x of pleuron width, anterior peritremal lobe slightly longer than posterior one, with evaporatorium small and transverse (Fig. 19). Abdomen ovate, dorsally flattened, intersegmental suture between III & IV, IV & V and V & VI recurved medially while between VI & VII and VII & VIII almost straight or slightly curved; with three pairs of dorsal glands as in nymphs; ventrally convexed, middle area shiny and never with white waxy secretion, devoid of any protuberance or punctures, single spiracles on II–VII segments and on VIII paratergite on each lateral side; with a pair of trichobothria on III–VII segment caudal to spiracles (Fig. 17). Male genitalia (Fig. 20) with pygophore (5.5 mm approx.) ventrally covered with sparse setae, lateral margins round, segment X, triangular and margins possess dense setae (Fig. 21); aedeagus with phallotheca large and almost cylindrical. Three pairs of conjunctival processes present; vesica very long, basally curved and bears sac–like ejaculatory reservoir and distally lying over the second pair of conjunctival processes (Fig. 22); parameres swollen and flattened distally and notched to project out into large sclerotized lobes and possess dense and long setae, with stem short and sclerotized (Fig. 23, 24). Female genitalia with plate- like ovipositor having VIII paratergites triangular and possess a spiracle near inner margin, paratergites IX approximately 2x than VIII, subquadrate and medially fused (Fig. 25); first pair of gonocoxae triangular, ventral surface with dense setae, with a rounded foveal impression towards posterior margin; spermatheca bulb globular and with pump enclosed within proximal and distal flanges (Fig. 26).

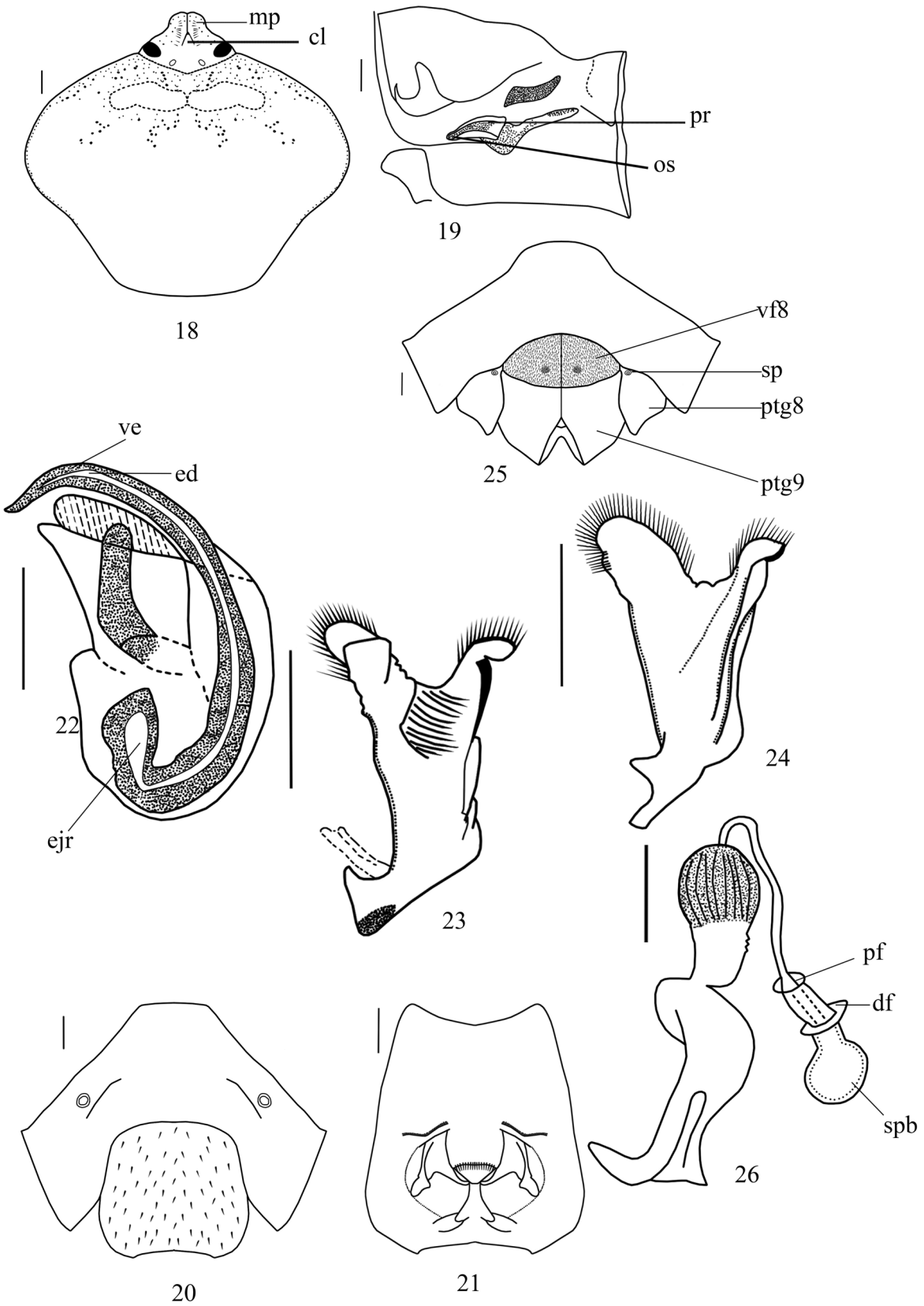

FIGURE 18–26. 18. Head and pronotum (dorsal view). 19. Exterior of metathoracic scent glands. 20. Pygophore (ventral view). 21. Pygophore (dorsal view). 22. Aedeagus. 23–24. Paramere in lateral view. 25. Ovipositor (ventral view). 26. Spermatheca. Abbreviations: cl; clypeus, df; distal flange, ed; endocephalic duct, ejr; ejaculatory duct, er; ejaculatory reservoir, mp; mandibular plates, os; ostiole, pf; proximal flange, pr; peritreme, ptg8; paratergite 8, ptg9; paratergite 9, sp; spiracle, ve; vesica and vf8; valvifer 8.

DNA barcoding. mtCOI DNA analyses of all the life stages namely the egg, first to fifth nymphal instars and adult male and female revealed similarity as anticipated. A comparison of the triplicate sequence showed no evidence of mismatch and indicated there were no sequencing errors. A total fragment of 658bp of the COI was analysed for all life stages (Fig. 25). Evidence of nuclear copies was not found, which was supported by the absence of stop codon within the sequence and base composition was similar with no indels. The sequences generated were deposited in the NCBI GenBank (Table 2) and these are also accessible in BOLD. Based on ML tree, two major clades were recognized and those clades differentiate two *Tessaratoma* spp. (Fig. 25). The *T. javanica* sequences were found clustered together and showed similarity with unidentified *Tessaratoma* sequences submitted in the GenBank. Further, the second clade revealed the separate clustering of *T. papilliosa.*

TABLE 2. *T. javanica* life stages with voucher and NCBI GenBank accession details.

S. No.	Life stages	Voucher No.	Accession No.
1	Egg	TJE01	KF534917
2	First instar	TJN02	KF534918
3	Second instar	TJN03	KF534919
4	Third instar	TJN04	KF534920
5	Fourth instar	TJN05	KF534921
6	Fifth instar	TJN06	KF534922
7	Adult male	TJM07	KF534923
8	Adult female	TJF08	KF534924

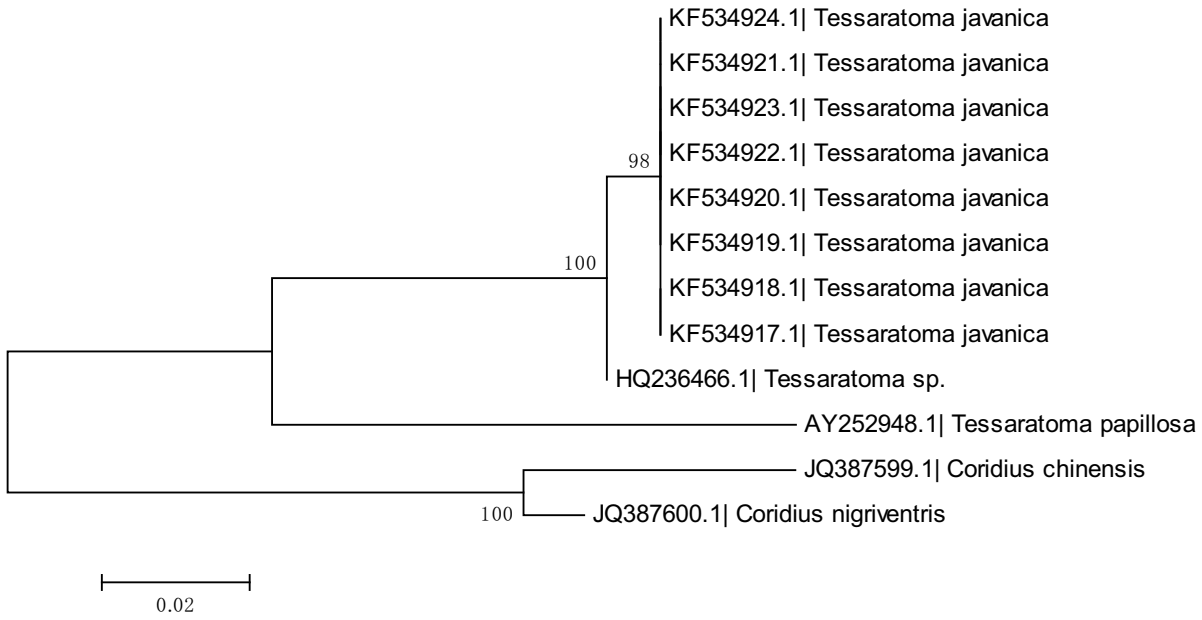

FIGURE 27. The ML tree showing the phylogenetic relationships of *Tessaratoma* species.

Discussion

Biology of *T. javanica* was earlier discussed by Mehra and Kapur (1955) on kusum and by Kumar and Singh (2007) on litchi. Life cycle stages are given in Table 1 which reveal that total life cycle of *T. javanica* takes 60.2 (±7.20) days for male and 72.6 (±9.30) for female. Likewise, moulting to second instar and third instar were found faster. The life span of fifth instar was longest than other life stages. Longevity studies showed that the lifespan of female is longer than male. Some results are in line with the earlier findings but some are different. Longevity of

females and males were found longer than the studies of Kumar Singh (2007). Similar trends were observed in case of incubation period as well as in all immature life stages. Total duration of development from egg to adult is 79.6 days which differs slightly from earlier findings i.e. 86.7 days by Mehra and Kapur (1955) and 66.6 days by Kumar and Singh (2007). In contrast to the five nymphal stages, Mehra and Kapur (1955) recorded six nymphal stages of *T. javanica.*

Tessaratoma species show a high degree of similarity in appearance, particularly in these immature stages and making species differentiation difficult. So far there are only few molecular studies like those of Lis *et al.* (2012) on the relationship between Tessaratomidae and Dinidoridae, and Song *et al.* (2013) who sequenced the complete mitochondrial genome of the tessaratomid *Eusthenes cupreus* (Westwood). The present study provides the mtCOI sequences as molecular identification tools for all life stages of *T. javanica* and incidentally adds to the molecular data of the Tessaratomidae. It was also observed that *T. javanica* shared 99% of mtCO1 identity with *Tessaratoma* sp. from Maharashtra, India and 98% identity with *T. papillosa* from USA, respectively, the sequences available in the NCBI GenBank. Also the ML tree of two *Tessaratoma* species produced two separate clades for *T. javanica* and *T. papilliosa*. It is the first study on *T. javanica* integrating biology, morphology, and the mtCO1 variations in the Indian populations. This study also contributes to the DNA barcode library for Heteroptera, with all life stage data. The present results provide an approach towards linking of the DNA barcodes with biology and morphology of the litchi bug and these might be critical in its diagnostics under special situations like plant quarantine or phytosanitary applications. Moreover, this enables the quick and accurate identification of immature stages during the import or export of litchi.

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