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ORIGINAL ARTICLE

The influence of stochastic and selective forces in the population divergence of female colour polymorphism in damselflies of the genus *Ischnura*

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Disentangling the relative importance and potential interactions of selection and genetic drift in driving phenotypic divergence of species is a classical research topic in population genetics and evolutionary biology. Here, we evaluate the role of stochastic and selective forces on population divergence of a colour polymorphism in seven damselfly species of the genus *Ischnura*, with a particular focus on *I. elegans* and *I. graellsii*. Colour-morph frequencies in Spanish *I. elegans* populations varied greatly, even at a local scale, whereas more similar frequencies were found among populations in eastern Europe. In contrast, *I. graellsii* and the other five *Ischnura* species showed little variation in colour-morph frequencies between populations. F_{ST}-outlier analyses revealed that the colour locus deviated strongly from neutral expectations in Spanish populations of *I. elegans*, contrasting the pattern found in eastern European populations, and in *I. graellsii*, where no such discrepancy between morph divergence and neutral divergence could be detected. This suggests that divergent selection has been operating on the colour locus in Spanish populations of *I. elegans*, whereas processes such as genetic drift, possibly in combination with other forms of selection (such as negative frequency-dependent selection), appear to have been present in other regions, such as eastern Europe. Overall, the results indicate that both selective and stochastic processes operate on these colour polymorphisms, and suggest that the relative importance of factors varies between geographical regions.
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Introduction

Ever since the start of the 'Modern Synthesis' (Wright, 1931, 1956; Fisher, 1958), the relative influence of different modes of selection and stochastic processes, like genetic drift, in driving phenotypic divergence of species has been a central research question in population genetics and evolutionary biology. In small populations, genetic drift might influence the divergence of adaptive phenotypic traits, particularly during periods of relaxed selection (Orr, 1998; Gray and McKinnon, 2007). Species with heritable colour polymorphisms are suitable model systems to study the interplay between selection and genetic drift in the phenotypic population divergence (Nevo, 1997; Hoffman *et al*., 2006; Gray and McKinnon, 2007; Svensson *et al*., 2009). Several different processes can affect the frequencies of colour morphs in populations, including genetic drift (Oxford, 2005; Gray and McKinnon, 2007), negative frequency-dependent selection (Svensson *et al*., 2005; Abbott *et al*., 2008) and directional selection in spatially and temporally heterogeneous habitats (Gray and McKinnon, 2007). If the balancing force of negative frequency-dependent selection is predominant, then populations are expected to reach similar morph frequencies at equilibrium, compared with a situation where genetic drift or diversifying selection is the dominant force (Abbott *et al*., 2008; Andrés *et al*., 2000, 2002). In contrast, if divergent selection operates and favours different colour morphs in heterogeneous environments, then the degree of population divergence in morph frequencies is expected to be higher than by drift alone (Wong *et al*., 2003).

Female colour polymorphism is common in odonates (dragonflies and damselflies), and more than 130 polymorphic odonate species have been described so far (Fincke *et al*., 2005). However, the degree of polymorphism within odonate families varies markedly. For example, 65% of the polymorphic species in the European fauna belong to the family Coenagrionidae (Cordero and Andrés, 1996), and many of these colour morphs are known to co-occur within the same local populations in the wild. In Coenagrionidae, morphs typically consist of one androchrome (male-mimicking) morph, and one or several discrete female-specific morphs, often called gynochromes (*sensu* Hilton, 1987).

Several adaptive hypotheses, based on sexual conflict over mating opportunities, have been proposed to explain the maintenance of colour polymorphisms in odonates. These hypotheses include either frequency- or

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density-dependent mechanisms, or both. The decisive factor under density-dependent selection is the number of females of each morph, or the number of males. Hinnekint (1987) proposed that males prefer to mate with the gynochrome females at low densities, and that the androchrome females are then at a disadvantage, because they risk to remain unmated. Conversely, at high densities, gynochrome females would be at a disadvantage due to the fitness costs resulting from high male mating harassment. The polymorphism is thus supposed to be maintained by cyclic fluctuations in population density (Hinnekint and Dumont, 1989; Cordero, 1992). On the other hand, under frequency-dependent selection, it is the female morph frequencies that are important (Robertson, 1985; Sherratt, 2001; Fincke, 2004; Svensson *et al*., 2005). As a rare female morph becomes more common in the population, males are expected to switch their attention to those morphs in a frequency-dependent manner, which will cause apostatic selection and a fitness disadvantage of the locally most frequent morph (Fincke, 2004; Svensson *et al*., 2005; Gosden and Svensson, 2009). However, because androchrome females mimic males, males should behave indiscriminately when most females are androchromes, as they would otherwise lose mating opportunities (Robertson, 1985; Sherratt, 2001; Cordero-Rivera and Sánchez-Guillén, 2007). These rapid frequency-dependent switches in female mating harassment are thought to be facilitated by plastic mate preferences (Miller and Fincke, 1999; Fincke, 2004).

In the last decade, different approaches have been utilised to examine the nature of selection operating on such heritable polymorphisms in the wild. Gillespie and Oxford (1998) proposed that insights into the relative importance of divergent and negative frequency-dependent selection can be obtained by contrasting the degree of genetic differentiation at a specific locus, in this case the colour-morph locus, to the degree of differentiation at neutral markers. Several recent studies on damselfly species have utilised this approach, but they have reached different conclusions regarding the relative importance of selection and drift. In both *Ischnura graellsii* (Andrés *et al*., 2000) and *Ceriagrion tenellum* (Andrés *et al*., 2002), the colour-morph locus divergence was lower, compared with the neutral divergence between populations, suggesting that negative frequency-dependent selection towards a common equilibrium does constrain population divergence. In contrast, a study by Wong *et al*. (2003) on *Nehalennia irene* found evidence for more pronounced population divergence at the morph locus than at neutral loci, suggesting that divergent selection has caused the pronounced differences in morph frequencies in local populations of this species. Finally, Abbott *et al*. (2008) studied populations of *Ischnura elegans* and found that some sort of balancing selection on the colour-morph locus operated in some years, whereas divergent selection was predominant in other years.

I. graellsii and *I. elegans* are two closely related species (Carchini *et al*., 1994) with very similar morphology (Monetti *et al*., 2002). In comparison, *I. graellsii* shows a limited distribution on the Iberian Peninsula and adjacent areas of northern Africa, whereas *I. elegans* has a wider distribution, encompassing both Europe and western Asia (Dijkstra and Lewington, 2006). As a result of their ecological and morphological similarities, the two species hybridise where their distributional ranges overlap (Monetti *et al*., 2002; Sánchez-Guillén *et al*., 2005). In *I. graellsii*, the female colour-morph frequencies are almost uniform among populations, with the *infuscans* (gynochrome) morph being the majority morph (Andrés *et al*., 2000; Sánchez-Guillén *et al*., 2005). In contrast, the female colour-morph frequencies in *I. elegans* vary substantially among populations, at both regional and continental scales (Gosden, 2008; Hammers and Van Gossum, 2008; Cordero-Rivera and Sánchez-Guillén 2007). Hammers and Van Gossum (2008) and Gosden (2008) studied the colour-morph frequency of European populations of *I. elegans* on a small and large scale, respectively. Although both studies found a geographic gradient in *androchrome* frequencies, Hammers and Van Gossum (2008) found an increase towards the north and east of the Netherlands and Belgium (Hammers and Van Gossum, 2008), whereas Gosden (2008) found a highly significant large-scale geographic cline towards the north. It was suggested that populations near the range limit might have had less time to reach an equilibrium of morph frequencies that would be expected under negative frequency-dependent selection, compared with those at the centre and in the southern part of the distributional range (Gosden, 2008). In addition, it has been proposed that morph frequencies in southern European populations of *I. elegans* are affected by a combination of various types of selection and genetic drift due to small population sizes, and/or recent founder events, and potentially also through hybridisation with *I. graellsii* (Gosden, 2008).

Here, we evaluate whether and how different modes of selection operate on the colour polymorphism in the sister species *I. elegans* and *I. graellsii*. We follow the approach suggested by (Kay *et al*. (2007) and compare morph-frequency divergence in different parts of the distributional range of *I. elegans*. We further review the literature on colour-morph frequencies in these two and five other species of the genus *Ischnura* to evaluate if colour-morph frequencies are generally stable among populations, and if the androchrome morph can also reach high frequencies in species other than *I. elegans*. We also use the F_{ST}-outlier procedure described by Beaumont and Nichols (1996), as modified by Excoffier *et al*. (2009), to generate null distributions of neutral F_{ST} values at different levels of variability and to evaluate signs of different types of selection at the colour locus.

Materials and methods

Colour-morph frequencies

To evaluate colour-morph frequency patterns in *Ischnura* species, we used colour-morph frequencies of populations from the literature, and we also collected data from additional populations (Tables 1 and 2). We analysed female morph frequencies from the literature in seven *I. graellsii* populations from Iberia (Andrés *et al*., 2000; Sánchez-Guillén *et al*., 2005), five *I. elegans* populations from eastern Europe (Gosden, 2008), four *I. senegalensis* populations from Japan (Takahashi and Watanable, 2009), four *I. fluviatilis* populations from Uruguay (Mckee *et al*., 2005), four *I. damula* (Johnson, 1964), three *I. ramburii* (Robertson, 1985; Sirot *et al*., 2003) and two

Table 1 Frequencies of female colour morphs in natural populations of *Ischnura elegans* and *I. graellsii*

Species	*Locality*	*Country*	*Date*	*Observed frequencies*				*Source*
				N	*A*	*I*	*O*	
I. elegans	Suchoi Limon*	Ukraine	May-2006	39	33.3	66.7	0.0	Gosden (2008)
I. elegans	Enmakov Island*	Ukraine	May-2006	64	31.3	68.8	0.0	Gosden (2008)
I. elegans	Lublin-Zemborzyce*	Poland	Jul-2007	45	22.2	71.11	6.7	Gosden (2008)
I. elegans	Zwięczyca Reszów*	Poland	Jul-2007	34	23.5	76.5	0.0	Gosden (2008)
I. elegans	Breznica*	Poland	Jul-2007	39	25.6	66.7	7.7	Gosden (2008)
I. elegans	Arreo*	Spain	Jul-2008	30	6.3	68.8	25.0	This study
I. elegans	Alfaro*	Spain	Jul-2007	33	69.7	27.3	3.0	This study
I. elegans	Baldajo*	Spain	Aug-2008	34	29.4	58.8	11.8	This study
I. elegans	Almoquera	Spain	Aug-2008	28	42.9	42.9	14.3	This study
I. elegans	Europa*	Spain	Jul-2008	30	16.7	6.7	76.7	This study
I. elegans	Amposta*	Spain	Jul-2008	30	3.3	33.3	63.3	This study
I. elegans	Marjal del Moro*	Spain	Sept-2008	25	36.0	20.0	44.0	This study
I. elegans	Caixanet	Spain	Sept-2009	27	25.9	25.9	48.1	This study
I. elegans	Doniños*	Spain	Jun-2007	40	20.0	75.0	5.0	This study
I. elegans	Louro*	Spain	Agu-2007	63	74.6	20.6	4.8	This study
I. elegans	Foz	Spain	Jun-2007	20	25.0	75.0	0.0	This study
I. graellsii	Ribeira de Cobres*	Portugal	Apr-2003	48	18.8	72.9	8.3	Sánchez-Guillén *et al*. (2005)
I. graellsii	Jaraiz de la Vera	Spain	Jun-2007	67	13.5	64.9	21.6	This study
I. graellsii	Doñana	Spain	Jun-2003	77	10.4	76.6	13.0	Sánchez-Guillén *et al*. (2005)
I. graellsii	La Cañas	Spain	Jul-2007	23	17.4	69.6	13.0	This study
I. graellsii	Alfaro	Spain	Jul-2007	23	13.0	60.9	26.1	This study
I. graellsii	Troi	Spain	Jul-2008	54	14.8	72.2	13.0	This study
I. graellsii	Córdoba*	Spain	Sept-2008	35	11.4	77.1	11.4	This study
I. graellsii	Puente de los Arenales	Spain	Sept-2008	33	3.0	78.8	18.2	This study
I. graellsii	Castelo	Spain	Jun-1999	42	11.9	76.2	11.9	Andrés *et al*. (2000)
I. graellsii	Corrubedo	Spain	Jun-1999	28	21.4	60.7	17.9	Andrés *et al*. (2000)
I. graellsii	Campus*	Spain	Jun-1999	68	1.5	89.7	8.8	Andrés *et al*. (2000)
I. graellsii	O Rosal	Spain	Jun-1999	68	8.8	85.3	5.9	Andrés *et al*. (2000)
I. graellsii	A Lanzada	Spain	Jun-1999	75	14.7	76.0	9.3	Andrés *et al*. (2000)
I. graellsii	Saidïa*	Morocco	Jun-2009	29	10.3	89.7	0.0	This study

The number of mature female examined in each locality is indicated (N). A: *androchrome*, I: *infuscans* and O: *infuscans-obsoleta* (*I. elegans*) or *aurantiaca* (*I. graellsii*)**.** Indicated populations (*) were used in the genetic analysis.

Table 2 Frequencies of female morphs in natural populations of five dimorphic *Ischnura* species: *I. senegalensis, I. fluviatilis, I. damula, I. ramburii* and *I. denticollis*

Species	*Country*	*Date*	*Observed frequencies*			*Source*
			N	*A*	*G*	
I. senegalensis	Japan	May-2007	37	21.6	78.4	Takahashi and Watanable (2009)
I. senegalensis	Japan	Jun-2007	42	35.7	64.3	Takahashi and Watanable (2009)
I. senegalensis	Japan	Jun-2006	42	26.2	73.8	Takahashi and Watanable (2009)
I. senegalensis	Japan	Nov-2005	??	22.9	77.1	Takahashi and Watanable (2009)
I. fluviatilis	Uruguay	Dec-2002	194	7.7	92.3	Mckee *et al*. (2005)
I. fluviatilis	Uruguay	Jan-2003	141	7.8	92.2	Mckee *et al*. (2005)
I. fluviatilis	Uruguay	Feb-2003	72	8.3	91.7	Mckee *et al*. (2005)
I. fluviatilis	Uruguay	Jan-2003	66	4.5	95.5	Mckee *et al*.(2005)
I. damula	North America	Apr-1963	72	11.1	88.9	Johnson (1964)
I. damula	North America	May-1963	60	11.7	88.3	Johnson (1964)
I. damula	North America	Dec-1963	43	16.3	83.7	Johnson (1964)
I. damula	North America	Nov-1962	51	15.7	84.3	Johnson (1964)
I. ramburii	North America	May-1983	90	31.1	68.9	Robertson (1985)
I. ramburii	North America	May-1983	44	25.0	75.0	Robertson (1985)
I. ramburii	North America	May-1998	94	36.2	63.8	Sirot *et al*. (2003)
I. denticollis	North America	??	94	24.0	76.0	Fincke *et al*. (2005)
I. denticollis	North America	??-1991	450	51.0	49.0	Córdoba-Aguilar (1993)

The number of mature female examined in each locality is indicated (N). A: *androchrome*, G: *gynochrome.*

I. denticollis (Fincke *et al*., 2005; Córdoba-Aguilar, 1993) populations from north America (Tables 1 and 2).

In addition, we estimated female colour-morph frequencies in six populations of *I. graellsii* from Iberia and northern Africa and in 12 populations of *I. elegans* from Spain (Table 1). These populations were visited during three consecutive years (2007, 2008 and 2009) between April and September on sunny days. Colour-morph frequencies were estimated by counting the number of each morph present and then dividing this by the total number of females in the population (ranging between 20 and 75 females, depending on the density of the

population). The smaller sample sizes are for populations with very low densities, and it should be kept in mind that uncertainty in the estimations of the population frequency will be induced by sampling errors (Kay *et al*., 2007). The sampling was carried out with entomological nets, and only single, solitary and mature females, which were not in tandem or mating, were counted. After the phenotype of the females was scored, each female was marked with a black dot on the wing, to avoid re-counting the same individual.

Genetic differentiation of populations of *I. elegans* and *I. graellsii*

For the genetic study, we collected *I. elegans* individuals from eight populations from Spain and five populations from eastern Europe. For *I. graellsii,* we selected three Iberian populations and one population from northern Africa, all of which were outside the distributional range of *I. elegans*, to avoid confounding the results due to hybridisation (Sánchez-Guillén *et al*., 2005) (Table 1). In each population, 20 males were collected for molecular analysis and preserved in ethanol. DNA was extracted with a standard phenol–chloroform protocol (Sambrook *et al*., 1989). Alleles at six microsatellite loci (I-002, I-015, I-041, I-053, I-095 and I-134) were amplified in *I. elegans* and at five loci in *I. graellsii* (I-002, I-015, I-041, I-053, I-095) by PCR using the protocol described by (Wellenreuther *et al*. (2010). These loci did not deviate from Hardy–Weinberg expectations and linkage equilibrium, and showed no evidence for the presence of null alleles in *I. elegans*, the species in which the microsatellites were characterised (Wellenreuther *et al*., 2010). The PCR products were separated and alleles were detected using an ABI PRISM 3730 capillary sequencer (Applied Biosystems, Stockholm, Sweden). GeneMapper 3.0 (Applied Biosystems) was used to determine the genotypes of the individuals for the neutral loci. Basic population statistics for the microsatellites were assessed in terms of expected heterozygosity (H_E), observed heterozygosity (H_O), number of alleles and allelic richness, using the program FSTAT version 2.9.3.2 (Goudet, 1995, 2001).

The inheritance system for female colouration has been reported for both species (Cordero, 1990; Sánchez-Guillén *et al*., 2005), and shows that the morphs are controlled by a single autosomal locus with three alleles that are under a hierarchical dominance: *androchrome* allele (A_{allele}) is dominant over both the *infuscans* (I_{allele}) and the *infuscans–obsoleta* (O_{allele}) alleles, and I_{allele} is dominant over O_{allele} (that is, $A_{allele} > I_{allele} > O_{allele}$). The *androchrome* morph has three possible genotypes ($A_{allele}A_{allele}$, $A_{allele}I_{allele}$, $A_{allele}O_{allele}$), the gynochrome morph *infuscans* represents two possible genotypes ($I_{allele}I_{allele}$, $I_{allele}O_{allele}$) and the other gynochrome morph (*infuscans–obsoleta* in *I. elegans* and *aurantiaca* in *I. graellsii*) represents a single genotype ($O_{allele}O_{allele}$) (Cordero, 1990; Sánchez-Guillén *et al*., 2005). Using our knowledge about the genetic basis of this colour polymorphism, colour-morph frequencies were used to estimate allelic frequencies, using the maximum probability estimates of Hedrick (1985) along with the phenotypic frequencies, while assuming Hardy–Weinberg equilibrium within populations. Lastly, allele frequencies were used to estimate genotypic frequencies, starting from which was considered the genetic differentiation degree by the program FSTAT Ver. 2.9.3.2 (Goudet, 1995).

For those populations for which we did not find any *infuscans–obsoleta* (or *aurantiaca*) specimens (see Table 1), presumably due to a combination of small-population sample sizes and low frequencies of the O_{allele}, we conducted the analyses assuming (i) that the O_{allele} was indeed absent (that is, O_{allele} had a frequency of 0%), and (ii) that the O_{allele} was present at low frequencies (5 and 10%, respectively). Calculations of the frequencies of the A and I alleles were then done for each of these populations using observed genotype frequencies and each of the assumed O_{allele} frequencies. The level of heterozygosity (H_E) and genetic differentiation among populations (F_{ST}) were calculated according to Weir and Cockerham, (1984) with the program FSTAT V. 2.9.3.2 (Goudet, 1995).

We compared the degrees of differentiation (the F_{ST} value) at neutral loci and at the colour locus using the F_{ST}-outlier procedure described by Beaumont and Nichols (1996) and modified by Excoffier *et al*. (2009). Expected distributions of F_{ST} values at different degrees of heterozygosity (H_E) under neutral conditions were obtained from coalescent simulations using the microsatellite data and hierarchical island models implemented in Arlequin 3.5 (Excoffier *et al*., 2009; http://anthro.unige.ch/software/arlequin/). These models were used to evaluate whether the colour locus acted as an F_{ST} outlier in *I. elegans* population from Spain and eastern Europe, and in the *I. graellsii* populations. In the hierarchical island models, we defined groups according to results from population structure analyses (Sánchez-Guillén *et al*., under review) in the program STRUCTURE (version 2.2.3, Pritchard *et al*., 2000): Spanish *I. elegans* were clustered into three groups (populations: 1, 2–5, 6–8; Table 3), the eastern European populations into two groups (populations: 9–11, 12–13; Table 3), and *I. graellsii* into three groups (populations: 14, 15–16, 17; Table 3).

We also tested for a correlation between the F_{ST} values of the colour morphs and the microsatellite loci in both *I. elegans* and *I. graellsii*. The degree of correlation can inform us about the importance of genetic drift and selection in maintaining colour-morph frequencies (Runemark *et al*., 2010). A strong correlation would suggest that the colour-morph frequency in local populations is largely affected by genetic drift, whereas a weak correlation would be indicative of selection. A moderate correlation would indicate a combination between drift genetic and selection (Runemark *et al*., 2010).

Results

Colour-morph frequencies

I. graellsii showed similar colour-morph frequencies among populations (Iberia and northern Africa), with the *infuscans* morph being always the most predominant morph (frequency range: 60–90%; Table 1) however, we found a marginal but significant difference in colour-morph frequencies among populations ($\chi^2 = 40.09$, d.f. $= 26$, $P = 0.038$; Figure 1).

In contrast, the colour-morph frequencies in the Spanish *I. elegans* populations varied widely and highly significantly among populations ($\chi^2 = 230.16$, d.f. $= 20$, $P < 0.0001$; Figure 1). The *infuscans–obsoleta* morph was the most common morph in the eastern part of Spain

Table 3 Genetic variation at microsatellite loci for each population of *I. elegans* and *I. graellsii*

Species	*Population*	*Region*	*N*	H_O	H_E	*A*	*K*
I. elegans	1. Doniños	North-West Spain	20	0.711	0.700	6.8	6.6
I. elegans	2. Louro	North-West Spain	15	0.712	0.729	5.3	5.8
I. elegans	3. Arreo	North Spain	15	0.631	0.761	8.3	7.8
I. elegans	4. Baldajo	Central Spain	17	0.603	0.795	8.0	7.7
I. elegans	5. Alfaro	Central Spain	20	0.663	0.758	8.3	7.0
I. elegans	6. Europa	North-East Spain	18	0.671	0.787	8.0	7.1
I. elegans	7. Amposta	East Spain	20	0.691	0.770	8.5	7.2
I. elegans	8. Marjal del Moro	East Spain	20	0.671	0.751	7.3	5.8
I. elegans	9. Breznica	South Poland	18	0.712	0.796	7.8	6.7
I. elegans	10. Zwięczyca Reszów	South Poland	11	0.668	0.827	8.7	7.3
I. elegans	11. Lublin-Zemborzyce	South Poland	14	0.751	0.797	10.0	8.1
I. elegans	12. Suchoi Limon	East Ukraine	20	0.719	0.791	7.5	6.5
I. elegans	13. Enmakov Island	East Ukraine	15	0.713	0.766	8.2	6.8
I. graellsii	14. Campus	North-West Spain	17	0.485	0.694	6.2	3.2
I. graellsii	15. Córdoba	South Spain	20	0.647	0.653	7.2	3.5
I. graellsii	16. Ribeira de Cobres	South Portugal	14	0.684	0.719	6.2	3.7
I. graellsii	17. Saïdia	North Morocco	13	0.490	0.677	5.0	3.1

Number of genotyped individuals (N), observed (H_O) and expected heterozygosity (H_E), average number of alleles (A) and allelic richness (K) at six loci in *I. elegans* and five loci in *I. graellsii* are shown.

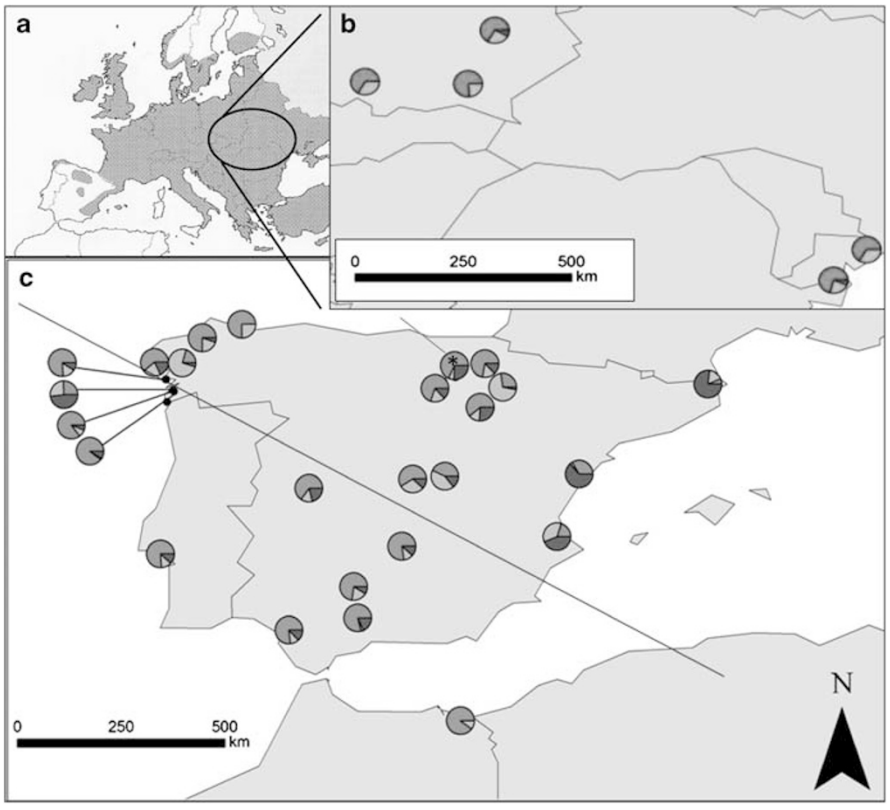

Figure 1 Female morph frequencies (%) in populations of *I. elegans* and *I. graellsii* in Europe. The figure shows the range of *I. elegans* (map **a**; Dijkstra and Lewington, 2006), and the frequencies of the three female colour morphs, namely the androchrome (gray), *infuscans* (clear gray) and *infuscans–obsoleta* morph (black) at each population. The frequencies of the eastern European populations of *I. elegans* in the Ukraine and in Poland are shown in map **b** and of Iberian populations of *I. elegans* and *I. graellsii* in map **c**. The line across Iberia indicates the rough distribution of *I. elegans* and *I. graellsii*, with *I. elegans* being most frequent above the line and *I. graellsii* below the line. For details of each population, see Table 1. A full color version of this figure is available at the *Heredity* journal online.

(Europe, Amposta, Barranco de Caixanet and Marjal del Moro, Figure 1), with frequencies between 44–77%. However, in central (Arreo, Las Cañas, Baldajo and Almoquera) and north-western (Doniños, Louro and Foz) Spain, either the androchrome or the *infuscans* morph dominated, whereas the *infuscans–obsoleta* was rare or even absent (Table 1, Figure 1). The colour-morph frequencies were comparably more stable in the eastern European populations of *I. elegans* ($\chi^2 = 12.39$, d.f. $= 8$, $P = 0.137$; Figure 1), where the *infuscans* morph dominated (range 67–77%; Table 1). *I. elegans* populations in Ukraine were characterised by a complete absence of

infuscans–obsoleta morphs, and in Poland the *infuscans–obsoleta* morph was only found in one of the three populations (Zwięczyca Reszów; Table 1).

In the case of the additional five dimorphic species in the *Ischnura* genus that were investigated (using data from the literature), four showed similar frequencies among populations (Table 2), with the *gynochrome* morph dominating (*I. senegalensis*: $\chi^2 = 2.48$, d.f. = 3, $P = 0.478$; *I. fluviatilis*: $\chi^2 = 0.94$, d.f. = 3, $P = 0.816$; *I. damula*: $\chi^2 = 1.026$, d.f. = 3, $P = 0.795$; *I. ramburii*: $\chi^2 = 1.41$, d.f. = 2, $P = 0.492$). In *I. denticollis*, where we compared only two populations, we found a highly significant difference in the colour-morph frequencies ($\chi^2 = 22.18$, d.f. = 1, $P < 0.0001$), with the *gynochrome* morph dominating in one population, whereas the morphs showed very similar frequencies in the other population (Table 2).

Genetic differentiation of populations of *I. elegans* and *I. graellsii*

The degree of genetic variation at the microsatellite loci was very high and similar between different *I. elegans* populations (Table 3) in terms of observed and expected heterozygosity (0.60–0.75 and 0.70–0.83, respectively), average number of alleles (5.3–10.0) and allelic richness (5.8–8.1) (Table 3). In contrast, *I. graellsii* populations showed moderate amounts of genetic variation (observed heterozygosity: 0.49–0.68; expected heterozygosity: 0.65–0.72; average number of alleles: 5.0–7.2; allelic richness 3.1–3.7; Table 3).

In *I. elegans*, the F_{ST} values between population pairs for the six microsatellite loci ranged between −0.002 and 0.145, and for the colour locus they ranged between −0.006 and 0.529, respectively (Table 4). In *I. graellsii*, we found an overall lower differentiation between populations, and the F_{ST} values between population pairs for five microsatellites and the colour locus ranged from −0.018 to 0.068, and from −0.010 to 0.259, respectively (Table 5).

Analysing the full sample of *I. elegans* populations (Spain and eastern Europe), the results from the F_{ST}-outlier analyses (using hierarchical island models implemented in Arlequin 3.5) showed that the colour locus ($F_{ST} = 0.271$) behaved as an outlier with an F_{ST} value larger than the generated null distribution of neutral F_{ST} values (Figure 2a). In addition, when we assumed low frequencies of the O_{allele} in populations where we did not observe any *infuscans–obsoleta* individuals, the F_{ST} values of the colour locus ($F_{ST} = 0.243$ and 0.223, assuming frequencies of the O_{allele} of 5 and 10%, respectively) fell outside the expected neutral F_{ST}-distribution (Figure 2a).

Similar results were found when we investigated only the Spanish populations of *I. elegans*. The analysis showed again that the colour locus was a clear F_{ST}-outlier ($F_{ST} = 0.231$; Figure 3b).

In contrast, in *I. elegans* populations from eastern Europe, the colour locus ($F_{ST} = 0.084$) did not deviate strongly from the neutral expectations, in particular, not when we assumed low frequencies of the O_{allele} (5 and 10%, respectively) in the populations where we did not observe any *infuscans–obsoleta* individuals ($F_{ST} = 0.046$ and 0.025, respectively; Figure 2c).

In *I. graellsii* populations, the F_{ST} value for the colour locus was 0.086, and if we assumed low frequencies for the O_{allele} (5 and 10%, respectively) in the populations where we did not observe any *aurantiaca* individuals, the obtained value for the colour F_{ST} was 0.064 and 0.040, respectively. Unlike for *I. elegans*, the F_{ST}-outlier analysis showed that the F_{ST} values of the colour locus did not deviate from the neutral expectations (Figure 2d).

The degree of differentiation at the set of microsatellite loci between all *I. elegans* populations (N = 13) did not correlate significantly with the degree of differentiation at the colour locus ($F_{1,74} = 0.135$, $P = 0.24$; $r^2 = 0.018$; Figure 3). In *I. graellsii*, the correlation between differentiation at the neutral loci and the colour locus for the four populations was slightly higher than in *I. elegans*; however, this correlation was also far from being significant ($F_{1,5} = 0.524$, $P = 0.28$; $r^2 = 0.27$; Figure 3).

Table 5 Degree of differentiation (F_{ST}) between *I. graellsii* populations for five neutral loci (above diagonal) and for the colour locus (below diagonal)

	pop 1	*pop 2*	*pop 3*	*pop 4*
1. Córdoba		0.018	0.003	0.065
2. Ribeira de Cobres	−0.010		0.017	0.068
3. Campus	0.001	0.007		−0.018
4. Saïdia	0.259	0.217	0.188	

Table 4 Degree of differentiation (F_{ST}) between *I. elegans* populations for six neutral loci (above diagonal) and for the colour locus (below diagonal)

	Spanish populations								*East European populations*				
	pop 1	*pop 2*	*pop 3*	*pop 4*	*pop 5*	*pop 6*	*pop 7*	*pop 8*	*pop 9*	*pop 10*	*pop 11*	*pop 12*	*pop 13*
1. Doniños		0.046	0.087	0.075	0.058	0.080	0.078	0.091	0.059	0.095	0.090	0.076	0.097
2. Louro	0.146		0.145	0.093	0.121	0.062	0.101	0.120	0.079	0.116	0.093	0.111	0.100
3. Arreo	0.066	0.356		0.007	0.006	0.029	−0.002	−0.011	0.062	0.062	0.083	0.043	0.102
4. Baldajo	0.105	0.018	0.300		0.003	0.015	0.005	−0.011	0.040	0.032	0.033	0.013	0.048
5. Alfaro	0.019	0.234	0.044	0.152		0.042	0.014	0.005	0.052	0.054	0.054	0.020	0.071
6. Europa	0.225	0.079	0.452	0.184	0.368		−0.003	0.017	0.020	0.043	0.036	0.028	0.060
7. Amposta	0.318	0.036	0.529	0.096	0.402	0.171		−0.009	0.037	0.053	0.063	0.036	0.087
8. Marjal del Moro	0.224	0.113	0.401	0.211	0.347	−0.011	0.209		0.050	0.047	0.057	0.034	0.078
9. Breznica	0.577	0.232	0.766	0.348	0.666	0.341	0.095	0.344		0.004	−0.006	0.008	0.013
10. Zwięczyca Reszów	0.540	0.200	0.728	0.321	0.630	0.285	0.080	0.300	−0.006		0.000	0.008	0.007
11. Lublin-Zemborzyce	0.564	0.224	0.728	0.345	0.640	0.318	0.090	0.328	−0.006	−0.010		−0.002	−0.006
12. Suchoi Limon	0.225	−0.002	0.436	0.047	0.311	0.107	0.001	0.146	0.155	0.129	0.146		0.009
13. Enmakov Island	0.266	0.012	0.471	0.069	0.349	0.129	−0.007	0.169	0.121	0.099	0.113	−0.010	

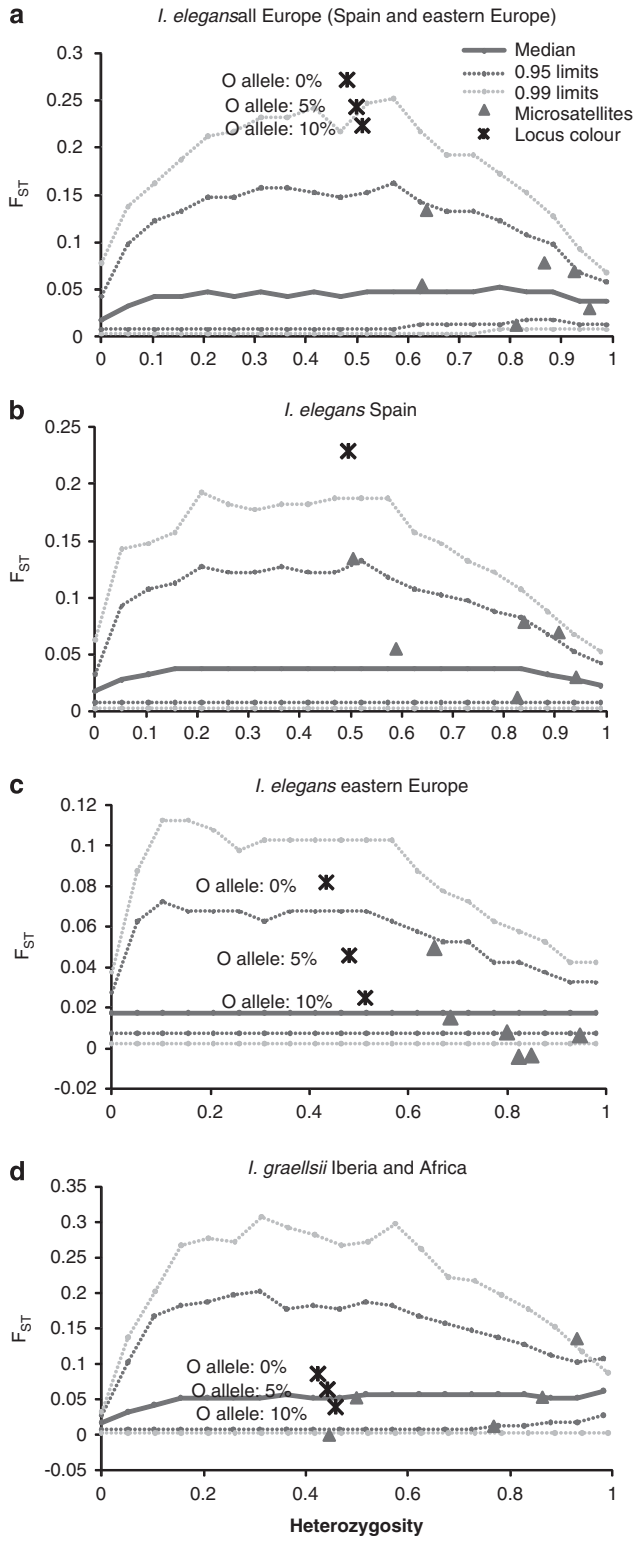

Figure 2 Summary of the results from the F_{ST}-outlier analyses (using hierarchical island models implemented in Arlequin 3.5) showing the F_{ST} values of the colour locus and the microsatellite loci, and the generated null distribution of neutral F_{ST} values. Data are for all *I. elegans* populations (**a**), for Spanish *I. elegans* (**b**), eastern European *I. elegans* (**c**), and all *I. graellsii* populations (**d**). Colour locus F_{ST} values were calculated assuming (in those populations where no *infuscans–obsoleta (I. elegans)* or *aurantiaca (I. graellsii)* females were sampled) that (i) the O_{allele} was indeed absent (that is, had a frequency of 0%), or (ii) the O_{allele} was present at low frequencies (5 and 10%, respectively). A full color version of this figure is available at the *Heredity* journal online.

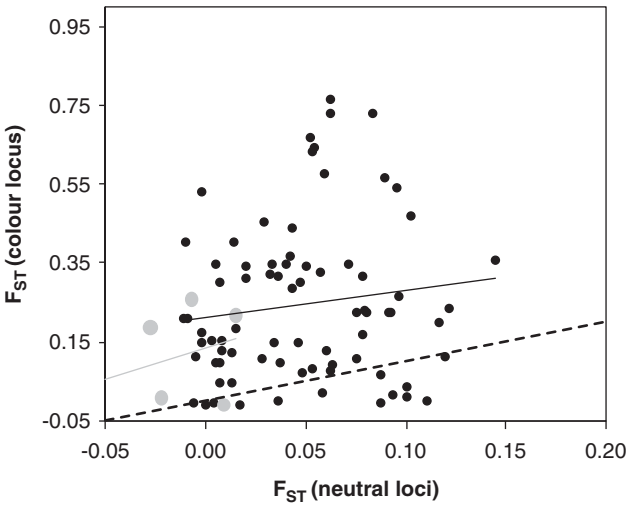

Figure 3 Correlation between colour-morph and neutral divergence between pairs of populations in *I. elegans* and in *I. graellsii*. In black, the values for *I. elegans* for the 13 studied populations (neutral data from six microsatellite loci), and in grey, the values for the four *I. graellsii* populations (neutral data from five microsatellite loci). The dotted line indicates the 1:1 relationship.

Discussion

Frequency of colour morphs in seven Ischnura species

Recently, Fincke *et al*. (2005) revised the frequency of androchrome colour morphs in polymorphic odonates, and found that in only 17% of the 134 studied species, the androchrome morph was the most common morph for at least one of the populations examined. In line with this, we found that the androchrome morph dominated only in some populations of *I. elegans*, whereas in the other *Ischnura* species (*I. graellsii*, *I. senegalensis*, *I. fluviatilis*, *I. damula* and *I. ramburii*) the gynochrome morph dominated consistently. The same was true for one of the two *I. denticollis* populations (Table 2), as well as in *I. genei* and *I. saharensis* (RA Sánchez-Guillén, personal observation).

Interestingly, the colour-morph frequencies in the Spanish populations of *I. elegans* varied widely, even at a local scale, whereas similar frequencies were found among populations in eastern Europe (Ukraine and Poland). The androchrome morph dominated in some Spanish populations (maximum frequency 75%), whereas the *infuscans* morph (maximum frequency 75%) and the *infuscans–obsoleta* morph (maximum frequency 77%) dominated in others. Similar patterns have been observed in previous studies of *I. elegans* at both regional and continental scales (Gosden, 2008; Hammers and Van Gossum, 2008; Sánchez-Guillén *et al*., 2005). It is particularly interesting that the degree of morph-frequency differentiation in *I. elegans* was most pronounced in the recently founded Spanish populations at the southern range limit of the species (Gosden, 2008; Sánchez-Guillén *et al*., 2005). As a mirror-image of this, population differentiation in morph frequencies was also substantial at the northern range limit in Sweden, which has been explained as a result of the combined action of genetic drift, non-equilibrium conditions, extinction–re-colonisation dynamics and harsh environmental conditions at the range limits that leads to strong divergent selection (Abbott *et al*., 2008; Gosden, 2008). In general, strong divergent selection in novel environments would

be expected to operate at the edges of a species range, often in combination with small effective population sizes (Kirkpatrick and Barton, 1997; Eckert *et al*., 2008).

In contrast to the pattern observed in *I. elegans*, *I. graellsii* showed more conserved colour-morph frequencies between populations in Iberia and northern Africa. The gynochrome *infuscans* morph was always the most common morph (maximum frequency 90%), although either the *aurantiaca* (gynochrome) or the androchrome morph was the least common one. A similar pattern was found for *I. senegalensis*, *I. fluviatilis*, *I. damula* and *I. ramburii,* wherein the gynochrome morph was always found to be the most predominant morph in the surveyed populations, and the proportion of colour morphs showed little variation among regions.

These results suggest that the frequencies of the colour morphs in the *Ischnura* species studied are usually similar between populations as well as species, and that the gynochrome morph is most often the predominant type. This stability of colour morphism frequencies indicates that some sort of balancing selection is likely to operate in these species (cf. Svensson *et al*., 2005; Abbott *et al*., 2008). An exception to this seems to occur in some regions of the distribution range of *I. elegans*, because our analyses detected substantial variation in colour-morph frequencies among populations, with the androchrome morph being in several instances the most frequent morph present. It would be interesting to investigate the colour-morph frequencies in other polymorphic species in the genus *Ischnura*, to test if *I. elegans* is the only *Ischnura* species where the *gynochrome* does not typically dominate.

Selection on the colour locus in *I. elegans* and *I. graellsii*

The correlation between F_{ST} values at the neutral markers and the colour locus was very weak in *I. elegans* ($r^2 = 0.018$), discarding that the morph-frequency variation between populations in this species is highly affected by genetic drift (cf. Runemark *et al*., 2010). The degree of differentiation between pairs of populations at the colour locus in *I. elegans* was in several cases considerably larger than at the neutral loci, suggesting that divergent selection can have important effects in shaping morph frequencies in *I. elegans*. Furthermore, the F_{ST}-outlier analyses supported the importance of divergent selection on the colour morph in *I. elegans* (cf. Beaumont and Nichols, 1996; Excoffier *et al*., 2009). The F_{ST} value of the colour morph was a sharp outlier when all populations of *I. elegans* were analysed together (Spain and eastern Europe; Figure 2a), and also when only the Spanish populations were considered (Figure 2b). Thus, although the colour morph-frequency variation differs largely between *I. elegans* regions, our results suggest that the patterns of morph frequencies might partly be affected by divergent selection favouring certain morphs in local populations that differ in ecology and abiotic factors. Potentially important factors might be temperature and precipitation or other local microclimatic factors that are likely to affect the different colour morphs in contrasting ways (Bots *et al*., 2009). For instance, Gosden (2008) found a highly significant north–south cline in the frequency of the androchrome female morph, that became much more common at higher latitudes in Europe, such as in Sweden. This might indicate that the androchrome female morph is more cold-adapted or cold-tolerant than the other morphs. Furthermore, a similar cline in the female morph frequency has been observed in another Coenagrionid in Canada, *Nehalennia irene*. This species shows a high androchrome frequency in its western distribution, where it is the majority morph, but the frequency decreases eastwards and is in minority in the east (Van Gossum *et al*., 2007; Iserbyt *et al*., 2010). This cline in the androchrome frequency has been related to some underlying selective agents, but also with genetic drift during recolonisation (Iserbyt *et al*., 2010). Significant clines in adaptive traits are usually considered as classical signature of selection (Endler, 1997).

Under a scenario of divergent selection (and if the stabilising force of negative frequency-dependent selection is not strong enough, relative to the strength of divergent selection), dispersal between populations, or temporal variation in factors that affect which morph is locally favoured, could contribute to the maintenance of the colour-morph polymorphism in local populations. Even if we assume that the recessive O allele, that codes for the *infuscans–obsoleta* morph, is present at low frequencies in populations where the *infuscans–obsoleta* morph was not found, the colour locus was still detected as an F_{ST}-outlier when all populations of *I. elegans* were analysed together (Figure 2a), but it fell inside the expected neutral distribution in the eastern European populations (Figure 2c). These results suggest that the female colour polymorphism might in some regions (for example, eastern Europe) be partly affected by stochastic factors such as genetic drift, perhaps in combination with different types of selection (for example, negative frequency-dependent selection), whereas divergent selection might be the predominant force in other regions of Europe (for example, Spain). It is also worth highlighting that the Iberian populations of *I. elegans* have been relatively recently founded, as this species has only in the last few years expanded its distributional range south-westwards (Monetti *et al*., 2002; Sánchez-Guillén *et al*., 2005). As *I. elegans* might be in the process of expanding into new geographic areas that are characterised by novel selection pressures, it is perhaps not surprising that divergent selection might be particularly strong in Spain. More generally, selection in peripheral populations of a species range might *a priori* be expected to be strong, although its effects on the allele frequencies in local populations might be counteracted by gene flow from better-adapted populations at the centre of a species range (Kirkpatrick and Barton, 1997; Bridle and Vines, 2007).

Populations of *I. graellsii* showed a similar pattern to the *I. elegans* populations from eastern Europe, and the F_{ST} value of the colour locus did not deviate from the expected neutral distribution (Figure 2d). Thus, we found no signature of divergent or balancing selection operating on the morph frequencies among Iberian *I. graellsii* populations. Rather, the morph frequencies might have been partly influenced by genetic drift, as indicated by the weak positive (non-significant) correlation between genetic differentiation at the colour locus and the neutral loci ($r^2 = 0.27$).

Field studies of selection have shown that selection often varies both spatially and temporally, and typically differ in magnitude, direction and/or form (Grant and Grant, 2002; Svensson and Sinervo, 2004; Abbott *et al*.,

2008; Gosden and Svensson, 2008). Thus, the possibility to detect balancing selection (for example, negative frequency-dependent selection) may depend on the geographical scale of the study. Andrés *et al*. (2000) and Abbott *et al*. (2008) suggested that balancing selection affected the colour-morph frequencies in Spanish *I. graellsii* and Swedish *I. elegans*, respectively (see also Gosden 2008). These results differ from the results concerning divergent selection in this study. Our study contained samples from a geographically extensive area (maximum distance between pair of populations = 960 km) compared with the relatively small spatial scale that was explored in Andrés *et al*. (2000) (maximum distances = 50 km) and Abbott *et al*. (2008) (maximum distances = 20 km). At such small local scales, the balance between the stabilising force of negative frequency-dependent selection and divergent selection due to abiotic environmental heterogeneity is likely to be tipped in favour of the former. The reason for this is that these closely located populations are expected to be quite similar in ecological and abiotic environmental conditions. In contrast, at a larger geographic scale, as in our study, overall morph frequencies are more likely to be influenced by divergent selection due to environmental heterogeneity, even if the frequencies could be similar between closely located populations. Clearly, the spatial and temporal scale of sampling should be considered carefully in studies like the current one when aiming to obtain indirect inferences about selection.

To conclude, the examination of morph frequencies in different *Ischnura* spp. (Tables 1 and 2) has shown that gynochrome morphs are typically dominant, with the exception of *I. elegans*. The relatively stable morph frequencies across species suggest that balancing selection is likely to operate and maintain colour-morph frequencies in these species, and that the role of genetic drift is relatively minor, which has also been suggested in other studies (Svensson *et al*., 2005). The differentiation in colour-morph frequencies between Spanish *I. elegans* populations was significantly higher than that would be expected by genetic drift alone (Figure 2), which suggests an influence of divergent selection. In contrast, colour-morph frequencies in populations of *I. elegans* in eastern Europe and in *I. graellsii* populations varied much less geographically and did not differ from neutral expectations. That we found no evidence for strong stabilising selection within species could have been caused by the geographic scale of our sampling and the relatively low neutral population divergence in these damselflies. Low neutral population divergence is one of several factors that contribute to make the detection of stabilising selection much more difficult than the detection of divergent selection (Excoffier *et al*., 2009). Thus, our results do not refute negative frequency-dependent selection, because the statistical power to detect this was low using these kind of indirect inferences about selection (Excoffier *et al*., 2009).

In summary, the results of this study together with other recent studies on the population biology of the genus *Ischnura* (Andrés *et al*., 2000; Abbott *et al*., 2008; Gosden, 2008) suggest that both stochastic and deterministic factors affect population divergence in the colour locus of these polymorphic damselflies. The results of this study have clear implications for explaining the maintenance and frequency distribution of colour morphs in odonates, and suggest that a single hypothesis is not enough to understand the evolution of this phenomenon, not even within species.

Conflict of interest

The authors declare no conflict of interest.

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