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

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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. hortfieldi* (Moore, 1860), *Rotunda rotundapex* (Miyata & Kishida, 1990), *Ernolatia moorei* (Hutton, 1865), *Triuncina 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
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 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 C01090. 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♀, Chunyun, 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 Sequencer 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 + Ψ + 1 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.
First-instar larva (Figs. 8–10, 12, and 22–34)

Body length $\bar{x} = 5.8 \pm 0.36$ (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

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### 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 ATC TGA CAG T 3′ | 52                    | 1000                | In this study           |
| COI    | Forward   | Skcox-2100  | 5′ TTT TGA TCC TGC 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           |
| CAD    | Reverse   | MiBo-3140   | 5′ TGT TCT ATT AAA GGA GAG GCT 3′ | 52                    |                    | In this study           |
| CAD    | Forward   | CAD79f1     | 5′ TTY GAR GAR GCA TTG CAA GC 3′ | 52                    | 650                 | Regier 2007             |
| EF-1α  | Forward   | EF15F       | 5′ CCG 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  | Forward   | EF843R      | 5′ TCT TG AAG AGG GTC TGC TGC CAT 3′ | 55                    |                     | Cho et al. 1995         |
| EF-1α  | Forward   | EF15.9      | 5′ CAR GAC GTA TAC AAA ATC AGG GC 3′ | 50                    | 511                 | Cho et al. 1995         |
| EF-1α  | Reverse   | EFrcM4      | 5′ ACA GCV ACK GTY TGY CTC ATR 3′ | 50                    |                     | Cho et al. 1995         |
| GAPDH  | Forward   | Frigga      | 5′ AAR GCT GGR GCT GAA TAT GT 3′ | 55                    | 691                 | Cho et al. 1995         |
| GAPDH  | Reverse   | Burre       | 5′ GWT TGA ATG TAC TGG ATR AGR TC 3′ | 55                    |                     | Wahlberg and Wheat 2008 |
| GAPDH  | Forward   | GAPDH-188F  | 5′ GCA CCC CCT GCT AAG GTC AT 3′ | 55                    |                     | In this study            |
| GAPDH  | Reverse   | GAPDH-494R  | 5′ GCG GCC TCT TGG ACC TTT TG 3′ | 55                    |                     | In this study            |
| RpS5   | Forward   | RpS5f       | 5′ ATG GCN GAR GAR GAR AAY TGG AAY GA 3′ | 55                    | 617                 | Wahlberg and Wheat 2008 |
| RpS5   | Reverse   | RpS5r       | 5′ CGG TTR GAY TTR GCA ACG C3′ | 55                    |                     | Wahlberg and Wheat 2008 |
| wingless | Forward  | LepWg1      | 5′ GAR TGY AAR TGY CAG GGY ATG TCT GG 3′ | 50                    | 400                 | Brower and DeSalle 1998 |
| wingless | 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 | COI GenBank number | CAD GenBank number | EF-1α GenBank number | GADPH GenBank number | RpS5 GenBank number | wgl GenBank number |
|-----------------------------|---------|----------|--------------------|--------------------|----------------------|----------------------|---------------------|------------------|
| *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*               | Taiwan  | Nantou   | AB083339           | EU141315           | EU136667             | EU141495             | NW_004582010       | EU141241          |
| *Erinolatia moorei*         | Taiwan  | Taipei   | MH817449           | MH822555           | MH822568             | MH822588             | MK67930            | MH822605          |
| *Gastresiota adoxima*       | Australia | Queensland | MH817459         | MH822565           | MH822578             | MH822590             | MH822602           | MH822615          |
| *Ocinara albicollis*        | Taiwan  | Kinmen   | MH817450           | MH822556           | MH822569             | MH822581             | MH822593           | MH822606          |
| *Rodontia mencia*           | 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 varisana*         | Taiwan  | Taipei   | MH817455           | MH822561           | MH822574             | MH822585             | MH822598           | MH822611          |
| *Cephoonodes bylas*         | Taiwan  | Taipei   | MH817456           | MH822562           | MH822575             | MH822586             | MH822599           | MH822612          |
| *Saturnia pyretonum*        | Taiwan  | Nantou   | MH817454           | MH822560           | MH822573             | MK67931              | MH822597           | MH822610          |
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 metathorax. 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);
Second-instar larva (Fig. 11)
Body length $\bar{x} = 9.7 \pm 0.84$ (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 $\pm$ 0.6 d.

Third-instar larva (Fig. 13)
Body length $\bar{x} = 14.6 \pm 1.49$ (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 $\pm$ 0.5 d.

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

Fifth-instar larva (Fig. 16)
Body length $\bar{x} = 45.5 \pm 5.38$ (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 $\pm$ 0.7 d.

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

Biology
Eggs were attached to the substrate and laid in small clusters, ranging from 19 to 21 ($\bar{x} = 20 \pm 1$ eggs, $n = 3$), in a compact row on thin twigs ($\bar{x} = 4.4 \pm 0.89$ mm in diameter, $n = 3$) of the larval host plant. During oviposition, the dorsal surface of the eggs
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). *Cephalotes 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 substitutions 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* + *Rondotia diaphana*). *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. *Monus 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. 2013), 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 vruca 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 Gastrodiota 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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