www.biotaxa.org/RSEA. ISSN 1851-7471 (online) Revista de la Sociedad Entomológica Argentina 79(3): 24-33, 2020

First record of *Cloeon dipterum* (L.) (Ephemeroptera: Baetidae) in Buenos Aires, Argentina

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Received *01 - V - 2020* | Accepted *26 - VIII - 2020* | Published *28 - IX - 2020*

https://doi.org/10.25085/rsea.790304

Primer registro de *Cloeon dipterum* (L.) (Ephemeroptera: Baetidae) en Buenos Aires, Argentina

RESUMEN. *Cloeon dipterum* (L.) (Ephemeroptera: Baetidae) está ampliamente distribuido en las zonas templadas de Eurasia, sin embargo, está restringido en América del Norte a tres estados de Estados Unidos y Canadá, y en América del Sur a Chile. Con el fin de confirmar nuestra hipótesis de que se introdujo recientemente en Argentina, el objetivo del presente estudio fue identificar la especie en base a análisis moleculares y caracteres morfológicos. Para ello se recolectaron ninfas de tres hábitats artificiales en diferentes localidades de la provincia de Buenos Aires y de dos arroyos afluentes del Río Luján, al noreste de Buenos Aires (Argentina). Todas las ninfas fueron identificadas como *C. dipterum*, lo que confirma la presencia de la especie en Argentina. La distribución de *C. dipterum* en Buenos Aires junto a su ausencia en los sitios previamente muestreados de manera regular, indicarían que los individuos registrados son introducidos y están bien establecidos en la zona de estudio.

PALABRAS CLAVE. Cuerpos de agua neotropicales. Especie exótica. Insectos acuáticos.

ABSTRACT. *Cloeon dipterum* (L.) (Ephemeroptera: Baetidae) is widely distributed in temperate areas of Eurasia, whereas, in North America, it is restricted to three states of the USA and Canada, and, in South America to Chile. To confirm our hypothesis that *C. dipterum* has been recently introduced in Argentina, the aim of the present study was to identify the species based on molecular analyses and morphological features. To this end, nymphs were collected from three artificial habitats located in different localities of Buenos Aires province and two tributary streams of the Luján River, northeast of Buenos Aires, Argentina. Nymphs were all identified as belonging to *C. dipterum* thus confirming the presence of the species in Argentina. The distribution of *C. dipterum* in Buenos Aires, together with its absence from previously sampled sites, would indicate that the individuals recorded are introduced and already well established in the study area.

KEYWORDS. Aquatic insects. Neotropical waterbody. Non-native species.

INTRODUCTION

The aquatic insect order Ephemeroptera has approximately 400 genera and over 3,000 species described worldwide (Barber-James et al., 2008). In South America, there are records of 671 species distributed in 14 families. In particular, in Argentina, there are records of 13 families, with most species belonging to the family Baetidae (Domínguez et al., 2019). In the province of Buenos Aires, only the genera *Americabaetis* Kluge and *Callibaetis* Eaton of this family have been reported (Hubbard et al., 1992; Ocon & Rodrígues Capítulo, 2004; Rigacci, 2009). Although Baetidae is a cosmopolitan family, its genera or species are not. In that sense, *Cloeon* Leach is almost an exception within the family. This genus was described by Leach in 1815, and, since then, numerous species, mainly from the Palearctic, Afrotropical Oriental realms, have been described (Leach, 1815; Salles et al., 2014). Rutschamnn et al. (2014) suggested that *Cloeon* is a monophyletic group, by reconstructing phylogenetic relationships with the genus *Baetis*. Due to their long imaginal stages (Degrange, 1960; Oehme, 1972), *Cloeon* species seem to be excellent candidates to colonize new habitats. Such is the case of *Cloeon dipterum* (L.), one of the most common and abundant mayflies distributed throughout the temperate areas of Eurasia, a region that includes Europe, Siberia, Mongolia and Northeast Asia (Sowa, 1975; Bae & Park, 1997), and oceanic islands, such as Azores and Canary Islands (Rutschmann et al., 2014). *Cloeon dipterum* is currently considered a species - complex that includes seven genetic groups as it is shown by genetic data from samples from East Asia, North America and Europe (Yano et al., 2019). In addition, it is a eurytopic species, with a wide range of tolerance to environmental conditions (Velasco et al., 1998; Menetrey et al., 2008; Lock & Goethals, 2011), such as water pH (Vilenica et al., 2016) and temperature (McKee & Atkinson, 2000). It can also survive at low concentrations of oxygen by using an anaerobic mechanism, after long-term acclimatization in waters at temperatures close to 0 ° C (Kamler, 1971; Nagell & Fagerstrom, 1978).

Since *C. dipterum* can be the main component in the diet of young predatory fish (Cianciara, 1979a; Mc Kee & Atkinson, 2000), its prevalence and abundance, particularly in spring and summer, can be of great ecological importance as an essential link in freshwater trophic networks. The nymphs of *C. dipterum* are swimmers, they colonize all kinds of still and standing waters and can be found among the riparian vegetation or bottom mud in ponds, lakes (Beketov & Lies, 2005; Jeffries, 2005) and peat bogs (Vilenica et al., 2016), as well as in artificial habitats (Gaino & Rebora, 2005; Salles et al., 2014; Vilenica et al., 2019).

Until a few years ago, *C. dipterum* was considered the only representative of the genus in the Western Hemisphere, restricted to the temperate zones of North America, in Illinois (Burks, 1953), Ohio (Traver, 1962), Pennsylvania (Sweeney et al., 2018) and Canada (Randolph et al., 2002). In South America, the first record of *Cloeon* was that by Salles et al. (2014) in the State of Espírito Santo in southeastern Brazil, where these authors recorded *Cloeon smaeleni* Lestage. Based on morphology and molecular evidence, they concluded that this species had been recently introduced in the country. Later, Vera et al. (2015) recorded *C. dipterum* in Limari and Maipo, Chile. They studied nymphs as well as male and female imagos, but omitted the evidence to confirm the first record of the species and date of collection.

Considering that *C. dipterum* had not been previously recorded in Argentina, the aim of the present study was to confirm the presence of this non-native species through molecular and morphological studies.

MATERIAL AND METHODS

Sampling of individuals

Nymphs were collected from three swimming pools, one located in Navarro (St1) and two in General Rodríguez (St2 and St3), and two tributary streams of the Luján River, located northeast of Buenos Aires province (Carabassa stream -St4- and Burgueño stream -St5-). The geographical records of the species were mapped with QGIS (version 3.4.9) (Fig. 1).

The swimming pools from which the nymphs were collected were located in private homes and were of different dimensions: two of base dimensions 4.45 × 2.25 m and 0.80 m deep and one of base dimensions 4 × 7 m and 1.5 m deep; the bottoms of the three swimming pools were covered with leaf litter of *Pinus* sp., *Populus* sp., *Juglans* sp., *Liquidambar* sp. and *Laurus nobilis* L. The Carabassa and Burgueño streams had slow or zero current sectors, and were colonized by submerged (*Egeria densa* Planch, *Potamogeton* sp.), floating (*Polygonum* sp., *Hydrocotile* sp., *Ludwigia* sp., *Sagittaria* sp.) and emerging (*Alternathera* sp.) macrophytes. Both streams had riparian vegetation with trees and shrubs. All collection sites were characterized by low current velocity, slow levels of nitrates and low dissolved oxygen values, except St5 (Table I).

Nymphs were collected using a hand net of 1 mm pore size, while the adults were obtained by maintaining nymphs and breeding in the laboratory. All voucher specimens are deposited in the *Colección de Invertebrados del Laboratorio de Ecología, Departamento de Ciencias Básicas de la Universidad Nacional de Luján*, Buenos Aires, Argentina.

Material examined

A total of 160 individuals (111 nymphs and 49 adults) collected from three private swimming pools (St1, St2 and St3) and two tributary streams of the Luján River (St4 and St5) were examined (Table II).

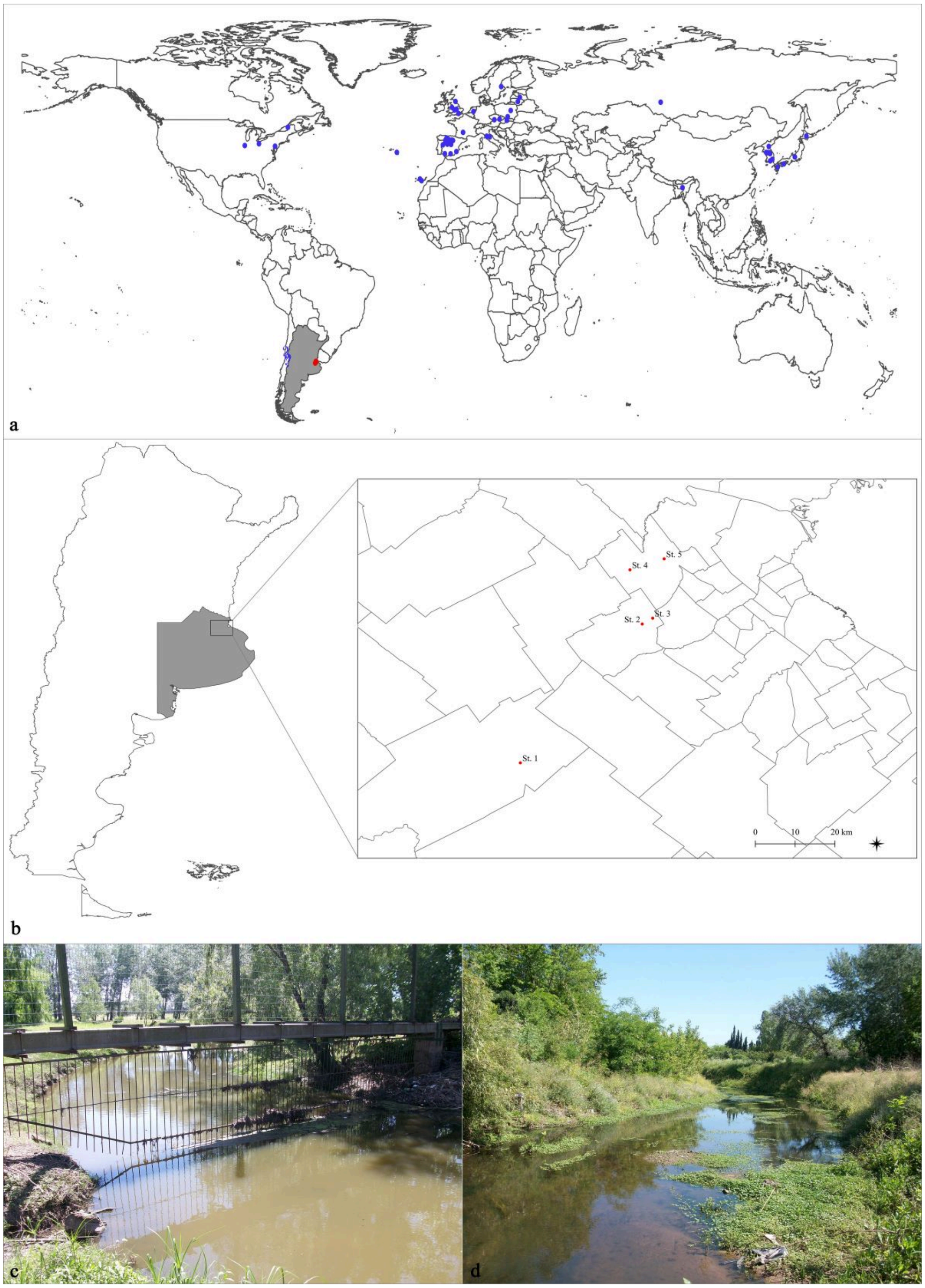

Fig. 1. Distribution of *Cloeon dipterum*. a. World map (grey, Argentina; blue circles, species distribution)*. b. Map of Argentina with detail of Buenos Aires province and collection stations (red circles) [St1: Navarro, St2 and St3: General Rodríguez, St4: Carabassa Stream (Pilar), St5: Burgueño Stream (Pilar)]. c. Carabassa Stream. d. Burgueño Stream.

* Based on Wingfield, 1939; Burks, 1953; Brown, 1961; Traver, 1962; Kjellberg, 1973; Sowa, 1975; Nagell, 1977; Nagell & Fagerstrom, 1978; Cianciara, 1979b; González del Tánago, 1984; Craig, 1990; Van Wijngaarden, 1993; Silina, 1994; Gupta et al., 1994; Bae & Park, 1997; Velasco et al., 1998; McKee & Atkinson, 2000; Randolph et al., 2002; Jeffries, 2005; Beketov & Liess, 2005; Cayrou & Céréghino, 2005; Gaino & Rebora, 2005; Menetrey et al., 2008; Lupetti et al., 2011; Lee et al., 2013; Camp et al., 2014; Rutschmann et al., 2014, 2016; Salles et al., 2014; Vera et al., 2015; Šupina et al., 2016; Vilenica et al., 2016, 2019; Rico et al., 2018; Sweeney et al., 2018; Almudi et al., 2019; Yano et al., 2019.

Site	Nitrates (mg/l)	Phosphates (mg/l)	pH	Temperature (°C)	Dissolved oxygen (mg/l)	Current velocity (m/s)
St1	7	0.50	7.8	16	4.8	0
St2	7.2	0.39	7.3	12	5.3	0
St3	7.5	0.44	7.2	10	5.5	0
St4	5.5	0.98	6	22.3	5	0.001
St5	0.1	0.35	6	11	9.2	0.005

Table I. Physicochemical variables measured in the sampled sites.

Site	Date	Coordinates	Nymph	Male imago	Female imago	Collector
St1	8/23/2019	59° 15’ 39.6” S 35° 0.3’ 18” W	27	4		Agustina Silvera
St2	5/15/2018	34° 36’ 58.9” S 58° 55’ 26.8” W	45	15	18	Pamela Banegas
St3	7/28/2017	34° 35’ 59.9” S 58° 53’ 42.2” W	32	7	5	Pamela Banegas
St4	10/25/2017	34° 27’ 52.5” S 58° 57’ 28.5” W	3			Luciana Rocha
St5	7/11/2017	34° 27’ 2.2” S 58° 52’ 29.8" W	4			Luciana Rocha

Table II. Data of the material examined from each site.

Molecular analyses

For the genetic identification of the nymphs, the DNA was extracted from four entire individuals fixed and preserved in 96% ethanol by the phenol-chloroform method (Sambrook et al., 1989). Fragments of the mitochondrial DNA Cytochrome Oxidase subunit I (COI) gene of approximately 650 bp were amplified using primers LCO 1490 (GGTCAACAAATCATAAAGATATTGG) and HCO 2198 (TAAACTTCAGGGTGACCAAAAAATCA) (Folmer et al., 1994). Each PCR had a reaction volume of 20 µl and contained 2 µl of 10X Pegasus Taq DNA Polymerase Buffer, 0.6 µl of 50 mM $MgCl_2$, 0.4 µl of 10 mM deoxynucleotide triphosphates, 1 µl of 10 µM forward and reverse primers, 0.1 µl of 5 U/µl Pegasus Taq Polymerase (EmbioTec), 5 µl of DNA extract and water to reach the final volume. The amplification protocol consisted of a single denaturation step at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 40 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min.

Amplification reactions were carried out in a MasterCycler (Eppendorf) thermal cycler. PCR products were resolved in 1% agarose gel electrophoresis and visualized under UV light. Then, 15 μl of each PCR product was purified using the enzymatic method EXO/SAP (Werle et al., 1994) and sent to an external laboratory (Macrogen Inc., Korea) for direct sequencing using the same oligonucleotide primers. The sequences obtained for the COI gene were manually edited using Chromas 2.6 software (http://www.technelysium.com.au/chromas.htm) and aligned using ClustalX 2.1 (Thompson et al., 1997). Then, they were compared with the sequences for different species of the family Baetidae available in GenBank by using the Basic Local Alignment Search Tool (BLAST). Finally, a phylogenetic analysis was performed by the Maximum Parsimony method using the Molecular Evolutionary Genetics Analysis (MEGA-X) software (Kumar et al., 2018) with 1,000 bootstrap replications. The COI sequences obtained were related to sequences representing each of the seven genetic haplogroups described for *C. dipterum* (Accession numbers: KU757091, KU757111-21-29-30-36, and LC223543; Yano et al., 2019) and sequences belonging to other *Cloeon* or Baetidae species available in GenBank: *Cloeon simile* Eaton, *C. smaeleni*, *Cloeon praetextum* Bengtsson, and *Callibaetis* sp. (Accession numbers: KY262243, HG935107, JN299150 and HM917067, respectively). A sequence belonging to *Baetis* sp. (Accession number HQ152334) was used as outgroup.

Morphological features of nymphs and imagos

For morphological identification of nymphs, specific publications of the genus and species of *Cloeon* were consulted (Sowa, 1975; Bae & Park, 1997; Webb & Suter, 2011; Salles et al., 2014) and the following characteristics were examined: number and shape of abdominal gills, presence of rows of lateral spines in segments VIII and IX, color pattern of terminal filaments, and number and shape of segments of the maxillary palps and labial palps. In the imagos, we examined the following: venation of the forewings, male genitalia, shape and coloration of eyes, and coloration of abdomen and terminal filaments.

Nymphs and imagos were measured with a ruler millimeter under a stereoscopic microscope. Length was measured from the apex of the head to the last segment of the abdomen.

RESULTS

Molecular analyses

Mitochondrial DNA COI gene fragments with a length of 620, 450 and 613 bp were obtained from three of the four analyzed samples (Accession numbers: MT786862, MT786863, MT786864). When these haplotypes were compared with those reported by Yano et al. (2019), they showed the highest values of percent identity with the genetic groups CT2-3 (range: 95.33 to 98.87% identity), intermediate values with the groups CT1 and JK (range: 92.01 to 92.43% identity) and the lowest values with the groups IS1-3 (range: 88.42 to 90.31% identity). The Maximum Parsimony phylogenetic tree showed that the haplotypes found in north-eastern Buenos Aires province (*C. dipterum* I, II and IV) constitute a clade of high reliability (99% bootstrap value) with the genetic groups CT2-3 (Fig. 2). All these sequences were then grouped with the rest of the *C. dipterum* sequences in another clade, with a high degree of reliability (99% bootstrap value). Moreover, the sequences from north-eastern Buenos Aires province showed a clear differentiation with the other *Cloeon* species and with *Callibaetis*, a genus with which it shares similar morphological features.

Morphological features of nymphs and imagos

The main diagnostic characteristics of the nymphs (5-7 mm) were: I) the terminal filaments, which have black rings located interspersed to the middle zone, located next to each other forming three black spots, one in each filament (characteristic of the species) (Fig. 3a), and II) the dorsal surface of the abdomen of mostly dark coloration with two small central yellow spots oriented obliquely backwards (Fig. 3b). Also, the presence of rounded double gills (similar to plates with tracheas) in segments I to VI, simple gills in segment VII (Fig. 3b), and rows of lateral spines in segments VIII and IX (Fig. 3c) were useful to distinguish this species. The morphological features of the labrum (Fig. 4a), hypopharynx (Fig. 4b), mandibles (Fig. 4c, d), and tarsal claws allowed us to separate *Cloeon* from *Callibaetis* found in the same collection sites as *C. dipterum.* Moreover, the maxillary and labial palps were composed of three segments (Fig. 4e, f), which is characteristic of the species of the genus *Cloeon* present in South America.

The main diagnostic characteristics of adults (6-8 mm) were the forewings, with single intercalary veins and absence of hindwings (Fig. 5a, c). The coloration of the body is orange-yellowish with rust-colored circular spots on the abdomen. The eyes in males are turbinate, from medium to large widened upwards (Fig. 5a), whereas in females the compound eyes are ornamented with two brown longitudinal bands. In the male genitalia the forceps are thin and their basal segment, whitish, is distinctly shorter than the second, being these strongly separated from the third. The base of the third segment has a small spur (Fig. 5b). The penis is a cone bent backwards, more strongly sclerotized on the ventral side, attached to the body with two arms, and can be attached back and move up and down. The terminal filaments of both sexes are clear with black rings.

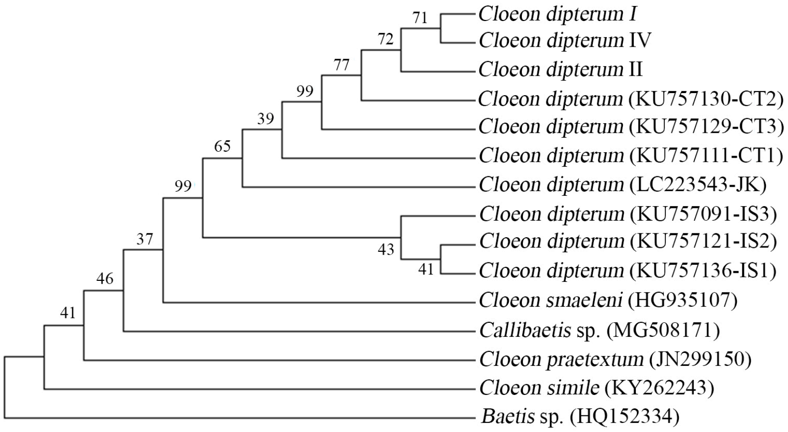

Fig. 2. Maximum Parsimony phylogenetic tree of the sequenced samples (*Cloeon dipterum* I, II and IV) compared with sequences of *C. dipterum* and other *Cloeon* and Baetidae species. GenBank accession numbers between brackets. The numbers around nodes indicate the bootstrap values.

DISCUSSION

Our results from molecular and morphological analyses confirmed the hypothesis that *C. dipterum* was introduced in the province of Buenos Aires (Argentina), representing the first record of the species in freshwaters habitats of slow current or stagnant waters in the country. The features of the body observed in nymphs and imagos, as well as the description of each of the mouthparts made in a previous study (Banegas et al., 2020), agree with those belonging to *C. dipterum* described by Brown (1961) and Sowa (1975). It is known that *C. dipterum* belongs to a complex of species, including species such as *Cloeon cognatum* Stephens, *Cloeon inscriptum* Bengtsson, *Cloeon rabaudi* Verrier, *Cloeon saharense* Soldán & Thomas and *Cloeon peregrinator* Gattolliat & Sartori (Rutschmann et al., 2016). Due to this, some authors have included some of these species within *C. dipterum* in studies of reconstruction of the phylogenetic relationships of Ephemeroptera (Rutschmann et al., 2014, 2016; Yano et al., 2019).

The haplotypes obtained through molecular analyses showed a high percentage of identity with the haplogroups CT2-3 described by Yano et al. (2019) for *C. dipterum*, which indicates that the samples analyzed belong to that species. The sequences of *C. dipterum* used in the phylogenetic tree were part of the same clade, with a high support (99% bootstrap value), which would indicate the monophyly of *C. dipterum*. It is remarkable, though, that *Callibaetis* sp. was recovered within the clade of the genus *Cloeon.* This could have been caused by an identification error in the only available sequence of *Callibaetis*, so this material should be reviewed to verify its identity, but this exceeds the aim of our work. It should also be noticed that nymphs of both genera have similar morphological features (Domínguez & Fernández, 2009; Webb & Suter, 2011; Salles et al., 2014): gills on abdominal segments I-VI with double and rounded lamellae and tarsal claws pointed towards

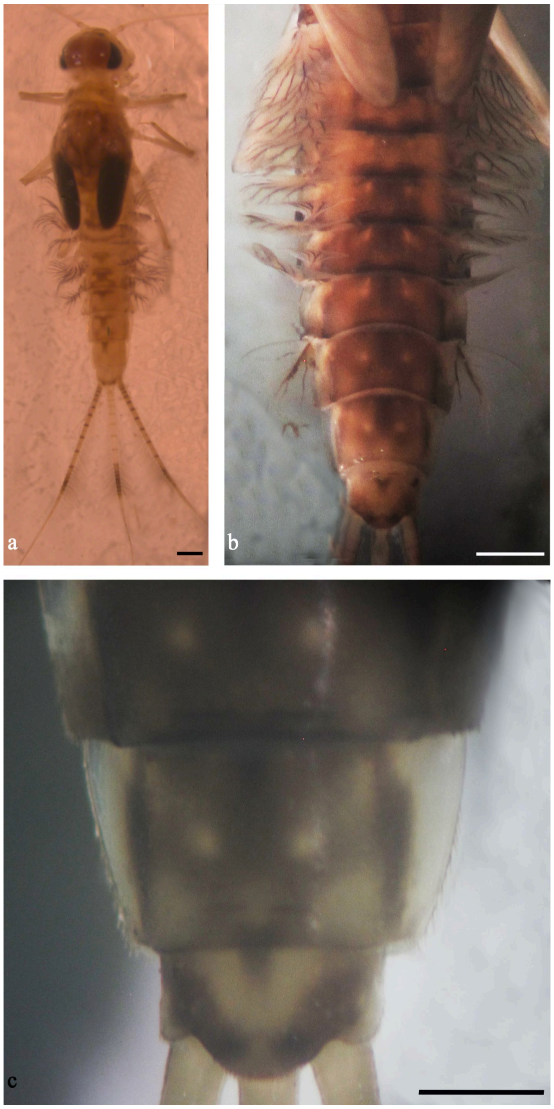

Fig. 3. *Cloeon dipterum*. a. Nymph. b. Male nymph, dorsal view. c. Detail of the male nymphal tergite IX. Scale bars: 500 µm.

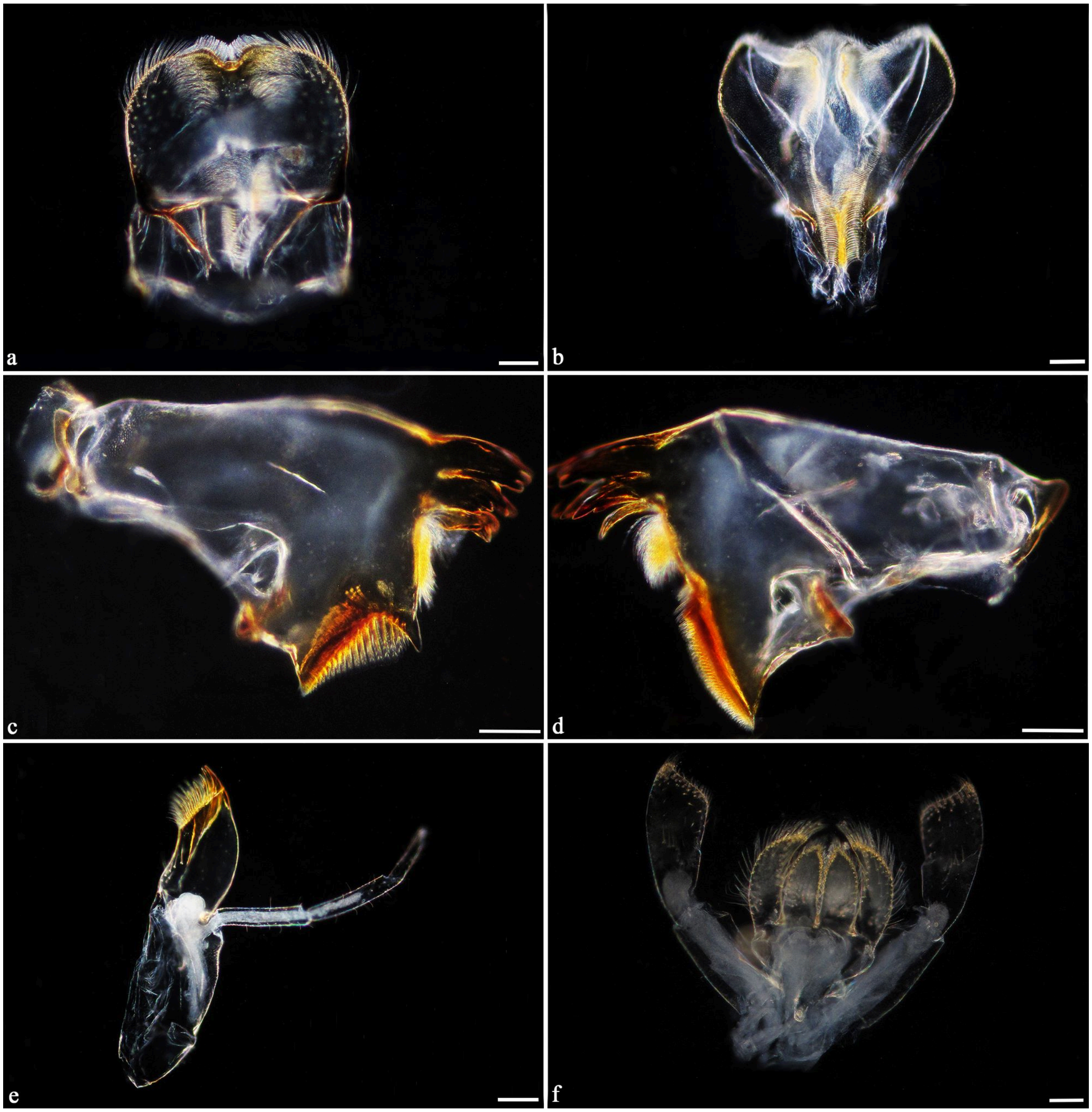

Fig. 4. ***Cloeon dipterum*, dorsal view. a. Labrum. b. Hypopharynx. c. Left mandible. d. Right mandible. e. Right maxilla. f. Labium.** Scale bars: 100 µm.

the apex with two rows of cylindrical denticles. Moreover, they share the same habitats: low current bodies of water (Nieto, 2008), and are both currently distributed in Buenos Aires province; they were found in the same sampling sites. However, there are not previous studies in which the phylogenetic relationships between *Callibaetis* and *Cloeon* are reconstructed. Therefore, we consider that the inclusion of a greater number of species is necessary to reconstruct the evolutionary histories between the different mayflies.

The distribution of *C. dipterum* in Buenos Aires and its absence from previously sampled habitats would indicate that the individuals collected are introduced. Yet, it is still unknown how and where this introduction has occurred. We could speculate that the ovoviviparity, the viability of female imagos and the great tolerance of *C. dipterum* nymphs to environmental factors would allow their establishment even in temporary water bodies, facilitating their dispersion (Sowa, 1975). In addition, female imagos may often be found quite far

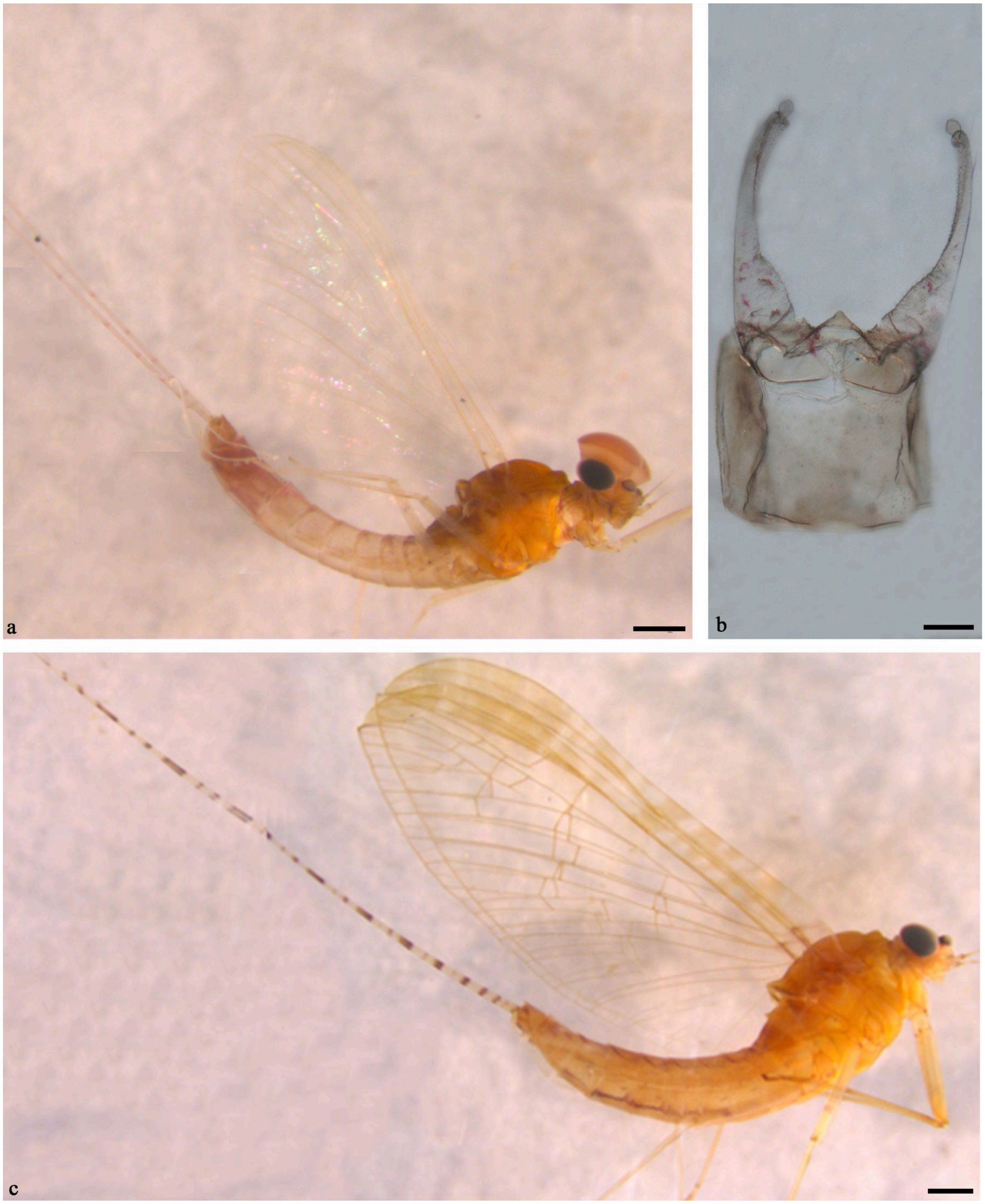

Fig. 5. *Cloeon dipterum*. a. Male imago, lateral view. b. Male genitalia. c. Female imago, lateral view. Scale bars: 500 µm (a-c); 30 µm (b).

from the water body where they developed as nymphs and are able to actively disperse into new habitats, all of which makes them a true colonizing species (Menetrey et al., 2008). *Cloeon dipterum* is abundant in temporary ponds, slow-moving streams, pools and peat bogs (Beketov & Liess, 2005; Jeffries, 2005; Vera et al., 2015; Vilenica et al., 2016), and imagos have even been collected from windows inside rooms (Vera et al., 2015).

In the swimming pools sampled, we found the nymphs exclusively among organic substrates such as leaf litter, and were very abundant in spring and summer.

As concluded by Salles et al. (2014), it is difficult to predict the impact caused by the presence of non-native species on the coast of Brazil or Argentina. However, it is clear that *Cloeon* species inhabit the same habitats as *Callibaetis* ones. Long-term studies of population dynamics are essential to evaluate the effect of non-native species on native species and their habitats.

ACKNOWLEDGMENTS

We are grateful to Soledad Byrne for her collaboration in molecular analyses, Dr. Frederico Salles and Dr. Carlos Molineri for their collaboration in the morphological identification and Agustina Silvera for collecting the nymphs. We thank Dr. Fernando Momo for reading and his comments of the manuscript. This work was funded by Departamento de Ciencias Básicas, Universidad Nacional de Luján (PI4 project, DISPCD-CBLUJ:0000503-18), Buenos Aires, Argentina.

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