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New species and findings of *Pagastia* Oliver (Diptera: Chironomidae: Diamesinae) from Central Asia, with DNA barcoding of known species of the genus

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Abstract

Chironomids of the genus *Pagastia* Oliver (Diamesinae, Diamesini) from the mountains of Central Asia are revised using both morphological characters and molecular data. Illustrated descriptions of the adult male *Pagastia* (*P.*) *caelestomontana* **sp. nov.** from Kirgizstan and Tajikistan, *P.* (*P.*) *hanseni* **sp. nov.** from Tajikistan, and record of a finding apparently a new species *P.* (*P.*) aff. *lanceolata* (Tokunaga) from Tajikistan as well as an updated a key to the determination of the adult males of all known species of *Pagastia* are provided. A phylogenetic framework is reconstructed based on two mitochondrial genes cytochrome oxidase subunit *I (COI)* sequences of 34 samples belonging to 7 species of the genus *Pagastia* and cytochrome oxidase subunit *II (COII)* available for most samples. Phylogenetic trees of some known species of the genus *Pagastia* were reconstructed using the combined dataset and Bayesian inference (BI) and Maximum Likelihood (ML) methods. The interspecific K2P distances between seven *Pagastia* species including *P.* (*P.*) *caelestomontana* **sp. nov.**, *P.* (P.) *hanseni* **sp. nov.** and undescribed *P.* (*P.*) aff. *lanceolata* (Tokunaga) are 6.3–13.2 which corresponding to species level.

Key words: Diptera, Chironomidae, Diamesinae, *Pagastia,* taxonomy, new species, key, DNA barcoding, phylogenetic relationships, Central Asia

Introduction

In this article we continue to publish the data obtained as a result of the revision of the subfamily Diamesinae, namely of the genus *Pagastia* Oliver (Makarchenko 2019, Makarchenko & Hansen 2020). The genus *Pagastia* was described by Oliver (1959) from North America with two included species, *P. orthogonia* (as new, and as the type species for the genus) and *P. partica* (Roback). Sublette (1967) erected the new genus *Hesperodiamesa* for *Prodiamesa sequax* Garrett, which later Serra-Tosio (1971, 1976) synonymized with *Pagastia.* Currently, *Pagastia* (*Hesperodiamesa*) includes a single Nearctic species, *P.* (*H.*) *sequax* (Garrett) (known only from Canada), while *Pagastia* s. str. includes ten valid species: *P. altaica* Makarchenko, Kerkis *et* Ivanchenko, 1997 (Altai Mountains, Russia), *P. hidakamontana* Endo, 2004 (Hokkaido, Japan), *P. lanceolata* (Tokunaga, 1936) (Japan, East Siberia, Russian Far East), *P. nivis* (Tokunaga, 1936) (Japan, Russian Far East), *P. orientalis* (Tshernovskij, 1949) (Siberia, Russian Far East, South Korea, China), *P. orthogonia* Oliver, 1959 (U.S.A., Japan, Russian Far East), *P. partica* (Roback, 1957) (Canada, U.S.A.) (Ashe & Connor 2009), *P. tianmumontana* Makarchenko *et* Wang, 2017 (South China), (Makarchenko & Wang 2017), *P. subletteorum* Makarchenko, 2019 (Canada, British Columbia) (Makarchenko 2019) and *P. donoliveri* Makarchenko et Hansen, 2020 (Makarchenko & Hansen 2020) (U.S.A., Wyoming). One additional undescribed species, known only from larvae, is recorded from Kirgizstan in Central Asia (Makarchenko & Makarchenko 2000), and two undescribed species are recorded from Nepal (Roback & Coffman 1987).

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Below we present a description of the adult males of *Pagastia* (*P.*) *caelestomontana* **sp. nov.** and *P.* (*P.*) *hanseni* **sp. nov.,** two species from Central Asia (Kirgizstan and Tajikistan). A record of an apparently a new species *P.* (*P.*) aff. *lanceolata* (Tokunaga) from Tajikistan is also reported, as well as a validation of these new species and a discussion of the evolutionary relationships of the genus *Pagastia.* The phylogeny is reconstructed and inferred from two molecular markers including mitochondrial cytochrome *c* oxidase subunit I (COI) and cytochrome *c* oxidase subunit II. Moreover, the estimation of K2P interspecific distances and ABGD analysis are used to confirm the species status of *P.* (*P.*) *hanseni* **sp. nov.**, *P.* (*P.*) *caelestomontana* **sp. nov.** and of the other species of the genus.

We also present an updated a key to the determination of the adult males of all known species of *Pagastia.* Unfortunately, a key to the determination of the larvae and pupae of these species is not yet feasible, due to their very close morphological similarity.

Materials and methods

The adults and preimaginal stages of chironomids were preserved in 96% ethanol for DNA-analysis and in 70% ethanol for further study of morphology. The material was slide-mounted in polyvinyl lactophenol following the recommendations of Moubayed and Langton (2019). The larvae and adults specimens of *Pagastia* (*P.*) *caelestomontana* **sp. nov. a**nd *P.* (*P.*) *hanseni* **sp. nov.** are associated by using of DNA barcoding as well as for comparing of these species with close related species. The morphological terminology and abbreviations used below generally follow Sæther (1980). For some structures of the hypopygium, however, the terminology of Hansen & Cook (1976) and Oliver (1989) is used. The photographs were taken using an Axio Lab.A1 (Karl Zeiss) microscope.

Holotypes and paratypes of the new species, as well as all other material, are deposited in the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia (FSCEATB FEB RAS).

Total genomic DNA is extracted from thorax by Qiagen DNeasy Blood & Tissue Kit and Invitrogen PureLink Genomic DNA Mini Kit, according to the manufacturer's instructions. Mitochondrial cytochrome oxidase subunit I and subunit II were amplified with Go Taq Green Master Mix (Promega Corp, Madison, WI, USA) and two pair of primers, LCO1490 - HCO2198 (Folmer *et al.* 1994) for COI and Mtd13-mod - Mtd20-mod (Ekrem *et al*. 2010) for COII. PCR reaction had 10 µL of final volume, containing 5 µL of Go Taq Green Master Mix (Promega Corp, Madison, WI, USA), 0.5 µM of each primer, and 1–2µL of eluted DNA. Profiles for PCR were as follow: pre-denaturation for 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 48°C, then 60s at 72°C and a final extension at 72°C for 5min. Amplification of PCR products is checked by electrophoresis using a 1,5% agarose gel in TBE buffer stained with Ethidium bromide and visualized on GelDoc XR+ imaging systems (BioRad). The amplified products are purified with Exonuclease I and Thermosensitive Alkaline Phosphatase (ThermoFisher Scientific, Waltham, MA, USA) and sequenced for both directions. Sequencing reaction was performed using BigDye® Terminator v3.1 Cycle Sequencing Kits and run on an ABI 3130xl Genetic Analyzer Sequencer (Applied Biosystems, Foster City, CA, USA).

Forward and reverse sequences are manually assembled and edited using Finch TV and MEGA 7 (Kumar *et al.* 2016). The obtained sequences are checked aligned at the nucleotide level using MUSCLE (Edgar 2004). Sequences of transfer RNAs (tRNAs) before and after COII gene (tRNA-Leu and tRNA-Lys) are discarded from further analyzes. Based on the Kimura-2-Parameter (K2P) model are calculated inter- and intraspecific genetic distances using MEGA7 (Kumar *et al.* 2016). ABGD analysis (wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html, Puillandre *et al.* 2012) is used for species delamination and establish taxonomic status of sequenced specimens, using relative gap width (X = 1.0) and intraspecific divergence (P) values between 0.005 and 0.100 with the K2P model according to Song *et al.* (2018). PartitionFinder 2.1.1 (Lanfear *et al.* 2012) is used to select the best-fit partitioning scheme and models separately for each codon position of protein coding genes using the greedy algorithm with linked branch lengths for the corrected Bayesian Information Criterion as the optimality criterion for model selection. The best models for the first codon position of COI and for second position of COII was F81 (Felsenstein 1981) while the best model for remaining positions was GTR+G (Tavaré 1986). Bayesian phylogenetic analyses was carried out using Markov Chain Monte Carlo (MCMC) randomization in MrBayes v3.2.7 (Ronquist *et al.* 2012). Four Markov chains (three heated chains, one cold) were run for 5 million generations, with the first 25% of sampled trees discarded as burn-in. ML analysis was performed in RAxML v. 8.2.4 using bootstrapping with 1000 replications

(Stamatakis 2006) and GTR+G model. Moreover, trace files of BI analysis were visually inspected in Tracer 1.7 (Rambaut *et al.* 2018) and then the BI and ML trees of is visualized in FigTree v. 1.4.4.

The obtained sequences have been deposited in GenBank under numbers MW691954-MW691968, KY640354-KY640373 (COI), and MW711802-MW711815, KY640337-KY640353 (COII).

Results

Genus *Pagastia* Oliver

Pagastia Oliver, 1959: 49.
Type species: *Pagastia orthogonia* Oliver, 1959, by original disignation.

Diagnosis. As in Oliver (1959, 1983, 1986, 1989), Oliver & Roussel (1982), Makarchenko (1985, 1994, 2006, 2019), Makarchenko & Makarchenko (2000), Sæther & Andersen (2003) and Endo (2004).

Key to the known species of *Pagastia* Oliver

Males

1. Eyes hairy. Aedeagal lobes reduced (**subgenus *Hesperodiamesa* Sublette**). Basal lobe of gonocoxite small . . . . *P.*(*H.*) *sequax* (Garrett, 1925) (Makarchenko 2019, Fig. 18)
\- Eyes bare or pubescent. One or two aedeagal lobes present (**subgenus *Pagastia* Oliver**). Basal lobe of gonocoxite large . . . 2
2. Both median and lateral aedeagal lobes present 3
\- Only lateral aedeagal lobe present 8
3. Anteponotum with dorsal and lateral setae in widely separated groups. Median aedeagal lobe digitated, widest in distal part or wedge-shaped and widest at base 5
\- Antepronotum completely covered with setae. Median aedeagal lobe widest medially, with apex forming a sharp hook or wedge-shaped, with fingerlike apex 4
4. Median aedeagal lobe widest medially, then tapering abruptly and with the apex forming a sharp hook . . . . *P.* (*Pagastia*) *partica* (Roback, 1957) (Makarchenko 2019, Figs.16–17)
\- Median aedeagal lobe wedge-shaped, with finger-like apex . . . . *P.* (*P.*) *hanseni* **sp. nov.** (Fig. 7)
5. Median aedeagal lobe digitated, widest in distal part 6
\- Median aedeagal lobe wedge-shaped, with toothed apex . . . . *P.* (*P.*) *caelestomontana* **sp. nov.** (Fig. 2)
6. Gonostylus subapical with “heel” . . . . *P.* (*P.*) *nivis* (Tokunaga, 1936) (Makarchenko 2006, Fig. 180, 5–10)
\- Gonostylus subapical without “heel” 7
7. Apex of the gonostylus is angled . . . . *P.* (*P.*) *orientalis* (Tshernovskij, 1949) (Makarchenko 2006, Fig. 181, 1–5)
\- Apex of the gonostylus is broadly rounded. . . . . *P.* (*P.*) *altaica* Makarchenko, Kerkis et Ivanchenko, 1997 (Makarchenko *et al.* 1997, Fig. 1)
8. Anal point absent. Alula as well as M_{1+2}, M_{3+4}, and Cu_1 of wing with setae . . . . *P.* (*P.*) *subletteorum* Makarchenko (Makarchenko 2019, Figs. 1–6, 19–20)
\- Anal point present. Alula and M_{1+2}, M_{3+4}, Cu_1 without setae 9
9. Anal point almost parallel-sided in dorsal view; lateral aedeagal lobe tapering to thin apex 10
\- Anal point tapering to apex; lateral aedeagal lobe broad in distal part 12
10. Anal point with rounded apex and without apical peg ... *P.* (*P.*) *orthogonia* Oliver, 1959 (Makarchenko 2019, Figs. 7–13, 21)
\- Anal point with pointed apical peg. 11
11. Gonostylus with small basal lobe in outer side; apical part rounded, without “heel”. AR 1.56–1.75 . . . . *P.* (*P.*) *hidakamontana* Endo, 2004 (Endo 2004, Figs. 1–6)
\- Gonostylus with rounded basal lobe in outer side and with lobe like “heel” in apical part. AR 1.22–1.26. . . . . *P.* (*P.*) *donoliveri* Makarchenko et Hansen, 2020 (Makarchenko & Hansen 2020, Figs. 4, 6–7, 9)
12. Anal point narrow, tapering to pointed apex, without apical peg; gonostylus in basal part with outer angle-shaped projection. AR 2.18–2.42 . . . . *P.* (*P.*) *tianmumontana* Makarchenko *et* Wang, 2017 (Makarchenko &Wang 2017, Fig. 17)
\- Anal point widest in basal part and thin apically, pointed and sometimes with apical peg; gonostylus without outer basal projection and with rounded apex. AR 1.81–2.10 . . . . *P.* (*P.*) *lanceolata* (Tokunaga, 1936) (Makarchenko 2006, Fig. 180, 1–4)

Descriptions

Subgenus *Pagastia* s. str.

Pagastia* (*P.*) *caelestomontana **Makarchenko, Semenchenko et Palatov, sp. nov.**

(Figs. 1–4, 8–9)

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Syndiamesa sp. 1, Kustareva & Ivanova 1980: 39, Fig. 9.
Pagastia (*P.*) sp. 1 Makarchenko & Makarchenko 2000: 175, Figs. 1–9.

Material. Holotype: adult male, Central Asia, Kirgizstan, Chuy Region, Alamudun district, Kyrgyz ridge of Tien Shan mountains, left tributary of the Ala-Archa River, 2.5 km above the cordon – the checkpoint of the Ala-Archa National Park, 27.V.2016, alt. 1717 m above sea level, N 42°37.743', E 74°29.293', leg. D. Palatov. Paratypes: 10 larvae, same data as holotype, same data as holotype except Ala-Archa River at the confluence of the Kara-bulak River, 28.V.2016, alt. 2061 m above sea level, N 42°36.200', E 74°28.819', leg. D. Palatov; 1 adult male, 4 larvae, Kirgizstan, Talas Region, Talas district, Talas valley, Otmek River (left source), under the Otmek pass, 12.VI.2016. alt. 2801 m above sea level, N 42°19.084', E 73°05.770', leg. D. Palatov; 18 larvae, Kirgizstan, Zailiyskiy Alatau, Turgen River, 20.VIII.1984, leg. N. Petrova. 3 larvae, Tajikistan, Gorno-Badakhshan Autonomous Region, Rushanskiy District, spring in front of the mouth of the Bartang River, 03.VII.2016, alt. 2025 m above sea level, N 37°55.493', E 71°36.183', leg. D. Palatov.

Derivatio nominis. From name of Tien-Shan mountains where was collected type material of a new species. Tien-Shan mountains in Chinese means "celestial mountains" and in Latin celestial is mean *caelestis,* mountains – *montanus*.

Adult male (n = 1).

Total length 4.5 mm. Total length/wing length 1.01.

Coloration. Head, thorax (except scutellum), legs, and abdomen brown to dark brown; antennae light brown; scutellum gray; wings greyish.

Head. Eyes bare and strongly extended dorsomedially. Temporal setae 29–34, including 4 coronals, 20 preoculars, 5–6 verticals, and 13 postorbitals. Clypeus with 42 setae. Antenna with 13 flagellomeres and well developed plume, these setae 840–1040 µm long; pedicel with 4 setae 38–40 µm long; terminal flagellomere with 1 subapical setae 56 µm long. AR 2.21. Palpomeres lengths (in µm): 112; 132; 200; 284; 340. Palpomere 3 in distal part with sensilla capitata (diameter 20 µm). Palpomeres 1–5 length/head width 1.21.

Thorax. Antepronotum with 4–5 dorsomedial and 12–18 ventrolateral setae. Acrostichals 15 (in middle part of mesonotum), dorsocentrals 26 (in 1–2 rows, in middle part), prealars 20–24 (in 2 groups), scutellars *ca* 60.

Wing. Length 4.44 mm; width 1.14 mm. Membrane without setae. R and R_1 with 39–40 setae; R_{4+5} with 15–16 setae. Costa extension 56 µm long. RM length/MCu length 2.83–3.2. Anal lobe well developed and protrude forward, rounded. Squama with 39–40 setae 100–180 µm long, in 1–2 rows. VR 0.85.

Legs. Spur of fore tibia 112 µm long; spurs of mid tibia 80 µm; of hind tibia 124 µm and 84 µm long. Hind tibial comb with 16 setae. Lengths and proportions of leg segments as in Table 1.

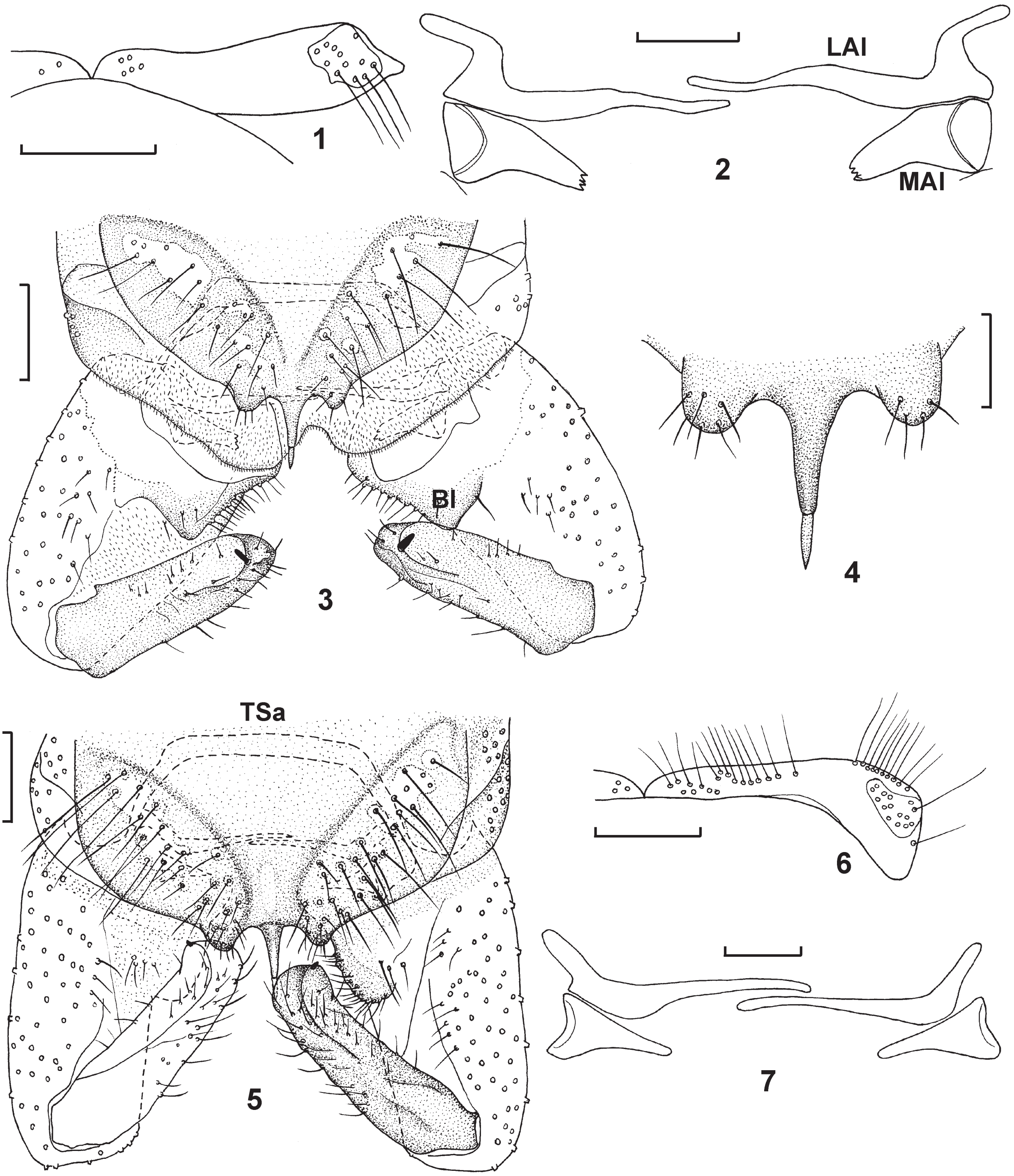
TABLE 1. Lengths (in µm) and proportions of leg segments of *Pagastia (P.) caelestomontana* **sp. nov.**, male (n=1).

	fe	ti	ta_1	ta_2	ta_3	ta_4	ta_5	LR	BV	SV	BR
P_1	1600	1840	1320	760	480	260	200	0.72	2.80	2.61	2.7
P_2	1640	1720	940	520	340	200	180	0.55	3.47	3.57	3.3
P_3	1600	2180	1320	680	440	240	180	0.52	3.31	2.86	3.2

Hypopygium (Figs. 2–4, 8–9). Tergite IX with 18–24 setae on each side and with an anal point 80 µm long, apically with peg 28 µm long. Tergite IX laterally from anal point on each side with rounded small lobe (Fig. 4). Laterosternite IX with 14–16 setae. Transverse sternapodeme trapezoidal, 312 µm long. Phallapodeme 52–56 µm long. Gonocoxite 292 µm long, basal lobe large, angular, with long setae along outer margin and with 2 setae at inner part (Figs. 3, 8). Median aedeagal lobe 80–88 µm long, wedge-shaped and with toothed apex; lateral aedeagal lobe narrow, 156–164 µm long (Fig. 2). Gonostylus 252–256 µm long, massive, with a rounded or rounded-angular protrusion basally along the outer edge, with megaseta 20 µm long. HR 1.14–1.16.

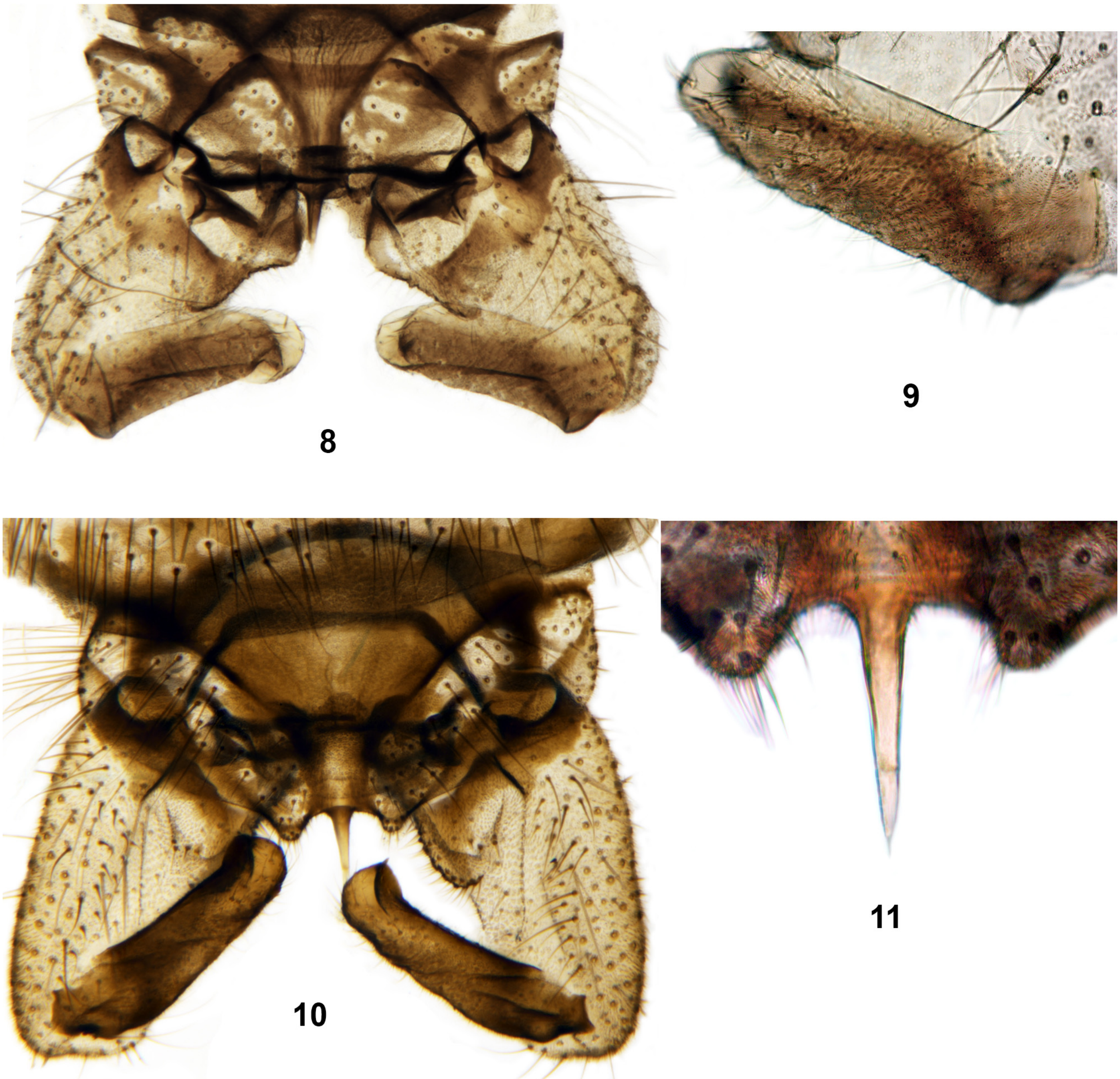
Pupa unknown.

Larva was described as *Pagastia* (*P.*) sp. 1 by Makarchenko & Makarchenko (2000).



FIGURES 1–7. *Pagastia* (*P.*) *caelestomontana* **sp. nov.** (1–4) and *P.* (*P.*) *hanseni* **sp. nov.** (5–7), males. **1, 6,** anteropronotum in dorsal view; **2, 7,** aedeagal lobes; **3, 5,** hypopygium in dorsal view; **4,** anal point. Bl, basal lobe of gonocoxite; LAl, lateral aedeagal lobe; MAl, median aedeagal lobe TSa, transverse sternapodeme. Scale bars for figures 1, 3, 5, 6 – 200 µm, for figures 2, 4, 7 – 50 µm.

Diagnosis. The male of this new species is most closely related to *P.* (*P.*) *hanseni* **sp. nov.** and can be separated in the following features: antepronotum with dorsomedial and ventrolateral setae; clypeus with 42 setae; AR 2.21; Dc 26 (in 1–2 rows), Pa 20–24; basal lobe of gonocoxite large and angular, median aedeagal lobe wedge-shaped and with toothed apex, lateral aedeagal lobe 156–164 µm long, gonostylus massive and with rounded or rounded-angular protrusion basally along the outer edge. The male of *P.* (*P.*) *hanseni* **sp. nov.** has antepronotum completely covered with setae; clypeus with *ca* 90 setae; AR 3.80; Dc 53–54 (in 2–4 rows), Pa 47–50; basal lobe of gonocoxite middle-sized and rounded, median aedeagal lobe wedge-shaped and with fingerlike apex, lateral aedeagal lobe 132–140 µm long; gonostylus slender, almost straight, of same thickness, sometimes slightly widened at the base, slightly curved apically. The larva of *P.* (*P.*) *caelestomontana* **sp. nov.** differs from all known species by the presence on the head capsule dorsal and dorsolateral dark markings (Makarchenko & Makarchenko 2000, Fig. 8–9). See also key above and results of DNA barcoding.



FIGURES 8–11. *Pagastia* (*P.*) *caelestomontana* **sp. nov.** (8–9) and *P.* (*P.*) *hanseni* **sp. nov.** (10–11), males. **8, 10,** hypopygium in dorsal view; **9,** gonostylus; **11,** anal point.

Ecology. Larvae were collected from stones and bowlders covered with algae and moss in mountain rivers, located at an altitude of 1717–2801 m, at a flow rate of 0.3–0.8 m/s, with water temperature *ca* 12°C and in spring at an altitude of 2025 m.

Distribution. Known only from the type locality in Tien Shan and Pamir Mountains (Kirgizstan and Tajikistan) (Figs. 12, 15).

Pagastia* (*P.*) *hanseni **Makarchenko, Semenchenko et Palatov, sp. nov.**
(Figs. 5–7, 10–11)
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Material. Holotype: adult male, Central Asia, Tajikistan, Gorno-Badakhshan Autonomous Region, Roshtqal'a District, Soktosh River, 2.3 km west of the ruins of the Derouj fortress, 2.VII.2016, alt. 3366 m above sea level, N 37°22.717', E 72°20.961', leg. D. Palatov. Paratypes: 1 adult male, 12 larvae, same data as holotype; 10 larvae, Tajikistan, Gorno-Badakhshan Autonomous Region, Shughnon District, aryk in the valley of the Toguzbulok River, at the beginning of the road to the Maysara Pass, 01.07.2016, alt. 3717 m above sea level, N 37°30.822', E 72°40.545', leg. D. Palatov.

Derivatio nominis. The species is named in honour of the American chironomid taxonomist of Diamesinae Dean Cyrus Hansen.

Adult male (n = 1).

Total length 5.0 mm. Total length/wing length 1.0.

Coloration. Head, thorax, legs, and abdomen brown to dark brown; antennae light brown or yellowish-brown; wings greyish.

Head. Eyes bare and extended dorsomedially. Temporal setae consisting of 4 coronals, 30–33 orbitals, 8 verticals, and 15–18 postorbitals. Clypeus with *ca* 90 setae. Antenna with 13 flagellomeres and a well developed plume; pedicel with 2–3 setae; terminal flagellomere with 1 subapical setae 48 µm long. AR 3.80. Palpomere lengths (in µm): 72; 136; 249; 304; 356. Distal part of palpomere 3 with sensilla capitata of 16–20 µm diameter. Palpomeres 1–5 length/head width 1.09.

Thorax. Antepronotum completely covered with 51 setae, 100–268 µm long (Fig 6). Acrostichals 14 (80–88 µm long); dorsocentrals 53–54 (168–260 µm long), in posterior part in 4 rows, in middle and anterior parts in 2–3 rows; prealars 47–50 (152–260 µm long); scutellars 73.

Wing. Length 4.96 mm; width 1.16 mm. Membrane without setae. R and R_1 with 21–22 setae; R_1 in distal part without setae; R_{4+5} without setae. Costa extension 72 µm long. RM length/MCu length 3.25. Anal lobe developed, rounded and slightly protrude. Squama with 36–44 setae in 2–3 rows. Alula without setae. VR 0.84.

Legs. Spur of fore tibia 128 µm long; spurs of mid tibia 92 µm and 100 µm long; spurs of hind tibia 136 µm and 92 µm long. Hind tibial comb with 12–13 setae. Lengths and proportions of leg segments as in Table 2.

TABLE 2. Lengths (in µm) and proportions of leg segments of *Pagastia (P.) hanseni* **sp. nov.**, male (n=1).

	fe	ti	ta_1	ta_2	ta_3	ta_4	ta_5	LR	BV	SV	BR
P_1	1440	1560	1120	640	400	240	240	0.72	2.72	2.68	2.8
P_2	1560	1440	740	460	320	200	240	0.51	3.07	4.05	2.4
P_3	1900	2080	1200	700	400	280	260	0.58	5.51	3.32	4.5

Hypopygium (Figs. 5, 7, 10–11). Tergite IX with 29–30 setae on each side and with an anal point 88 µm long, apically with peg 28 µm long. Tergite IX laterally from anal point on each side with rounded small lobe (Fig. 11). Laterosternite IX with 11–12 setae. Transverse sternapodeme trapezoidal, 212 µm long. Phallapodeme 48–52 µm long. Gonocoxite 384–392 µm long; basal lobe of medium size, rounded, with long setae along inner margin and with 4 setae at inner part, 28–32 µm long (Figs. 5, 10). Median aedeagal lobe 72 µm long, wedge-shaped, with fingerlike apex, 72 µm long; lateral aedeagal lobe narrow, 132–140 µm long (Fig. 7). Gonostylus 280 µm long, slender, almost straight, of same thickness, sometimes slightly widened at the base, slightly curved apically with megaseta 16–20 µm long. HR 1.37–1.40.

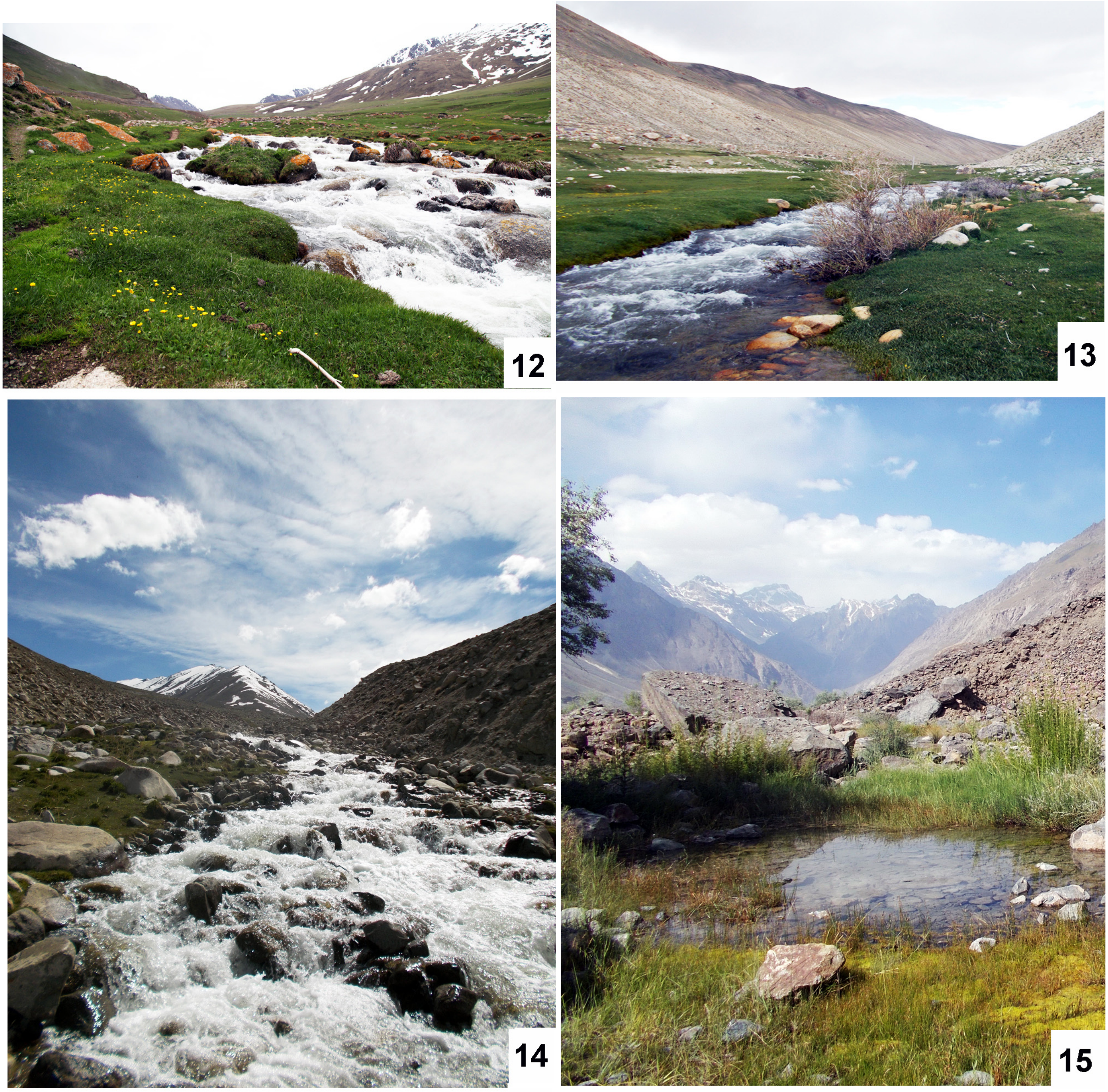
Pupa unknown.

Larva not separable from *P.* (*P.*) *caelestomontana* **sp. nov.,** can be distinguished only by molecular genetic data.

Diagnosis. The male of this new species is most closely related to *P.* (*P.*) *caelestomontana* **sp. nov.** See the diagnosis of this species for details, the key and results of DNA barcoding.

Ecology. Larvae were collected from stones and bowlders sometimes covered with algae and moss in mountain rivers, located at an altitude of 2016–3717 m, at a flow rate of 0.4–1.0 m/s, with water temperature *ca* 12°C.

Distribution. Known only from the type locality in Pamir Mountains (Tajikistan) (Fig. 13).



FIGURES 12–15. Type localities of *Pagastia* (*P.*) *caelestomontana* **sp. nov. (12)**, *P.* (*P.*) *hanseni* **sp. nov. (13)**, and *P.* (*P.*) aff. *lanceolata* (Tokunaga) **(14–15)**. **12,** Otmek River, Talas district, Kirgizstan (photo by E.S. Chertoprud); **13,** Soktosh River, Gorno-Badakhshan Autonomous Region, Roshtqal'a District, Tajikistan (photo by D.M. Palatov); **14,** Tuzumtaikul River, Gorno-Badakhshan Autonomous Region, Shughnon District, Tajikistan (photo by M.V. Vinarski); **15,** spring in front of the mouth of the Bartang River, Gorno-Badakhshan Autonomous Region, Rushanskiy District, Tajikistan (photo by M.V. Vinarski).

Pagastia (*P.*) aff. *lanceolata* (Tokunaga, 1936)

Material. 1 larva, Central Asia, Tajikistan, Gorno-Badakhshan Autonomous Region, Shughnon District, Tuzumtaikul River within the Jelondy village, about 400 m above the mouth, alt. 3560 m above sea level, 01.VII.2016, N 37°34'26.56", E 72°34'48.29", leg. D. Palatov; 2 larvae, the same region, Rushanskiy District, spring in front of the mouth of the Bartang River, near the bridge to Rushan Town. 03.VII.2016, alt. 2025 m above sea level, N 37°55.493', E 71°36.183', leg. D. Palatov.

Remarks. The larvae collected in mountain river and spring of Pamir do not differ in morological characteristics from P. (*P.*) *lanceolata* (Tokunaga), but molecular genetic data allow us to assert about an independent and possibly new species of *Pagastia* (Fig. 16).

Ecology. Larvae were collected from stones and bowlders covered with moss in mountain river, located at an altitude of 3569 m, at a flow rate of 0.3–1.0 m/s, with water temperature 5°C and in spring from stones covered with algae, at an altitude of 2025 m, at a flow rate of 0.2–0.5 m/s.

Distribution. Known only from one river and spring in Pamir Mountains (Tajikistan) (Figs. 14–15).

Results of DNA barcoding and phylogenetic relationships

Overall, we have sequenced fragments of the cytochrome oxidase subunit I (658 bp) and complete cytochrome oxidase subunit II with two tRNAs, tRNA-Leu and tRNA-Lys (741-790 bp) of 15 samples and 14 samples respectively. The list of samples consists of five *Pagastia* (*P.*) *orientalis*, three *P.* (*P.*) *nivis*, one *P.* (*P.*). *caelestomontana*, three *P.* (*P.*) aff. *lanceolata* (two for COII) and two of *P.* (*P.*) *lanceolata*. As an outgroup for phylogenetic trees, we also sequenced one samples of *Pseudodiamesa stackelbergi* (Goetghebuer).

To perform the final dataset, we added COI sequences of *Pagastia* (*P.*) *orthogonia* from GenBank under numbers KR641660, KR643221 and KR641487 (Hebert *et al.* 2018). Dataset consist of 35 specimens belonging to eight *Pagastia* species and including the outgroup *P. stackelbergi.*

In COI dataset excluding *P. stackelbergi* nucleotide frequencies of A, T, G, and C were 26.3%, 38.6%, 17.1%, and 18.1%, respectively. Dataset contained 156 (23.7%) variable sites 146 (22.2%) of which was parsimony informative. The results for COII were as follows: 33.1%, 38.0%, 13.7%, 15.2% for A, T, G, and C respectively. Variable and parsimony informative were 170 and 153 respectively. The K2P sequence divergence within and between seven *Pagastia* species shown in the Table 3, the obtained interspecific K2P corresponds to species level (Montagna *et al.* 2016). The high differences between seven *Pagastia* species were also confirmed by ABGD analysis, which yielded 7 operational taxonomic units (OTU) using a 0.0050–0.0097 intraspecific divergence of COI gene.

TABLE 3. Intraspecific and interspecific K2P nucleotide distances of seven *Pagastia* species estimated using COI and COII sequences. Mean values of interspecific COI distances are under the diagonal and COII distances above diagonal.

Species	Intraspecific		Interspecific					
	COI	COII	1	2	3	4	5	6
1. *P.*(*P.*) *caelestomontana* **sp. nov.**	0.55	0.55		11.39	13.30	14.31	12.52	12.03
2. *P.* (*P.*) *hanseni* **sp. nov.**	1.01	0.57	9.4		12.08	13.19	10.56	10.75
3. *P.* (*P.*) *lanceolata* (Tokunaga)	0.61	0.00	10.8	12.1		8.68	12.94	12.32
4. *P.* (*P.*) aff. *lanceolata* (Tokunaga)	1.13	0.59	14.5	13.2	10.0		12.95	12.72
5. *P.* (*P.*) *nivis* (Tokunaga)	1.44	0.88	11.9	12.3	13.2	12.6		7.48
6. *P.* (*P.*) *orientalis* (Tshernovskij)	1.33	0.81	11.3	11.1	12.7	11.8	6.2	
7. *P.* (*P.*) *orthogonia* Oliver	0.72	n/a	11.0	11.4	9.3	11.9	11.1	10.5

Bayesian inference (BI) phylogeny revealed a two strongly supported clades (PP = 1.00) at the base of the tree. The first clade (PP = 0.94, ML = 66) includes *P.* (*P.*) *orthogonia* as the earliest branching lineage and a later branching *P.* (*P.*) *lanceolata* and *P.* (*P.*) aff. *lanceolata* (PP = 0.84, ML = 60). The similarity of the last two species is also confirmed by the identity of the structure of their larvae (see above). The monophyly of second clade was well supported (PP = 1, ML = 67). Clade formed by *P.* (*P.*) *caelestomontana* **sp. nov.**, *P.* (*P.*) *hanseni* **sp. nov.** and *P.* (*P.*) *nivis* + *P.* (*P.*) *orientalis* as sister groups. This clade is inconsistent with morphological data, since the similar structure of the larva between *P.* (*P.*) *caelestomontana* **sp. nov.** and *P.* (*P.*) *hanseni* **sp. nov.** is not confirmed by their monophyly. In turn, morphologically closely related species *P.* (*P.*) *nivis* and *P.* (*P.*) *orientalis* (Makarchenko & Makarchenko 2000) are sister and their clade is highly supported (PP = 1, ML = 99).

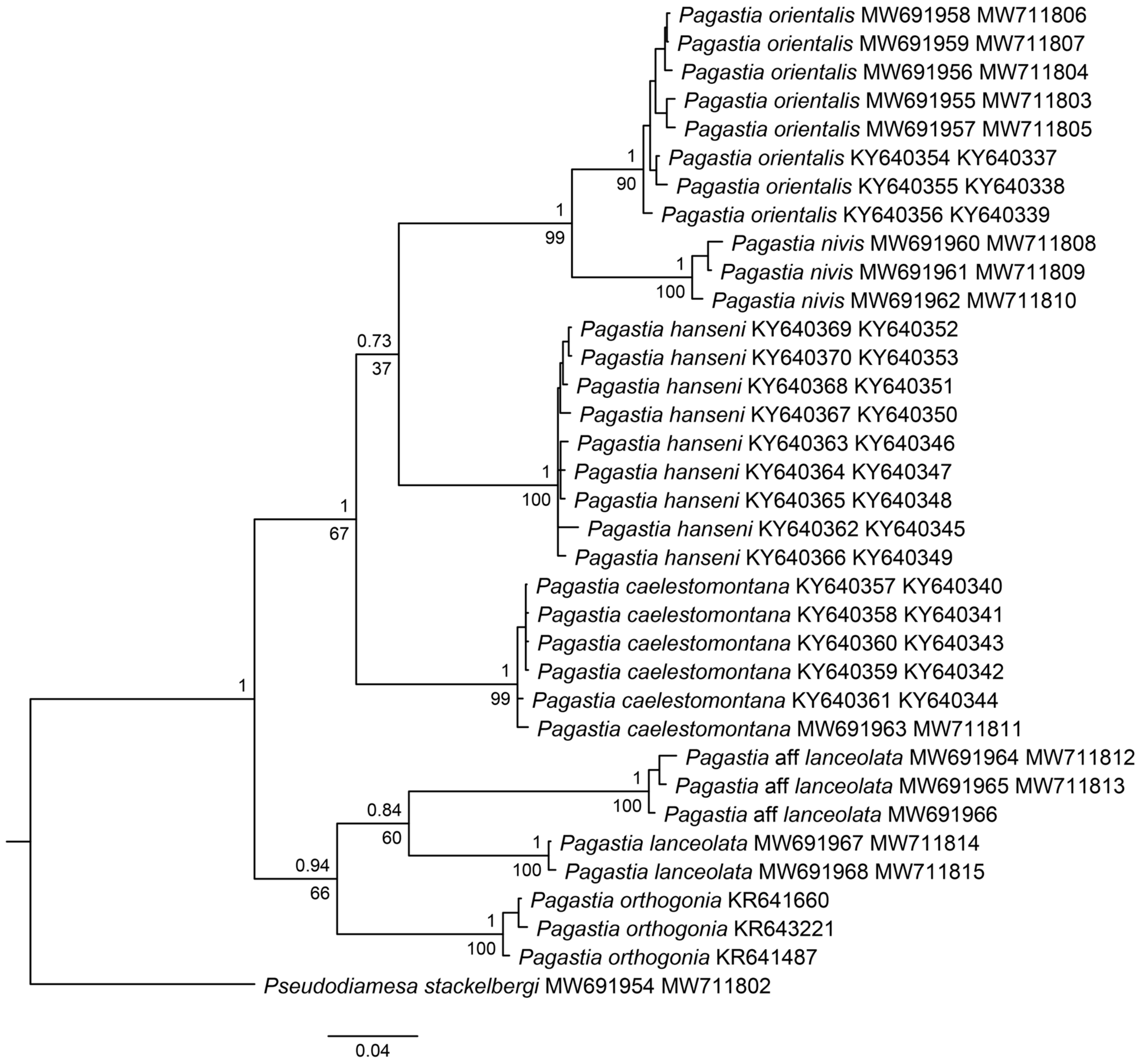


FIGURE 16. Bayesian tree based on mitochondrial COI and COII genes for available members of the genus *Pagastia* Oliver with *Pseudodiamesa stackelbergi* (Goetghebuer) as outgroup. Bayesian posterior probabilities (PP) are given above tree nodes and bootstrap support values found in the ML analysis are shown below nodes.

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