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Research

The Immature Stages, Biology, and Phylogenetic Relationships of *Rotunda rotundapex* (Lepidoptera: Bombycidae)

Rung-Juen Lin,[1] Michael F. Braby,[2,3] and Yu-Feng Hsu[1,4,]

[1]Department of Life Science, National Taiwan Normal University, 88 Ting-Chow Rd, Sec 4, Taipei 116, Taiwan, [2]Division of Ecology and Evolution, Research School of Biology, The Australian National University, Acton, ACT 2601, Australia, [3]The Australian National Insect Collection, National Research Collections Australia, GPO Box 1700, Canberra, ACT 2601, Australia, and [4]Corresponding author, e-mail: t43018@ntnu.edu.tw

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Abstract

The life history, morphology, and biology of the immature stages and phylogenetic relationships of *Rotunda rotundapex* (Miyata & Kishida, 1990) are described and illustrated for the first time. The species is univoltine: eggs hatch in spring (March or April) and the life cycle from egg to adult is completed in about 3 wk, with larvae developing rapidly on young leaves of the host plants, *Morus australis* and to a lesser extent *Broussonetia monoica* (Moraceae), and adults emerging in April–May. Eggs are laid in clusters on twigs of the host plant, are covered by scales during female oviposition, and remain in diapause for the remainder of the year (i.e., for 10–11 mo). Larvae (all instars) are unique among the Bombycidae in that they lack a horn on abdominal segment 8. A strongly supported molecular phylogeny based on six genes (5.0 Kbp: COI, EF-1α, RpS5, CAD, GAPDH, and wgl) representing seven genera of Bombycinae from the Old World revealed that *Rotunda* is a distinct monotypic lineage sister to *Bombyx*. This phylogenetic position, together with morphological data of the immature stages (egg and larval chaetotaxy), supports the current systematic classification in which the species *rotundapex* has been placed in a separate genus (*Rotunda*) from *Bombyx* in which it was previously classified.

Key words: Bombycinae, life history, molecular phylogeny, *Morus*, oviposition

Bombycids are among the most well-known group of moths because of the famous silkmoth *Bombyx mori* (Linnaeus 1758), which has important economic value. The silk of this species has been farmed by humans for at least 5,000 yr, and there are currently more than 1000 strains of domesticated silkworms harvested throughout the world. *Bombyx mori* has become a model insect, attracting wider attention from fields such as genetics, physics, and biochemistry (Goldsmith *et al.* 2005). It is also the first species of Lepidoptera in which the whole genome was sequenced (Xia *et al.* 2009).

The Bombycidae contain four subfamilies: Bombycinae, Apatelodinae, Phiditiinae, and Prismostictinae according to Lemaire and Minet (1998). Bombycinae includes two tribes: Bombycini and Epiine. Most Bombycini occur in the Indo-Australian region, with only a few species in the Palaearctic of Asia and Afrotropical region of central Africa (Congo, Ethiopia, Kenya, and Tanzania) and Madagascar. The Epiini are exclusively Neotropical. The Oriental Bombycidae comprise several genera that have been delineated on characters concerning the wing venation and male genitalia.

In Taiwan, there are six genera and eight species of Bombycinae, including *B. mori*, *B. mandarina* (Moore, 1872), *B. horsfieldi* (Moore, 1860), *Rotunda rotundapex* (Miyata & Kishida, 1990), *Ernolatia moorei* (Hutton, 1865), *Trilocha varians* (Walker, 1855), *Triuncina brunnea* (Wileman, 1911), and *Ocinara albicollis* (Walker, 1862). The larvae of Bombycinae primarily feed on plants in the family Moraceae (Dierl 1978, Holloway *et al.* 1987, Common and Edwards 1991). The larvae of *Bombyx* feed on *Morus* (mulberries), whereas those of *E. moorei*, *T. varians*, *T. brunnea*, and *O. albicollis* feed on *Ficus* (figs) (Barlow 1982, Holloway 1987, Lin 2005, Daimon *et al.* 2012, Navasero *et al.* 2013, Wang *et al.* 2015). For *R. rotundapex* (Figs. 1–4), Moraceae has been reported as the larval host plant (Wang *et al.* 2015), but the basic natural history is fragmentary and details of host specificity have hitherto remained unknown. Wang *et al.* (2015) illustrated the final instar larva of *R. rotundapex* and noted that "The larvae are quite variable in color with numerous black dots all over the body," but provided few other details.

Rotunda rotundapex, the subject of this study, is distributed in mainland China, Korea, Myanmar, and Taiwan. It was first collected and described from Taiwan (type locality is Nantou) by Miyata and Kishida (1990). In Taiwan, it occurs in montane areas between 800

Figs. 1–4. Adults of *R. rotundapex*: 1, dorsal view of male (TAIWAN: Nantou County:Xitou, 1100 m, 3-III-2014,Y. C. Lin, reared from *M. australis*, emgd. 3-IV-2014); 2, ventral view of male (same data as Fig. 1); 3, dorsal view of female (same data as Fig. 1), reared from *M. australis*, emgd. 3-IV-2014; 4, ventral view of female (same data as Fig. 1). Scale bars = 10 mm.

and 2100 m (Wang *et al.* 2015). *Rotunda rotundapex* was originally assigned to the genus *Bombyx* based on wing pattern elements and features of the male genitalia in which the uncus is long and forked. However, Wang *et al.* (2015) erected the monotypic genus *Rotunda* to accommodate the species *rotundapex*. They distinguished the genus by its rounded wings, the narrow and forked uncus of the male genitalia, and lack of a small horn on the eighth abdominal segment of the mature larva.

The aim of this study is to document the larval host plants, morphology, and biology of the immature stages of *R. rotundapex* based on field and laboratory work conducted in Taiwan. We then compare the immature stage morphology and biology with related genera in the subfamily. We also reconstruct a well-supported phylogenetic hypothesis of the Old World Bombycidae using a multigene data set to determine the phylogenetic relationships of *Rotunda*. In particular, the status of *Rotunda* as a distinct genus and its relationship to *Bombyx* have not previously been investigated in the context of their evolutionary history.

Materials and Methods

Field Observations

The immature stages (eggs and larvae) of *R. rotundapex* were collected in winter and spring on the larval host plants from several localities in Taiwan, including 1) Nantou County, Luku township, Xitou (23°67′N, 120°79′E); 2) Nantou County, Renai township, Chunyang (24°02′N, 121°14′E); and 3) Nantou County, Luku township, Shanlinxi (23°66′N, 120°77′E). Immatures of *R. rotundapex* were collected by examining leaves and twigs of potential host plants in the mulberry family (Moraceae). The immatures were discovered and collected by searching the host plant. During the investigation, most immatures of were found on *Morus australis* except one from *Broussonetia monoica* (the rearing database HSUM lot 18D46); both are native plants commonly distributed in low to moderate elevations in Taiwan (Chang *et al.* 2014, Chung *et al.* 2017); and both are confirmed as host plants of the moth in nature. Larvae were reared in plastic containers (150 × 80 × 45 mm) on fresh cuttings of the host plant, which was changed daily, until pupation. Male and female adults that emerged from their cocoons were kept inside the rearing containers for mating. Fertilized females inside the containers were then given twigs of the host plant on which to lay their eggs. These eggs were transported back to the field (locality 1, Xitou) and the twigs attached to the host plant with wire. The eggs were then monitored every month for 15 mo, from March 2013 to May 2014, to ascertain the time of hatching. The temperature and relative humidity of habitat records (Supp. Fig. 1 [online only]) were taken from Central Weather Bureau Observation Data Inquire System (https://e-service.cwb.gov.tw/HistoryDataQuery) data set, the automatic weather station C0I090. When the eggs hatched, the first-instar larvae were brought back to the laboratory for rearing.

Laboratory Rearing

Immature stages of *R. rotundapex* collected from the field were brought to the laboratory in National Taiwan Normal University for rearing. The early instar larvae (instars I and II) were initially reared in small plastic containers (80 × 55 × 30 mm) and then, as later instars, transferred to larger plastic containers (150 × 80 × 45 mm). Fresh cuttings of the larval host plant were replaced every 3 to 4 d. Its development time was determined in the laboratory under controlled conditions (maintained at constant room temperature of around 24–25°C, 65% relative humidity, and 16:8 [L:D] h). Rearing codes follow the system of Powell and De Benedictis (1995) where the code 'HSUM13B11' refers to the name of rearing database (HSUM), the year (13 for 2013), the month (B for February), and the sequential number of collection (11) for the eleventh collection in February 2013. Morphological descriptions of the immature stages were based on the following rearing cohorts: 13B11, 13C06, 13C14, 13D05, 13D06, 13D08, 14C04, and 15E18. In total, 15 males and 16 females were reared from the larval samples. Voucher material is deposited in the Department of Life Science, National Taiwan Normal University, Taipei (NTNU).

Adult wing length, cocoon, all stages of larval body length, and twigs of the host plant (*M. australis*) were measured with a Digimatic caliper (ABSOLUTE Digimatic Caliper Series 500-196-30, Mitutoyo, Japan). In total, 31 individuals first-instar, 31 second-instar, 34 third-instar, 40 fourth-instar, 41 fifth-instar larvae, and 19 cocoons were measured. Measurements of 31 eggs and general aspects of morphology were observed using a Leica MZ6 stereomicroscope equipped with a micrometric scale. Scanning electron microscopy (SEM) was conducted using a HITACHI S-3500N SEM (Chiayi, Taiwan). Using SEM, images of three first-instar larvae, eggs, and hatched eggs were obtained. The photographs of SEM provided the details of the chaetotaxy. Larvae and eggs were placed in boiling water for 2 min and then transferred to 70% ethanol for 30 min; dehydration was achieved by transferring to successive alcohol concentrations for 3 h, viz., 80, 85, 95, and 100% ethanol and then finally acetone for 12 h. Samples were prepared by critical point drying in a HITACHI HCP-2 critical point dryer (Chiayi, Taiwan) attached to stubs and gold coated with PELCO SC-6 sputter coater (Chiayi, Taiwan). Terminology for larval chaetotaxy follows Stehr (1987).

Material Examined

The following adult voucher specimens are deposited in NTNU: 6♂, 3♀, Xitou, Nantou, Taiwan. 1000 m, 28.II.2013, reared from *M. australis*, HSUM lot 13B11 (Y. C. Lin & R. J. Lin, NTNU); 1♂, 1♀, Xitou, Nantou, Taiwan. 1000 m, 10.III.2013, reared from *M. australis*, HSUM lot 13C06 (Y. C. Lin, K. W. Hsiao & R. J. Lin, NTNU); 8♂, 8♀, Chunyung, Nantou, Taiwan. 1200 m, 27.III 2013, reared from *M. australis*, HSUM lot 13C14 (C. L. Huang, L. H. Wang, R. J. Lin & K. W. Hsiao, NTNU); 1♂, Xitou, Nantou, Taiwan. 13.IV.2013, reared from *M. australis*, HSUM lot 13D05 (Y. C. Lin & R. J. Lin, NTNU); 1♂, 8♀, Shanlinxi, Nantou, Taiwan. 1600 m, 14.IV.2013, reared from *M. australis*, HSUM lot 13D06 (Y. C. Lin & R. J. Lin, NTNU); 1♂, 1♀, Shanlinxi, Nantou, Taiwan. 1800 m, 14.IV.2013, reared from *M. australis*, HSUM lot 13D08 (C. L. Huang, Y. C. Lin, R. J. Lin & K. W. Hsiao, NTNU); 29♂, 22♀, Xitou, Nantou, Taiwan. 1000 m, 3.III.2014, reared from *M. australis*, HSUM lot 14C04 (Y. C. Lin & R. J. Lin, NTNU); 1♂, 1♀, Sinbaiyang, Taroko National Park, Hualien, Taiwan. 1650 m, 10.V.2015, emgd. 20.V.2015, HSUM lot 15E18 (L. H. Wang & R. J. Lin, NTNU); 1♂, Fuxing, Taoyuan, Taiwan. 25.IV.2018, reared from *B. monoica*, HSUM lot 18D46 (Y. M. Hsu, NTNU).

Molecular Data

To infer the phylogenetic position of *R. rotundapex* within the Bombycini, our data set included 11 species (12 samples) representing seven Old World genera from the family of Bombycidae. Two species—one Sphingidae and one Saturniidae—were used as outgroup taxa in accordance with Zwick *et al.* (2011). DNA was extracted from legs using Qiagen tissue extraction kit (Qiagen, Valencia, CA). DNA amplification primers followed the list in Wahlberg and Wheat 2008. All primers were listed in Table 1. The following six genes were sequenced: cytochrome oxidase subunit I (COI) from the mitochondrial genome, and Elongation factor 1 alpha (EF-1α), Ribosomal protein S5 (RpS5), Carbamoyl phosphate synthetase domain protein (CAD), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and wingless (wgl) from the nuclear genome. Each polymerase chain reaction (PCR) was carried out in a final volume of 30 µl, with 0.2 µM of each primer. The following PCR settings were adopted: 4 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 48–60°C, and 0.5–1 min at 72°C. The final elongation step was continued for 10 min at 72°C and stopped at 16°C. If the above conditions failed, we amplified the fragments using a touchdown method: 4 min at 95°C, then following by 20 cycles of 30 s at 95°C, 30 s at 65°C decreasing 0.5°C degree each cycle, 0.5–1 min at 72°C, and then followed by 25 cycles of 30 s at 95°C, 30 s at 50°C, and 0.5–1 min at 72°C. The final elongation step was continued for 10 min at 72°C and stopped at 16°C. The PCR products were run on 1.0% agarose gels in 1X TBE buffer to ensure that the lengths of PCR fragments were correctly amplified.

Sequence Alignment and Phylogenetic Analysis

Molecular sequences of COI, CAD, EF-1α, GADPH, RpS5, and wgl genes were checked and assembled into contigs using Sequencher 4.8 (GeneCode, Boston, MA). Primer regions were cropped. The data sets were aligned according to amino sequence similarity by MUSCLE implied in MEGA6 (Tamura *et al.* 2013). Missing data and ambiguities were designated as IUPAC codes. All sequences used in the present study were submitted to GenBank with the accession numbers included *B. mori* from NCBI (listed in Table 2).

Phylogenetic analyses were based on the combined DNA sequence data set for the six genes. Nucleotide substitution models and partition schemes were determined with PartitionFinder v.2.1.1 (Lanfear *et al.* 2017). The data set was analyzed using maximum likelihood (ML) and Bayesian inference. For ML, we used RAxML (Stamatakis, 2014) in CIPRES (Miller *et al.* 2010), with the GTR + Γ + I substitution model; nodal support was assessed using 1,000 bootstrap replicates. For Bayesian inference, we used the program MrBayes v.3.2.5 (Ronquist *et al.* 2012); two independent runs were implemented simultaneously for 5 million generations and sampled every 1,000 generations. We removed the first 25% burn-in parts and the remainder was used to generate a 50% majority consensus tree. We then evaluated the parameters and convergence of two runs with the software Tracer v.1.7 (Rambaut *et al.* 2018). The trees were read by FigTree v.1.4.3.

Results

Morphology

Egg (Figs. 5–7 and 18–21)

Approximately 1.2 ± 0.27 mm in diameter, 0.5 ± 0.05 mm in height (n = 31); flat-shaped, smooth, and 'polished' when scales removed, surface with poriform structures, yellow when laid, then changing to pale orange before hatching.

Table 1. Primers used in this study

Gene	Direction	Primer name	Primer sequence	Annealing temperature	Product length (bp)	Source
COI	Forward	Zcox-1530	5′ CAA CAA ATC ATA AAG ATA TTG G 3′	52	1000	Folmer et al. 1994
COI	Reverse	Zcox-2530	5′ CTC CTG TTA ATC CTC CTA CAG T 3′	52		In this study
COI	Forward	Skcox-2100	5′ TTT TGA TCC TGC AGG AGG AGG 3′	52	1000	[Wu et al. 2010]
COI	Forward	Chcox2200	5′ ACC AGG ATT TGG TAT AAT TTC CCA 3′	52		In this study
COI	Reverse	MiBo-3140	5′ TGT TCT ATT AAA GGA GAG GCT 3′	52		In this study
CAD	Forward	CAD791f	5′ TTY GAR GAR GCN TTY CAR AAR GC 3′	52	650	Regier 2007
CAD	Reverse	CAD1057r	5′ CTC AWR TCA TAA TCW GTR CTH AC 3′	52		Zwick 2008
EF-1α	Forward	EF15F	5′ CGG ACA CGT CGA CTC CGG 3′	55	750	Cho et al. 1995
EF-1α	Forward	EF266F	5′ CAC AGA GAT TTC ATC AAG AAC A 3′	55		Cho et al. 1995
EF-1α	Reverse	EF843R	5′ TCYTG GAGAG CYTCG TGGTG CAT 3′	55		Cho et al. 1995
EF-1α	Forward	EF51.9	5′ CAR GAC GTA TAC AAA ATC GG 3′	50	511	Cho et al. 1995
EF-1α	Reverse	EFrcM4	5′ ACA GCV ACK GTY TGY CTC ATR TC 3′	50		Cho et al. 1995
GAP-DH	Forward	Frigga	5′ AAR GCT GGR GCT GAA TAT GT 3′	55	691	Cho et al. 1995
GAP-DH	Reverse	Burre	5′ GWT TGA ATG TAC TTG ATR AGR TC 3′	55		Wahlberg and Wheat 2008
GAP-DH	Forward	GAPDH-188F	5′ GCA CCC CTT GCT AAG GTC AT 3′	55		In this study
GAP-DH	Reverse	GAPDH-494R	5′ GCG GCC TCT TTG ACC TTT TG 3′	55		In this study
RpS5	Forward	RpS5f	5′ ATG GCN GAR GAR AAY TGG AAY GA 3′	55	617	Wahlberg and Wheat 2008
RpS5	Reverse	RpS5r	5′ CGG TTR GAY TTR GCA ACA CG 3′	55		Wahlberg and Wheat 2008
wing-less	Forward	LepWg1	5′ GAR TGY AAR TGY CAY GGY ATG TCT GG 3′	50	400	Brower and DeSalle 1998
wing-less	Reverse	LepWg2	5′ ACT ICG CAR CAC CAR TGG AAT GTR CA 3′	50		Brower and DeSalle 1998

Each row represents gene name, PCR primers used, and resulting sequence length of the six genes in this study.

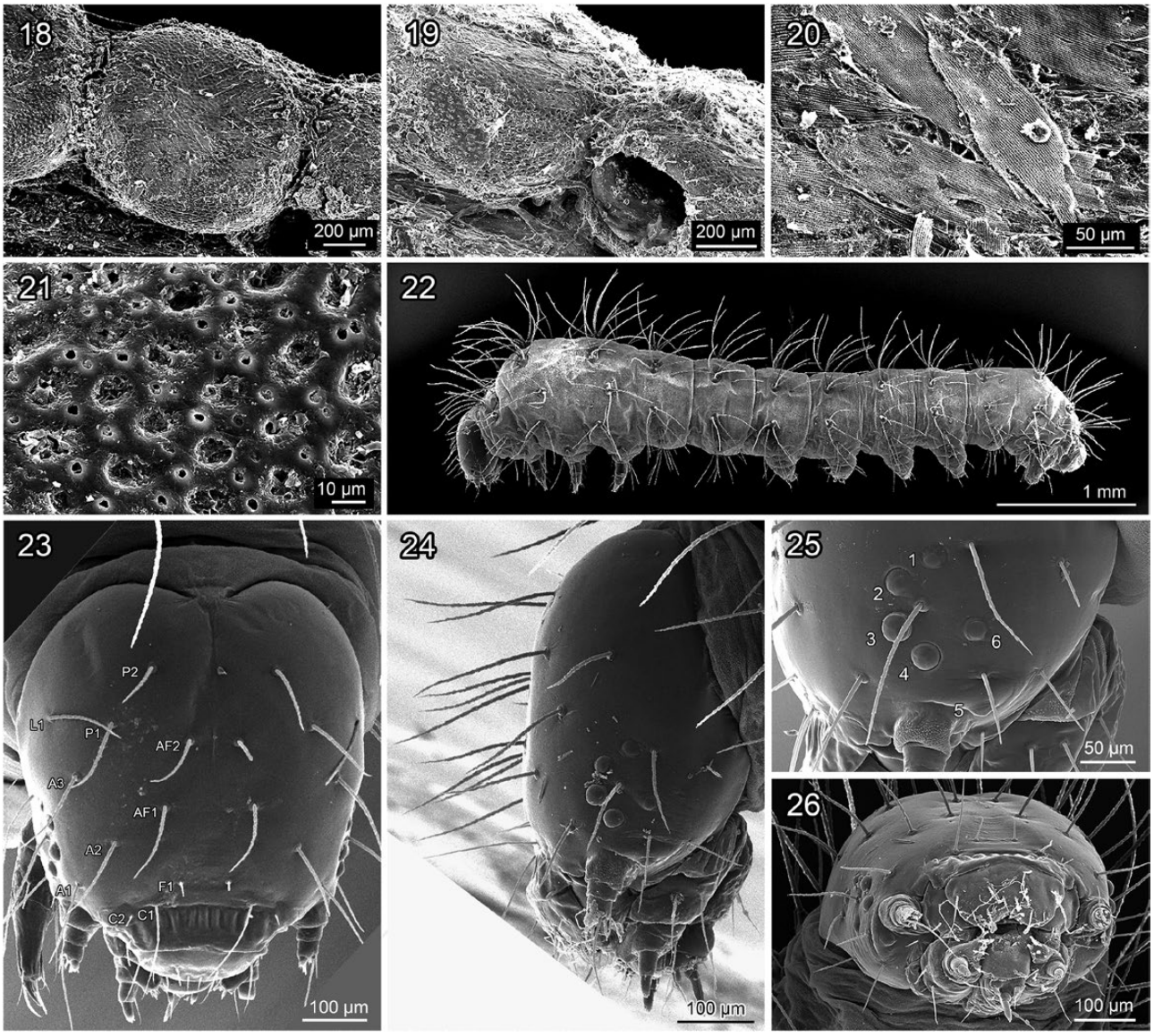
Table 2. Specimens used for sequencing of phylogenetic analysis in this study

Species	Region	Location	GenBank number					
			COI	CAD	EF-1α	GADPH	RpS5	wgl
Bombyx huttoni	China	Yunnan	MH817457	MH822563	MH822576	MH822587	MH822600	MH822613
Bombyx lemeepauli	China	Guangxi	MH817452	MH822558	MH822571	MH822583	MH822595	MH822608
Bombyx mandarina	China	Sichuan	MH817451	MH822557	MH822570	MH822582	MH822594	MH822607
Bombyx mandarina formosana	Taiwan	Nantou	MH817448	MH822554	MH822567	MH822580	MH822592	MH822604
Bombyx mori			AB083339	EU141315	EU136667	EU141495	NW_004582010	EU141241
Ernolatia moorei	Taiwan	Taipei	MH817449	MH822555	MH822568	MH822588	MK567930	MH822605
Gastridiota adoxima	Australia	Queensland	MH817459	MH822565	MH822578	MH822590	MH822602	MH822615
Ocinara albicollis	Taiwan	Kinmen	MH817450	MH822556	MH822569	MH822581	MH822593	MH822606
Rodontia menciana	China	Beijing	MH817458	MH822564	MH822577	MH822589	MH822601	MH822614
Rondotia diaphana	China	Yunnan	MH817453	MH822559	MH822572	MH822584	MH822596	MH822609
Rotunda rotundapex	Taiwan	Nantou	MH817447	MH822553	MH822566	MH822579	MH822591	MH822603
Trilocha varians	Taiwan	Taipei	MH817455	MH822561	MH822574	MH822585	MH822598	MH822611
Cephonodes hylas	Taiwan	Taipei	MH817456	MH822562	MH822575	MH822586	MH822599	MH822612
Saturnia pyretorum	Taiwan	Nantou	MH817454	MH822560	MH822573	MK567931	MH822597	MH822610

First-instar larva (Figs. 8–10, 12, and 22–34)
Body length $\bar{x}$ = 5.8 ± 0.36 (SD) mm (n = 31). Head: rounded, rather flat in front, hypognathous. Thorax: T2 and T3 both conspicuously humped. T1 shield and anal plate are weakly sclerotized, with brown color (Figs. 8–9). Head chaetotaxy: the surface smooth with long primary setae (Figs. 23–26). Six stemmata arranged on each side of the

Figs. 5–17. Immature stages and adult of *R. rotundapex*: 5, eggs with scale coverings; 6, eggs with scales removed; 7, lateral view of eggs showing exit hole of first-instar larva; 8, lateral view of first-instar larva; 9, dorsal view of first-instar larva; 10, anterior view of first-instar larva; 12, posterior view of first-instar larva; 11, lateral view of second-instar larva; 13, lateral view of third-instar larva; 15, lateral view of fourth-instar larva; 16, lateral view of fifth-instar larva; 14, cocoon; 17, adult. Scale bars = 1 mm.

head; stemma 5 is separated from the others and ventrally located near the antennal scape; all stemmata of same size, stemmata 2, 3, 4, and 5 all distinctly different from and elevated above stemmata 1 and 6 (Fig. 25). Seventeen pairs of long primary setae: anterior (A1, A2, and A3), stemmatal (S1, S2, and S3), substemmatal (SS1, SS2, and SS3), lateral (L1), posterodorsal (P1 and P2), frontal (F1), adfrontal (AF1 and AF2), and clypeal (C1 and C2) setae. Thorax: most of primary setae arranged on verrucae (Fig. 30). Prothoracic shield lightly sclerotized along anterior margin and surround XD, SD, D1, and D2 setae (Fig. 27). XD bears five setae, SD bears four setae, seta D1 and D2 solitary (Fig. 28); L bears four setae; SV bears three setae. Meso- and metathroax. D bears seven setae on segment of T2; bears six setae on segment of T3 (Fig. 29), SD1 bears four setae, L3 solitary above L1 + L2, verruca L1 + L2 bears five setae, SV bears three setae. Abdomen: segments A1–A8 verruca D1 bears four setae, seta D2 is isolated posterior to D1, SD1 bears four setae, verruca L1 is posterior to seta L2, L1 bears three setae, SV bears three setae, and seta V is present. Verruca D1 and seta D2 are shorter than all segments on A7 (Figs. 31–33). A9 with only one D verruca and one L seta, SD verruca is absent. L group is absent on A10. D1 + SD verruca on anal shield bears five pairs of setae on A10. Paraproctal group (PP) on the posterior margin of the anal lobe, PP bears four pairs of setae. Crochet on ventral prolegs uniordinal arranged in a lateropenellipse (Fig. 34).



Figs. 18–26. Scanning electron micrographs of egg and first-instar larva of *R. rotundapex*: 18, ovum; 19, dorsal view of ovum; 20, scales on ovum; 21, pore on dorsal egg shell; 22, lateral view of larva; 23, anterior view of head capsule; 24, lateral view of head of capsule; 25, stemmata of head capsule; 26, ventral view of head capsule.

Second-instar larva (Fig. 11)
Body length $\bar{x}$ = 9.7 ± 0.84 (SD) mm (n = 31). Head black. Body covered with wax, slightly enlarged in T2 and A8, ground color yellow with dark spots throughout whole body, except white color in T3 to A3. Spiracles black. Mean duration of second instar 2.4 ± 0.6 d.

Third-instar larva (Fig. 13)
Body length $\bar{x}$ = 14.6 ± 1.49 (SD) mm (n = 34). Head dark black in lower half, white in upper-half covered with wax. Body covered with wax, slightly enlarged in T2 and A8, ground color white with black markings throughout whole body, thin yellow transversal line in A3-A5. Spiracles black. Mean duration of third instar 2.4 ± 0.5 d.

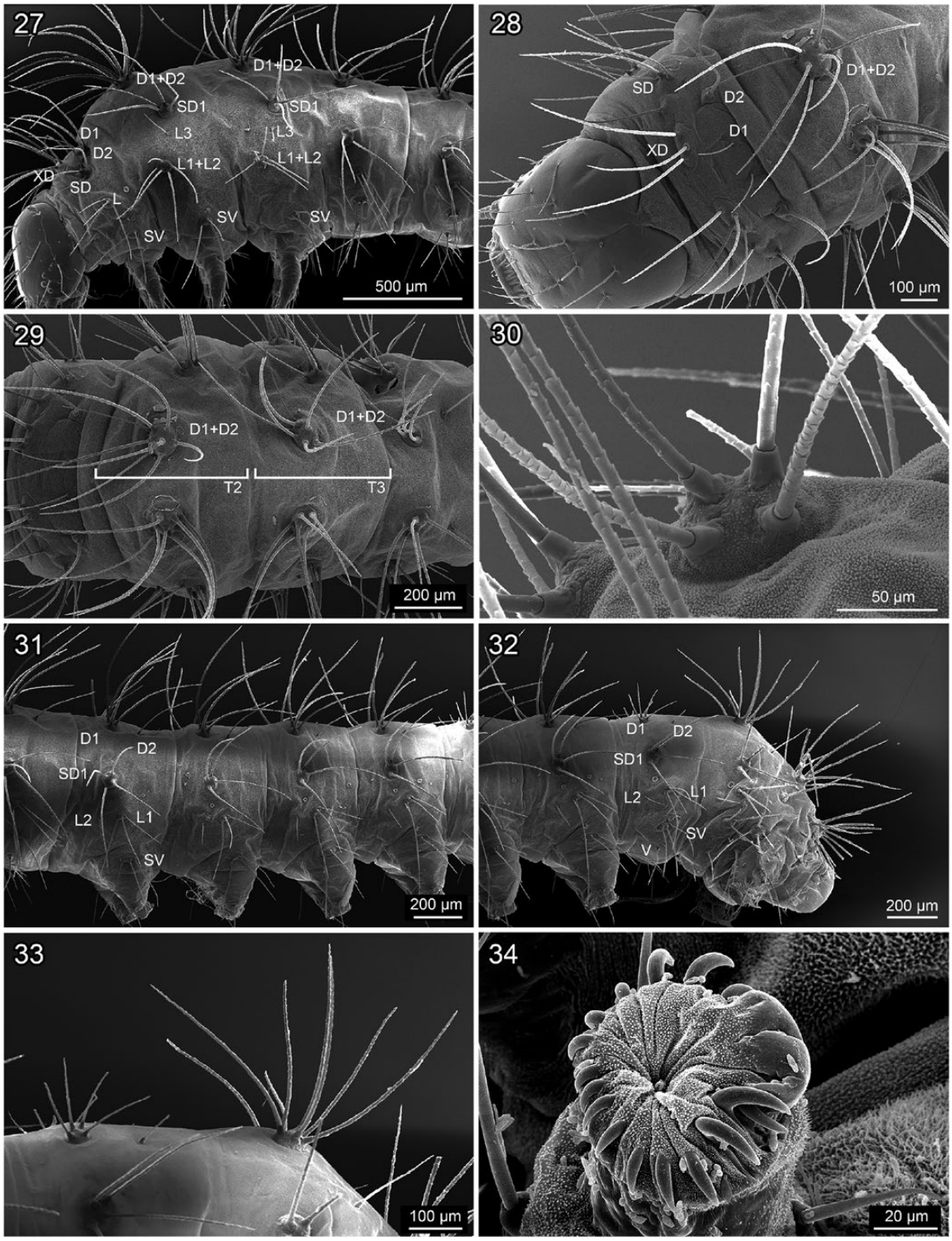
Fourth-instar larva (Fig. 15)
Body length $\bar{x}$ = 24.6 ± 2.67 (SD) mm (n = 40). Head and body color similar to third instar. Mean duration of fourth instar 2.9 ± 0.7 d.

Fifth-instar larva (Fig. 16)
Body length $\bar{x}$ = 45.5 ± 5.38 (SD) mm (n = 41). Head dark black in lower half, yellow in upper-half covered with wax. Body covered with wax, slightly enlarged in T2 and A8, ground color yellow, with black spots and markings throughout whole body. Spiracles black. Mean duration of fifth instar 5.5 ± 0.7 d.

Cocoon (Fig. 14)
Silk yellow, $\bar{x}$ = 19.9 ± 2.44 (SD) mm in length, $\bar{x}$ = 9.2 ± 1.35 (SD) mm in width (n = 19).

Biology

Eggs were attached to the substrate and laid in small clusters, ranging from 19 to 21 ($\bar{x}$ = 20 ± 1 eggs, n = 3), in a compact row on thin twigs ($\bar{x}$ = 4.4 ± 0.89 mm in diameter, n = 3) of the larval host plant. During oviposition, the dorsal surface of the eggs



Figs. 27–34. Scanning electron micrographs of first instar larva of *R. rotundapex*: 27, lateral view of T1–T3; 28, dorsal view of T1 shield and XD group; 29, dorsal view of T2–T3; 30, primary setae arranged on verrucae of T3; 31, lateral view of A3–A6; 32, lateral view of A7–A10; 33, D group on A7–A8; 34, ventral view of proleg on the segment of A6.

was covered in numerous dark brown scales, which were glued by secretions from the female accessory gland. The scales closely resemble the color of the host twig and thus the eggs were well concealed. On hatching, the first-instar larvae emerged from the lateral surface of the eggs, without consuming the chorion. The larvae fed on young soft leaves, and sometimes fruits. When not feeding or molting, they usually resided on the underside of the midrib of the leaf. Prior to pupation, the final instar larvae spun silken cocoons between two leaves.

Our field observations indicate that *R. rotundapex* is univoltine. The life cycle from larva to adult was completed in approximately 4 wk (larva: 12–14 d; prepupal stage: 1–2 d; pupa: 10–13 d). Mean

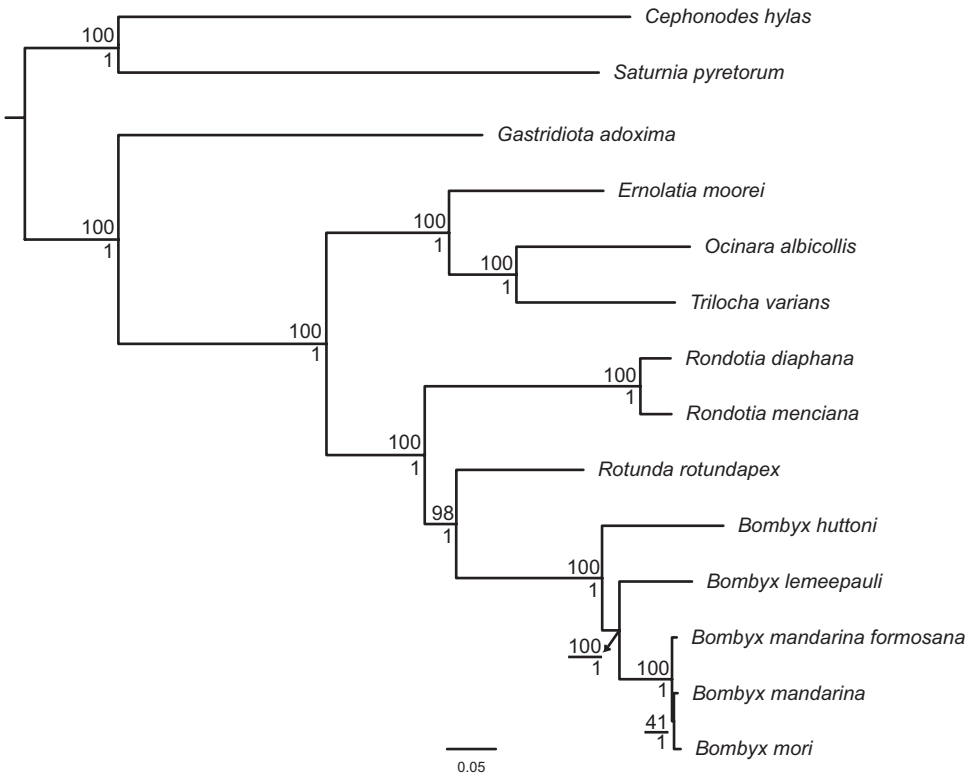


Fig. 35. Phylogenetic tree of the Bombycidae for seven genera (11 species) from the Old World constructed using maximum likelihood and Bayesian inference for the combined data set (5009 bp: COI, CAD, EF-1α, GADPH, RpS5 and wgl). *Cephonodes hylas* (Sphingidae) and *Saturnia pyretorum* (Saturniidae) were used as the outgroup samples in the analysis. Support values are indicated by bootstrap and Bayesian probability above and below each node, respectively. Branch lengths are proportional to inferred subsititutions rate.

larval development was 15.4 ± 1.4 d from egg hatching to pupation (n = 37), whereas the mean duration of the pupal stage was 13.5 ± 0.7 d (n = 51). Adults were recorded in April and May at mid-elevation forests in Taiwan (1,000–1,800 m). However, the moth was rarely collected, and to date only males have been captured at light traps. In captivity, females laid their eggs in mid May, but the eggs entered diapause and the first-instar larvae did not hatch until the following spring (March or April). Larvae developed rapidly and pupation occurred in April or May.

Molecular Phylogeny

The 14 selected taxa were sequenced successfully for each of the six genes (COI, CAD, EF-1α, GADPH, RpS5, and wgl). The combined data set of aligned sequences contained a total of 5009 bp, corresponding to 1459 bp of COI, 612 bp of CAD, 1229 bp of EF-1α, 706 bp of GADPH, 618 bp of RpS5, and 385 bp of wgl (Table 2). Phylogenetic analysis using ML and Bayesian inference yielded congruent topologies, with relationships of all ingroup taxa strongly supported (Fig. 35). The phylogenetic tree showed that the Old World bombycid taxa comprised three major clades: *Gastridiota*, *(Ernolatia + (Ocinara +Trilocha)), and (Rondotia* + (*Rotunda* + *Bombyx*)). *Rotunda* was recovered as a distinct lineage closely related to *Bombyx*—the mean average genetic distance between the two genera was 10.84% compared with a mean pairwise distance of 5.34% within *Bombyx*. Interestingly, the 'basal' lineage of the subfamily was recognized as *Gastridiota* from the mid-elevation subtropical rainforests of the eastern Australian coast.

Discussion

The lifecycle of *R. rotundapex* is tightly synchronized with the phenology of its larval host plant. *Morus australis* is deciduous in winter (Chang 2006), and thus, foliage is not available for the larvae at this time. During this period, the eggs overwinter and egg hatching coincides with the time when the leaf buds begin to sprout in spring. Larval growth is rapid, completing development in 2 wk. The phenomenon of larvae specializing on new soft leaf growth and having fast maturation when leaves are available for only a limited period has been recorded in many species of Lepidoptera that are univoltine (Zalucki *et al.* 2002, Saeed *et al.* 2010).

The behavior of gluing scales on the surface of eggs by *R. rotundapex* is an interesting oviposition strategy in the Bombycidae. This behavior has not been documented in *Bombyx* or other *Ficus*-feeding silkworm species (Wang *et al.* 2015), and so far has only been recorded in *Rondotia menciana* from China (Xu *et al.* 1994). Egg coverings with scales, hairs, frass, or foam by females are well-known in other Lepidoptera (Gross 1993, Renwick and Chew 1994, Greeney *et al.* 2012). It has also been demonstrated that egg coverings effectively reduce rates of parasitism because they increase the searching time by parasitoids and thus enhance the overall survival rate of eggs (Floater 1998, Rodriguez *et al.* 2004). *Rondotia menciana* is univoltine in northern populations, but has two or three generations in southern populations in China (Xu *et al.* 1994). Interestingly, egg coverings in this species occur in both univoltine and multivoltine populations, but only among the overwintering generations—eggs of the spring-summer generations in the southern populations are devoid of scales. This strongly suggests that the scales in both *Rotunda* and *Rondotia* have a protective function to enhance survival during embryonic diapause.

Several studies have demonstrated the importance of first-instar larval morphology in elucidating phylogenetic relationships (Freitas and Brown 2004, Duarte *et al.* 2005). Character states of the first instar larva that may be considered synapomorphic for the Bombycidae include 1) D1 always stronger than D2, 2) unequal L setae on all segments, and 3) D1 and D2 are fused on one verruca on

segment A9 (Dierl 1978, Common 1991). In the later instars, all verrucae and nearly all primary setae are lost. In *B. mori*, the first-instar larva has long primary setae situated on proper scoli, not on simple chalazae as in *Ocinara*.

Our phylogenetic tree suggests that simple chalazae is plesiomorphic because the 'basal' lineage *Gastridiota* and *Ocinara* are the only genera that have this character state. In contrast, first-instar larvae of *Rotunda* and *Bombyx* possess many primary setae arranged on verrucae, which suggests that this character may be a synapomorphy for this pair of genera. However, *Rotunda* is the only genus that is known to lack a dorsal horn on segment A8 within the entire Bombycinae. It remains to be established if this represents a secondary loss or the dorsal horn has evolved independently in the other lineages—our phylogeny suggests at least four times (Fig. 35).

In summary, our phylogenetic reconstruction of the Old World Bombycidae recovered a close relationship between *Rotunda* and *Bombyx*, supporting the current classification that the species *rotundapex* comprises a separate lineage, and thus belongs in a separate genus (*Rotunda*), closely allied to *Bombyx*. Further evidence in support of this classification comes from our detailed study of the life history and morphology of the immature stages—both *Rotunda* and *Bombyx* specialize primarily on *Morus*, but *Rotunda* possesses characteristic features distinct from the latter, such as eggs covered in scales, first-instar larvae with primary setae on verrucae, and the final instar larva lacking a dorsal horn on the abdomen.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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