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Integrative analysis of the egg chorion ultrastructure combined with multilocus phylogeny sheds light on the taxonomy of the subgenus *Thersamolycaena* Verity, 1957 of the genus *Lycaena* Fabricius, 1807 (Lepidoptera: Lycaenidae)

Интегративный анализ ультраструктуры хориона яйца в сочетании с мультилокусной филогенией проясняет систематику подрода *Thersamolycaena* Verity, 1957 рода *Lycaena* Fabricius, 1807 (Lepidoptera: Lycaenidae)

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КЛЮЧЕВЫЕ СЛОВА. Lycaeninae, Lycaenini, Монголия, Палеарктика, биогеография, интегративная систематика, молекулярная филогенетика, морфология, сканирующая электронная микроскопия.

ABSTRACT. The subgenus *Thersamolycaena* Verity, 1957 of the genus *Lycaena* Fabricius, 1807 is revised using integrative analysis of the ultrastructure of the chorion of the eggs combined with multilocus phylogenetic analysis. It is shown that the *L.* (*T.*) *splendens* (Staudinger, 1881) species group comprises five species, *L.* (*T.*) *splendens*, *L.* (*T.*) *violacea* (Staudinger, 1892), *L.* (*T.*) *dabrerai* Bálint, 1996, *L.* (*T.*) *odbayar* (Churkin, 2004) and *L.* (*T.*) *ratushinskayae* Churkin et Kolesnichenko, 2019, although molecular phylogenetic analysis based on two genes confirms species status of only two species of the group, *L.* (*T.*) *splendens* and *L.* (*T.*) *ratushinskayae*. Other species differ from each other by the size and ultrastructure of the chorion of the eggs, namely by the number and conformation of the lateral ribs and the number of primary cells in the micropyle area. The most recent common ancestor of the whole subgenus *Thersamolycaena* splitted in the late Miocene in Eurasia, the ancestor of the *L.* (*T.*) *splendens* group diversified in the late Pliocene in Central Asia, and the *L.* (*T.*) *violacea* clade diversified in the Pleistocene in the mountains of Mongolia, probably due to the Pleistocene climate oscillations.

РЕЗЮМЕ. Подрод *Thersamolycaena* Verity, 1957 рода *Lycaena* Fabricius, 1807 ревизован с использованием интегративного анализа ультраструктуры хориона яиц в сочетании с мультилокусным филогенетическим анализом. Показано, что группа видов *L.* (*T.*) *splendens* (Staudinger, 1881) включает пять видов, *L.* (*T.*) *splendens*, *L.* (*T.*) *violacea* (Staudinger, 1892), *L.* (*T.*) *dabrerai* Bálint, 1996, *L.* (*T.*) *odbayar* (Churkin, 2004) и *L.* (*T.*) *ratushinskayae* Churkin et Kolesnichenko, 2019, хотя молекулярно-филогенетический анализ на основе двух генов подтвердил видовой статус лишь двух видов группы, *L.* (*T.*) *splendens* и *L.* (*T.*) *ratushinskayae*. Другие виды отличаются друг от друга размером и ультраструктурой яиц, а именно числом и формой латеральных рёбер и числом первичных ячеек в микропилярной области. Ближайший общий предок подрода *Thersamolycaena* разделился на территории Евразии в позднем миоцене,

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предок группы видов *L.* (*T.*) *splendens* эволюционировал в Средней Азии в позднем плиоцене, а клада *L.* (*T.*) *violacea* диверсифицировала в плейстоцене в горах Монголии предположительно в результате плейстоценовых изменений климата.

Introduction

Traditional morphological methods such as the study of the genitalia and wing pattern often fail to discriminate species, especially in evolutionarily recently diversified groups of butterflies; these difficulties sometimes lead to unjustified taxonomic treatments and description of so-called "bad species" [Descimon, Mallet, 2009]. On the other hand, conventional DNA-based methods of species delimitation such as DNA barcoding [Hebert *et al*., 2003] sometimes also lead to such results, especially when applied independently from morphology or more complicated molecular phylogenetic analyses, due to such phenomena as incomplete lineage sorting or interspecific hybridization resulting in mitochondrial introgression [Talavera *et al*., 2013; Shapoval *et al*., 2021; Krupitsky *et al*., 2023]. In such cases, an integrative approach combining study of morphological characters with analysis of chromosomal, unlinked molecular markers and ecological characters is required [Lukhtanov *et al*., 2015; Marabuto *et al*., 2023].

One of the cases requiring an integrative approach is the taxonomy of the coppers (subfamily Lycaeninae of the family Lycaenidae) of the subgenus *Thersamolycaena* Verity, 1957. This subgenus was erected for *Lycaena dispar* (Haworth, 1802) as a replacement name for *Disparia* Verity, 1943 (homonym of *Disparia* Nagano, 1916, Lepidoptera: Erebidae) described earlier by the same author. In addition to *L.* (*T.*) *dispar*, which is widely distributed across the Palaearctic, the subgenus *Thersamolycaena* comprises five closely related species constituting the *L.* (*T.*) *splendens* (Staudinger, 1881) species group distributed in Kazakhstan, Kyrgyzstan, Russia, Mongolia and China [Churkin, Kolesnichenko, 2019].

Lycaena (*Thersamolycaena*) *splendens* (type locality: Lepsy valley, Dzhungarsky Alatau Mountains, Kazakhstan) is distributed in the Dzhungarsky Alatau and the Tian Shan Mountains in Kyrgyzstan, Kazakhstan and China.

The most widespread species of the group is *L.* (*T.*) *violacea* (Staudinger, 1892) (type locality: Bolshaya Kudara vill., Republic of Buryatia, Russia). Previously [Lukhtanov, Lukhtanov, 1994] it was often considered as a subspecies of *L.* (*T.*) *splendens*, but analysis of the male genitalia revealed its specific status [Zhdanko, 1993; Bálint, 1996]. The range of the nominotypical subspecies covers Dahuria, Tuva, the Altai and Sayan mountains, northern Mongolia around Lake Khovsgol and the Khangai Range; an isolated population was found in the Gurvan-Saikhan Range [Bozano, Weidenhoffer, 2001; Churkin, 2004; Churkin, Kolesnichenko, 2019]. Additionally, two geographically isolated subspecies of *L.* (*T.*) *violacea* are recognized: *L.* (*T.*) *violacea labrangi* Bozano et Weidenhoffer, 2001 (type locality: Xiahe, Gansu Province, China) distributed in the southern Qilian Mountains in Gansu Province, China, and *L.* (*T.*) *violacea chunhaoi* Huang et Bi, 2016 (type locality: Donglingshan, Beijing, China), known from Beijing Municipality and Hebei Province. Both subspecies differ in details of colouration, while the male genitalia are rather similar to those of the nominotypical subspecies [Huang, Bi, 2016].

Lycaena (*Thersamolycaena*) *naryma* Zhdanko, 2014, described from the Narymsky Ridge, South Altai Mountains, Kazakhstan based on a single aberrant male, does not demonstrate any considerable differences from *L.* (*T.*) *violacea* and probably represents a synonym of the latter.

Lycaena (*Thersamolycaena*) *dabrerai* Bálint, 1996 (type locality: Mongolia, Bayankhongor Province, Tsagan-Bogd Mountains), *L.* (*T.*) *odbayar* (Churkin, 2004) (type locality: Mongolia, Govi-Altai Province, 30 km south Biger somon) and *L.* (*T.*) *ratushinskayae* Churkin et Kolesnichenko, 2019 (type locality: Mongolia, Khovd Province, Baitag Mountains) are united by a peculiar reduced pattern of the ventral side of the wings. This complex was recently revised by Churkin, Kolesnichenko [2019]. Species of the group are allopatrically distributed in Southern Mongolia and prefer different habitats and altitudes: *L.* (*T.*) *dabrerai* is known from the Trans-Altai Gobi and the Dzhungarian Gobi and inhabits deserts and desert foothills, *L.* (*T.*) *odbayar* occurs at the southern edges of the Mongolian Altai and inhabits alpine meadows, and *L.* (*T.*) *ratushinskayae* is known only from the type locality, the Baitag Mountains, where it inhabits high altitude dry slopes. According to observations in nature made by Churkin, Kolesnichenko [2019], at least *L.* (*T.*) *dabrerai* and *L.* (*T.*) *ratushinskayae* feed on different species of *Rheum* (Polygonaceae). The above-mentioned species are characterized by differences in wings pattern and subtle but stable differences in the structure of the male genitalia, especially the juxta, and obviously represent a complex of closely related species.

In order to reveal additional differences between species of this taxonomically complicated group of copper butterflies, we use a relatively novel approach, the study of the chorion of the egg. The characters of the eggs were considered to be of taxonomic value in various groups of butterflies (e.g., Llorente-Bousquets *et al*. [2018]; Kolesnichenko, Kotlobay [2020]; Nieves-Uribe *et al*. [2025]). Despite its taxonomic and evolutionary significance, ultrastructure of the egg chorion is still applicated to the taxonomy of Lycaenidae rather rarely. Several mostly descriptive publications are devoted to the Holarctic [Downey, Allin, 1981, 1984; Wright, 2021] and South African [Clark, Dickson, 1971] lycaenid species. Some studies are devoted to the discrimination of closely related species using the ultrastructure of the eggs chorion in the lycaenid subfamily Polyommatinae [Forister *et al*., 2006; Steiner *et al*., 2006; Pérez-Fernández, Rodríguez, 2018]. In fact, only one paper is devoted to the ultrastructure of the eggs of the Palaearctic species of the genus *Lycaena* s.l. [Munguira *et al*., 2015].

In addition to the study of the egg ultrastructure, for the first time we present a multilocus phylogeny of the subgenus *Thersamolycaena*, covering all of the described and accepted species to date, combined with dated multilocus molecular phylogenetic analysis to test morphology-based taxonomic hypotheses and reveal phylogeny and historical biogeography of the group.

Material and methods

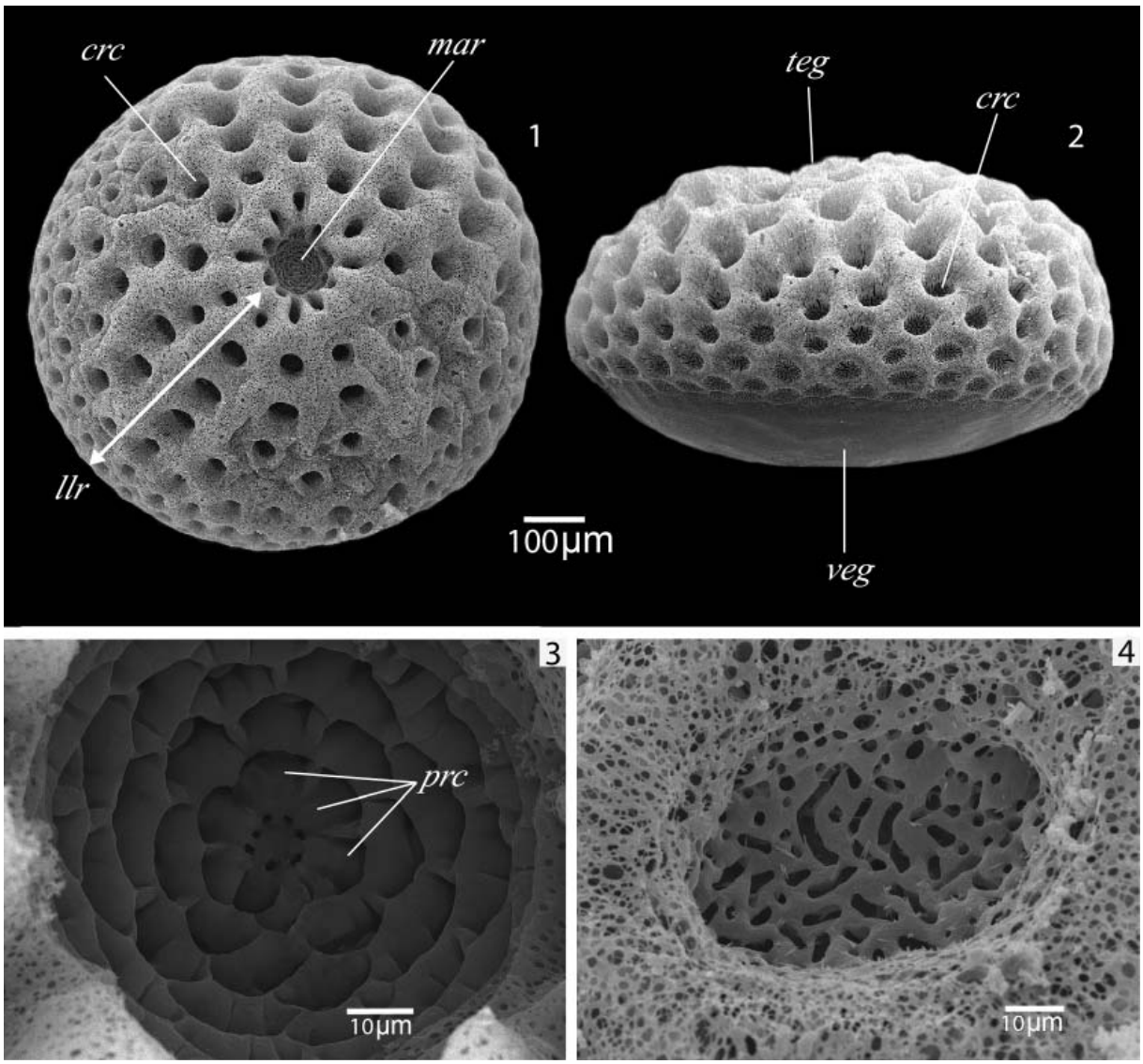
Morphology

The material for this study was mostly collected by the second author during his field studies in Russia, Mongolia and Kyrgyzstan. The studied female specimens are deposited in the collection of the Department of Entomology, Lomonosov Moscow State University.

The eggs were extracted from abdomens of mounted specimens by enzymatic digestion method described by Junker *et al*. [2006] using an Olympus SZ-ET stereomicroscope.

For further study using SEM, the eggs were fixed in 75% ethanol, cleaned, dehydrated in a graded series of ethanol solutions and acetone, dried in a Hitachi NCP-1 (Hitachi Corp., Japan) critical point dryer, coated with gold in a Hitachi IB-3 (Hitachi Corp., Japan) ion spraying unit, and examined using a JEOL JSM-6380 (JEOL Ltd., Japan) scanning electron microscope.

We use the terminology of Salkeld [1984] and Munguira *et al*. [2015] to describe the morphology of the eggs chorion (Figs 1–4).



Figs 1–4. Ultrastructure of the chorion of the egg of the subgenus *Thersamolycaena*: 1 — egg, dorsal view; 2 — egg, lateral view; 3 — micropyle area at high magnification; 4 — crater-like appearance cell at high magnification; *crc* — crater-like appearance cell; *llr* — lateral longitudinal rib; *mar* — micropyle area; *prc* — primary cells of micropyle rosette; *teg* — top of the egg; *veg* — ventral surface of the egg.

Рис. 1–4. Структура хориона яиц подрода *Thersamolycaena*: 1 — яйцо, вид сверху; 2 — яйцо, вид сбоку; 3 — микропилярная область при большом увеличении; 4 — кратерообразная ячейка при большом увеличении; *crc* — кратерообразная ячейка; *llr* — боковое ребро; *mar* — микропилярная область; *prc* — первичные ячейки микропилярной розетки; *teg* — вершина яйца; *veg* — нижняя часть яйца.

Molecular phylogeny

The molecular phylogenetic analysis was based on 39 specimens covering most of the species of the genus *Lycaena* s.l. including 15 specimens of all species of the subgenus *Thersamolycaena* known and accepted to date. Sequences of two species, *Helleia helle* (Denis et Schiffermüller, 1776) and *Tharsalea arota* (Boisduval, 1852), were selected as outgroups. Thus, the final dataset included 41 specimens of 24 species of the tribe Lycaenini.

Our molecular analysis was based on a DNA matrix having a length of 5997 bp and consisting of a concatenation of the mitochondrial cytochrome c oxidase I gene, *COI* (positions 1–1225) and five nuclear genes, elongation factor 1-alpha, *EF-1a* (positions 1226–2262), wingless, *wg* (positions 2263–2656), carbamoyl phosphate synthetase/aspartate transcarbamylase/dihydroorotase, *CAD* (positions 2657–4690), malate dehydrogenase, *MDH* (positions 4691–5400), and ribosomal protein S5, *RpS5* (positions 5401–5997). In creating this matrix, we followed the recommendation of Talavera *et al*. [2022] to combine a dataset consisting of multiple DNA barcodes with a multilocus backbone dataset consisting of a selection of taxa with multigene sampling. In order to resolve higher-level nodes, the backbone set should include at least one individual with multigene data per genus, and the DNA barcode set should represent all or nearly all species-level taxa [Talavera et al., 2022].

Most of the sequences were obtained from GeneBank (accession numbers are given on Fig. 1), and 13 sequences of *COI* and four sequences of *EF-1a* of the focus species of our study were sequenced prior the analysis.

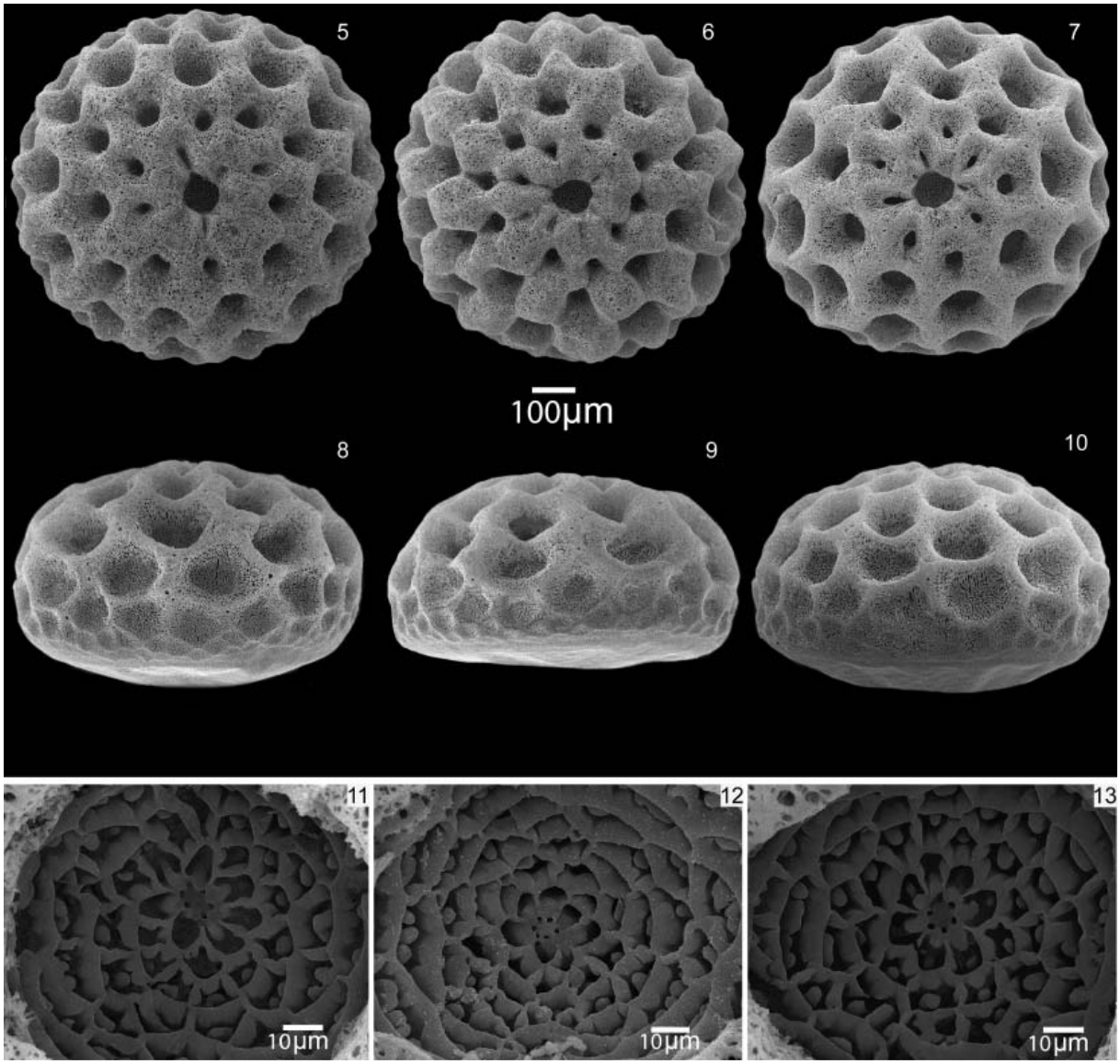
DNA was extracted from two legs using PALL AcroPrep 96-well purification plates supplied by PALL Corp., Port Washington, New York, USA [Ivanova *et al.*, 2006], following the manufacturer's protocol, at the Department of Invertebrate Zoology, Biological Faculty, Moscow State University (Moscow, Russia). Extracted DNA was used as a template for the amplification of partial *COI* and *EF-1a* genes. Polymerase chain reaction amplifications were carried out in a 20 μL reaction volume, which included 4 μL of 5× Screen Mix (Eurogen, Russia), 0.5 μL of each primer (10 μM stock), 1 μL of genomic DNA and 14 μL of ddH_2O. The target fragment of *COI* was amplified using the primers LCO/HCO [Folmer *et al*., 1994] or the pair primer combination LCO/MH-MR1 and MH-MF1/HCO [Hajibabaei *et al*., 2006] under the following profile: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. The target fragment of *EF-1a* was amplified using the primer combination ef44/ELF1R [Kim *et al*., 2010] under the following profile: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. All sequences obtained in the present study were deposited at GenBank (Table 1). The amplified fragments were separated using an automated sequencing machine (Applied Biosystems 3500).

The obtained chromatograms were analyzed with Geneious v.10.0.9 [Kearse *et al.*, 2012], and the sequences were aligned with MUSCLE algorithm implemented in the latter program. The data were partitioned by genes. Nucleotide substitution models for each dataset were estimated using PartitionFinder v.2.1.1 [Lanfear *et al.*, 2012] based on the Bayesian information criterion (BIC) for each partition. The following models were selected: GTR+I+G for *COI*, SYM+I+G for *EF-1a* and *RpS5*, GTR+I for *wg* and *CAD*, and HKY+I for *MDH*.

The Bayesian estimation (hereinafter BI) of posterior probability was performed in MrBayes v.3.2.5 [Ronquist, Huelsenbeck, 2003], applying the selected evolutionary models for partitions. Markov Chains were sampled at intervals of 500 generations. Two runs of ten million generations with four chains (one cold and three heated) were performed. Maximum

Table 1. Specimens sequenced for the molecular phylogenetic analysis and their corresponding GenBank numbers.
Таблица 1. Экземпляры, отсеквенированные для молекулярно-филогенетического анализа, и соответствующие номера последовательностей из базы данных GenBank.

Voucher No.	Species	Locality	*COI*	*EF-1a*
LYC032	*L.* (*T.*) *dabrerai*	MONGOLIA, Govi-Altay Province, 8 km E Bum vill.	PX123774	PX123637
LYC033	*L.* (*T.*) *dabrerai*	MONGOLIA, Govi-Altay Province, 8 km E Bum vill.	PX123775	–
LYC034	*L.* (*T.*) *dabrerai*	MONGOLIA, Govi-Altay Province, 10 km ESE Altai somon	PX123776	–
LYC035	*L.* (*T.*) *dabrerai*	MONGOLIA, Govi-Altay Province, 10 km ESE Altai somon	PX123777	–
LYC036	*L.* (*T.*) *violacea*	MONGOLIA, Govi-Altay Province, 17 km S Dzhargalan t.	PX123778	–
LYC037	*L.* (*T.*) *violacea*	MONGOLIA, Govi-Altay Province, 17 km S Dzhargalan t.	PX123779	–
LYC038	*L.* (*T.*) *ratushinskayae*	MONGOLIA, Khovd Province, Baitag Mts., Buduun Khargaityn R.	PX123780	PX123638
LYC039	*L.* (*T.*) *ratushinskayae*	MONGOLIA, Khovd Province, Baitag Mts., Buduun Khargaityn R.	PX123781	PX123639
LYC040	*L.* (*T.*) *violacea*	RUSSIA, Republic of Buryatia, Mondy	PX123782	–
LYC041	*L.* (*T.*) *violacea*	RUSSIA, Republic of Buryatia, Mondy	PX123783	–
LYC042	*L.* (*T.*) *violacea*	MONGOLIA, Kentei Province, Kerulen R., 45 km S Baganur	PX123784	–
LYC043	*L.* (*T.*) *odbayar*	MONGOLIA, Govi-Altay Province, Biger	PX123785	–
LYC044	*L.* (*T.*) *violacea*	MONGOLIA, E Gobi Province, 100 km SE Khuvsgul somon	PV358539	PV344753



Figs 5–13. The eggs of *L.* (*T.*) *splendens* (Staudinger, 1881), Kirgyzstan, Kirgyzsky Mts.: 5–7 — dorsal view; 8–10 — lateral view; 11–13 — micropyle area.
Рис. 5–13. Яйца *L.* (*T.*) *splendens* (Staudinger, 1881), Киргизия, Киргизский хребет: 5–7 — вид сверху; 8–10 — вид сбоку; 11–13 — микропилярная область.

Likelihood (hereinafter ML) analysis was performed using IQ-TREE web server [Trifinopoulos *et al*., 2016]. Node supports were assessed both by ultrafast bootstrap (UFBoot) analysis [Hoang *et al*., 2018] with 1000 replications and Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT) [Guindon *et al*., 2010]. The final phylogenetic tree images were rendered in FigTree v.1.4.0 [Rambaut, 2012].

To infer a dated phylogeny, we used BEAST v.2.6.2 software [Bouckaert *et al.*, 2014] with a Relaxed Log Normal clock model. Tree prior was set to the Birth Death model, while all the remaining parameters were left as default. Four calibration points with corresponding 95% highest posterior density (HPD) range for the distribution of node ages were selected from the dated phylogeny published by Marabuto *et al*. [2023]: age of the clade uniting the subgenera *Heodes* and *Thersamonia* (10.9 Mya), age of the subgenus *Thersamonia* (6.4 Mya), age of the clade uniting *L. tityrus*, *L. bleusei*, *L. ottomana* and *L. virgaureae* (8.5 Mya), and age of the clade uniting *L. tityrus* and *L. bleusei* (4.8 Mya). The analysis was run two times for 60 million generations of MCMC, sampling every 1000 iterations. The parameters of all three runs were compared in Tracer v.1.5 [Drummond, Rambaut, 2007], in which we also checked the model convergence (effective sample size > 200). Trees from all three runs were combined by LogCombiner v.1.8.4 [Drummond *et al.*, 2012], and 10% of trees were discarded as burn-in. The maximum credibility tree was selected using TreeAnnotator v.1.8.4 [Drummond *et al.*, 2012]. The final phylogenetic tree was rendered in FigTree v.1.4.0 [Rambaut, 2012].

Classification

In the present paper, we follow generic and subgeneric classification after Lukhtanov *et al*. [2025].

Results

Morphology

Lycaena (*Thersamolycaena*) *splendens* (Staudinger, 1881)
Figs 5–13.

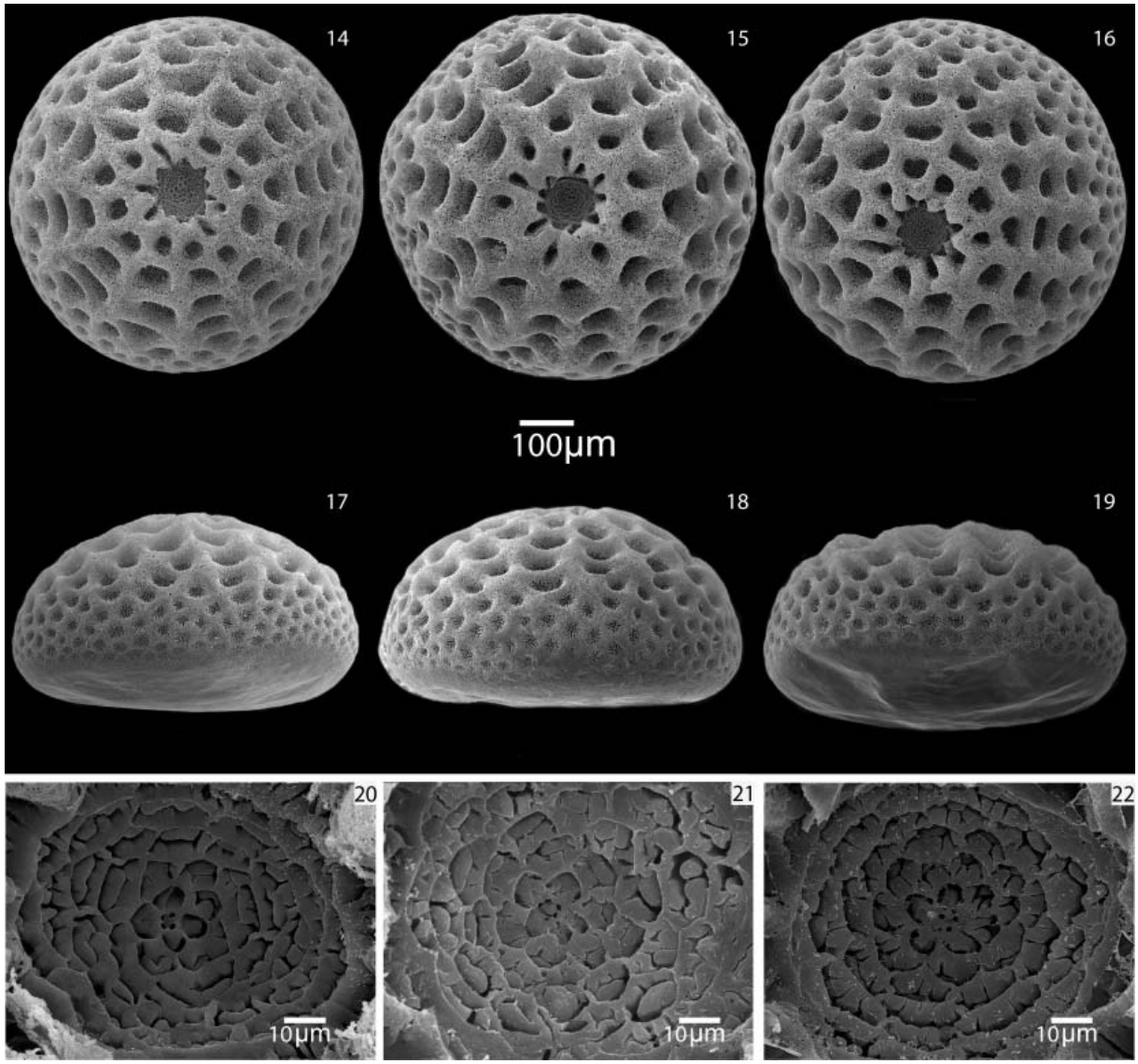
MATERIAL EXAMINED: 2 specimens, 18 eggs. Kyrgyzstan, Kirgyzsky Mts., Alamedin R., Uzun-Gyr, 2500 m, 29.VI.1998, K. Kolesnichenko leg.

The height of the egg is about 500 µm; the width is about 870 µm. The diameter of the micropyle area in the widest part is on average 100 µm. The micropyle rosette is formed by 6–7 primary rounded quadro-pentahedral cells of various lengths and widths. There are 9–10 lateral longitudinal ribs with the width of more than 80 µm in the micropyle region which fall to the base of the egg surface.

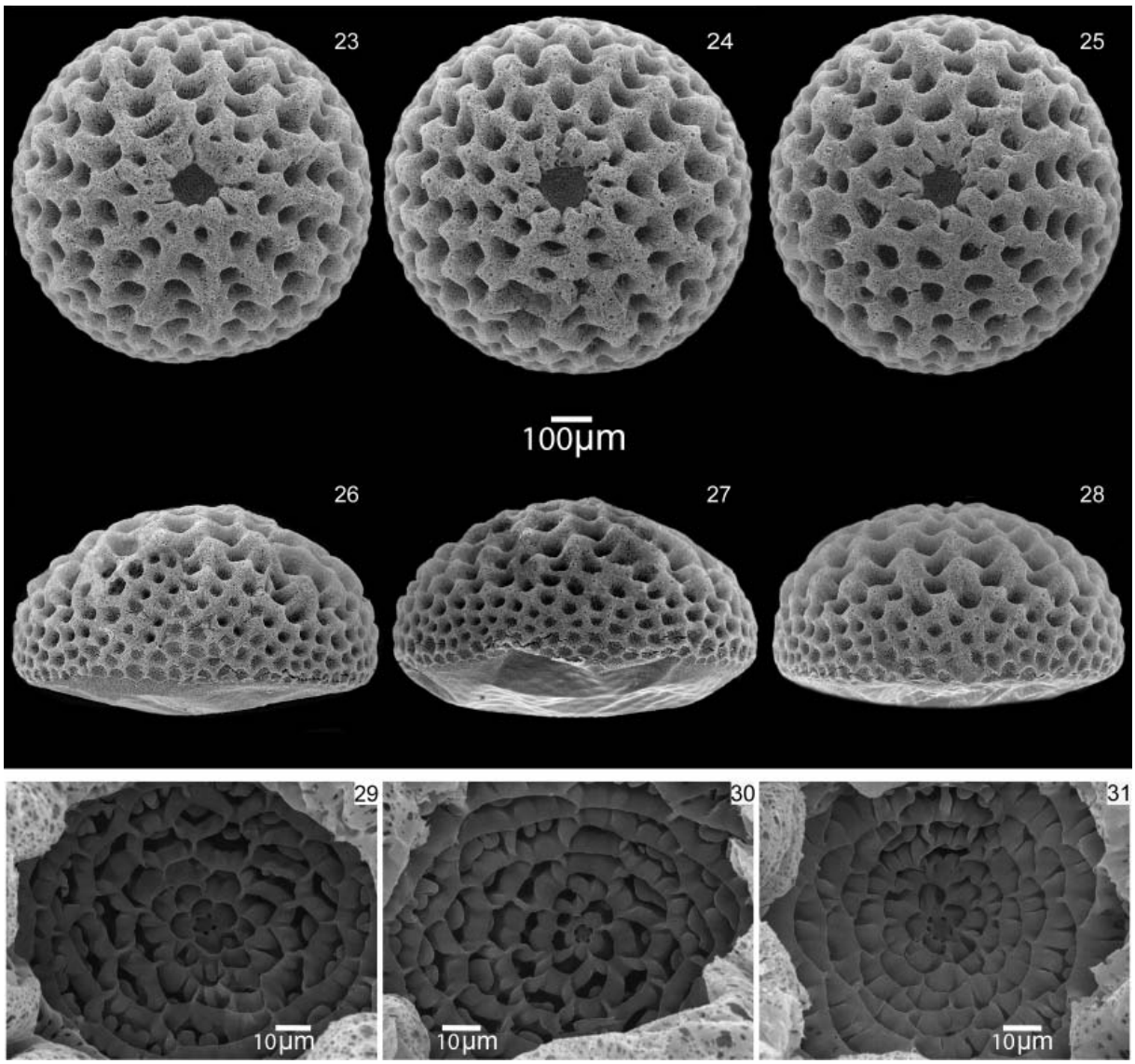
Lycaena (*Thersamolycaena*) *dabrerai* Bálint, 1996
Figs 14–22.

MATERIAL EXAMINED: 2 specimens, 30 eggs. Mongolia, Gobi-Altai Prov., 56 km NE Altai somon, 8 km S Boom v., 1700 m, 19.VII.2013, K. Kolesnichenko leg.

The height of the egg is about 400 µm; the width is about 700 µm. The diameter of the micropyle area in the widest part is on average 100 µm. The micropyle rosette is formed by 5–6 primary rounded quadra-pentahedral cells of various lengths and widths. There are 10–12 lateral longitudinal ribs with the width of more than 30 µm in the micropyle region which fall to the base of the egg surface.



Figs 14–22. The eggs of *L.* (*T.*) *dabrerai* Bálint, 1996, Mongolia, Gobi-Altai Province: 14–16 — dorsal view; 17–19 — lateral view; 20–22 — micropyle area.
Рис. 14–22. Яйца *L.* (*T.*) *dabrerai* Bálint, 1996, Монголия, Гоби-Алтайский аймак: 14–16 — вид сверху; 17–19 — вид сбоку; 20–22 — микропилярная область.



Figs 23–31. The eggs of *L.* (*T.*) *odbayar* (Churkin, 2004), Mongolia, Gobi-Altai Province: 23–25 — dorsal view; 26–28 — lateral view; 29–31 — micropyle area.
Рис. 23–31. Яйца *L.* (*T.*) *odbayar* (Churkin, 2004), Монголия, Гоби-Алтайский аймак: 23–25 — вид сверху; 26–28 — вид сбоку; 29–31 — микропилярная область.

Lycaena (*Thersamolycaena*) *odbayar* (Churkin, 2004)
Figs 23–31.

MATERIAL EXAMINED: 2 specimens, 22 eggs. South Mongolia, Gobi-Altai Prov., 30 km south Biger somon, 2700–3000 m, 3–10.VII.2002, S. Churkin leg.

The height of the egg is about 500 µm; the width is about 830 µm. The diameter of the micropyle area in the widest part is on average 100 µm. The micropyle rosette is formed by 5–6 primary rounded quadro-pentahedral cells of various lengths and widths. There are 11–12 lateral longitudinal ribs with the width is on average 36 µm in the micropyle region which fall to the base of the egg surface.

Lycaena (*Thersamolycaena*) *ratushinskayae* Churkin et Kolesnichenko, 2019
Figs 32–40.

MATERIAL EXAMINED: 2 specimens (paratypes), 20 eggs. SW Mongolia, Khovd Prov., Baitag Mountains, Buduun Khargaityn River, 2600–2800 m, 2–3.VII.2018, K. Kolesnichenko leg.

The height of the egg is about 424–430 µm; the width is about 760 µm. The diameter of the micropyle area in the widest part is on average 90 µm. The micropyle rosette is formed by 4–5 primary rounded quadra-pentahedral cells of various lengths and widths. There are 11–12 lateral longitudinal ribs with the width of on average 36 µm in the micropyle region which fall to the base of the egg surface.

Lycaena (*Thersamolycaena*) *violacea* (Staudinger, 1892) (population from Zabaikalsky Krai)
Figs 41–49.

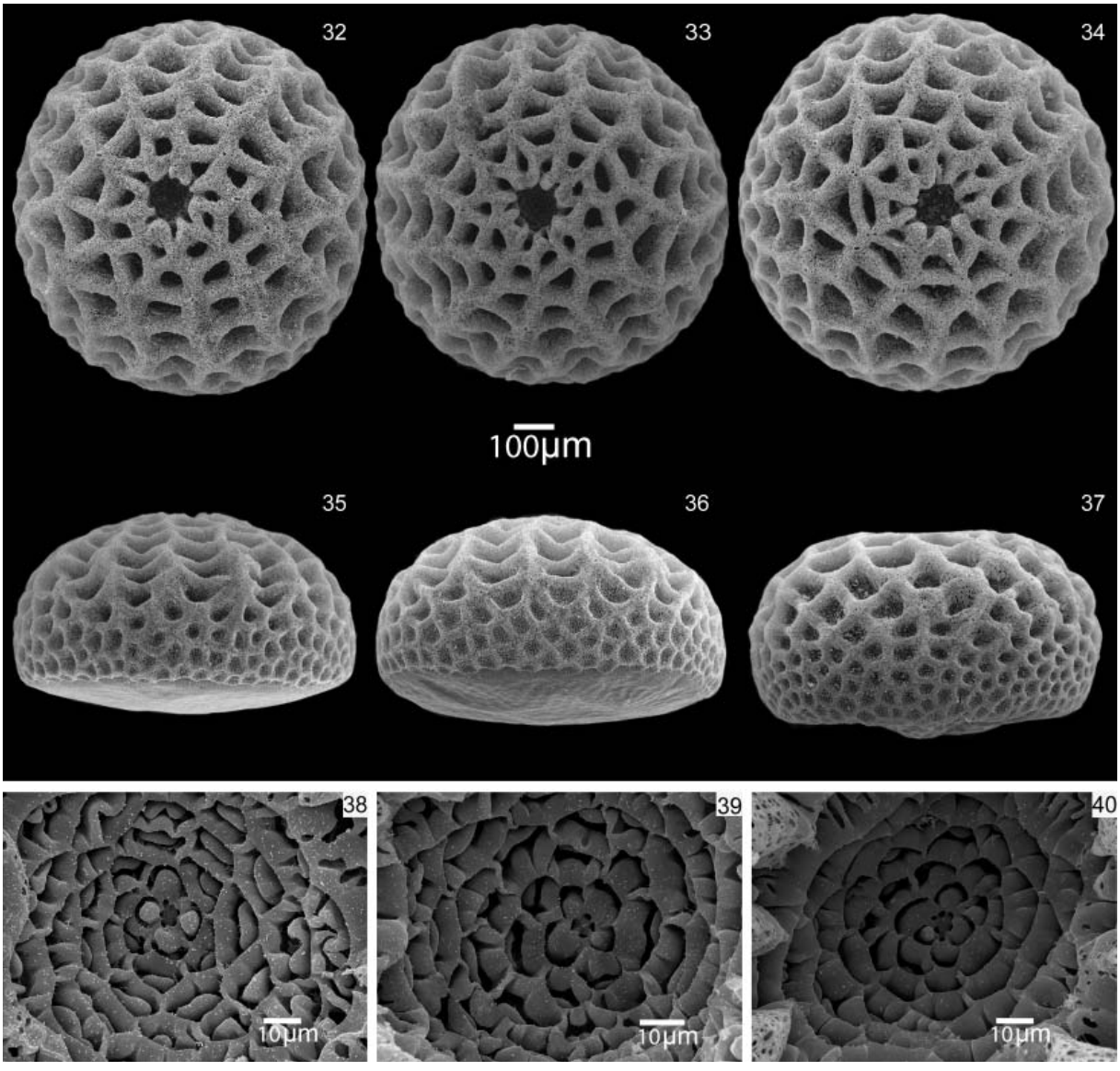
MATERIAL EXAMINED: 2 specimens, 33 eggs. Zabaikalsky Krai, Krasnokamensky Distr., Urulungui Range, Margutsek village, 19–23.VII.2003, K. Kolesnichenko leg.

The height of the egg is about 435 µm; the width is about 750 µm. The diameter of the micropyle area in the widest part is on average 82 µm. The micropyle rosette is formed by 4–5 primary rounded quadra-pentahedral cells of various lengths and widths. There are 10–12 lateral longitudinal ribs with the width of more than 50 µm in the micropyle region which fall to the base of the egg surface.

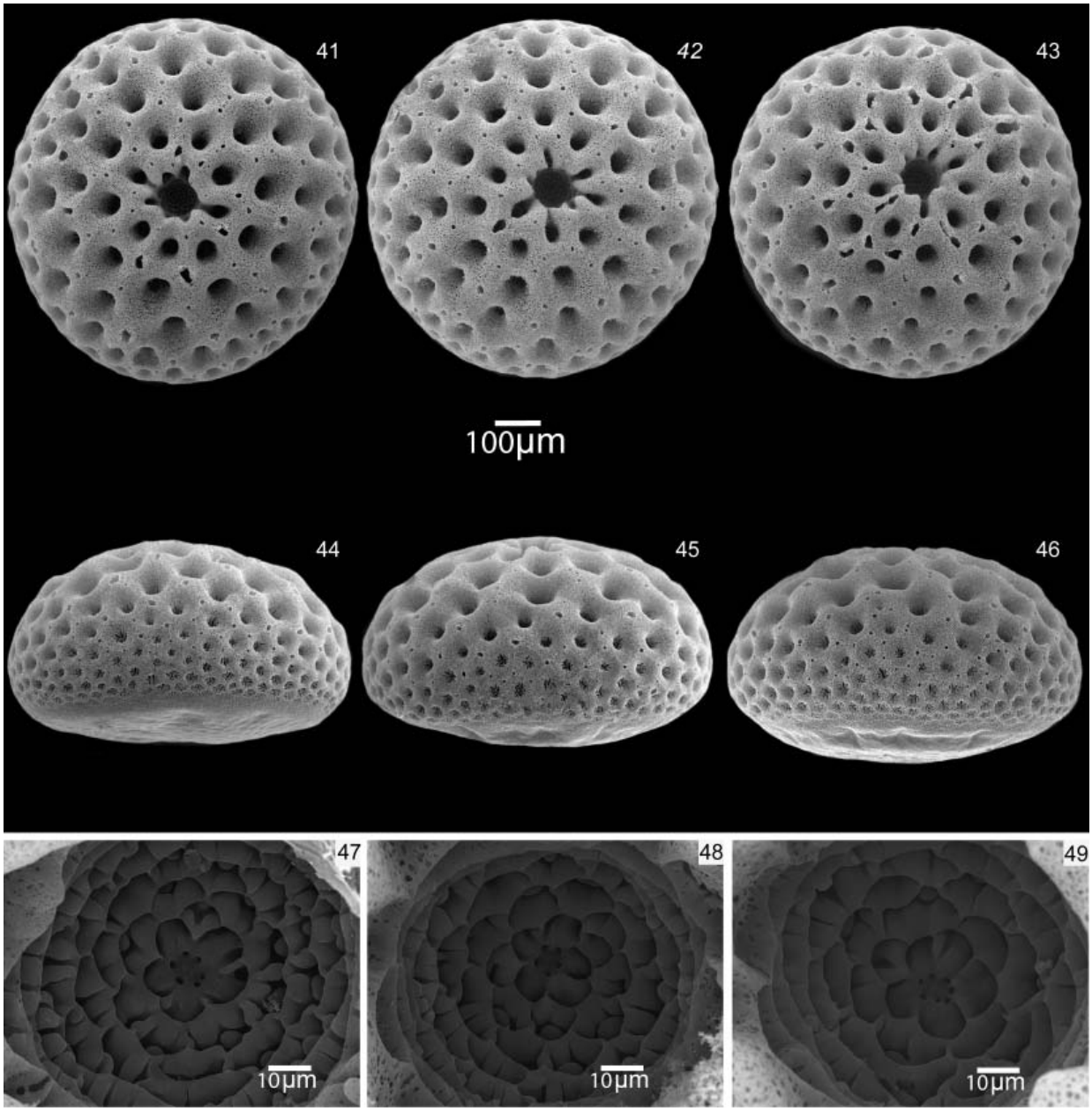
Lycaena (*Thersamolycaena*) *violacea* (Staudinger, 1892) (population from Mongolia)
Figs 50–58.

MATERIAL EXAMINED: 1 specimen, 11 eggs. Mongolia, Kentei Prov., Kerulen River, 45 km S Baganur, 1400 m, 12.VIII.2014, K. Kolesnichenko leg.

The height of the egg is about 441 µm, the width is about 837 µm. The diameter of the micropyle area in the widest part is on average 90 µm. The micropyle rosette is formed by 4–6 primary rounded quadra-pentahedral cells of various lengths and widths. There are 12 lateral longitudinal ribs with the width of on average 67 µm in the micropyle region which fall to the base of the egg surface.



Figs 32–40. The eggs of *L.* (*T.*) *ratushinskayae* Churkin et Kolesnichenko, 2019, Mongolia, Khovd Province: 32–34 — dorsal view; 35–37 — lateral view; 38–40 — micropyle area.
Рис. 32–40. Яйца *L.* (*T.*) *ratushinskayae* Churkin et Kolesnichenko, 2019, Монголия, Ховд аймак: 32–34 — вид сверху; 35–37 — вид сбоку; 38–40 — микропилярная область.



Figs 41–49. The eggs of *L.* (*T.*) *violacea* (Staudinger, 1892), Zabaikalsky Krai: 41–43 — dorsal view; 44–46 — lateral view; 47–49 — micropyle area.
Рис. 41–49. Яйца *L.* (*T.*) *violacea* (Staudinger, 1892), Забайкальский край: 41–43 — вид сверху; 44–46 — вид сбоку; 47–49 — микропилярная область.

The basic parameters of the eggs are summarized in the Table 2.

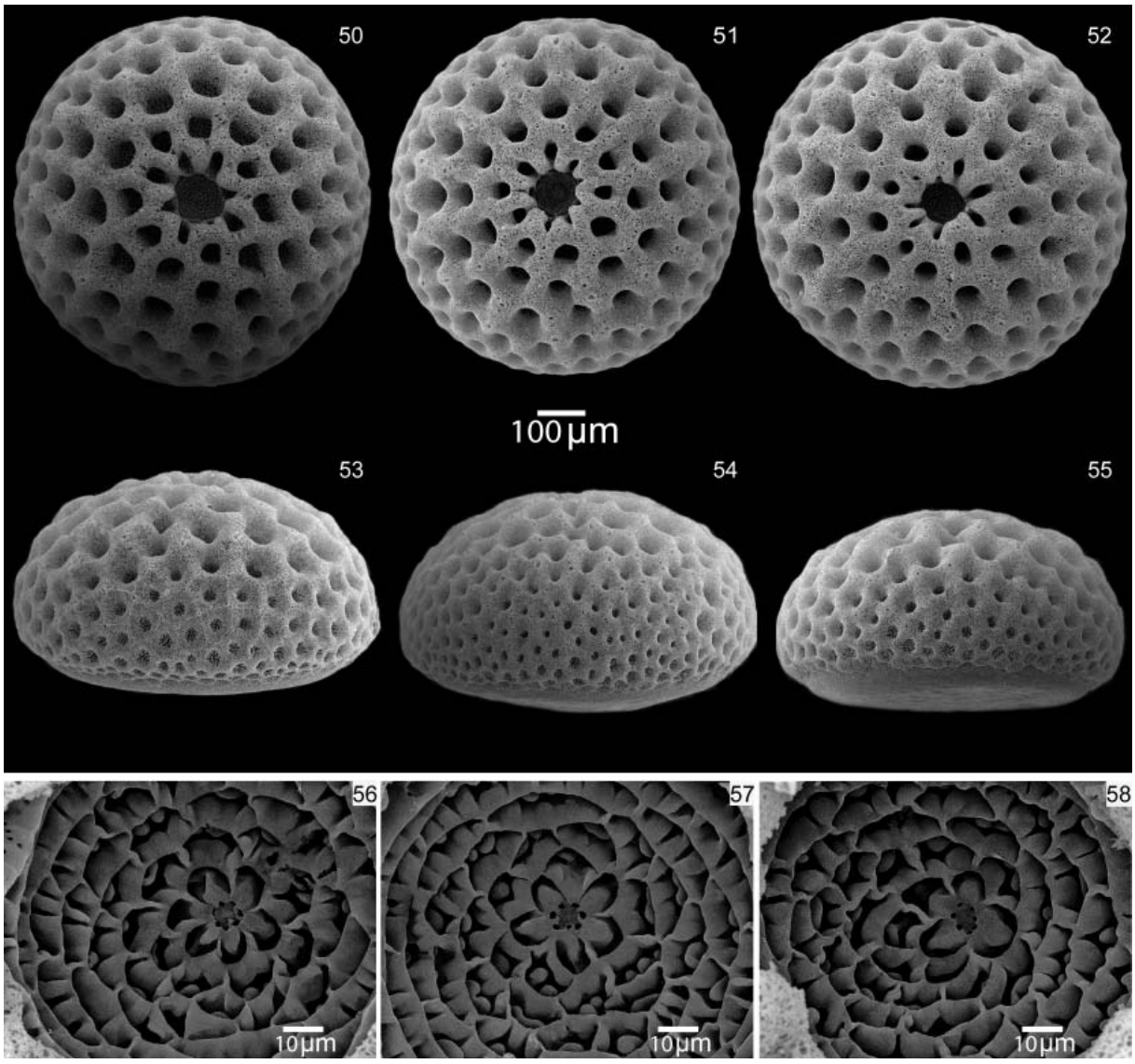
Molecular phylogenetic analysis

Both BI and ML reconstructions gave generally same topologies, accounting to clade supports (Fig. 59). The genus *Lycaena* was rendered as strongly supported monophyletic group, and most clades corresponding to subgenera or species groups were completely supported or received high supports at least in one of the analyses. The subgenus *Thersamolycaena* was reconstructed as a sister lineage to the rest of *Lycaena* species in all the analyses. *Lycaena* (*Thersamolycaena*) *dispar* was reconstructed as a sister taxon to the *L.* (*T.*) *splendens* species group, which also received high supports. Within the latter, a clade uniting the taxa *violacea*, *dabrerai* and *odbayar* received high supports in ML analysis but it was not supported in BI analysis. These taxa are genetically overlapped in both studied gene fragments (only *COI* in the case of *L.* (*T.*) *odbayar*), while *L.* (*T.*) *ratushinskayae* was placed as an independent sister lineage, although this node received low supports in all the analyses.

Table 2. Basic parameters of the eggs of the species of the subgenus *Thersamolycaena*. Average values are given in bold, standard deviations are in regular font.

Таблица 2. Основные параметры яиц изученных видов подрода *Thersamolycaena*). Средние значения приведены жирным шрифтом, стандартные отклонения обычным шрифтом.

Species	Egg height (μm)	Egg width (μm)	Micropile rosette diameter (μm)	Lateral rib width (μm)	Number of lateral ribs	Number of primary cells
L. (*T.*) *splendens*	**500.7** ± 2.2	**872.3** ± 5.2	**100.6** ± 1.7	**82.4** ± 7.5	**9.6** ± 0.5	**6.8** ± 0.4
L. (*T.*) *dabrerai*	**393.8** ± 1.5	**706.2** ± 1.6	**104.2** ± 1.2	**32.6** ± 3.0	**11.2** ± 0.6	**5.6** ± 0.5
L. (*T.*) *otbayar*	**498.2** ± 2.3	**835.4** ± 2.3	**100.5** ± 2.0	**36.7** ± 3.1	**11.3** ± 0.5	**5.6** ± 0.5
L. (*T.*) *ratushinskayae*	**426.5** ± 2.0	**761.6** ± 8.4	**90.1** ± 1.1	**36.0** ±3.8	**11.3** ± 0.5	**4.7** ± 0.4
L. (*T.*) *violacea* (Zabaikalsky Krai)	**435.8** ± 1.7	**750.3** ± 2.9	**82.8** ±2.0	**51.3** ± 6.9	**10.7** ± 0.9	**4.8** ± 0.5
L. (*T.*) *violacea* (Kentei Prov.)	**441.7** ± 19.5	**837.1** ± 18.7	**90.3** ±3.3	**67.7** ± 13.8	**11.7** ± 0.5	**5.2** ± 0.8



Figs 50–58. The eggs of *L.* (*T.*) *violacea*, Mongolia, Kentei Province: 50–52 — dorsal view; 53–55 — lateral view; 56–58 — micropyle area.

Рис. 50–58. Яйца *L.* (*T.*) *violacea*, Монголия, Кентей аймак: 50–52 — вид сверху; 53–55 — вид сбоку; 56–58 — микропилярная область.

Estimation of diversification time

The Bayesian phylogenetic tree obtained with BEAST (Fig. 60) resulted in the same topology as in BI and ML analyses. According to BEAST, the primary divergence of the genus *Lycaena* occurred ca. 14.44 Mya (95% HPD 18.67–10.24 Ma). The divergence between the ancestor of *L.* (*T.*) *dispar* and an ancestor of the *L.* (*T.*) *splendens* species group occurred ca. 9.03 Mya (95% HPD 13.66–4.76 Ma), while that between *L.* (*T.*) *splendens* and the rest taxa occurred ca. 3.07 Mya (95% HPD 5.41–1.21 Ma). Finally, the ancestor of the remaining species of the group diverged ca. 1.73 Mya (95% HPD 2.91–0.58 Ma).

Discussion

A detailed morphological description of the egg chorion of several West European copper species, *Helleia helle* (previously considered within the genus *Lycaena*), *L.* (*Lycaena*) *phlaeas* (Linnaeus, 1761), *L.* (*Heodes*) *tityrus* (Poda, 1761), *L.* (*Heodes*) *bleusei* (Oberthür, 1884),

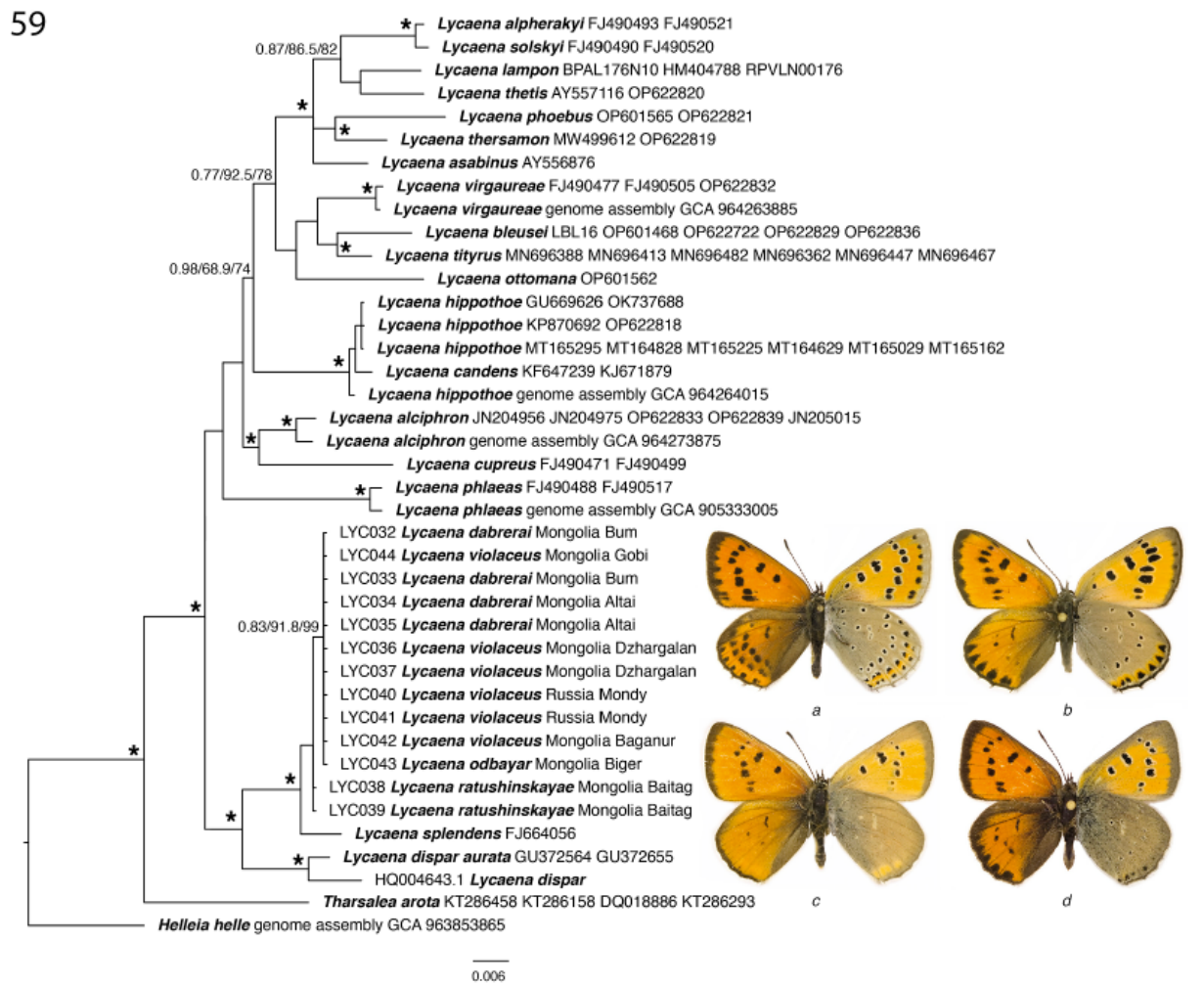


Fig. 59. Bayesian molecular phylogenetic tree of the genus *Lycaena* s.l. obtained from the concatenated dataset of the *COI*, *EF-1a*, *wg*, *CAD*, *MDH* and *RpS5* gene fragments. Numbers at nodes indicate Bayesian posterior probabilities/SH-aLRT/UFboot values, respectively. Nodes completely supported in all analyses are marked with asterisk, completely unsupported nodes are not marked. Scale bar = 0.006 substitutions per position. Inserted photos of the specimens: a — *L.* (*T.*) *violacea*, Mongolia, Khangai Mts., b — *L.* (*T.*) *dabrerai*, Mongolia, Gobi-Altai Prov., Adzh-Bogd Mts., c — *L.* (*T.*) *odbayar*, Mongolia, Khovd Prov., Khazhingiin Nuruu Mts., d — *L.* (*T.*) *ratushinskayae*, paratype, Mongolia, Khovd aimak, Baitag Mts. (photos by K. Kolesnichenko).

Рис. 59. Молекулярно-филогенетическое дерево, построенное на основе Байесова анализа объединённого выравнивания фрагментов генов *COI*, *EF-1a*, *wg*, *CAD*, *MDH* и *RpS5*. Значения в узлах показывают Байесовы апостериорные вероятности/SH-aLRT/UFboot-поддержки, соответственно. Полностью поддержанные узлы отмечены звездочкой, полностью неподдержанные не отмечены. Масштаб = 0,006 замен на позицию. Вставленные фотографии экземпляров: a — *L.* (*T.*) *violacea*, Монголия, Хангай, b — *L.* (*T.*) *dabrerai*, Монголия, Гоби-Алтайский аймак, горы Аж-Богд, c — *L.* (*T.*) *odbayar*, Монголия, Ховд аймак, горы Хажингиин Нуруу, d — *L.* (*T.*) *ratushinskayae*, паратип, Монголия, Ховд аймак, горы Байтаг (фотографии К. Колесниченко).

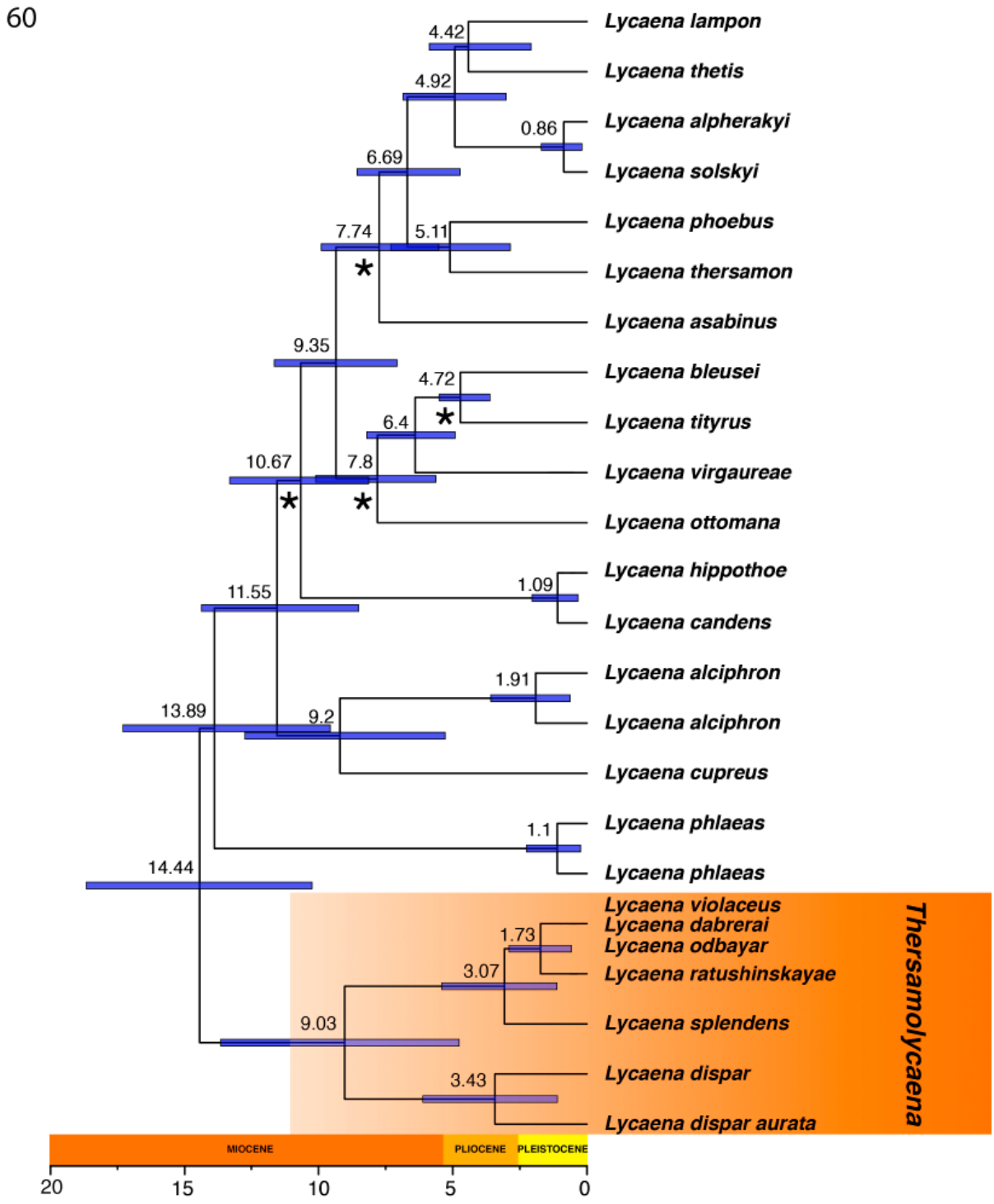


Fig. 60. BEAST calibrated molecular phylogenetic tree. Numbers at nodes indicate divergence times in million years ago. Calibration points are marked with asterisk. Bars over nodes indicate the 95% HPD range for the posterior distribution of node ages.

Рис. 60. Откалиброванное молекулярно-филогенетическое дерево, полученное в программе BEAST. Значения в узлах показывают времена дивергенции в миллионах лет назад. Точки, использованные для калибровки, отмечены звёздочками. Отрезки показывают 95% интервал наибольшей постериорной вероятности для возраста узлов.

L. (*Heodes*) *virgaurae* (Linnaeus, 1758), *L*. (*Alciphronia*) *alciphron* (Rottemburg, 1775), *L*. (*Paleochrysophanus*) *hyppothoe* (Linnaeus, 1761), was given by Munguira *et al*. [2015]. These authors considered the presence of crater-like cells on the surface of the egg as the main character of the egg chorion of the genus *Lycaena*. In addition, they mentioned the micropyle to be deeply set within a cavity.

The results of our study of five *Lycaena* (*Thersamolycaena*) species distributed in Central Asia and Mongolia generally confirm those published by Munguira *et al*. [2015].

The eggs of all studied species have a well-defined semicircular shape in lateral view; in dorsal view, they are noticeably rounded (Figs 1–2). The egg chorion is characterized by crater-like cells on the surface (Fig. 4). Such formations, in our opinion, is produced by intersection of massive and wide longitudinal lateral stiffening ribs (Fig. 1), connected by jumpers of the same thickness. Both ribs and jumpers form the reticulation of the egg chorion. In addition to this feature, the eggs of all studied species are characterized by the deeply submerged micropyle region above the general surface of the egg (Fig. 3). The number of primary cells (Fig. 3) has diagnostic significance. Based on our data and the results of Munguira *et al*. [2015], the above-mentioned features should be considered as diagnostic characters of the genera *Lycaena* and *Helleia*.

Compared to the eggs of the subgenus *Thersamolycaena*, the eggs of the species of the genus *Lycaena* s.l. studied by Munguira *et al*. [2015] are characterized by thinner stiffening ribs. They form cribrate structure without lateral longitudinal ribs and look like jumpers surrounding crater-like cells on the surface of the egg.

Among the studied species, *L*. (*T.*) *splendens* and *L*. (*T.*) *odbayar* are characterized by the largest eggs (over 500 μm high and 872 μm wide and over 498 μm high and over 835 μm wide, respectively), whereas eggs of *L*. (*T.*) *dabrerai* are characterized by smaller size (about 394 μm heigh and 706 μm wide). The size of the eggs of *L*. (*T.*) *violacea* and *L*. (*T.*) *ratushinskayae* is intermediate (height 436–442 and 426.5 μm, and width 750–837 and 762 μm, respectively). The micropyle area diameter does not depend on the egg size. In the eggs of *L*. (*T.*) *splendens* and *L*. (*T.*) *odbayar*, comparable in size, this indicator is more than 100 μm, whereas in *L*. (*T.*) *dabrerai* the micropyle area diameter is about 104 μm, while the height of the egg does not exceed 394 μm, and the width is not more than 706 μm. Both the micropyle area diameter of *L*. (*T.*) *ratushinskayae* and *L*. (*T.*) *violacea* from Kentei Province (Mongolia) is approximately the same, over 90 μm.

Estimation of the egg size variability for *L*. (*T.*) *ratushinskayae*, *L*. (*T.*) *odbayar* and *L*. (*T.*) *dabrerai* from different localities is rather difficult, since these endemic species are extremely local and known to date mainly from the type localities. Nevertheless, our data suggest that the egg sizes range has low variability in *L*. (*T.*) *splendens*, *L*. (*T.*) *ratushinskayae*, *L*. (*T.*) *odbayar* and *L*. (*T.*) *dabrerai*, while specimens of the widespread *L*. (*T.*) *violacea* from different localities demonstrate some variability in the egg size. This is especially noticeable for the egg width. Thus, the egg diameter in the Mongolian *L*. (*T.*) *violacea* is slightly larger on average than the same for the specimens from Zabaikalsky Krai (837 and 750 μm, respectively), although the standard deviation of these values for Mongolian specimens of *L*. (*T.*) *violacea* is quite high. A similar situation is observed with the ratio of the micropyle area diameters (90 μm in specimens from Mongolia and 83 μm in specimens from Zabaikalsky Krai).

The number of primary cells in the micropyle rosette apparently has a diagnostic significance for the species in question. *Lycaena* (*Thersamolycaena*) *splendens* is characterized by seven (rarely six) primary cells. For *L*. (*T.*) *violaceous* and *L*. (*T.*) *ratushinskayae*, the number of primary cells in the micropyle rosette is the smallest: it varies from four to five. For *L*. (*T.*) *odbayar* and *L*. (*T.*) *dabrerai*, the number of primary cells in the micropyle area varies from five to six.

The broadest lateral ribs are found in the eggs of *L*. (*T.*) *splendens* (about 80 μm). The rib width in the eggs of *L*. (*T.*) *dabrerai*, *L*. (*T.*) *odbayar* and *L*. (*T.*) *ratushinnskayae* is approximately the same (on average, 33, 36 and 36 μm, respectively). The lateral rib width of the eggs of *L*. (*T.*) *violaceous* is intermediate and ranges from 51 to 68 μm, depending on the specimen locality.

The number of longitudinal ribs in *L*. (*T.*) *dabrerai*, *L*. (*T.*) *odbayar*, *L*. (*T.*) *violacea* and *L*. (*T.*) *ratushinskayae* eggs is approximately the same (from 10 to 12), and for *L*. (*T.*) *splendens* this number is the smallest among studied species (from 9 to 10).

Thus, considering the above-mentioned data, *L*. (*T.*) *splendens* is the most peculiar species among the studied *Lycaena* (*Thersamolycaena*) species by the features of the egg chorion structure. The egg of this species differs from eggs of other species in size, number of lateral ribs and number of primary cells in the micropyle area. *Lycaena* (*Thersamolycaena*) *dabrerai*, *L*. (*T.*) *odbayar*, *L*. (*T.*) *ratushinskayae* and *L*. (*T.*) *violacea* are the most closely related species but all of them slightly differ from each other in the egg chorion structure.

The exochorion of the eggs of the species of the subgenus *Thersamolycaena* is characterized by the similar structure including rather strong and wide stiffening ribs and the lateral longitudinal ribs clearly visible dorsally. This combination of characters is especially pronounced in *L*. (*Thersamolycaena*) *dispar* (Haworth, 1802). Based on photographs of the eggs of this species made by Stradomsky and Fomina [2011], it can be noted that the egg bears six or seven convex lateral longitudinal ribs, with crater-like cells located between them. A tendency to increasing of the number of lateral longitudinal ribs is observed in more recently diversified complex of species, namely *L*. (*T.*) *dabrerai*, *L*. (*T.*) *odbayar*, *L*. (*T.*) *ratushinskayae* and *L*. (*T.*) *violacea*. Their eggs are characterized by on average 11 lateral ribs, while in the eggs of *L*. (*T.*) *splendens* the number of these ribs is reduced to 9–10.

The eggs of other species of the genus *Lycaena* s.l. studied by Munguira *et al*. [2015] are characterized

by thinner stiffening ribs. They form cribrate structure without lateral longitudinal ribs and look like jumpers surrounding crater-like cells on the surface of the egg.

Results of our molecular phylogenetic analysis are generally concordant with previously published reconstructions based on multilocus [Lukhtanov *et al*., 2025] and phylogenomic [Zhang *et al*., 2020] analyses, where the subgenus *Thersamolycaena* is rendered as a monophyletic lineage sister to the rest of the diversity of the genus *Lycaena*. It is noteworthy that the inclusion in the analysis of the Chinese species *L. standfussi*, for which the monotypic subgenus *Fussia* Grishin, 2022 was recently described [Zhang *et al*., 2022], drastically changes topology: *Thersamolycaena* and *Fussia* turn out to be sister lineages, and deep relationships of the whole genus *Lycaena* become unresolved due to very low bootstrap values.

Among the target taxa of our study, an integrative analysis confirms the species status of *L*. (*T*.) *splendens* and *L*. (*T*.) *ratushinskayae*, while other taxa differ from each other by the egg structure and genetically indistinguishable based on our analysis of two genes. Based on the phylogenomic reconstruction by Zhang *et al*. [2022], *L*. (*T*.) *violacea* and *L*. (*T*.) *odbayar* are somewhat different; we can suggest that single molecular markers do not necessarily discriminate species in this group, which is not an uncommon phenomenon among Lycaenidae [Talavera *et al*., 2013; Krupitsky *et al*., 2023; MacDonald *et al*., 2025], and further genomic analyses are required.

The position of Central Asian *L*. (*T*.) *splendens* as sister to the Mongolian complex of taxa indicates that Central Asia was the ancestral region for this group of species. As our dated analysis indicated, this complex probably diversified during the Pleistocene, ca. 1.73 Mya. The analysis confirms the hypothesis by Churkin, Kolesnichenko [2019] on the longer isolation of *L*. (*T*.) *ratushinskayae* compared to other species of the group. We can suggest that the previously united range of the ancestor of the Mongolian taxa was fragmented during the Pleistocene due to climate fluctuations that led to the forming of three different species, *L*. (*T*.) *dabrerai*, *L*. (*T*.) *odbayar* and *L*. (*T*.) *ratushinskayae*, in the disjointed regions of the Gobi Altai, the Mongolian Altai and the Baitag Mountains in the Dzhungarin Gobi, respectively. Being ecologically more plastic species, *L*. (*T*.) *violacea* probably significantly expanded its range in the mountains of Mongolia and China during the Pleistocene due to the development of grassland biomes caused by Pleistocene climate oscillations [Barbolini *et al*., 2020].

Taking into account integrative data, namely differences in the male genitalia and the ultrastructure of the egg chorion supplied with the results of molecular phylogenetic and phylogenomic analyses, we consider the taxa of the *L*. (*T*.) *splendens* group as recently diversified but distinct species with different ecological requirements. Our analysis demonstrates that characters of the egg chorion may discriminate species in evolutionarily recently diversified groups.

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Competing interests. The authors declare no competing interests.

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