

First Comprehensive Tadpole Description of the Relict and Endemic Mountain Frog *Chrysopaa sternosignata* (Murray 1885) from Afghanistan

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ABSTRACT: The Baluch Mountain Frog, *Chrysopaa sternosignata* (Murray 1885), is one of the least known amphibian species in the Hindu Kush–Himalayan region. It is endemic to an area on the edge of the Palearctic region with harsh environment and with the long-term complicated security situation, where biodiversity research is difficult or virtually impossible. Thus, very little is known about the life history, ecology, and distribution of this frog, representing the monotypic genus *Chrysopaa* (Dicroglossidae). Similarly, data about the larva of this taxon are scarce. Thus, we provide the first detailed imagery and description of the larval stage of *C. sternosignata* (Murray 1885) from Afghanistan. One tadpole was obtained from Jabul Saraj, Charikar, Afghanistan. Morphological and genetic analysis of mitochondrial and nuclear DNA confirmed the identity of the larva as *C. sternosignata*. Tadpole characters were illustrated by photos. Basic measurements and details on oral apparatus provide relevant characteristics to delimit the larva of this species from other spiny frogs. This information should facilitate the identification of *C. sternosignata* larvae in museum collections and in the field.

Key words: Dicroglossidae; DNA; Endemism; Hindu Kush; Identification; Larva; Murray's frog

BALUCH Mountain Frogs, *Chrysopaa sternosignata* (Murray 1885), belong to the tribe Paini (Dicroglossidae), a group of Asian frogs that occur in the Hindu Kush–Himalayan region, particularly in mountains of Afghanistan, Pakistan, and northern India, through Nepal, Sikkim, and Bhutan, and in the valleys of southern and eastern Tibet, eastwards to eastern China, and southwards to the mountains of Indochina (Frost 2022). Phylogenetic data suggest an origin of the Paini tribe in Paleogene East or Southeast China (Che et al. 2010; Hofmann et al. 2021a). Currently the tribe is formed by the genera *Quasipaa* Dubois 1992 (11 species), *Nanorana* Günther 1896 (around 30 species), *Allopaa* with possibly two species and which should be treated as *Nanorana* (see Hofmann et al. 2023; we follow this taxonomic arrangement), and the monotypic *Chrysopaa* Ohler and Dubois 2006. The latter is endemic to parts of the Hindu Kush and the Sulaiman Mountain range of Pakistan and Afghanistan, north of the Indus River valley, and represents the westernmost taxon of the Paini (Fig. 1). The genus *Chrysopaa* seems to be exclusively aquatic, active during the day and night, and occupies predominantly regions in the colline zone with warm-temperate, arid climate (Hofmann et al. 2023). The species has been reported to be common in pools and water-channels in the Quetta and Pishin district of Balochistan in Pakistan from 1500 up to 1800 m above sea level (a.s.l.; Boulenger 1920) as well as in other areas of southern, western, and central Afghanistan (Wagner et al. 2016) where it occupies different habitats (i.e., both rivers and/or lakes; D. Jablonski, personal observation). Males are characterized by dark, keratinous, nuptial spines, scattered on the first two fingers and the inner carpal tubercle (Khan 2004). Phylogenetically, *Chrysopaa* is placed basally relative to *Nanorana*, and probably evolved in the

Oligocene, more than 20 Mya (Hofmann et al. 2021a). Interestingly, this frog shows no close phylogenetic relationship to geographically neighboring Himalayan spiny frogs, indicating a migration of the ancestor from the eastern margin of the Himalaya–Tibet–Orogen (HTO) across paleo-Tibet into the far western area of the HTO (Hofmann et al. 2021a).

Chrysopaa sternosignata was originally described from the Sindh province in Pakistan (Murray 1885). The syntypes, presumably at the Karachi Museum, are apparently lost (Frost 2022); two further syntypes are noted in the BMNH (1947.2.1.21–22; Boulenger 1920), with the type localities “Mulleer [= Malir] near Kurrachee [= Karachi]; Zandra and Quetta, in South Afghanistan” (Frost 2022; all localities actually in Pakistan). Given the geographically and climatically distinct position of Malir (arid areas of southern Pakistan), we consider the occurrence of the species at this locality as unlikely (Hofmann et al. 2023; see also Mertens 1969). Little is known about the life history, ecology, and distribution of *Chrysopaa* and far less recent data are available about the larva of this taxon. The main distribution area of the species is located within a country that has been inaccessible for zoological studies for 40 yr as a result of armed conflicts (Jablonski et al. 2021). Although, some information (of varying levels of completeness) on the larval stage of *Chrysopaa* exists in the older literature (under *Nanorana sternosignata*; Annandale and Rao 1918; Boulenger 1920; Khan 2004; Ohler and Dubois 2006), to date no comprehensive description supported with molecular data and photographs is available.

Thus, we provide the first detailed photographs, and a detailed description, of a *Chrysopaa sternosignata* tadpole from Afghanistan. We used mitochondrial and nuclear DNA sequence data to validate the identity of our specimen by assigning it to existing *Chrysopaa* sequences.

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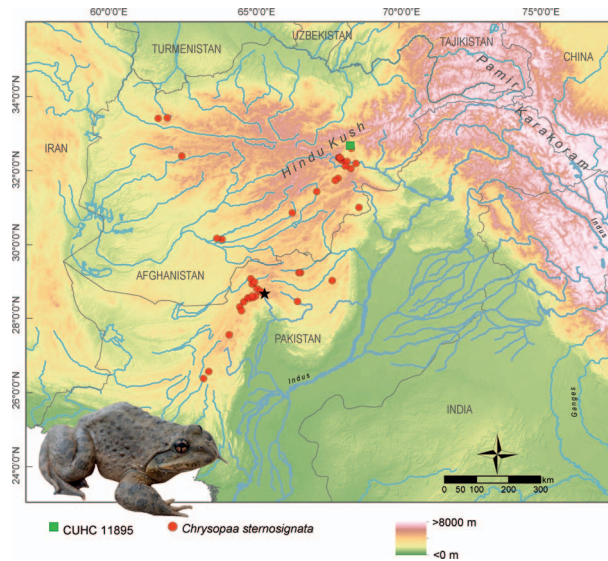


FIG. 1.—Map showing the sample location of the *Chrysopaa sternosignata* specimen CUHC 11895 and known records (for details of records, see Hofmann et al. 2023). The type locality of *C. sternosignata* is indicated by a black star. The inserted photo at the lower left shows an adult male from Qargha Dam, Kabul, Afghanistan (photo credit: D. Jablonski). A color version of this figure is available online.

MATERIAL AND METHODS

Sampling, Illustrations, and Character Assessment

One larva (CUHC 11895; Comenius University Herpetological Collection) was collected by D. Jablonski in August 2022 during daytime in Charikar, Afghanistan ($35^{\circ}00'15.9''\text{N}$ $69^{\circ}11'56.4''\text{E}$, datum = WGS84 in all cases, 1,510 m a.s.l.; Fig. 1). The collected tadpole was found in a small garden pond (Fig. 2) as part of another, bigger pond where two more adult individuals were also observed. The artificial ponds had a size of approximately 5×2 m, and 0.5×0.5 m, 1 m deep. The surrounding vegetation consisted of vine and eucalyptus trees. The overall habitat can be characterized as urban environment in the valley formed by villages with clay buildings. The tadpole was collected using a net. After taking photographs, the specimen was anaesthetized with ethyl acetate, fixed in 80% ethanol for 4–6 h, and then later transferred to 70% ethanol for permanent storage. Before preservation, a blood sample was taken from the specimen, transferred into absolute ethanol, and stored at -20°C . The study was conducted according to the regulations for the protection of terrestrial wild animals under the permits for access to genetic resources and export (Permit nos. 12455 and 12429 to D. Jablonski) issued by the National Environmental Protection Agency of the Islamic Emirate of Afghanistan. The specimen is deposited in the herpetological collection of the Comenius University Bratislava, Slovakia.

Digital photos of the tadpole were taken in the field before preservation with a Nikon D810 digital camera and 105-mm macro lens. At the lab, the specimen was then staged according to Gosner (1960), and morphologically investigated under a stereo-microscope. Standardized lateral, dorsal, and ventral views were photographed with a Nikon D750 digital camera, a 105-mm macro lens, and a teleconverter 2.0 \times for detail shots. Morphometric measure-



FIG. 2.—The collection site the *Chrysopaa sternosignata* tadpole (CUHC 11895) from Jabul Saraj, Charikar, Afghanistan (photo credit: D. Jablonski). A color version of this figure is available online.

ments of external characters were taken with a digital caliper (accuracy ± 0.1 mm). Tadpole terminology follows Altig and McDiarmid (1999); the following measurements were obtained: TL (total length), BL (body length), TAL (tail length), TMH (tail muscle height at tail base), TMW (tail muscle width at tail base), IOD (interorbital distance), IND (internarial distance), EN (eye–nostril distance), ODW (oral disc width). Characteristics of the oral apparatus were described according to Altig (1970). The arrangement of keratodonts (= labial tooth rows) is presented according to Altig and McDiarmid (1999), with the anterior (A-) and posterior (P-) rows indicating gaps in brackets and a backslash separating the upper and lower jaw sheaths (Schulze et al. 2015). This information is summarized in a Labial Tooth Row Formula (LTRF) and as Keratodont Row Formula (KRF) in accordance with Dubois (1995). Additional data on size and KRF of other Painsi tadpoles were compiled from literature.

DNA Extraction, Sequence Alignment, and Phylogenetic Reconstruction

Genomic DNA was isolated from blood tissue using the E.Z.N.A.[®] Tissue DNA Kit (Omega Bio-tek, Inc.) following the manufacturer's protocol. Approximately 570 base-pairs (bp) of 16S ribosomal RNA (16S), 539 bp of the cytochrome oxidase I (COI), and 1207 bp of recombination activating gene 1 (Rag1) were amplified following conditions and primers of Hofmann et al. (2019). Polymerase chain reaction products were purified using the ExoSAP-IT enzymatic

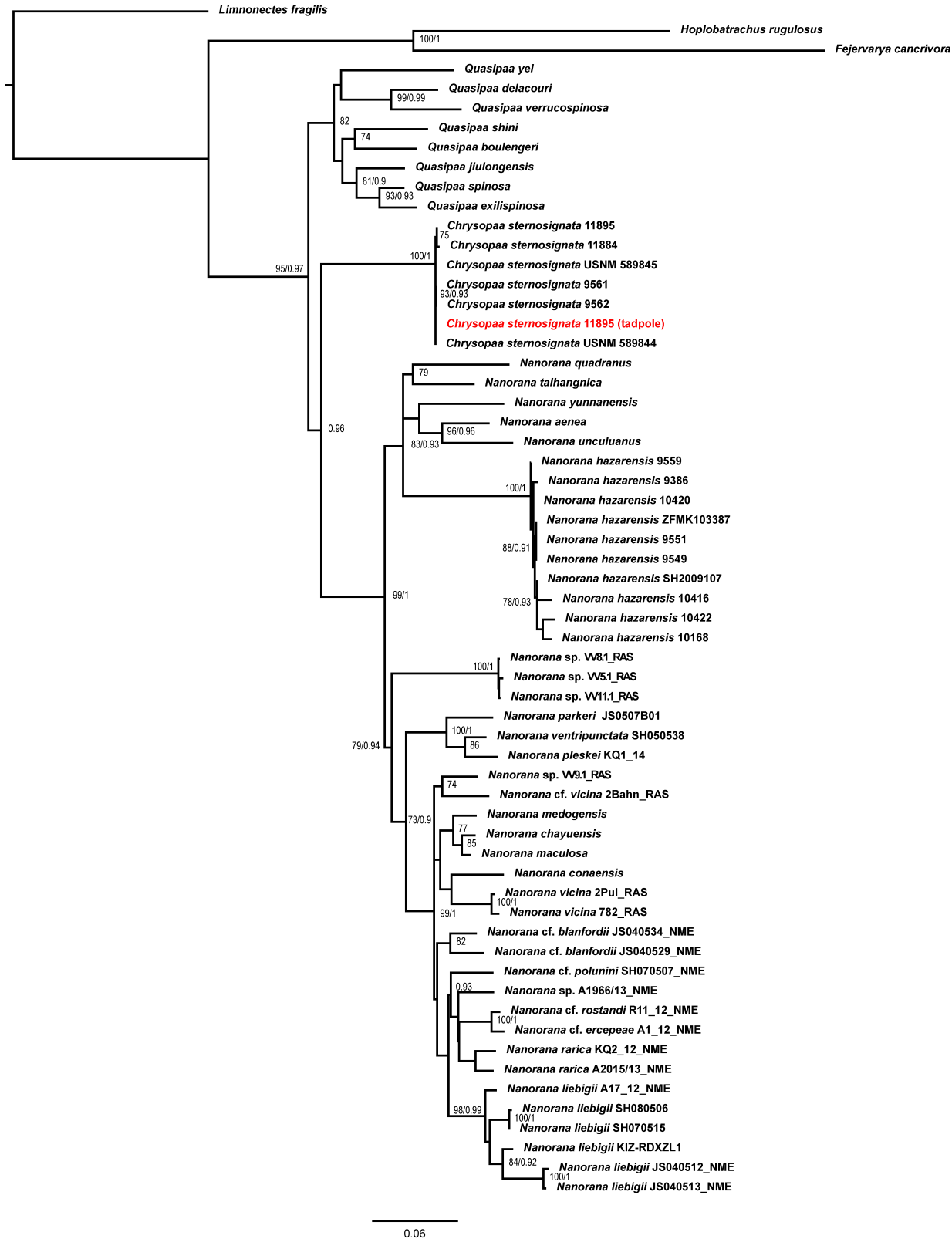


FIG. 3.—Maximum-likelihood tree based on concatenated mtDNA+nuDNA sequence data. Numbers at branch nodes refer to Felsenstein's bootstrap values $\geq 70\%$ and Transfer Bootstrap Expectation ≥ 0.9 . Taxon name is followed by specimen or voucher ID if available; ID of the tadpole CUHC 11895 described herein is indicated red. A color version of this figure is available online.

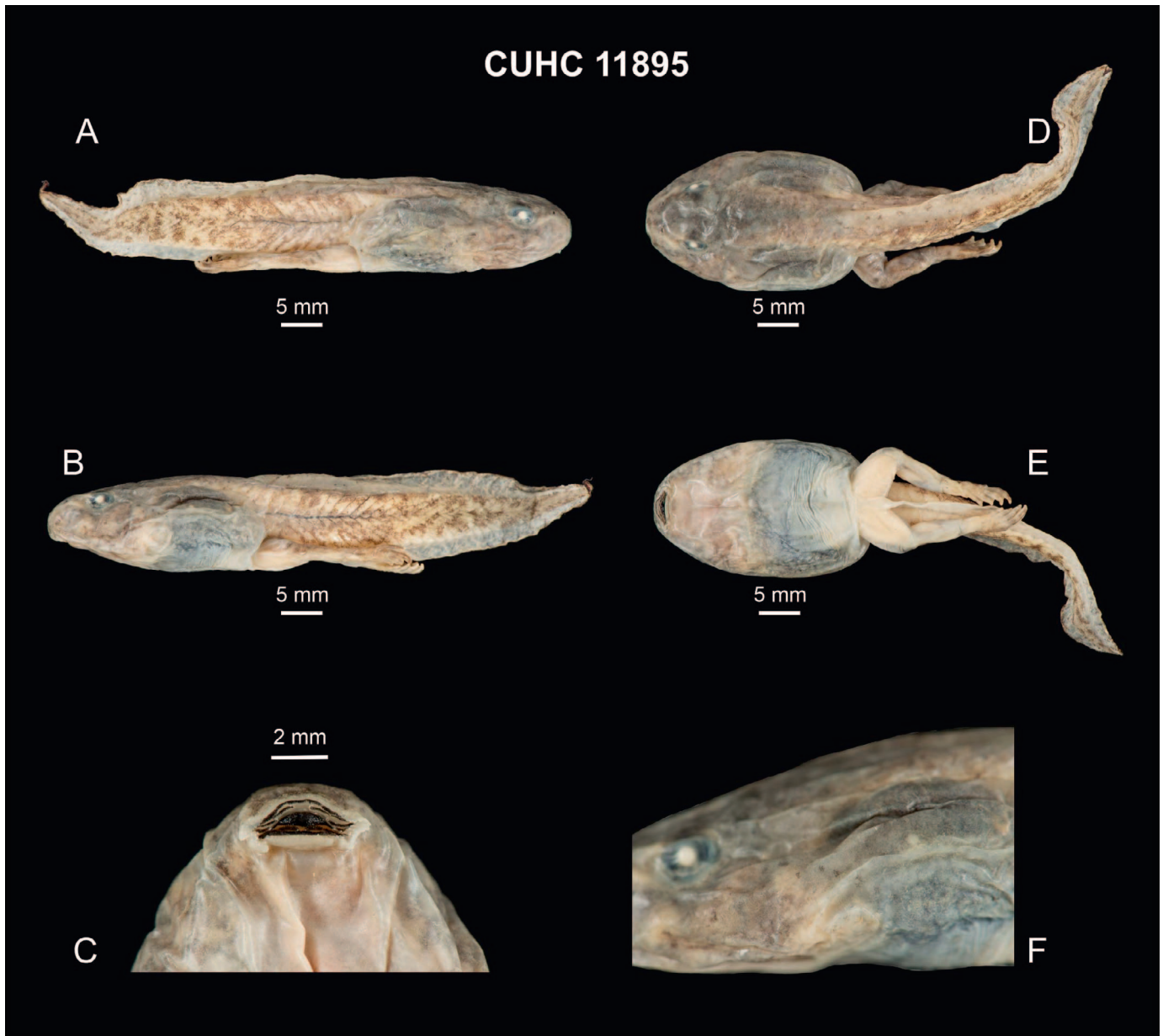


FIG. 4.—Fixed tadpole at Gosner stage 38 of *Chrysopaa sternosignata* (CUHC 11895). (A, B) ventral views; (C) mouthpart; (D, E) dorsal and ventral views; (F) spiracle. A color version of this figure is available online.

clean-up (USB Europe GmbH, Staufen, Germany) and sequenced (for nuclear *Rag1* in both directions) by Macro-gen Europe (Amsterdam, The Netherlands; see <https://www.macro-gen-europe.com/>).

We aligned the newly generated sequences (GenBank accession numbers: 16S, OP861627–OP861629; COI, OP866780–OP866782; *Rag1*, OP902369–OP902371) to selected data available from our previous studies (Hofmann et al. 2019, 2021b), including DNA sequence data of *Chrysopaa* (for accession numbers see Supplemental Table S1, available online). The final concatenated sequence data set consisted of 61 sequences and contained 2316 bp. We inferred a maximum-likelihood tree using RAxML-NG v1.1.0 (Kozlov et al. 2019) using the data set partitioned by genes and codons, and applying 20 random and 20 parsimony starting trees, 10,000 bootstrap replicates. We

specified the Felsenstein's bootstrap as well as the recently introduced Transfer Bootstrap Expectation (Lemoine et al. 2018) as branch support metrics.

RESULTS

Phylogenetic Assignment

Our tadpole specimen (11895) nested with high support in the clade of *Chrysopaa sternosignata* that includes GenBank sequences of this frog collected from Afghanistan and used in our previous study (Fig. 3; Supplemental Table S1; Hofmann et al. 2023). Sequence identity between our tadpole sequences and those additional *Chrysopaa* sequences available in GenBank (16S, MG700153–MG700155; COI, MG699937, MG699938) was >99%. Specimens CUHC 9561, 9562, 11884, and 11894 originated from Paghman

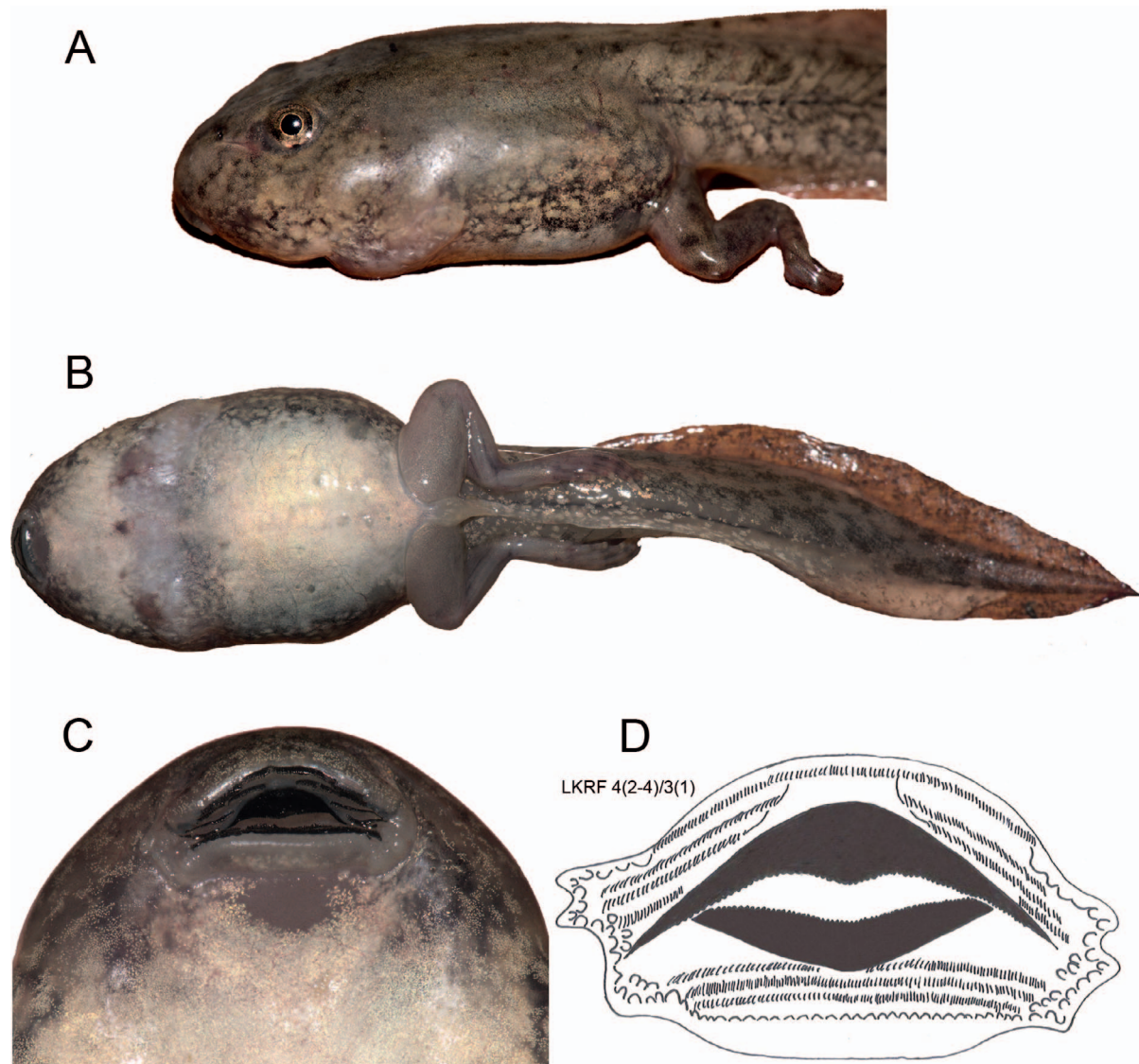


FIG. 5.—Views on the tadpole CUHC 11895 (Gosner Stage 38) of *Chrysopaa sternosignata* from Jabul Saraj, Charikar, Afghanistan, 1,510 m, photographed in the field; (A) lateral, and (B) ventral view; (C) oral apparatus (photo credit: D. Jablonski); (D) drawing of oral apparatus (drawing credit: S. Hofmann). A color version of this figure is available online.

(34°36'51.12"N 68°54'45.72"E), Qargha Dam, Kabul (34°33'35.28"N 69°1'57"E), and Jabul Saraj (35°5'17.88"N 69°12'23.76"E), respectively, ca. 51, 52, and 10 km distant from the collection site of the tadpole CUHC 11895. Sequences of specimens USNM:Herp:589844 and USNM:Herp:589845 are from Parvan, Bagram Air Force Base, Afghanistan (34°56'46.0"N 69°15'54.0"E), ca. 10 km from the tadpole collection site.

Tadpole Description.—*Chrysopaa sternosignata* (Murray 1885)

Material examined.—CUHC 11895 (GenBank: 16S, OP861629; COI, OP866782; Rag1, OP902371), Gosner Stage 38, Afghanistan, Jabul Saraj, Charikar, (35°00'15.9"N 69°11'56.4"E, 1,510 m a.s.l.), 08.08.2022, leg. D. Jablonski.

Description.—Large tadpole with longer, muscular tail (Fig. 4); body ovoid in dorsal and ventral view, compressed in lateral view; snout rounded in dorsal and ventral view,

blunt in lateral view. Eyes and nostrils small, positioned dorsolaterally; broader tail fins, both starting from tail base at trunk–tail junction; tail tip pointed; low sinistral spiracle, below body's midline; oral disc anteroventrally; LTRF 4(2–4)/3(1), KRF 1:3+3/1+1:2; dorsal gap in papillation present; posterior lip with one row of uniformly triangular papillae. Submarginal papillae present and cumulated laterally, in the wrinkle between labia (Fig. 4C, D); dark, robust, serrated jaw sheaths; upper jaw slightly arc-shaped with a small medial projection; lower jaw broadly V-shaped. Measurements: TL 68.0 mm, BL 25.5 mm, TAL 43.6 mm, TMH 6.6 mm, TMW, 6.9 mm, IOD 6.8 mm, IND 3.0 mm, EN 3.8 mm, ODW 5.6 mm.

Color in preservative.—Light grayish brown dorsally and laterally, venter light grayish-beige, body and tail densely and irregularly mottled with darker brownish spots, tail fins mottled, dorsal brownish with dark spots, ventral fin translucent with few spots.

TABLE 1.—Comparison of size and keradont row formula (KRF) of Painsi tadpoles. *C.* = *Chrysopaa*, *N.* = *Nanorana*, *Q.* = *Quasipaa*, TTL = total tadpole length.

Species	Gosner stage	TTL (mm)	KRF	Source
<i>C. sternosignata</i>	38	68	1:3+3/1+1:2	This study
<i>C. sternosignata</i>	40	78–80	1:4+4/3	Khan 2004
<i>C. sternosignata</i>	Not reported	up to 90	1:4+4/1+1:2	Boulenger 1920
<i>N. aenea</i>	26–29	16.8–36.10	1:1+1/1+1:2	Chuaynkern et al. 2018
<i>N. blanfordii</i>	Not reported	60	1:4+4/1+1:2	Boulenger 1920;
	32–33	52	2:3+3/1+1:2	Fei et al. 2012
<i>N. chayuensis</i>	30–31	50	2:4+4 or 2:5+5/1+1:2	Fei et al. 2012
<i>N. conaensis</i>	35–37	65	2:3+3/1+1:2	Fei et al. 2012
<i>N. hazarensis</i>	26	61.6–76.5	1:6+6 or 1:7+7/1+1:2	Hofmann et al. 2021b;
	36–42	65.3–76.8	1:6+6 or 1:7+7/1+1:2	Dubois and Khan 1979
<i>N. liebighii</i>	Not reported	Not reported	2:5+5 or 2:6+6 or 3:4+4 or 3:5+5/1+1:2	Boulenger 1920;
	31–33	57	1:4+4 or 2:3+3/1+1:2	Fei et al. 2012
<i>N. maculosa</i>	26–27	47	2:4+4 or 2:5+5/1+1:2	Fei et al. 2012
<i>N. medogensis</i>	36–38	81	2:4+4/1+1:2	Fei et al. 2012
<i>N. parkeri</i>	36	51	1:2+2 or 1:3+3/1+1:2	Fei et al. 2012
<i>N. pleskei</i>	29–34	27	1:2+2/1+1:2	Fei et al. 2012
<i>N. quadranus</i>	38–41	86	2:6+6 or 2:7+7/1+1:2	Fei et al. 2012,
<i>N. sichuanensis</i>	36–39	72	1:4+4/1+1:2	Fei et al. 2012
<i>N. taihangnica</i>	27–28	51	2:6+6 or 1:7+7/1+1:2	Fei et al. 2012
<i>N. unculuanus</i>	35	37.0	2:4+4/1+1:2	Fei et al. 2012
<i>N. ventripunctata</i>	31–34	49	1:1+1 or 1:2+2/1+1:2	Fei et al. 2012
<i>N. vicina</i>	Not reported	Not reported	2:3+3/1+1:2	Gill et al. 2020
<i>N. yunnanensis</i>	30–34	52	1:4+4/1+1:2	Fei et al. 2012;
	29–35	Not reported	1:3+3/1+1:2	Thongproh et al. 2023
<i>N. zhaoermii</i>	27–40	49.3–76.7	1:2+2 or 2:3+3 or 2:2+2 or 1:4+4/1+1:2	Shuo et al. 2019
<i>Q. boulengeri</i>	36–38	52	1:4+4/1+1:2	Fei et al. 2012
<i>Q. exilispinosa</i>	30–36	58	1:3+3 or 1:4+4/1+1:2	Fei et al. 2012
<i>Q. fasciculispina</i>	28,37	71.9,77.7	2:5+5/1+1:2	Inthara et al. 2009
<i>Q. robertingeri</i>	27–35	41–63	1:4+4/1+1:2	Fei et al. 2012
<i>Q. shini</i>	36–38	66.00	1:5+5/1+1:2	Fei et al. 2012
<i>Q. spinosa</i>	34–38	59	1:4+4/1+1:2	Fei et al. 2012
<i>Q. verrucosipinosa</i>	27–29	71.1–75.4	1:5+5/1+1:2 or 2:4+4/1+1:2	Inger et al. 1999

Color in life.—Light brown with dark speckling; venter light grayish-beige, branchial chamber area with reddish-brownish spots; oral disc translucent gray; pupil black and rounded (Fig. 5).

DISCUSSION

To date, very little is known about the tadpole morphology and ecology of *Chrysopaa sternosignata*. According to Khan (2006), tadpoles of this species are largest and stoutest among all tadpoles of frogs and toads of the Hindu Kush region. They live in sympatry with other larvae of different taxa (e.g., *Euphylyctis adolfi* or *Bufo* spp.) and feed on algal vegetation present in water bodies. Tadpoles of *Chrysopaa* can overwinter in water and metamorphosize early the next summer (Khan 2004), similar to what has been suspected for the geographically neighboring spiny frog *Nanorana hazarensis* (Hofmann et al. 2021b).

Our new data mainly agree with previous short reports (Boulenger 1920; Khan 2004) characterizing *C. sternosignata* larvae as large (78–80 mm at Stage 40, Khan 2004; up to 90 mm long, Boulenger 1920), with a strong muscular tail. The ventrally located oral disc is prominent, bordered by long papillae with a larger gap on the upper lip, and the serrated jaws are massive. Three labial tooth rows are present in the posterior part with P_1 being discontinuous. In contrast, Khan (2004) report all posterior tooth rows as continuous. He did not confirm tadpole identity with molecular data but by association with adult frogs. The number of keratodonts varies between four and five in the anterior part of the mouth with A_2 – A_n being discontinuous

rows (this study; Boulenger 1920; Khan 2004). In contrast, Ohler and Dubois (2006) report a keradont formula of 7/3 for *Chrysopaa*. None of the *Chrysopaa* specimens investigated by Ohler and Dubois (2006) were confirmed by molecular data and the number of tadpole specimens they included in their study is unclear.

Based on the morphology, *Chrysopaa* seems more similar to *Quasipaa* than to the neighboring Himalayan *Nanorana*. The genus *Quasipaa* occurs far east of the HTO, in southern and southwestern China to central Vietnam, northern and southeastern Laos, southeastern Thailand, and southwestern Cambodia (Frost 2022).

Our tadpole differs significantly from those of *Nanorana hazarensis*, and *N. vicina* (Stoliczka 1872), which are endemic to the Kashmir Himalaya (*N. hazarensis*), and uplands in Pakistan and India (*N. vicina*). Compared with *N. hazarensis*, the number of tooth rows on the upper labia of *Chrysopaa* is smaller (4–5 vs. 7–8; no difference on lower labia; Hofmann et al. 2021b); in *N. vicina* the first two anterior tooth rows are continuous (Gill et al. 2020), while in *Chrysopaa* it is only A_1 . In both, *N. hazarensis* and *N. vicina*, the oral apparatus is much more prominent than in *Chrysopaa*.

The tooth row formula of the upper lip of *Chrysopaa* also differs from that of *Chaparana* (*N. aenea*, *N. quadranus*, *N. taihangnica*, *N. unculuanus*), several taxa of *Paa* (*N. chayuensis*, *N. conaensis*, *N. maculosa*, *N. medogensis*), as well as the alpine species *N. pleskei*, and *N. ventripunctata*, and also *Quasipaa fasciculispina*, *Q. shini*, and *Q. verrucosipinosa* (Table 1). In contrast, upper labium formula equals

that of *N. sichuanensis*, *N. yunnanensis*, *N. zhaoermii*, *Quasipaa boulengeri*, *Q. exilispinosa*, *Q. robertingeri*, and *Q. spinosa* (Table 1). However, these comparisons must be treated with caution because in most of these studies, the identity of the larvae have not been confirmed with molecular data, except in Shuo et al. (2019), Hofmann et al. (2021b), and Thongproh et al. (2023).

The illustration and description of the *Chrysopaa* tadpole should facilitate the identification of this species (e.g., in museum collections). Given the sparse knowledge about the genus and many other taxa endemic to the Hindu Kush region, biodiversity research is desperately needed in Afghanistan, even more because this country may hold a large number of hidden species and unknown genetic diversity that might be crucial for understanding the biogeography of High Asia.

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SUPPLEMENTAL MATERIAL

Supplemental material associated with this article can be found online at <https://doi.org/10.1655/Herpetologica-D-22-00046.s1>.

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