

Taxonomic status of the Central Asian damselfly *Calopteryx samarcandica* Bartenev, 1912 (Odonata: Calopterygidae)

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Research Article

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All relevant data are within the paper and its [Supporting Information files](#).

Abstract. The taxonomic status of the Central Asian damselfly *Calopteryx samarcandica* was determined on the basis of molecular and phenotypic data from the Kugitang Mountains, SE Uzbekistan. Molecular analyses (COI and 18S rDNA, ITS1-5.8S rDNA-ITS2) revealed that *C. samarcandica* forms part of a clade that includes taxa treated in the literature as subspecies of *Calopteryx splendens* and/or related species. However, both mitochondrial and nuclear DNA confirmed that *C. samarcandica* deserves separate species status. The COI distance to *C. splendens* (including *C. xanthostoma*) is moderate (3.47%, K2P), but much greater than the internal distances between the sequences qualified in *C. splendens* (0.4%); this difference is supported by the low values of the barcoding gap in this genus. The rather distant separation of *C. samarcandica* from both *C. splendens* (*ancilla*, *taurica*, *intermedia*) and *C. orientalis* and the length of the *samarcandica*-branch recognisable in nuclear rDNA strengthen the independent position of the first species. The phylogenetic position of *C. samarcandica* in the genus *Calopteryx* remains unclear due to the low variability of nuclear rDNA markers and insufficient and partly incomparable data for other taxa. The molecular data support the phenotypic and ecological specificity of *C. samarcandica*, defined by a diagnostic combination of its traits (male and female wing colours and large size and basal diffusion of the wing spot in males) and the conservative association with groundwater-fed natural watercourses in the mountains and foothills fringing the vast Central Asian mountain block.

Key words. Dragonfly, Zygoptera, Central Asia, Uzbekistan, COI, DNA barcoding, ITS, 18S rDNA, phenotypic characteristics, taxonomy

Introduction

Damselflies of the genus *Calopteryx* have always attracted a great deal of attention due to their spectacular colours and behaviour. This research interest did not necessarily lead, however, to clarity with regard to species distinctness and taxonomical classification. In contrast to many other odonates, some aspects of functional morphology, e.g., of the abdominal appendages, simply appeared not to be diagnostic traits for species identification and classifying taxa in this group. This situation is a consequence of the fact that instead of mechanical (lock-antilock) mechanisms, colour-flashing and premating displays play a decisive role in their reproductive isolation (Anders & Ruppel, 1997; Dumont et al., 1987, 2005). However, the focus of researchers on colour pattern, especially of the wings, resulted in the identification of many Palaearctic *Calopteryx* taxa being viewed differently

by various specialists in odonatological history. The understanding of their taxonomic status evolved, but even today, in the era of molecular studies, several taxa, such as *xanthostoma* or *orientalis*, are traditionally treated as separate good species (e.g., Paulson et al., 2023) or as subspecies/forms of *Calopteryx splendens* (e.g., Sadeghi et al., 2010; Sadeghi & Dumont, 2014). In this confusing situation, the specific status of each taxon similar to *C. splendens* or its close relatives may raise doubts. One such taxon is *C. samarcandica* Bartenev, an endemic species from Central Asia. Its specific status was never questioned, but it was never studied in detail either. Apart from the original description of the species, published in Russian by Bartenev (1912) and based on several specimens, data on its traits are scarce (e.g., Dumont et al., 1997; Schmidt, 1961). Illustrative material is poor, as published images of the species are scarce as well. *C. samarcandica* is believed to be common between southern Kazakhstan and northern Afghanistan, in the lower mountains and foothill regions of the western Tian Shan, Pamir-Alay, and the northern part of the Hindu Kush, but it is in fact known from relatively few localities, reflecting a patchy knowledge of the odonates of this large area (Boudot et al., 2021).

The molecular analysis of the material collected during an expedition undertaken by R. Bernard, B. Daraž and T. Makhkamov to SE Uzbekistan in 2019 now allows us to address questions concerning the taxonomic status of *C. samarcandica*. We also provide a description of selected morphological traits of this species, based on the collected individuals and enriched with photographic images, in order to verify the original species description.

Materials and methods

Scientific names of taxa are given according to the newest version of the World Odonata List (Paulson et al., 2023). The subspecies name *Calopteryx splendens johanseni* originally used in Dumont et al., 2005 and in GenBank (AJ459188) was replaced with *C. splendens ancilla*, as the former is a synonym of the latter (Bernard & Kosterin, 2010; Malikova & Kosterin, 2019).

Phenotypic descriptions of *C. samarcandica* are based on eight individuals, four males and four females, collected during the above-mentioned expedition. Dimensions are given in millimetres. The total length includes the cerci, and the length of the abdomen excludes the appendages. Abdominal segments 1–10 are abbreviated as S1–10. The individuals studied are in the collection of Rafał Bernard in the Nature Collections at the Faculty of Biology, Adam Mickiewicz University in Poznań, Poland.

Molecular analysis

Genomic DNA was extracted from the legs using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Hilden, Ger-

many). A DNA-barcode fragment of the cytochrome c oxidase subunit I (COI) was amplified with the primers bcdF01 (CATTTCCHACTAAYCATAARGATATTGG) and bcdR04 (TATAAACYTCDDGGATGNCCAAAAA) (Dabert et al., 2010). Primers used for PCR amplification of the 18S ribosomal DNA gene (18S rDNA) were 18SF (CTTGTCTCAAAGATTAAGCCATGCA) and 18SR930 (GACGGTCCAAGAATTTTAC) (Dabert et al., 2010). Primers used for amplifying the internal transcribed spacer region of the nuclear ribosomal DNA (ITS1-5.8S rDNA-ITS2) were ITS1_18S (AGAGGAAGTAAAAGTCGTAACAAG) and ITS2_28S (ATATGCTTAAATTCAGGGGG) (Navajas et al., 1999).

PCR was performed in a total reaction volume of 10 µl, containing 2 µl of HOT FIRE Pol Blend Master Mix (Solis Biodyne, Tartu, Estonia), 0.5 µM of primers for COI amplification, 0.25 µM of primers for rDNA amplification and 4 µl of DNA template. The thermocycling profile used for the reaction was one cycle of 12 min at 95°C, followed by 35 steps of 15 s at 95°C, 1 min at 50°C, and 1 min at 72°C, with a final elongation step of 5 min at 72°C. PCR products were purified with thermostable Exonuclease I and FastAP Alkaline Phosphatase (Thermo Scientific, Waltham, MA, USA) and sequenced with BigDye Terminator v3.1 on an ABI Prism 3130XL analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were published in GenBank under accession numbers OQ429301–7 (18S rDNA), OQ429308–14 (ITS1-5.8S rDNA-ITS2) and OQ433899–905 (COI), respectively.

Sequence and phylogenetic analyses

Analysis of COI data (658 nps) was conducted using the sequences isolated from seven *Calopteryx samarcandica* individuals from the new collection, 77 published sequences for seven *Calopteryx* species (Behrens-Chapuis, 2021; Curry et al., 2011; deWaard et al., 2019; Futahashi, 2011; Galimberti et al., 2021; Geiger et al., 2021), three sequences for *Calopteryx japonica* published in GenBank, and a sequence of *Atrocalopteryx atrata* also published in GenBank was used as an outgroup (Table 1). Comparison of rDNA sequences, in total 1499 nps, including the 5'-fragment of the 18S rDNA (876 nps) and the ITS1-5.8S rDNA-ITS2 region (623 nps), was conducted with the use of sequences isolated from seven *C. samarcandica* individuals (three males and four females) from new collection and 21 published sequences for 17 *Calopteryx* taxa (species/subspecies) and for *A. atrata* as an outgroup (Dumont et al., 2005) (Table 2). We did not use the published sequence of *C. splendens* (X98502.1) from Slovenia due to its ambiguous mutations at conservative nps. All sequences were aligned using the MUSCLE 5.1 software (Edgar, 2004) incorporated in Geneious Prime 2023.0.2 (Biomatters Ltd., Auckland, New Zealand). Phylogenetic analysis of COI sequences for species delimitation was carried out with the neighbour-joining (NJ) method as incorporated in MEGA7 (Kumar et al.,

Table 1. Taxa, geographical origin, reference, and GenBank accession numbers of the COI sequences used in this study. Each sequence number refers to one individual. *C.* – *Calopteryx*, *A.* – *Atrocalopteryx*.

Taxon	Genbank accession number	Country	Reference
<i>C. samarcandica</i>	OQ433899, OQ433900, OQ433901, OQ433902, OQ433903, OQ433904, OQ433905	Uzbekistan	This study
<i>A. atrata</i>	MN206457	Republic of Korea	National Science Museum, Daejeon, Republic of Korea
<i>C. aequabilis</i>	JN419412, JN419413, JN419414, JN419415, JN419416, JN419417, JN419418, JN419419, JN419420, JN419421, JN419422, JN419423, JN419424, JN419425, JN419427, JN419428	Canada	Curry et al., 2011
<i>C. aequabilis</i>	MG511540	Canada	deWaard et al., 2019
<i>C. haemorrhoidalis</i>	MT298277, MT298278, MT298279, MT298280, MT298281, MT298282, MT298283, MT298284	Italy	Galimberti et al., 2021
<i>C. haemorrhoidalis</i>	MW490203	Italy	Geiger et al., 2021
<i>C. japonica</i>	MN609569, MN609570, MN609571	Republic of Korea	National Science Museum, Daejeon, Republic of Korea
<i>C. japonica</i>	AB708330, AB708331	Japan	Futahashi, 2011
<i>C. maculata</i>	JN419443, JN419445, JN419446, JN419448, JN419449, JN419451, JN419453, JN419454, JN419455, JN419456, JN419457, JN419458, JN419459, JN419460, JN419461, JN419463, JN419464, JN419465, JN419466, JN419467, JN419468	Canada	Curry et al., 2011
<i>C. splendens</i>	MT298286, MT298287, MT298288, MT298289	Italy	Galimberti et al., 2021
<i>C. splendens</i>	MT298285	Montenegro	Galimberti et al., 2021
<i>C. splendens</i>	MW490124, MW490134, MW490199, MW490230, MW490324, MW490332, MW490487, MW490510	Germany	Geiger et al., 2021
<i>C. splendens</i>	MW490111, MW490143, MW490204, MW490329, MW490397	Poland	Geiger et al., 2021
<i>C. virgo</i>	MW459503	Germany	Behrens-Chapuis, 2021
<i>C. virgo</i>	MT298290, MT298291, MT298293, MT298294, MT298295	Italy	Galimberti et al., 2021
<i>C. virgo</i>	MT298292	Montenegro	Galimberti et al., 2021
<i>C. virgo</i>	MW490428	Poland	Geiger et al., 2021
<i>C. xanthostoma</i>	MT298296, MT298297	Italy	Galimberti et al., 2021

2016). Pairwise distance calculations between nucleotide sequences were computed as per Kimura's 2-parameter (K2P) distance model (Kimura, 1980) for all codon positions with MEGA7. The COI sequence data were also analysed using the Assemble Species by Automatic Partitioning (ASAP) method (Puillandre et al., 2021) for finding best species partitions. The online ASAP version was used (<https://bioinfo.mnhn.fr/abi/public/asap/>) with default settings and the K2P distance model. The approximately-maximum-likelihood phylogenetic tree for rDNA fragments was constructed with FastTree 2.1.11 software (Price et al., 2010), using the Generalized-Time-Reversible (GTR) model. The phylogenetic tree for rDNA was also reconstructed by means of the Bayesian inference (BI) in MrBayes 2.2.4 (Huelsenbeck & Ronquist, 2001) incorporated in Geneious Prime 2023.0.2. The best-fit model of DNA evolution chosen by PartitionFinder2 (Lanfear et al., 2012) was JC+G for all codon positions.

Results

Individuals collected and locality

Eight individuals of *C. samarcandica*, four males and four females, were collected on 28 June 2019 by R. Bernard and B. Daraž; one male with wing spot colours that were slightly less saturated appeared to be immature. They were encountered at a permanent stream in the Kugitang Mountains, at 38.1551° N, 66.7275° E, 1577 m a.s.l., in Surxondaryo viloyati (Surkhondaryo Province), SE Uzbekistan. This locale is situated on a mountain plateau that gently descends towards the Turkmenistan border, in an open valley between mountain slopes loosely vegetated with juniper forests (Fig. 1a). The mountain stream is up to 5 m wide, with stronger currents and calmer sections, in many places stony and poorly vegetated. However, the section hosting *Calopteryx samarcandica* was different: 2.5 m wide and up to 40 cm deep, anastomosing and fed by seepages, with

Table 2. Taxa, geographical origin, reference, and GenBank accession numbers of the rDNA sequences (18S rDNA, ITS1-5.8S rDNA-ITS2) used in this study. Each row refers to one individual; in some individuals, the study fragment is merged from two GenBank sequences. *C.* – *Calopteryx*, *A.* – *Atrocalopteryx*.

Taxon	GenBank accession number	Country	Reference
<i>C. samarcandica</i>	OQ429301, OQ429308	Uzbekistan	This study
<i>C. samarcandica</i>	OQ429302, OQ429309	Uzbekistan	This study
<i>C. samarcandica</i>	OQ429303, OQ429310	Uzbekistan	This study
<i>C. samarcandica</i>	OQ429304, OQ429311	Uzbekistan	This study
<i>C. samarcandica</i>	OQ429305, OQ429312	Uzbekistan	This study
<i>C. samarcandica</i>	OQ429306, OQ429313	Uzbekistan	This study
<i>C. samarcandica</i>	OQ429307, OQ429314	Uzbekistan	This study
<i>A. atrata</i>	Y12889, AJ458982	Japan	Dumont et al., 2005
<i>C. aequabilis</i>	Y12888, AJ308360	USA	Dumont et al., 2005
<i>C. amata</i>	AJ458977, AJ308361	Canada	Dumont et al., 2005
<i>C. cornelia</i>	Y12890, AJ458981	Japan	Dumont et al., 2005
<i>C. exul</i>	Y12891, AJ308346	Morocco	Dumont et al., 2005
<i>C. haemorrhoidalis</i>	AJ458976, AJ308362	Algeria	Dumont et al., 2005
<i>C. haemorrhoidalis</i>	Y12892, AJ308347	Morocco	Dumont et al., 2005
<i>C. splendens intermedia</i>	AJ459191	Iran	Dumont et al., 2005
<i>C. japonica altaica</i>	AJ459193	Russia, Altai	Dumont et al., 2005
<i>C. japonica japonica</i>	Y12893, AJ458980	Japan	Dumont et al., 2005
<i>C. maculata</i>	AJ459198	USA	Dumont et al., 2005
<i>C. orientalis</i>	AJ459192	Iran	Dumont et al., 2005
<i>C. splendens ancilla</i>	AJ459187	Belarus	Dumont et al., 2005
<i>C. splendens ancilla</i>	AJ459188	Russia, Altai	Dumont et al., 2005 (as <i>C. s. johanseni</i>)
<i>C. splendens caprai</i>	AJ458966, AJ308371	Italy	Dumont et al., 2005
<i>C. splendens taurica</i>	Y12894, AJ458979	Ukraine	Dumont et al., 2005
<i>C. syriaca</i>	AJ459190	Jordan	Dumont et al., 2005
<i>C. virgo</i>	X98503	Slovenia	Dumont et al., 2005
<i>C. virgo virgo</i>	AJ458968, AJ308359	France	Dumont et al., 2005
<i>C. xanthostoma</i>	AJ458972, AJ308354	France	Dumont et al., 2005
<i>C. xanthostoma</i>	AJ458971, AJ308353	Italy	Dumont et al., 2005

a fairly strong central current and broad and shallowly flooded streambanks with trickling water, and partially to fully overgrown by abundant herbaceous vegetation, mostly grass and rushes (Fig. 1b). Syntopic odonate species were dominated by very large numbers of *Ischnura forcipata* Morton and *Orthetrum brunneum* (Fonscolombe), but several individuals of *Onychogomphus lefebvrei* (Rambur), mostly teneral and originating from a shallow stony section upstream, a single male of *Ischnura evansi* Morton, and a juvenile female of *Ischnura pumilio* (Charpentier) were present here as well.

Phenotypic characteristics

Male (Figs 1c,d, 2a,b)

- Wing spot dark brown with iridescent metallic dark blue veins, glistening with rich dark blue in full light;
- wing spot large, covering ca. 75% of the wing length, reaching the wing tip distally and ca. 40% of the length between nodus and wing base; wing spot broadly diffused, i.e., paling and not sharply defined

basally, reaching farther in the anterior and central parts (covering 15–18 antenodal cells in the subcostal space and ending ca. 5–8 cells before the quadrilateral) than along the posterior margin;

- the unspotted basal part of the wing suffused with yellowish amber as in female wings;
- thorax, abdomen and dorsal parts of head (vertex, frons and postclypeus) metallic and glistening with dark blue and green;
- ‘tail-light’ white to yellowish white, including the paraprocts, S10, S9 and the distal margin of S8:
 - paraprocts white ventrally, blackening towards tips;
 - S10 ventrally white with cream admixture;
 - S9 with various ventral patterns: from white or yellowish with brown spots and suffusion to black with two proximal dull white spots;
- white spots in the frontal parts of the head (with cream admixture or a greenish/bluish tint locally):
 - two longitudinal spots as ‘tears’ streaming down the pedicels of the antennae;
 - a small central spot on the anteclypeus;

- two large lateral spots on the labrum; two large spots on the mandibles (on the mandibular cheeks) and two small and narrow spots on their bases;
- the spots on the pedicels, labrum and mandibular cheeks are bright and conspicuous, the remaining spots less eye-catching;
- cream-white or dull yellow colours also occur on the posterior parts of the coxa, ventral parts of the synthorax, as an anterior stripe on the metepimeron along the metapleural suture (in the lower two thirds of its length, plus a narrow spot above in some individuals), and along the posterior margin of the metepimeron combined with the adjacent post-sternum;
- black legs;
- occipital tubercles lacking or at most barely discernible;
- total length (four individuals) 46.5–48.8, abdomen length 36.0–38.0, hindwing length 29.8–31.2, hindwing width 9.9–10.3;
- number of complete Ax (hindwings): in costal space 22–25, in subcostal space 29–32;
- venation denser in males than in females, with many anastomosing crossveins.

Female (Figs 1e, 2c,d)

- Wings suffused throughout with an amber-brown background, glistening gold and copper in bright light; costa metallic green, other veins yellowish brown to brown, some of them slightly glistening in bright light;



Figure 1. The natural habitat (a, b) and individuals (c, d – male, e – female) of *Calopteryx samarcandica* on the mountain plateau in the Kugitang Mountains in SE Uzbekistan. Photos by R. Bernard (a) and B. Daraž (b–e)

- fairly large white pseudopteroostigmas, 6–10 (mostly 7–9) cells long and swollen, but weakly defined, slightly longer on forewings;
- head, thorax and abdomen metallic, glistening with green, gold and copper;
- the pale frontal spots on the head situated as in males: those on pedicels, labrum and mandibular cheeks light yellow, yellowish white on the anteclypeus and mandible bases;
- the light yellow pattern on thorax and abdomen brighter and more expansive than in a male, occurring on the coxa (partly), the lower edge of the prothorax, the ventral parts of the synthorax (extending to the lowest parts of the thorax sides), along the metapleural suture (completely or in the lower two thirds along its length and including adjacent stripes, posterior on the metepisternum and anterior on the metepimeron), along the posterior margin of the metepimeron combined with the adjacent post-sternum, and in distal parts of the abdomen (especially on S7–10 ventrally and laterally, and in a dorsal broad line from the most distal part of S7–10);
- dorsal ridge on the distal half of S10, ending with a spine protruding distally (broken in some individuals);
- legs black;
- total length (four individuals) 43.1–46.7, abdomen length 33.4–36.1, hindwing length 31.9–32.5, hindwing width 9.0–10.2;
- number of complete Ax (hindwings) 19–25 in the costal space, 25–29 in the subcostal space.

Molecular analysis

Among the three males and four females of *C. samarcandica* sequenced for the COI barcoding fragment (Table 1), we found only one COI haplotype. The final alignment for species delimitation comprised 658 nucleotide positions (nps) for 88 sequences, representing eight species of *Calopteryx* and *A. atrata* as an outgroup (Table 1). In our dataset, 440 nps out of 658 were invariable (66.9%), and the transition to transversion ratio (R) amounted to 2.16 (without the outgroup: 461 nps, 70.1%, R = 2.67). NJ analysis clustered the *Calopteryx* COI sequences in well-supported clades corresponding to the known species, except for *C. xanthostoma* that was embedded in *C. splendens* (Fig. 3). This analysis revealed the lack of a clear internal structure of 20 sequences grouped in the clade of *C. splendens*, representing unnamed but certainly various—judging from their geographical locations—phenotypic forms (subspecies) of this species, and including *C. xanthostoma*, often considered to be a separate species (Fig. 3). Excluding the outgroup, the mean genetic distance between the COI sequence groups recovered in the NJ analysis was 13.07% K2P (SD = 1.04). Excluding the value 0.26% (SD = 0.08) between *C. splendens* and *C. xanthostoma*, the distance ranged from 3.28% (SD = 0.73) between *C. samarcandica* and *C. xanthostoma* to 22.70% (SD = 2.38) between *C. japonica* and *C. maculata* (Supplementary Table S1).

C. samarcandica differed from its closest relatives, *C. splendens* (including *C. xanthostoma* and without

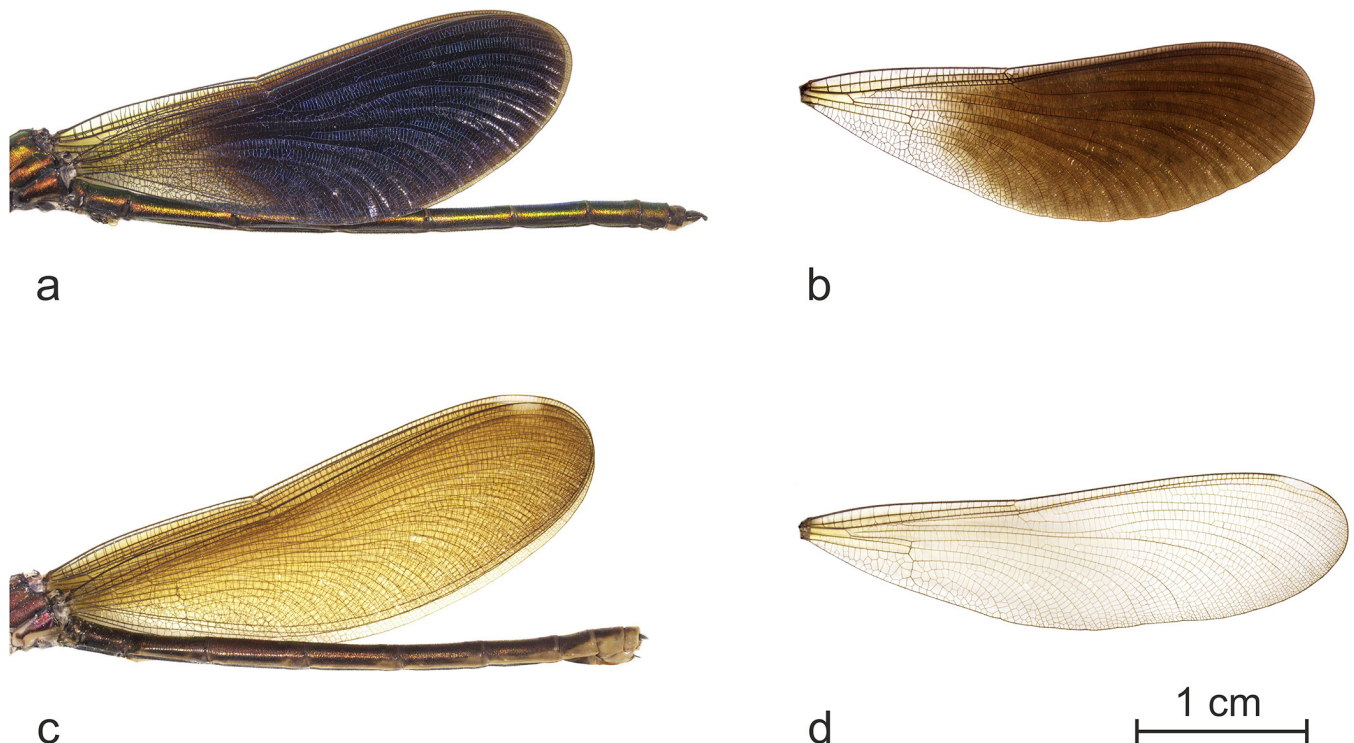


Figure 2. The wing colour and pattern of *Calopteryx samarcandica* from the Kugitang Mountains, Uzbekistan. a, b – male, c, d – female. Specimens taken out of their ethylene storage and photographed dry, except for a single male wing (b) that was left submerged in ethylene. Photos by B. Daraž

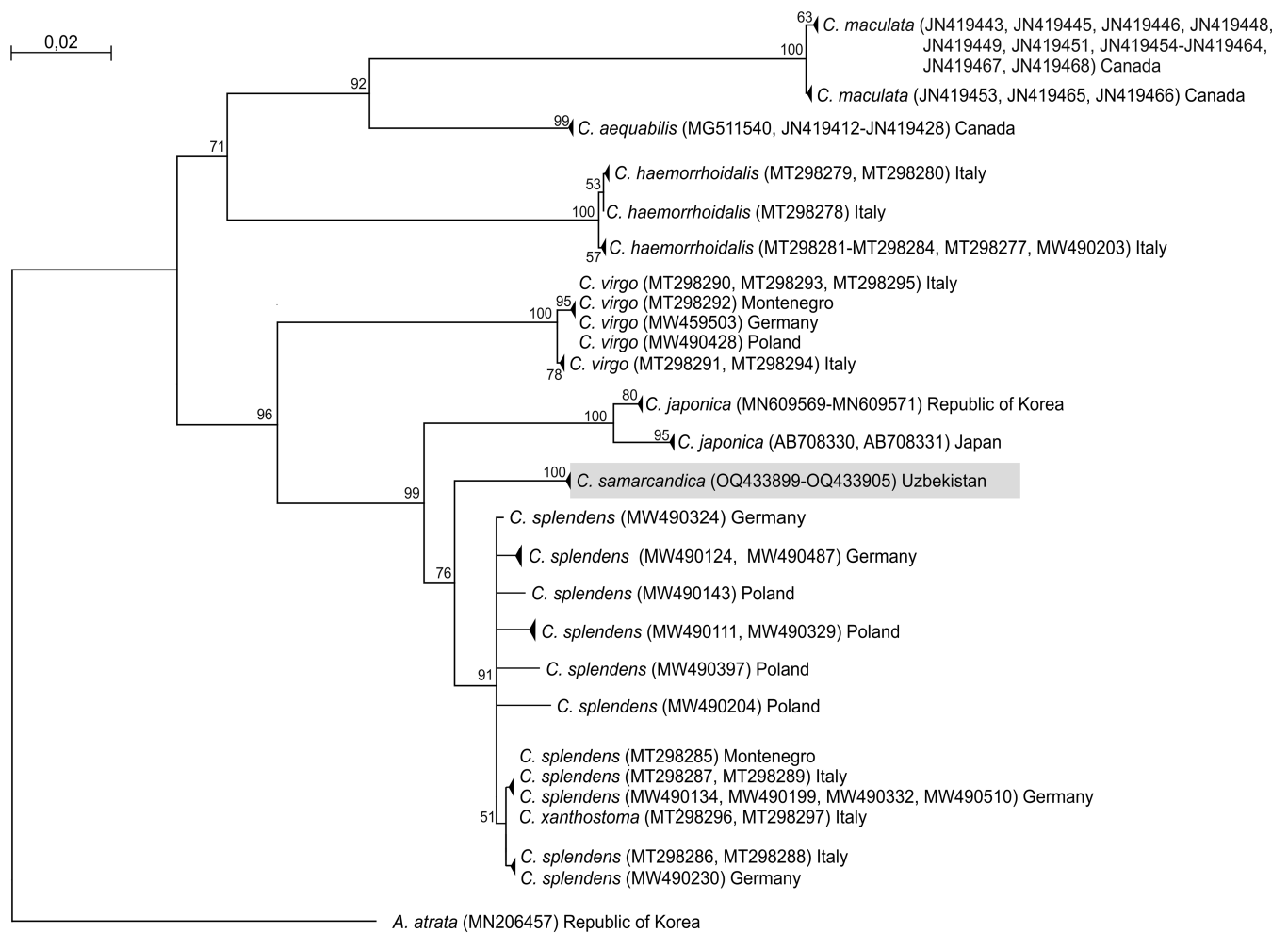


Figure 3. Neighbour-joining (NJ) tree based on COI sequences of *Calopteryx* species. Some clades were compressed for clarity of the graphic, except for those in the *C. splendens* clade in order to show their lack of order in relation to their geographical origins. The numbers at the branches indicate bootstrap values (1000 replications); only $\geq 50\%$ indicated.

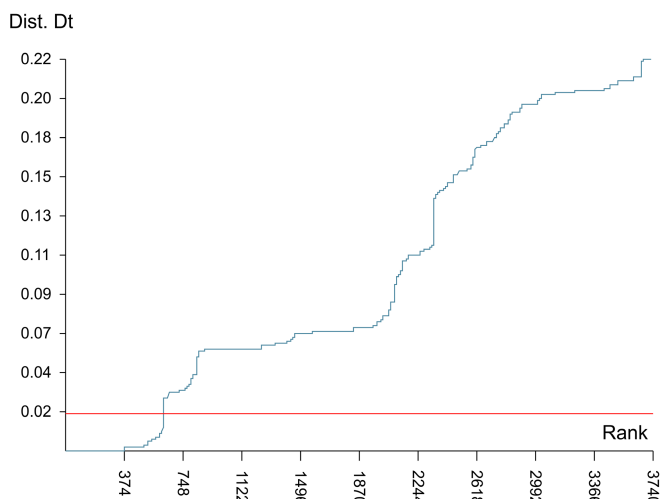


Figure 4. Barcode gap in the distribution of ranked pairwise differences found by the Assemble Species by Automatic Partitioning (ASAP) for COI sequences of the genus *Calopteryx* (results from <https://bioinfo.mnhn.fr/abi/public/asap/>).

it) by 3.47% (SD = 0.72) and 3.49% (SD = 0.73), respectively, and from *C. xanthostoma*, treated as a separate species or as a subspecies of *C. splendens*, by 3.28% (SD = 0.73) (Supplementary Table S1). These values were more than sevenfold higher than the mean distance among sequences in the broad *C. splendens* clade including *C. xanthostoma* (0.40%, SD = 0.12) (Supplementary Table S1). The distance between *C. samarcandica* and *C. japonica* was 8.19% (SD = 1.19). The ASAP analysis revealed a barcoding gap at ca. 1.9% (between 1.1 and 2.6) in the pairwise-distance distribution (Fig. 4) and thus supported the partition of the *Calopteryx* sequences studied into the same seven species as those revealed by the NJ analysis, with *C. xanthostoma* included in *C. splendens*.

Among the same three males and four females of *C. samarcandica* sequenced for nuclear rDNA (Table 2), we also found only one type of rDNA sequence. The final alignment for species delimitation comprised 1499 nucleotide positions (nps) for 28 sequences represent-

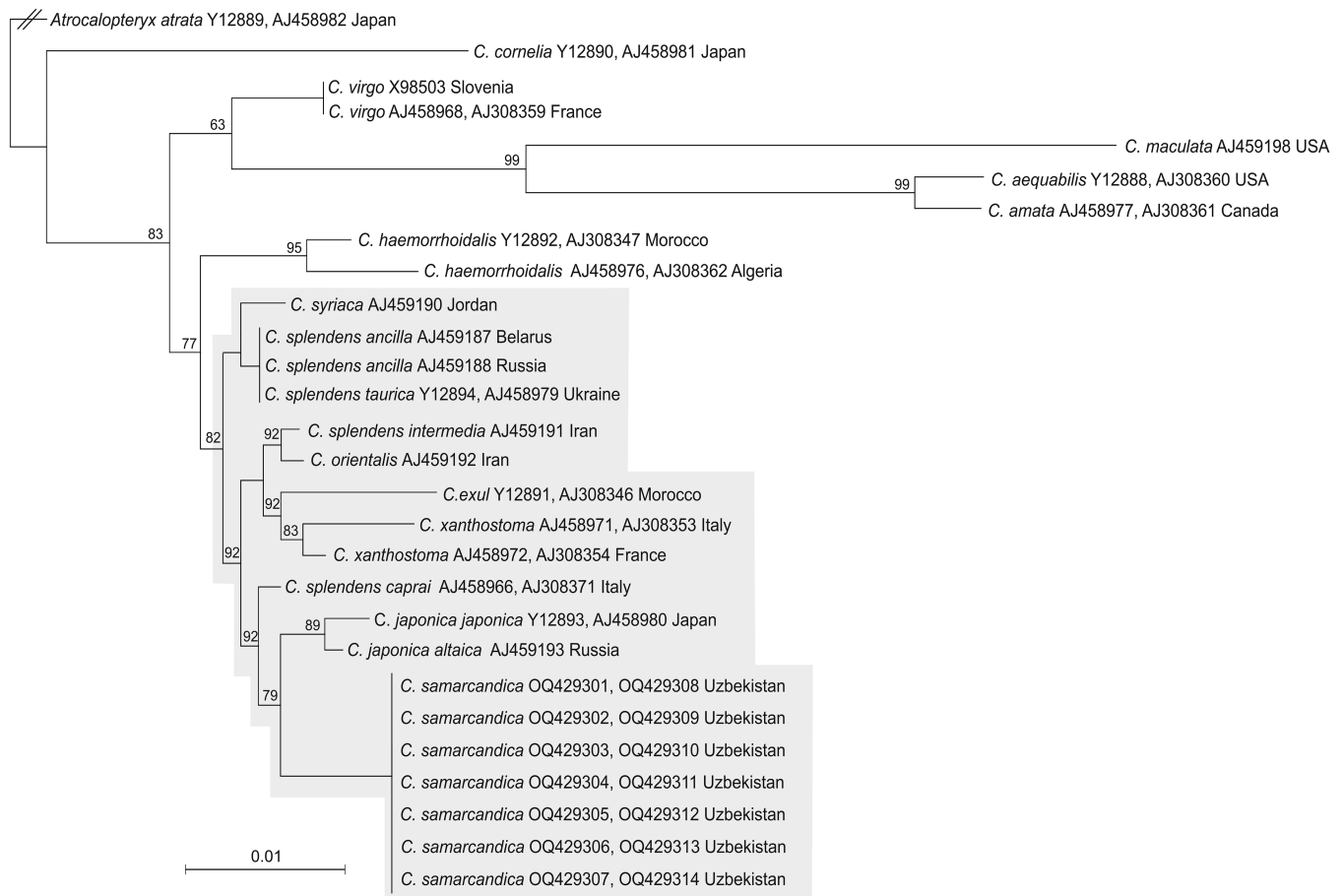


Figure 5. Phylogeny of *Calopteryx* taxa based on the 18S rDNA, ITS1-5.8S rDNA-ITS2 sequences as recovered with the use of FastTree, i.e., the approximately-maximum-likelihood method. The numbers at the branches indicate the SH local support values (only $\geq 50\%$ indicated). The clade of *Calopteryx splendens* and its relatives is shaded with grey.

ing 13 species (16 taxa including subspecies) of *Calopteryx* and *A. atrata* as an outgroup (Table 2). In the dataset, 1307 nps out of 1499 were invariable (87.2%), and the transition to transversion ratio (R) amounted to 0.82 (without the outgroup: 1373 nps, 91.6%, R = 0.80).

The Bayesian analysis of rDNA sequences yielded a mostly polytomous tree (not illustrated here) with unresolved phylogenetic relationships. The Fast Tree method on the same data placed *C. samarcandica* within the *C. splendens* complex, which was recovered as monophyletic (SH = 0.82) (Fig. 5.) and including: (a) forms commonly accepted as subspecies of *C. splendens* (*ancilla*, *taurica*, *intermedia*, *caprai*); (b) taxa variously treated as subspecies or, more often, separate species (*xanthostoma*, *syriaca*, *orientalis*); and (c) forms commonly treated as separate species (*exul*, *japonica*, *samarcandica*). *C. samarcandica* is situated there on a relatively long branch and distinctly separate from both *C. splendens* (*ancilla*, *taurica*, *intermedia*) and *C. orientalis*. Phylogenetic analysis of the nuclear rDNA sequences revealed *C. samarcandica* as a sister species to *C. japonica* with moderate support (SH = 0.79) (Fig. 5) and we found this clade to be sister to *C. splendens caprai* from Italy (AJ458966, AJ308371) (SH = 0.92).

Discussion

Both our mitochondrial and nuclear DNA analyses have confirmed that *C. samarcandica* deserves separate species status (Figs 3, 5). The mitochondrial sequence data point to its close relationship with *C. splendens* collected in Europe. Dumont et al. (1997) even mentioned presumed hybrid forms between *C. samarcandica* and: (a) Central Asian *C. splendens* in the Syr Darya catchment and east of it; and (b) *C. orientalis* to the south and east of the Kopet Dag in Turkmenistan, in the Harirud-Tedzhen and Murghab catchments, where they take on more *samarcandica*-like phenotypic traits. However, our COI and rDNA results clearly indicate a distinct specific status of *C. samarcandica* from the Amu Darya River Basin. The COI genetic distance between *C. samarcandica* and European *C. splendens* is moderate, but much greater (by another order of magnitude) than internal distances between the sequences qualified in *C. splendens*, including sequences unnamed but certainly representing different infraspecific forms from Central Europe, Italy, and the Balkans, as well as *C. xanthostoma* (Fig. 3). This difference is also supported by the location of the barcoding gap in this genus at the comparatively low level of the genetic distance (Fig. 4); additionally, the NJ and ASAP analyses

suggest a subspecific status of *C. xanthostoma* within *C. splendens*, although these results still require confirmation from analyses using a greater and wider sample size. The rather distant separation of *C. samarcandica* from both *C. splendens* (*ancilla*, *taurica*, *intermedia*) and *C. orientalis* and the length of the *samarcandica*-branch recognisable in the nuclear rDNA (Fig. 5) strengthen the independent position of the first species.

The results of our phylogenetic analysis of rDNA sequences, e.g., the sister positions of *C. samarcandica* and *C. japonica* and the close proximity of the Italian *C. splendens caprai*, grouped with *C. japonica* and *C. samarcandica* rather than with other subspecies of *C. splendens*, raise doubts whether it reflects true phylogenetic relationships. It seems that the relative paucity of data, both qualitative and quantitative, e.g., including only several sequences from the rich group of *C. splendens* and its relatives, is probably the reason for this unconvincing picture. Thus, we interpret it as strongly influenced by the taxon sampling used, and we do not consider it a resolved phylogeny of the complex. These doubts, however, do not subtract from the greater closeness of *C. japonica* to *C. splendens* than to the superficially similar *C. virgo*, which is recognisable in both trees for mitochondrial COI and nuclear rDNA (Figs 3, 5).

The differences between *C. samarcandica* and *C. splendens* are also ecological: the former is mostly associated with groundwater-fed natural watercourses in the mountains and foothills, while the latter is with lowland watercourses, also artificial ones, such as canals (Borisov & Haritonov, 2007; Boudot et al., 2021; Kosterin, 2020). Flowing on a mountain plateau and fed by numerous seepage springs the stream from which our sample originated perfectly matches the habitat preferences of *C. samarcandica*. The conservative association of this species with stable fringes of the vast and high Central Asian mountain block, surrounded by plains deficient in water, appears to be both a basis for its well-established occurrence and a restriction for its dispersal. Against this background, the more lowland-adapted *C. splendens* seems to be a less stenoecious species, benefiting from the human impact of providing open water in the plains adjacent to the mountains. The new locality record of *C. samarcandica*, situated in the Kugitang, the southernmost mountain ridge of the Pamir-Alay system, also falls into the known zoogeographical range of this species (Borisov & Haritonov, 2007; Boudot et al., 2021; Kosterin, 2020). It is located ca. 25 km from the locality in the same mountains on the Turkmen side of the border, from which Dumont et al. (1997) presented one of the first photographs of the species.

Considerations of the phenotypic differences between *C. samarcandica* and *C. splendens* and its relatives are focussed on wing traits. Our data confirm and complement earlier data (Bartenev, 1912; Dumont et al., 1997), e.g., the wing pattern of the males collected by us perfectly corresponds to that illustrated in Bartenev's original description. The species-specific combination of male traits includes: (a) the proximal margin of the wing spot

broadly diffused, i.e., paling and not as sharply defined as in most taxa of the *C. splendens*-complex (cf. Figs 1 & 2 in the present paper versus, e.g., Fig. 1 in Sadeghi & Dumont, 2014); (b) the intensively dark brown background colour of the wing spot encrusted with metallic dark blue veins; although glistening dark blue, the wing spot appears to be darker and more subdued than the brilliant spot in *C. splendens*; (c) the large size of the wing spot, covering ca. 75% of the wing length and reaching the wing top; in some forms of *C. splendens*, the expanse of the wing spot is similar, but it is slightly smaller proximally and has a transparent wing top in the coexisting or territorially adjacent *splendens*-populations in Uzbekistan, Tajikistan, Kyrgyzstan, and Kazakhstan (see Sadeghi & Dumont, 2014); (d) the unspotted basal part of the wing suffused with yellowish amber, like in female wings. The traits 'a' and 'b' seem to have the greatest diagnostic value. Even freshly emerged and juvenile males in which the wing spot is not (or hardly) discernible can be easily identified by their brownish-tinged wings (Dumont et al., 1997, and a photo in Boudot et al., 2021). The males with spots without the effect of metallic blue veins (as provided by Schmidt [1961] from Afghanistan under the synonymous name *C. maracandica*) must also have been immature (or badly preserved?) individuals. The presence of diagnostic brown or brownish wing colours is not restricted to males, though: the female wings of *C. samarcandica* are suffused throughout with an amber-brown background with most veins showing in yellowish brown to brown (Figs 1 & 2), and with the strong glistening gold and copper effect in bright light. In contrast, the wings of female *C. splendens* are quite clear and greenish in a general view.

The status of *C. samarcandica* as a separate species, as revealed on the basis of our molecular data and supported by phenotypic and ecological specificity of this taxon in comparison to *C. splendens* in Central Asia, still requires further research, however. To assess the advancement of the interspecies divergence it would be interesting to check with molecular means whether the coexistence in the contact zones of *samarcandica* and *splendens* (and *orientalis*) really produces interspecific hybrids, as was suggested by Dumont et al. (1997) on the basis of wing spots, and whether this hybridization occurs in a wide intergradation zone or is only a rare phenomenon. It would be helpful in such study to also check for (in)consistency between COI and nuclear rDNA data and possibly to use other DNA markers as microsatellite loci. What would be especially helpful in understanding the relationships between these species would be to study the courtship flights of *C. samarcandica* versus *C. splendens*. Anders and Rüppell (1997) convincingly outlined differences in the wingbeat frequency and phase relationship between hindwings and forewings between *C. splendens*, *C. virgo* and *C. haemorrhoidalis*, but found these differences to lack between *C. splendens* and *C. xanthostoma* (the next similar parameter in the last pair of species). Is it possible that the species-specific courtship flight signal already evolved in *C. samarcandica*?

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Supplementary Material

Supplementary Table S1. COI genetic distances between species in the genus *Calopteryx*.