# The evolutionary history of colour polymorphism in *Ischnura* damselflies (Odonata: Coenagrionidae)

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**Abstract.** A major challenge in evolutionary biology concerns how genetic and phenotypic variation is created and maintained. In this study, we investigated the origin(s) and evolutionary patterns of the female-limited colour polymorphism in *Ischnura*. This involves the presence of one to three colour morphs: one androchrome morph with coloration that resembles that of the male, and two gynochrome morphs (infuscans and aurantiaca) with a female-specific coloration. We documented the colour of 44 and mating system of 36 of the 76 species within *Ischnura* to investigate the ancestral state of both traits and the correlated

evolution and to infer directionality of trait-state transitions. The ancestral state reconstructions suggest that the most recent common ancestor of the ischnuran damselflies was most likely polymorphic and polyandrous. Our results give some support to the evolutionary correlation between female-limited colour polymorphism and mating system in *Ischnura*. That correlation is consistent with the idea that sexual selection through sexual conflict over the frequency of matings has selected for polymorphic females to reduce the overall intensity of male mating harassment, and our finding that the same phenotypic morphs have evolved multiple times (convergent evolution) suggests that several species in this genus might be experiencing similar selective pressures.

**Further key words.** Dragonfly, Zygoptera, ancestral state, correlated evolution, colour polymorphism, mating system

## Introduction

Understanding how genetic variation arises and is maintained is one of the main goals of evolutionary biology. Genetic polymorphisms, such as heritable colour polymorphisms, constitute ideal systems to study the processes that maintain genetic variation over time. Female-limited colour polymorphisms are ubiquitous in nature (Roulin 2004), from insects, *e.g.*, dragonflies and damselflies (Fincke et al. 2005) and butterflies (Kunte 2009), to crustaceans (Fava 1988), reptiles (Forsman & Shine 1995; Vercken et al. 2008) and birds (Galeotti et al. 2003).

In odonates, female-limited colour polymorphisms are surprisingly common, occurring in more than 100 Holarctic species (Fincke et al. 2005), although the degree of intra-family polymorphism varies markedly (Cordero & Andrés 1996). Some coenagrionid damselfly genera like *Argia*, *Coenagrion*, *Enallagma* and *Ischnura* show a strikingly high prevalence of colour polymorphism (Fincke et al. 2005); for example, *Ischnura* includes at least 33 polymorphic species (Table 1). Female-limited colour polymorphism in *Ischnura* consists of two or three mature colour morphs, where one (the androchrome morph) resembles the male in coloration, whereas the other morphs (gynochrome morphs: aurantiaca and/or infuscans) have a more cryptic coloration. Colour variation in *Ischnura* is pronounced, and some of this variation is related also to ontogeny and pruinescence, *i.e.*, pale bluish coloration caused by epicuticular wax crystals (Henze et al. 2019). Genetic studies have shown that female-limited colour polymorphism in *Ischnura* is heritable, and explained by a single Mendelian locus segregating with two alleles in the dimorphic species *I. damula* (Johnson 1964),

*I. demorsa* (Johnson 1966) and *I. senegalensis* (Takahashi et al. 2014) and three alleles in the trimorphic species *I. elegans* (Sánchez-Guillén et al. 2005), *I. genei* (Sanmartín-Villar & Cordero-Rivera 2016) and *I. graellsii* (Cordero 1990a). Several studies have proposed that female morphs use colour in avoiding male harassment (Johnson 1975; Robertson 1985; Hinnekint 1987; Utzeri 1988; Cordero 1992; Sherratt & Forbes 2001; Sirot & Brockmann 2001; van Gossum et al. 2001).

In *Ischnura* the mating system is highly diverse in terms of mating frequency, mating duration and oviposition type. 'Monandrous' species mate more than once only if their sperm loads are low (Fincke 1987), and they appear to mate prior to the development of pruinescence (Johnson 1975). On the other hand, polyandrous females routinely mate more than once, with highly promiscuous females mating several times (5.83 times in average the gynochrome females of *I. elegans*; Sánchez-Guillén et al. 2013a). Mating duration is highly variable, with monandrous females mating for briefer periods than polyandrous females (Robinson & Allgeyer 1996). For instance, polyandrous species as *I. elegans* and *I. graellsii* can mate for several hours (more than five) while monandrous species, such as *I. hastata* or *I. kellicotti,* complete mating within 20 minutes (Robinson & Allgeyer 1996). Oviposition can take place alone, both in species with long copulations, which function as a mechanism of 'in-copula' guarding (Cordero 1990b), and also in monandrous species (because females do not re-mate), or with the male guarding the female in tandem (Robinson & Allgeyer 1996).

Robinson & Allgeyer (1996) were pioneers in addressing the correlation between mating system (polyandry/monandry) and intraspecific colour variation (polymorphism/monomorphism) in several *Ischnura* species. The association between mating rates and colour diversity implies a causative link between high mating rates, *i.e.*, strong male mating harassment due to polyandry, and diversity in coloration, *i.e.*, species with polymorphic females. Empirical evidence has linked female colour morphs to several life history traits in *Ischnura* such as: (i) mating system (Robinson & Allgeyer 1996); (ii) fecundity (Banham 1990; Takahashi & Watanabe 2010; Sánchez-Guillén et al. 2017); (iii) parasite resistance (Sánchez-Guillén et al. 2013b); and (iv) behaviour and male harassment avoidance (van Gossum et al. 2001; Sirot et al. 2003; Galicia-Mendoza et al. 2017; Sánchez-Guillén et al. 2017).

In ischnuran damselflies female-limited colour polymorphism indicates the presence of alternative reproductive strategies (Sánchez-Guillén et al. 2017), as in polymorphic butterflies (Ellers & Boggs 2002), fish (Craig & Foote 2001) and reptiles (Galeotti et al. 2013). Androchrome females are often the minority morph (Fincke et al. 2005; but see Sánchez-Guillén et al. 2011), and commonly experience lower levels of harassment and have lower short-term fecundity (Sánchez-Guillén et al. 2017). On the other hand, gynochrome females tend to have higher fecundity and can thus persist as the majority morph in the population (Sánchez-Guillén et al. 2017), but have low fecundity when male harassment is high (Galicia-Mendoza et al. 2017). The occurrence of female alternative strategies over time and space allows a rapid response of the population to the prevalent ecological conditions (Sánchez-Guillén et al. 2017). For this reason, colour polymorphisms are frequently under multiple selection pressures, which modulate the covariance between phenotypes and fitness, and can affect speciation rates, either positively or negatively (Hugall & Stuart-Fox 2012). The 'morph speciation hypothesis' (West-Eberhard 1986) predicts that the loss of one morph can lead to character release. Thus, this hypothesis predicts that polymorphism is the ancestral state with the monomorphic species at terminal phylogenetic positions (West-Eberhard 1986).

In this study, we reconstructed a phylogeny of *ca* 60 % of all species in the genus *Ischnura* (44 of the 76 described species). Using this phylogeny, we investigated the ancestral state of colour polymorphism (monomorphic/ polymorphic), ancestral colour (androchrome/gynochrome/ andro-gynochrome), and mating system (monandrous/polyandrous). Moreover, we estimated character correlation using phylogenetic comparative approaches to determine if mating system is associated with colour polymorphism in this genus.

## Material and methods

**Data collection on distribution, colour, and mating system descriptions**
We included 44 taxa (42 species and 2 subspecies) for which molecular sequences or DNA were available from five biogeographical regions (Nearctic, Neotropical, Palaearctic, Afrotropical and Australasian/Indomalayan).

We compiled information about: i) species distributions; ii) female mating frequency (polyandrous/monandrous); iii) number of mature colour morphs; iv) type of mature colour morphs (androchrome, aurantiaca or infuscans); and v) genetic system of colour (Table 1). We obtained information from a variety of sources: publications, field guides, web pages and fellow scientists.

**Species distribution**

To determine the biogeographical region for each species, we compiled information by using multiple databases providing open access to biodiversity data, viz. the 'Global Biodiversity Information Facility' (https://www.gbif.org/) and 'The Dragonfiles system' (https://medusa.jcu.edu.au/Dragonflies/home.php).

**Female colour morphs**

We compiled colour information for 44 species. When colour polymorphism was not previously described for a taxon, we gathered available colour information from multiple sources. We did our data search in Google Scholar using the following terms: i) *Ischnura*; ii) colour/color with the Operators (and, or). Our search was done in both Spanish and English. We also obtained information from publications, field guides, web pages and damselfly experts, who both confirmed the information from the search and provided new information, including personal unpublished observations.

  Female-limited colour polymorphism in *Ischnura* is complex, with three genetically determined mature colour morphs and numerous immature colour forms due to colour ontogeny and pruinescence (*cf.* Fincke et al. 2005). In this study, we use four terms (androchrome, gynochrome, aurantiaca and infuscans) to describe female-limited colour polymorphism. The term androchrome refers to male-like females, *i.e.*, mature females that have the same thoracic coloration and black patterning as males. Figures 1a–c show mature *Ischnura* males, Figure 1d shows a juvenile androchrome female with a mid-dorsal black line on the thorax and two humeral black lines, and Figure 1e shows a mature androchrome female with the same thorax coloration and black patterning as males, including the blue spot at the end of the abdomen (except in the *pumilio*-clade).

**Table 1.** List of studied *Ischnura* species, with biogeographical regions in which the species occurs, mating system (pa – polyandrous, ma – monandrous), colour (pm – polymorphism, mm – monomorphism), type of colour morph(s) present (A – androchrome, Au – aurantiaca; I – infuscans), genetic system controlling colours and references. Na – data were not available; ? – not included in the analyses. Ref. (References): 1 – Clausnitzer & Dijkstra (2005); 2 – Yanybaeva et al. (2006); 3 – Haritonov (1988); 4 – Dumont (1996); 5 – Schmidt (1954); 6 – Sugimura et al. (2001); 7 – Tajima & Watanabe (2010); 8 – Robinson & Allgeyer (1996); 9 – Marrón et al. (2015); 10 – Fincke et al. (2005); 11 – Vilela et al. (2017); 12 – Dickerson et al. (1992); 13 – Realpe (2010); 14 – Johnson (1964); 15 – Johnson (1966); 16 – Sánchez-Guillén et al. (2005); 17 – Boudot et al. (2009); 18 – Askew (1989); 19 – Katbeh-Bader et al. (2004); 20 – Schmidt (1954); 21 – Marzoq (2005); 22 – Ozono et al. (2012); 23 – McKee et al. (2005); 24 – Bota-Sierra et al. (2019); 25 – Kosterin (2015); 26 – Dumont (1991); 27 – Garrison & Hafernik (1981); 28 – Sanmartín-Villar & Cordero-Rivera (2016); 29 – Cordero (1989); 30 – Huang et al. (2012); 32 – De Knijf et al. (2016); 33 – Joshi et al. (2020); 34 – Paulson (1999); 35 – Novelo-Gutiérrez & Peña-Olmedo (1989); 36 – van Gossum & Mattern (2008); 37 – Lam (2004); 38 – Dunkle (1990); 39 – Cordero Rivera & Andrés Abad (1999); 40 – Robertson (1985); 41 – Rowe (2010); 42 – Sanmartín-Villar et al. (2016); 43 – Takahashi & Watanabe (2009); 44 – von Ellenrieder & Garrison (2007); 45 – Fincke (1987); 46 – Milena et al. (2015); 47 – Kunz (2015); 48 – Montoya-Q. et al. (2014); 49 – Schröter (2010); 50 – Paulson (2009); 51 – Robinson & Jordan (1996); 52 – Deviche (2010); *1 – M.I. Velásquez-Vélez (unpubl.); *2 – R.A. Sánchez-Guillén (unpubl.); *3 – R. & D. Sparrow (pers. comm.); *4 – I. Sanmartín-Villar (unpubl.); *5 – N. von Ellenrieder (pers. comm.); *6 – C.A. Bota-Sierra (pers. comm.); *7 – J. Cuéllar-Cardoso (pers. comm.).

| Species | Regions | mating system | Colours | Type-colours | Gen. system | Ref. |
|---|---|---|---|---|---|---|
| *I. abyssinica* Martin, 1908 | Afrotropical | Na | pm | A, Au | Na | 1 |
| *Ischnura* sp. "a" | Neotropical | pa | pm | A, Au | Na | *1 |
| *I. aralensis* Haritonov, 1979 | Palaearctic | Na | pm | A, Au | Na | 2, 3, 4 |
| *I. asiatica* (Brauer, 1865) | Palaearctic | pa | mm | Au | Na | 5, 6, 7 |
| *I. aurora* Brauer, 1865 | Indomalayan-Australasian | ma | mm | I | Na | 5, 6, 8 |
| *I. barberi* Currie, 1903 | Nearctic | pa | pm | A, Au | Na | 9, 10, 50, 52 |
| *I. capreolus* (Hagen, 1861) | Neotropical | pa | pm | A, Au | Na | 10, 11, *7 |
| *I. cervula* Selys, 1876 | Nearctic | pa | pm | A, Au | Na | 8, 10, 12 |
| *I. chingaza* Realpe, 2010 | Neotropical | ma | pm | A, Au | Na | 13, *6 |
| *I. cruzi* De Marmels, 1987 | Neotropical | pa | pm | A, Au | Na | 48, *7 |
| *I. cyane* Realpe, 2010 | Neotropical | pa | pm | A, Au | Na | *1, 46 |

| Species | Regions | mating system | Colours | Type-colours | Gen. system | Ref. |
|---|---|---|---|---|---|---|
| *I. damula* Calvert, 1902 | Nearctic | pa | pm | A, Au | Au>A | 8, 14 |
| *I. demorsa* (Hagen, 1861) | Nearctic | ma | pm | A, Au | Au>A | 8, 15 |
| *I. denticollis* (Burmeister, 1839) | Nearctic | pa | pm | A, Au | Na | 8, 10 |
| *I. elegans* (Vander Linden, 1820) | Palaearctic | pa | pm | A, Au, I | A>I>Au | 8, 10, 16 |
| *I. e. ebneri* Schmidt, 1938 | Palaearctic | pa | pm | A, Au, I | Na | 17, 18, *2 |
| *I. erratica* Calvert, 1895 | Nearctic | pa | pm | A, Au | Na | 8, 10 |
| *I. evansi* Morton, 1919 | Palaearctic | pa | pm | A, Au, I | Na | 19, 20, 21 |
| *I. ezoin* (Asahina, 1952) | Palaearctic | Na | mm | Au | Na | 22 |
| *I. fluviatilis* Selys, 1876 | Neotropical | pa | pm | A, Au | Na | 11, 23, 24 |
| *I. foylei* Kosterin, 2015 | Indomalayan | Na | pm | A, Au | Na | 25 |
| *I. forcipata* Morton, 1907 | Palaearctic | pa | mm | Au | Na | 5, 49 |
| *I. fountaineae* Morton, 1905 | Palaearctic | pa | pm | A, Au | Na | 26, *2 |
| *I. gemina* (Kennedy, 1917) | Nearctic | pa | pm | A, Au | Na | 8,27 |
| *I. genei* (Rambur, 1842) | Palaearctic | pa | pm | A, Au, I | A>I>Au | 10, 28, *2 |
| *I. graellsii* (Rambur, 1842) | Palaearctic | pa | pm | A, Au, I | A>I>Au | 8, 10, 29 |
| *I. hastata* (Say, 1839) | Neotropical-Nearctic | ma | mm | Au | Na | 8, 10 |
| *I. heterosticta* (Burmeister, 1839) | Australasian | pa | mm | I | Na | 30 |
| *I. indivisa* (Ris, 1918) | Neotropical | Na | pm | A, Au | Na | 24 *1 |
| *I. intermedia* Dumont, 1974 | Palaearctic | pa | mm | Au | Na | 32, *3 |
| *I. kellicotti* Williamson, 1898 | Nearctic | ma | mm | A | Na | 8, 10 |
| *I. nursei* (Morton, 1907) | Palaearctic | Na | pm | A, I | Na | 33, 47 |
| *I. perparva* Selys, 1876 | Nearctic | ma | pm | A, Au | Na | 8, 34, 49 |
| *I. p. atezca* Novelo & Pena, 1989 | Nearctic | ma | mm | I | Na | 35, 37 |
| *I. posita* (Hagen, 1861) | Nearctic | ma | mm | I | Na | 8, 37 |
| *I. prognata* (Hagen, 1861) | Nearctic | Na | mm | Au | Na | 36, 37, 38 |
| *I. pumilio* (Charpentier, 1825) | Palaearctic | pa | pm | A, Au | Na | 8, 39 |
| *I. ramburii* (Selys in Sagra, 1857) | Neotropical-Nearctic | pa | pm | A, Au | Na | 8, 40 |
| *I. rubilio* (Selys, 1876) | Indomalayan-Australasian | pa ? | mm | I | Na | 41 |
| *I. rufostigma* Selys, 1876 | Palaearctic | pa | pm | A, Au | Na | 42, *4 |
| *I. saharensis* Aguesse, 1958 | Palaearctic | pa | pm | A, Au, I | Na | 17, 18, *2 |
| *I. senegalensis* (Rambur, 1842) | Palaearctic-Afrotropical-Indomalayan | pa | pm | A, Au | Au>A | 43, 51 |
| *I. ultima* Ris, 1908 | Neotropical | ma | pm | A, Au | Na | 44, *5 |
| *I. verticalis* (Say, 1839) | Nearctic | ma | pm | A, Au | Na | 8, 45 |
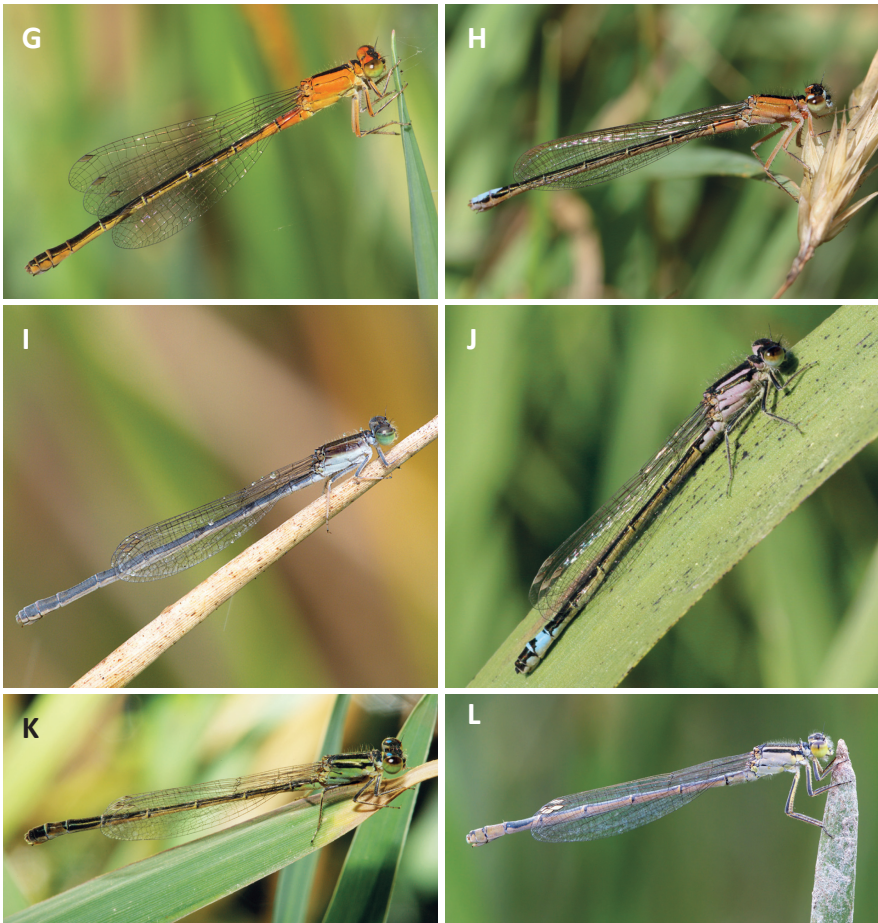
**Figure 1.** Colour diversity in ischnuran damselflies. A – *Ischnura senegalensis* mature male (Namibia). B – *Ischnura chingaza* mature male (Colombia). C – *Ischnura aurora* mature male (Australia). D – *Ischnura elegans* juvenile androchrome (violet thorax coloration) (Spain). E – *Ischnura elegans* mature androchrome (blue thorax coloration) (Spain). F – *Ischnura capreolus* juvenile aurantiaca (citron-fluorescent thorax coloration) (Peru). G – *Ischnura pumilio* juvenile aurantiaca (orange thorax coloration) (Azores, Portugal). H – *Ischnura chingaza* juvenile aurantiaca (orange thorax coloration) (Colombia). I – *Ischnura hastata* mature aurantiaca (body pruinescent coloration) (Azores, Portugal). J – *Ischnura graellsii* juvenile infuscans (light violaceous thorax coloration) (Spain). K – *Ischnura genei* mature infuscans (green-brown thorax coloration) (Sardinia, Italy). L – *Ischnura heterosticta* mature infuscans (green-grey thorax coloration) (Australia). Photos by ACR

We used the term gynochrome for all non-male-like females (aurantiaca and infuscans). The term aurantiaca morph was used to denote gynochrome females with an orange thorax coloration (but sometimes yellow or even pink) when juvenile, and in many cases with only one mid-dorsal black line on the thorax, with or without the blue spot on the abdomen (Figures 1f–h). Upon maturation, aurantiaca females become brownish, sometimes with greenish tones, and lose the blue spot on the abdomen (Figure 1i). Also, when more than 7–10 days old, some females may develop two deep brown (rarely black) humeral lines, resembling the black patterning of males. This morph is therefore orange when immature, but not when mature. Exceptionally, in some species (*e.g.*, *I. pumilio*), the juvenile aurantiaca coloration changes to blue in androchromes or brown in gynochrome females.

The term infuscans refers to gynochrome females with a mid-dorsal black line on the thorax and two black humeral lines. They therefore have a black patterning similar to male and androchromes, and usually have a blue spot on the tip of the abdomen when juvenile (Figure 1j). Before maturation, female juvenile thoracic coloration is highly variable and can be white, violet, light blue, or green. Upon maturation, these females become greenish and finally brown and lose the blue tip (Figures 1k–l) and, in many species, are indistinguishable from the mature aurantiaca females. We treated *I. heterosticta* as a monomorphic species because, while it was defined as a dimorphic species consisting of the androchrome and infuscans morph (*cf.* Tillyard 1905), Huang & Reinhard (2012) found that the androchrome morph is in fact the immature ontogenetic colour stage of the infuscans morph. Thus, *I. heterosticta* is a genetically monomorphic species when mature.

### Mating frequency (mating system)

We compiled information about mating frequency (mating system) for 36 species. When the mating system was not previously described, we determined field mating frequency again via a data search in Google Scholar using the following terms: i) *Ischnura*; ii) mating; iii) copula; and iv) alone, with the Operators (and, or). Our search was done in Spanish and English. We also obtained information from publications, field guides, web pages and damselfly experts to confirm and expand our dataset.

In our study, we used copulation frequency as an indicator of mating system (*cf.* Robinson & Allgeyer 1996; Fincke 1987). These authors considered all species to be polyandrous when females were seen to mate more than once, even at a low rate, and monandrous for all species that rarely mate after maturity (only if their sperm load is low). Additionally, Robinson & Allgeyer (1996) defined those species commonly seen in copula as polyandrous, and those species rarely seen in copula as monandrous. Robinson & Allgeyer (1996) identified several life characters in the monandrous *vs* polyandrous species: i) smaller individuals; ii) greater sexual size dimorphism; iii) shorter copulation duration; and iv) no male biased operational sex ratios at aquatic sites, compared to polyandrous species. Sixteen species were previously categorized by Robinson & Allgeyer (1996) as monandrous (*Ischnura aurora*, *I. demorsa, I. hastata*, *I. kellicotti*, *I. perparva* and *I. posita posita*) and polyandrous (*Ischnura cervula*, *I. damula, I. denticollis*, *I. elegans*, *I. erratica*, *I. gemina*, *I. graellsii*, *I. pumilio, I. ramburii* and *I. senegalensis*). One more species, *I. verticalis*, was classified as monandrous by Fincke (1987) since mature females mated only if their sperm loads were low. Following that methodology, we comprehensively reviewed information about mating frequency (mating system), operational sex ratio at the aquatic sites, for a further 19 species (see Results).

**DNA extraction and sequencing**
Previous phylogenetic studies included 15 Nearctic and Neotropical species (Chippindale et al. 1999) and 24 old-world species (mainly Palaearctic species; Dumont 2013) and a recent phylogenetic reconstruction added five new species from the Australasian region (Willink et al. 2019). We included 44 species in our study and used two mitochondrial genes, cytochrome oxidase II (COII) and cytochrome b (CYTB), and a part of two nuclear genes, the small sub-unit 18S nrDNA (ITS1) and 5.8S (ITS2), and the large sub-unit rDNA 28 nrDNA (18S-ITS). For species lacking published sequences (*Ischnura* sp. "a", *I. chingaza*, *I. cruzi*, *I. cyane*, *I. foylei*, and *I. indivisa*), DNA was extracted and sequenced. Details are given in supplementary material; for GenBank accession numbers see Table S1 of Supplementary File 1. The inclusion of *Ischnura* sp. "a", *I. chingaza*, *I. cruzi, I. cyane* and *I. indivisa* represents a completely new clade from the Neotropical region

that has not previously been studied, while including *I. foylei* increases the number of Australasian species.

**Phylogeny**

Sequence data were aligned using MAFFT v.7 (Katoh & Standley 2013) and then uploaded to CIPRES v3.3 (Miller et al. 2010) for phylogenetic reconstruction. All aligned sequences are given in Supplementary File 2. *Enallagma basidens* and *E. civile* were designated as outgroup species (Bybee et al. 2008; Dumont et al. 2010).

   Phylogenies were estimated under Bayesian and maximum likelihood frameworks. Maximum likelihood (ML) analysis was carried out using RAxML (Stamatakis 2014) under GTR+GAMMA model. Nodal support was estimated with 1 000 bootstrap replicates. Bayesian phylogenetic analysis was performed using BEAST v2.4.8 (Bouckaert et al. 2014) assuming relaxed molecular clock model, under GTR+GAMMA model of DNA substitution. The most unconstrained form of GTR family substitution model (implemented in BEAST and RAxML) was chosen because it is preferred over other more restricted models (*e.g.*, HKY or JC) (Abadi et al. 2019). A log-normal distributed secondary root calibration (*Ischnura-Enallagma* split = 44 Ma; Toussaint et al. 2019) was enforced with mean (in real space) of 44 Ma and standard deviation of 0.25. Additionally, a second log-normal distributed secondary calibration prior was assigned for *Ischnura* crown group (mean in real space = 30, standard deviation = 0.25, with monophyly enforced) following the date estimates of *Ischnura* in Toussaint et al. (2019). Multiple runs of 30 000 000 MCMC iterations were performed and checked for convergence using the program Tracer v.1.6.0 (available at http://beast. community/). The sampled posterior trees (Supplementary File 3) were used for further downstream analyses (Bayesian posterior probabilities of clades, ancestral state estimation and correlated character evolution).

**Phylogenetic signal, ancestral state estimation and correlated character evolution**

To evaluate the presence of a phylogenetic pattern in our traits of interest (female-limited colour polymorphism and mating system), where trait states/values are more prevalent in certain clades but not in others, we estimated

the phylogenetic signal of each trait using the maximum clade credibility (MCC) tree obtained from the BEAST analysis. Since we were interested in the presence or absence of polymorphism (monomorphic/polymorphic) and mating system (polyandry/monandry), we classified species as possessing (1) or not (0) possessing a polymorphism and based on the type of mating system observed (1 = polymorphic/polyandry; 0 = monophormic/monandry). We measured the phylogenetic signal of both traits separately by calculating the D statistic, specifically designed for binary traits (Fritz & Purvis 2010). Phylogenetic signal is considered when D values are around 0 or negative, *i.e.*, clumped or extremely clumped, respectively, according to Brownian motion (BM) evolution. Conversely, D values around 1 or higher (random or overdispersed pattern, respectively, according to BM evolution) imply a lack of phylogenetic signal in the binary trait. We used 1 000 permutations to calculate the probability of the observed D value under simulated BM and under a null model of no phylogenetic structure as implemented in the R package 'caper' (Orme et al. 2013).

To estimate the ancestral states of female-limited colour polymorphism and mating system types for the *Ischnura* crown, a Bayesian Stochastic Mapping (Huelsenbeck et al. 2003) was used as implemented in the R package 'phytools' (Revell 2012). For female-limited colour polymorphism, the morph types were grouped into (i) two categories: monomorphic *vs* polymorphic; and (ii) three categories: androchrome *vs* gynochrome *vs* androgynochrome. Mating system was categorized into binary traits (monandry, polyandry). One thousand stochastic maps were simulated on the MCC tree from BEAST, and based on weighted Akaike Information Criteria (Akaike 1974), the character state transition models applied were the "equal rates" model for the binary state coding (colour polymorphism grouping (i) above and mating system) and the "symmetrical" model for the polymorphic data (colour polymorphism grouping (ii) above).

An evolutionary correlation test between female-limited colour polymorphism and mating system was carried out in 'phytools' on the MCC tree using Pagel's binary character correlation test (Pagel 1994) with a likelihood ratio test to determine the model fit of either independent or dependent rate transition models (Pagel & Meade 2006) and to test whether the evolution of one trait was dependent on the other.

## Results

### Distribution, female-limited colour polymorphism and mating frequency

The 44 *Ischnura* species studied came from five biogeographical regions. Of these, 37 came from one biogeographical region (13 species from the Nearctic, eight from the Neotropical, 15 from the Palaearctic, one from the Afrotropical, one from the Indomalayan and two from the Australasian); four from two biogeographical regions, two of them from the Neotropical/Nearctic and two from Indomalayan/Australasian; and one from three biogeographical regions, Palaearctic/Afrotropical/Indomalayan (Fig. 2 and Table S2 of Supplementary File 1). We included 44 out of the currently 76 described species; the species that appear in several biogeographic regions were included in all the regions in which they occur to calculate the percentages of the colour polymorphism by biogeographical region: the majority of the Nearctic (100 %), Neotropical [71 % (14)], Palaearctic [75 % (20)]; half of the Afrotropical [50 % (4)]; and a small proportion of the Indomalayan-Australasian [14 % (29)] (Fig. 2 and Table S2 of Supplementary File 1).

Out of the 44 species for which colour morphs have already been described, 12 were monomorphic (6 aurantiaca, 5 infuscans and 1 androchrome), distributed in all except the Afrotropical region. Twenty-six were dimorphic (22 with the androchrome and the aurantiaca morphs, and one taxon with the androchrome and infuscans morph), distributed in all biogeographical regions; and six species were polymorphic, presenting the three colour morphs (androchrome, aurantiaca and infuscans) and being distributed exclusively in the Palaearctic region (Table 1 and Fig. 3).

For the mating system, we included information for 36 of the 44 species studied. Out of the 36 species, seventeen species were previously categorized by Robinson & Allgeyer (1996) and Fincke (1987; Table 1), and 19 species were for the first time categorized in this study, based on published data (7 species); personal communication from expert colleagues on the topic (3 species); or unpublished data from the authors (9 species; details in Table 2). Out of the 36 species, twenty-six were polyandrous, and the remaining ten species were monandrous (Tables 1 and 2).

**Phylogenetic reconstruction**

The phylogenetic tree estimated using Bayesian methods (Fig. 3) showed no major topological incongruence for the clades discussed below. The clades supported in the ML analysis (Fig. S1 of Supplementary File 1) (BS ≥60 %) were also supported in the Bayesian analysis (Bayesian posterior probability, BPP ≥0.95), consistent with the two earlier phylogenies published for this genus. Chippindale et al. (1999) focused on the North American species and included 15 Nearctic and Neotropical species. More recently, Dumont (2013) focused on the old-world species, including 24 mainly Palaearctic species. Our phylogeny included 10 additional species: six Neotropical (*Ischnura* sp. "a", *I. chingaza*, *I. cruzi*, *I. cyane*, *I. indivisa* and *I. ultima*), one Australasian (*I. foylei*), and three Palaearctic (*I. e. ebneri*, *I. genei* and *I. intermedia*). All our newly added ten species were resolved within the *Ischnura* phylogeny, which is a sister group to *Enallagma*. Our phylogenetic analysis was able to group 41 out of the 44 species into nine clades. Six geographically structured clades (clades 1–6) and remaining three clades (clades 7–9) that included species from different distributions (Fig. 3).

The Neotropical and Nearctic clade 1, named by Chippindale et al. (1999) as the *hastata*-clade, was strongly supported (BPP = 1; Fig. 3) and included two monomorphic (aurantiaca morph) species and three dimorphic species (androchrome and aurantiaca morphs; Table 1): one Nearctic-Neotropical (*I. hastata*), one Nearctic (*I. prognata*) and three Neotropical species (*I. chingaza, I. cruzi* and *I. ultima).* This clade includes at least three monandrous (*I. hastata, I. ultima* and *I. chingaza*) and one polyandrous (*I. cruzi*) species (Table 1).

The Nearctic clade 2 (*denticollis*-clade) was strongly supported (BPP = 1; Fig. 3) and comprised 11 species with Nearctic distribution. Our phylogenetic reconstruction reconfirms the close relationship between *I. gemina* and *I. cervula*, and among *I. damula*, *I. verticalis*, *I. perparva*, *I. p. posita*, *I. denticollis*, *I. demorsa* and *I. p. atezca*, consistent with Chippindale's et al. (1999) phylogenetic reconstruction. We were also able to corroborate the finding that *I. p. atezca* is more closely related to *I. demorsa* than to *I. p. posita* (Chippindale et al. 1999) supporting the species status of *I. p. atezca*. The *denticollis*-clade includes three monomorphic species; one of them with the androchrome morph (*I. kellicotti*), and two with the infuscans morph (*I. p.*

*posita* and *I. p. atezca*); and eight dimorphic species, with the androchrome and the aurantiaca gynochrome morph (Table 1). In this clade, the three monomorphic species are monandrous, while only three out of the eight polymorphic species are monandrous (*I. demorsa, I. perparva* and *I. verticalis*; Table 1).

The Palaearctic clade 3 (*pumilio*-clade) was strongly supported (BPP = 0.99; Fig. 3) and included five Palaearctic species: *I. asiatica*, *I. pumilio*, *I. ezoin*, *I. forcipata* and *I. intermedia*. In agreement with a recently published phylogeny (De Knijf et al. 2016) the *pumilio*-clade is a well-supported clade. *Ischnura pumilio* is a dimorphic species that consists of the androchrome and aurantiaca morphs. This species, together with *I. kellicotti* have a unique androchrome female morph that shows an indistinguishable immature coloration from the immature aurantiaca morph coloration (Table 1). The remaining four species (*I. asiatica*, *I. ezoin*, *I. forcipata* and *I. intermedia*) are monomorphic for the aurantiaca morph. The four species for which mating system is known are polyandrous (Table 1).

The Indomalayan-Australasian clade 4 (*aurora*-clade) was strongly supported (BPP = 1; Fig. 3) and included two Indomalayan-Australasian species (*I. aurora* and *I. rubilio*), both of them with a similar and unique abdominal coloration. In these species, the first five male abdominal segments are orange, followed by the sixth black and the seventh and eighth blue segments (Papazian et al. 2007; Schneider et al. 2015). There are slight colour differences between the two. Females have a typical black abdomen and are monomorphic for the infuscans (gynochrome) morph (Table 1). The mating system in *I. aurora* is unusual: In Australia (A.G. Orr pers. comm.) and New Zealand (Rowe 1978), females mate only once (monandrous) immediately after emergence. In India, females formerly identified as *I. aurora*, only mate when fully mature and are polyandrous (Andrew 2001). However, these populations were regarded as *I. rubilio* and not as *I. aurora* by Papazian et al. (2007). Given these taxonomic uncertainties, we have refrained from including *I. rubilio* in our analysis of mating systems, waiting for a critical evaluation of the *aurora*-clade.

The Neotropical clade 5 (*capreolus*-clade) was strongly supported (BPP = 1; Fig. 3) and comprised four recently diverged Neotropical species. *Ischnura cyane* is the most basal species, from which *I. capreolus, I. indivisa* and one

not yet described species (*Ischnura* sp. "a") have recently evolved and they together form the youngest clade in the genus. All four species are dimorphic (Table 1). Coloration is different from other *Ischnura* clades, in that all immature females have bright coloration, with intense orange in the aurantiaca morph, and intense green in the androchrome morph (Realpe 2010). All species for which reproductive behaviour is described are polyandrous; only the mating system of *I. indivisa* is unknown (Table 1).

Clade 6 (weakly supported; BPP = 0.81; Fig. 3) forms the *ramburii*-clade and included two Nearctic-Neotropical (*I. ramburii*, *I. fluviatilis*) and one Australasian (*I. foylei*) species. In a previous phylogeny by Willink et al. (2019), *I. foylei* was resolved in clade 8; that inconsistency could be due to the weak support of clade 6 in our reconstruction. All species have dimorphic females consisting of the androchrome and gynochrome aurantiaca morphs (Table 1) and the two species for which we have information about the reproductive system, *I. ramburii* and *I. fluviatilis*, are polyandrous.

The Palaearctic clade 7 (*elegans*-clade) was strongly supported (BPP = 0.99; Fig. 3). It included seven Western Palaearctic species (*I. elegans*, *I. e. ebneri*, *I. evansi*, *I. fountaineae*, *I. genei*, *I. graellsii* and *I. saharensis*) and is a recent radiation from the Mediterranean basin. In a previous phylogeny by Dumont (2013), *I. evansi* was resolved in the *I. elegans* clade but not in the core group, however that relationship had very low support (50 %). Those species are closely related and almost completely reproductively isolated (Sánchez-Guillén et al. 2013c), but where ranges overlap, hybridization takes place between *I. elegans* and *I. graellsii* in Spain, between *I. elegans* and *I. genei* in Sardinia, and between *I. graellsii* and *I. saharensis* in Morocco (Sánchez-Guillén et al. 2014a, b). All species except for *I. fountaineae*, which has two colour morphs (androchrome and aurantiaca), have the three colour morphs and are highly polyandrous (mean = 5.83 matings in gynochromes of *I. elegans*; Sánchez-Guillén et al. 2013b), so that this clade contains all trimorphic species in the genus *Ischnura* (Table 1).

Clade 8 (strongly supported, BPP = 1; Fig. 3), the *senegalensis*-clade, comprises only two species: one widely distributed (Palaearctic, Afrotropical and Indomalayan; *I. senegalensis*); and one Australasian (*I. heterosticta*). Both species are polyandrous but *I. heterosticta* is monomorphic (infuscans morph) while *I. senegalensis* is dimorphic (androchrome and aurantiaca).
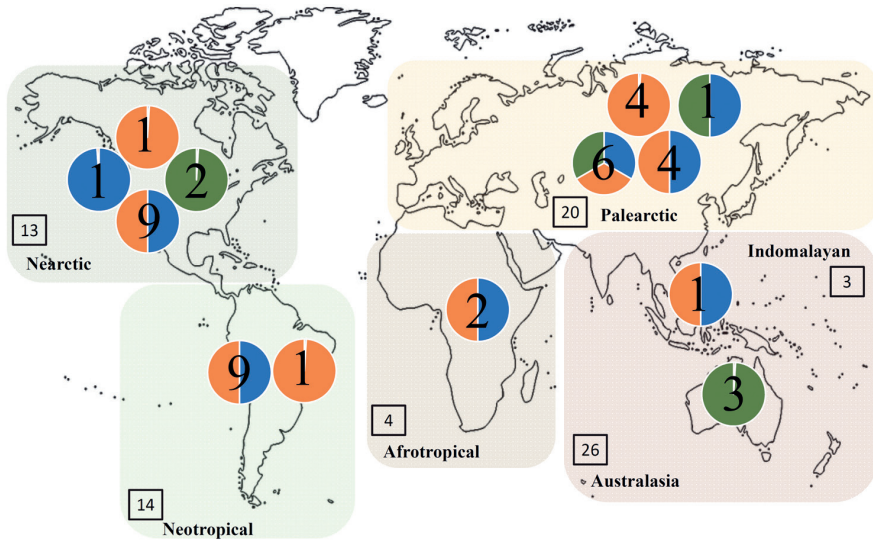
**Figure 2.** Distribution by geographic regions of the female colour diversity of the 44 *Ischnura* species sampled. In each geographic region, colour combinations for each species are shown. Blue colour in circles represents the androchrome morph, orange colour the aurantiaca morph, and green colour the infuscans morph. Nearctic region (13 species): one monomorphic species for the androchrome morph, one monomorphic species for the aurantiaca morph, two monomorphic species for the infuscans morph, and nine dimorphic species with the aurantiaca and the androchrome morphs. Neotropical and Neotropical-Nearctic region (10 species): one monomorphic species for the aurantiaca morph and nine dimorphic species with the aurantiaca and the androchrome morphs. Afrotropical and Afrotropical-Palaearctic-Indomalayan region (2 species): two dimorphic species with the aurantiaca and the androchrome morphs. Palaearctic region (15 species): four monomorphic species for the aurantiaca morph, four dimorphic species with the androchrome and the aurantiaca morphs, one dimorphic species with the androchrome and infuscans morphs, and six trimorphic species. Indomalayan region (1 species): one dimorphic species with the infuscans and the androchrome morphs. Australasian-Indomalayan region (3 species): all with monomorphic species for the infuscans morph. Numbers in squares indicate the number of described species for each biogeographical region. Species present in several regions are included in each region.
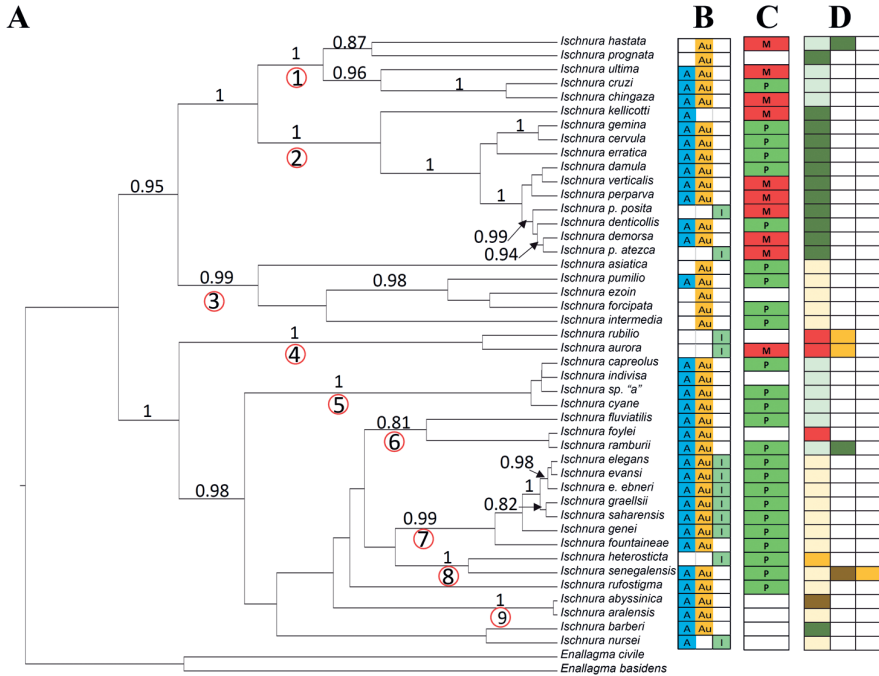
**Figure 3.** A – Maximum-clade credibility tree of COII, CYTB, and ITS sequences for the 44 *Ischnura* species, and two *Enallagma* species (*E. basidens and E. civile*) as outgroups. Values above branches represent the Bayesian posterior probability (only those which have a posterior probability >80 %). Red circles below branches indicate some highlighted *Ischnura* clades. B – Type and number of female colour morphs: Blue rectangle – androchrome; orange rectangle – aurantiaca; green rectangle – infuscans. Females with a rectangle with single colour are monomorphic, females with a rectangle with two colours are dimorphic, and females with a rectangle with three colours are polymorphic. C – Mating system: Green – polyandrous; red – monandrous. D – Geographical affinities of species indicated by colour. Dark green – Nearctic region; pale green – Neotropical region; cream – Palaearctic region; brown – Afrotropical region; red – Indomalayan region; orange – Australasian region.

**Table 2**. List of *Ischnura* species for which reproductive behaviour has been inferred based on mating frequencies and sex ratio. We did our data search in Google Scholar using the following terms: i) *Ischnura*; ii) mating and iii) copula, with the Operators (and, or). * – Mature females seen in tandem or mating, but pairs rarely seen considering the abundance of the females. Ref. (References): 1 – Robinson & Allgeyer (1996); 2 – Rowe (1978); 3 – Paulson (2009); 4 – Vilela et al. (2017); 5 – Dickerson et al. (1992); 6 – Galindo (2018); 7 – Johnson (1964); 8 – Johnson (1975); 9 – Córdoba-Aguilar (1992); 10 – Parr & Palmer (1971); 11 – Cannings & Doerksen (1979); 12 – Paulson & Cannings (1980); 13 – Katbeh-Bader et al. (2004); 14 – McKee et al. (2005); 15 – Schröter (2010); 16 – Hafernik & Garrison (1986); 17 – Cordero (1989); 18 – Huang et al. (2012); 19 – De Knijf et al. (2016); 20 – Bick & Bick (1957); 21 – Robinson (1983); 22 – Cordero Rivera & Andrés Abad (1999); 23 – Robertson (1985); 24 – Furtado (1972); 25 – Fincke (1987). *1 – J. Cuéllar Cardozo (pers. comm.); *2 – F. Palacino-Rodríguez (pers. comm.); *3 – N. Galindo (pers. comm.); *4 – N. von Ellenrieder (pers. comm.).

| Species | ♀ Alone | Tandems | Matings | Mating-Freq. | Sex ratio | Locality/Country | Ref. |
|---|---|---|---|---|---|---|---|
| *I. asiatica* | 191 | | 78 | Common | (260/191) Equal | China | This study |
| *I. aurora* | Many | 1 | 0 | Rare | – | New Zealand | 1, 2 |
| | 7 | 0 | 0 | Rare | (9/7) Equal | China | This study |
| *Ischnura* sp. "a" | >100 | – | 30 | Common | – | Colombia | This study |
| *I. barberi* | – | | Many | Common | | West, USA | 3 |
| *I. capreolus* | Minority | | Majority (30) | Common | – | Colombia | *1 |
| *I. capreolus* | 316 | | 33 | Common | – | Brazil | 4 |
| *I. cervula* | 96 | | 34 | Common | (592/96) Male | California, U.S.A. | 1, 5 |
| *I. chingaza* | 1118 | | 10 | Rare | Female | Colombia | *2 |
| *I. cruzi* | Minority | | Majority (46) | Common | – | Colombia | *3 |
| *I. cyane* | >100 | | 18 | Common | – | Colombia | 6 |
| *I. damula* | >500 | 220 | – | Common | – | New Mexico, USA | 1, 7, 8 |
| *I. demorsa* | >500 | 140 | – | Rare* | – | New Mexico, USA | 1, 7, 8 |
| *I. denticollis* | >100 | – | 36 | Common | Male | Veracruz, Mexico | 1, 9 |
| *I. elegans* | 2073 | | 158 | Common | Male | Wales, UK | 1, 10 |

| Species | ♀ Alone | Tandems | Matings | Mating-Freq. | Sex ratio | Locality/Country | Ref. |
|---|---|---|---|---|---|---|---|
| *I. erratica* | Common | | | | | | 1, 11, 12 |
| *I. evansi* | | Common | | Common | | | 13 |
| *I. fluviatilis* | 107 | | 921 | Common | (2078/921) Male | Uruguay | 14 |
| *I. forcipata* | | | Common | Common | – | Kyrgyzstan | 15 |
| *I. fountaineae* | >20 | – | >5 | Common | | Morocco | This study |
| *I. gemina* | 51 | – | 228 | Common | Male | San Francisco, USA | 1, 16 |
| *I. genei* | 555 | – | 294 | Common | (879/555) Male | Sicily, Sardinia | This study |
| *I. graellsii* | 1727 | – | 732 | Common | Male | Galicia, Spain | 1, 17 |
| *I. hastata* | >500 | 0 | 0 | Rare | Female | Arlington, USA | 1 |
| *I. heterosticta* | 382 | | 41 | Common | 387/382 Equal | Australia | 18 |
| *I. intermedia* | 78 | 7 | 24 | Common | (1478/78) Males | Cyprus | 19 |
| *I. kellicotti* | >500 | 0 | 0 | Rare | Female | Arlington, USA | 1 |
| *I. perparva* | – | | | Rare * | | West, USA | 1, 3 |
| *I. posita* | 248 | 0 | 0 | Rare | (184/248) Female | Louisiana, USA | 1, 20 |
| | 1080 | 0 | 0 | Rare | (1135/1080) Equal | Arlington, USA | 21 |
| *I. pumilio* | 100 | – | 143 | Common | (±400/142) Male | Galicia, Spain | 1, 22 |
| *I. ramburii* | 90 | – | 164 | Common | Male | Florida, USA | 1, 23 |
| *I. rufostigma* | 192 | – | 19 | Common | (272/192) Male | China | This study |
| *I. saharensis* | 217 | – | 131 | Common | (217/251) Equal | Morocco | This study |
| *I. senegalensis* | 300 | – | 12 | Common | – | Malaysia | 1, 24 |
| | 65 | – | 5 | Common | 145/111 (Equal) | China | This study |
| *I. ultima* | Common | 2 | 0 | Rare | – | Argentina | *4 |
| *I. verticalis* | | | | Rare | Female | Michigan, USA | 25 |

Clade 9 (strongly supported, BPP = 1; Fig. 3), also included two species, *I. aralensis* with Palaearctic distribution and *I. abyssinica* with Afrotropical distribution. Both species are dimorphic for the androchrome and the aurantiaca (gynochrome) coloration. No information is available about mating system in both species.

### Phylogenetic signal, ancestral state reconstruction and correlated character evolution

Both female-limited colour polymorphism and mating system of *Ischnura* species showed significant phylogenetic signal. D values for both traits were around 0 (-0.03 and 0.06, respectively) and significantly different from those expected under a random distribution of traits in the phylogeny ($p = 0.003$, for both traits).

The results of the ancestral state estimations based on a combination of 1 000 Bayesian stochastic maps for female-limited colour polymorphism and mating system are shown in Figures 4 and 5. Ancestral state estimation of colour morphs was polymorphic with a probability of 0.63 and on average there were 14.33 changes between the two states, divided into 5.57 changes from mono- to polymorphic and 8.76 *vice versa*. Over the evolutionary history of the genus, the mean proportional time spent in the polymorphic state was 0.63 and 0.37 in the monomorphic state. For the mating system, the ancestral state was estimated as polyandrous with a probability of 0.71 and on average, there were 13.11 changes between the two states, divided into 7.46 changes from monandrous to polyandrous and 5.65 *vice versa*. The mean proportional time spent in the polyandrous state was 0.69 and 0.31 in the monandrous state.

Based on the evolutionary correlation tests between female-limited colour morph and mating system as was previously proposed by ROBINSON & ALLGEYER (1996), the likelihood ratio of the independent *vs* dependent model fit was 5.76 (p = 0.056) in favour of the dependent model (dependent model log-marginal likelihood: -38.86 and independent model log-marginal likelihood: -41.74) (Fig. S2 of Supplementary File 1). For the models testing the dependence of one trait on the other, female colour morph was found to be dependent on the mating system (LR: 4.26, $p = 0.039$; Fig. S3 of Supplementary File 1), but not *vice versa* (LR: 1.71, $p = 0.19$; Fig.  S2 of Supplementary File 1).

## Discussion

Heritable colour polymorphisms, frequently associated with other life history traits, are ideal systems to study the processes that maintain genetic variation over time (Roulin 2004; Gray & McKinnon 2007; Wellenreuther et al. 2014). Ancestral state reconstructions of *Ischnura* suggest that their most recent common ancestor was most likely polymorphic and polyandrous, and there were probably several independent evolutionary transitions to colour monomorphism and monogamy. Our results also confirmed the evolutionary correlation between female-limited colour polymorphism and mating system in *Ischnura*, as was previously proposed by Robinson & Allgeyer (1996), and further showed that colour depends on mating system but not *vice versa*.

### Phylogenetic reconstruction
Our phylogenetic trees based on both Bayesian and ML methods showed no major topological incongruences with previous published reconstructions focused on the North American species (Chippindale et al. 1999), the Palaearctic species (Dumont 2013), and the phylogeny of Willink et al. (2019), which added five new species from the Australasian region. Our phylogenetic trees have added, to previous phylogenetic reconstructions, six Neotropical, three Palaearctic and one Australasian species. However, more effort to include species from the Indomalayan-Australasian region (currently only 14 % of the species) is needed to provide a better balance with the remaining biogeographical regions.

### Phylogenetic signal and ancestral state of colour and mating system traits
Closely related species tend to exhibit similarities in their traits (phylogenetic signal), including life-history among others such as morphological, behavioural and ecological characteristics (Harvey & Pagel 1991). However, it is recommended that such phylogenetic signal should not be assumed *a priori* but instead explicitly tested using trait values and a phylogeny (Blomberg et al. 2003; Revell et al. 2008). In our study we found that both binary traits, female-limited colour polymorphism and mating system, showed a significant phylogenetic signal with values of the D statistic indicating a clumped structure (instead of random) following Brown-

ian motion evolution. As such, closely related species do tend to exhibit a similar female-limited colour polymorphism and mating system. In fact, six clades included only polymorphic species and one clade only monomorphic species, while only two clades clustered both polymorphic and monomorphic species. Similarly, monandry was more prevalent in the Nearctic and Neotropical species (with 3/5 species in the *hastata*-clade and 6/11 species in the *denticollis*-clade) than in the Palaearctic, Afrotropical, Australasian and Indomalayan species with one monandrous species in the six clades (28 species).

A robust inference of an ancestral trait state not only requires an appropriate choice of optimality criterion but also a complete species-level sampling (Litsios & Salamin 2012). Although we have included a high number of species for female colour polymorphism (44 species, 59 % of the genus) and for mating system (36 species, 48 % of the genus), including species from the five biogeographical regions where the genus occurs, our phylogenetic reconstruction remains incomplete. However, it still covers more species than previous studies seeking to infer the ancestral state of female colour polymorphism and mating system (Fincke et al. 2005; van Gossum & Mattern 2008). Our ancestral state reconstruction revealed that the most recent common ancestor of *Ischnura* was most likely polymorphic (polymorphic: 63 %, monomorphic: 36 %). Robinson & Allgeyer (1996) assumed that polymorphism is the ancestral condition arguing that in *Ischnura*, as in many other damselflies, the ancestral mating system included mate guarding by postcopulatory tandem where the male remains in tandem during oviposition (Group 1), then evolved to male mate guarding by extending time in copula, without postcopulatory tandem (Group 2), and then to monandrous species which mate for short periods and whose monomorphic females also oviposit unguarded (Group 3). This assumption, partly supported by our findings, is consistent with the 'morph speciation hypothesis' (West-Eberhard 1986) that predicts that polymorphism is the ancestral state with the monomorphic species at terminal phylogenetic positions. West-Eberhard (1986) proposed that alternative mating strategies (alternative adaptations) are likely to be involved in speciation and that they can even speed up the process itself. Her postulated morph speciation hypothesis predicts that the loss of one morph can lead to a character release.

This is because the loss of a mating morph strategy breaks an evolutionary constraint (on the genome of a polymorphic species) imposed by the ancestral state comprising multiple phenotypes. Once the constraint is broken, West-Eberhard (1986) proposed that new alleles can spread in the population and lead to a phenotypic specialization of the remaining morphs. Finally, this can exert a direct effect on traits for mate recognition via sexual selection and can thus ultimately lead to a generation of genetic divergence and reproductive isolation (West-Eberhard 1983; Rieseberg et al. 1996).

Results from sampling 1000-character histories detected 14.33 changes between the two states and suggest that colour polymorphism most likely evolved 5.57 times and was subsequently lost 8.76 times throughout the evolutionary history of *Ischnura*. Similarly, the most recent common ancestor of *Ischnura* was most likely polyandrous (71%) rather than monandrous (29%), with 13.11 changes between the two states suggesting that polyandry most likely evolved 7.46 times and was subsequently lost 5.65 times throughout their evolutionary history. In *Ischnura*, female-limited polymorphism is controlled by a single autosomal Mendelian locus segregating with two or three alleles, with sex-limited expression probably due to differential gene expression: In *I. elegans* melanin, pteridine and ommochrome colour pathways have already been identified, and two of them, the ommochrome and melanin pathways, showed evidence for differential expression between the sexes (Chauhan et al. 2016). Accordingly, few changes are required to express or suppress a trait in both sexes and thus many losses and gains are expected, as our data have demonstrated.

**Evolutionary history of the colour polymorphism**

A large body of work has related colour polymorphism to several other traits, ranging from life history traits to behaviour (McKinnon & Pierotti 2010). Detailed eco-evolutionary work has revealed several ways by which the occurrence of two or more alternative colour morphs can influence the ecological success and evolutionary dynamics of a species (Forsman et al. 2008). For example, individuals from different morphs have been shown to occupy different subsets of available resource dimensions, such that when summed across alternative morphs the species as a whole occupies a wider niche (van Valen 1965; Galeotti & Rubolini 2004; Svanbäck et al. 2008;
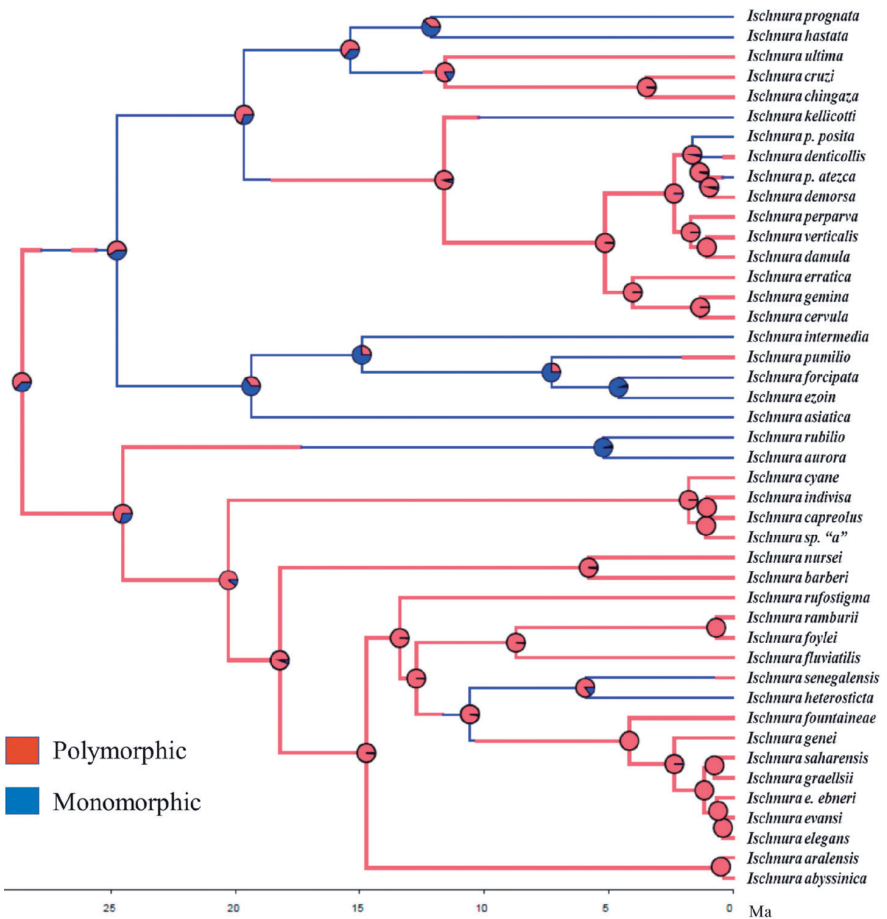
**Figure 4.** Maximum-clade credibility tree of *Ischnura* species with a random Bayesian stochastic map of female-limited colour morphs along the branches and ancestral character states shown at the nodes. Pies at the nodes represent the relative probabilities of each character state (polymorphic *vs* monomorphic) obtained by averaging over 1 000 Bayesian stochastic maps.
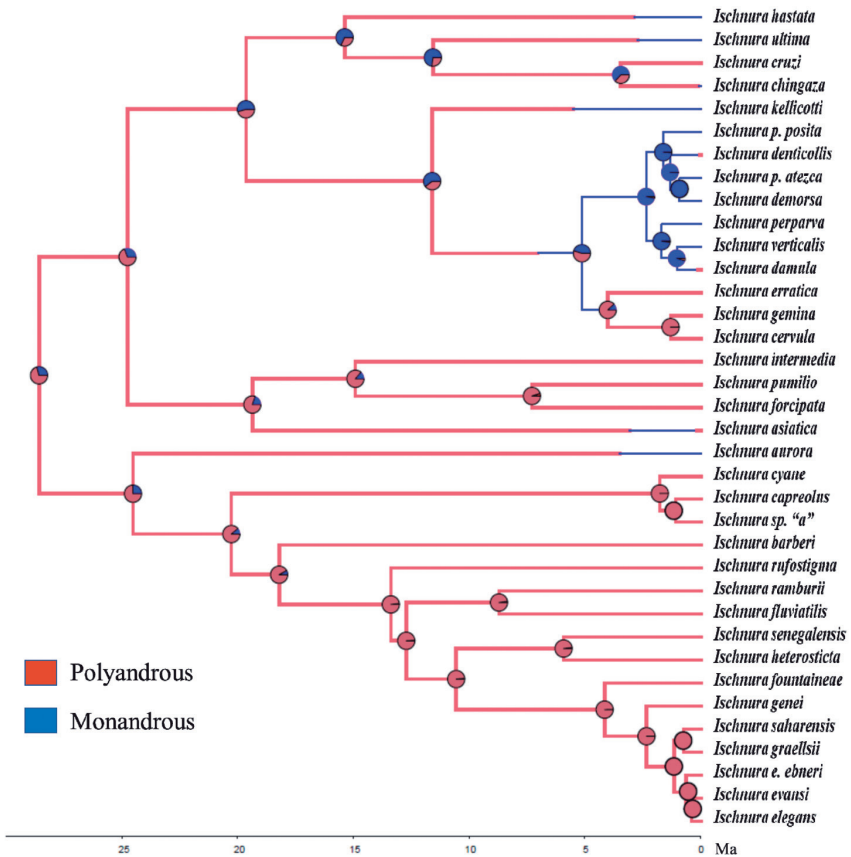
**Figure 5.** Maximum-clade credibility tree of *Ischnura* species with a random Bayesian stochastic map of mating strategy along the branches and ancestral character states shown at the nodes. Pies at the nodes represent the relative probabilities of each character state (polyandrous *vs* monandrous) obtained by averaging over 1 000 Bayesian stochastic maps.

Karpestam & Forsman 2011). The wide utilisation of resources is thought to be due to colour associated effects on performance and fitness, because individuals that differ in coloration utilize different microhabitats that best match their needs, such as temperature, humidity and background matching (Forsman et al. 2008). At a species level, the degree of experienced niche variation of monomorphic *versus* polymorphic species is therefore thought to differ, with polymorphic species occupying a wider fundamental niche compared to monomorphic species (Forsman et al. 2008; Forsman & Wennersten 2016). Accordingly, the presence of polymorphism has been suggested to increase speciation rates (Gray & McKinnon 2007; Gavrilets et al. 2000). Together, previous studies suggest that colour polymorphism in *Ischnura* may confer some advantages in terms of long-term survival and resilience. This agrees with our finding that the polymorphic state is more common than the monomorphic state in *Ischnura*. Other damselfly genera with colour polymorphism, like *Argia, Ceriagrion*, *Coenagrion*, *Enallagma* or *Mesamphiagrion*, are geographically more restricted than *Ischnura*, to one or two biogeographical regions, and apparently show less variation in reproductive strategies. In fact, we are unaware of species described as 'monandrous' in these genera. We hypothesize that the high prevalence of polymorphism in *Ischnura*, and its association with male harassment, has been an engine for behavioural diversification in this genus.

Our correlation test suggests (marginally significant) the view that coloration diversity is associated with the type of mating system (colour depends on mating system but not *vice versa*). A significant association between female-limited colour polymorphism and mating system supports that the phenotypic evolution of additional colour morphs, and thus the diversification at the morph level of the clade could be linked to the intense sexual selection and conflict in this group of damselflies. Indeed, female-limited colour polymorphism has frequently been proposed to be an evolutionary response to sexual conflict over the mode and frequency of matings, and male mating harassment may hence have selected for polymorphic females to reduce harassment levels (Cordero-Rivera & Egido Pérez 1998; Fincke et al. 2005; van Gossum & Sherratt 2008; Sánchez-Guillén et al. 2017). Morph-specific female behaviour and fecundity combined with context-dependent male behaviour can maintain long-term polymorphic states, even

in populations that differ in morph frequencies (Sánchez-Guillén et al. 2017; Galicia-Mendoza et al. 2017). In *I. elegans,* regional variation in stochastic (genetic drift) and deterministic factors (divergent/balancing selection) is responsible for the highly differentiated female morph frequencies, from 3.3 to 74.6 % for the androchrome morph (Sánchez-Guillén et al. 2011), whereas the stable and similar frequencies over the whole distribution of *I. graellsii* (Andrés et al. 2000; Sánchez-Guillén et al. 2005), *I. damula*, *I. demorsa*, *I. ramburii* and *I. denticollis* (Sánchez-Guillén et al. 2011) indicate that some sort of uniform selection is acting over a wide distributional range.

## Conclusions

Our results support the evolutionary correlation between female-limited colour and mating system in *Ischnura*, as proposed by Robinson & Allgeyer (1996), and investigated by Fincke et al. (2005) and van Gossum & Mattern (2008) in *Enallagma* and *Ischnura*. Ancestral state reconstructions suggest that the most recent common ancestor of the ischnuran damselflies was most likely polymorphic and polyandrous. The correlation between female-limited polymorphism and mating system is consistent with idea that sexual selection through sexual conflict over the frequency of matings has selected for polymorphic females to reduce the overall extent of male mating harassment.

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**Appendix I: Supplementary data**

Supplementary data to this article (supplementary files 1–3) can be found online at https://doi.org/10.5281/zenodo.4066815 and on the Odonatologica homepage at http://www.odonatologica.com/supplementary-files/