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Genetic divergence predicts reproductive isolation in damselflies

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sexual and natural forces;
speciation clock.

Abstract

Reproductive isolation is the defining characteristic of a biological species, and a common, but often untested prediction is a positive correlation between reproductive isolation and genetic divergence. Here, we test for this correlation in odonates, an order characterized by strong sexual selection. First, we measure reproductive isolation and genetic divergence in eight damselfly genera (30 species pairs) and test for a positive correlation. Second, we estimate the genetic threshold preventing hybrid formation and empirically test this threshold using wild populations of species within the *Ischnura* genus. Our results indicate a positive and strong correlation between reproductive isolation and genetic distance using both mitochondrial and nuclear genes cytochrome oxidase II (COII: $r = 0.781$ and 18S–28S: $r = 0.658$). Hybridization thresholds range from −0.43 to 1.78% for COII and −0.052–0.71% for 18S–28S, and both F_1-hybrids and backcrosses were detected in wild populations of two pairs of *Ischnura* species with overlapping thresholds. Our study suggests that threshold values are suitable to identify species prone to hybridization and that positive isolation–divergence relationships are taxonomically widespread.

Introduction

Reproductive isolation is widely accepted as an irreversible point along the evolutionary trajectory towards the origin of species (cf. reticulate evolution, e.g. Arnold *et al.*, 2010), and this has led some to propose a general relationship between genetic divergence (as a surrogate for time) and reproductive isolation (e.g. Coyne & Orr, 1989, 1997). A positive relationship between the strength of reproductive isolation and genetic distance was first detected by Zouros (1973) and Ayala (1975) when working on closely related species of *Drosophila*. A little over a decade later, Coyne & Orr (1989) argued that if the time since species splitting affects genetic distance, then a general relationship with the degree of reproductive isolation should be expected. Indeed, comprehensive work by Coyne & Orr (1989, 1997) detected a positive correlation between the strength of prezygotic (sexual/behavioural) and post-zygotic isolation (hybrid sterility and inviability) and genetic divergence in a meta-analysis of 174 pairs of *Drosophila* species. Although some exceptions to this rule have since been found (Lessios & Cunningham, 1990 species of echinoderms, Scopece *et al.*, 2007 species of orchids), the vast majority of studies have documented consistent results in frogs (Sasa *et al.*, 1998 46 species), butterflies (Presgraves, 2002 182 species), birds (Price & Bouvier, 2002 368 species) and angiosperms (Moyle *et al.*, 2004 191 species). These studies are in line with the hypothesis that reproductive isolation is a by-product of gradual genetic divergence, that is, a phenomenon commonly referred to as the 'speciation clock' (Coyne & Orr, 1989, 1997).

However, some species may remain genetically isolated without approaching full reproductive isolation as, for example, seen when the geographical ranges of related species are connected by stable hybrid zones

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(Hewitt, 2001) or where species show introgressive hybridization over large spatial scales (e.g. Sánchez-Guillén *et al.*, 2011). Variation in the association between reproductive isolation and genetic divergence may also occur if the strength of selective forces that govern the speciation process differs. One long-standing idea is that species with strong sexual selection might evolve reproductive isolation more rapidly (Darwin, 1871), and this idea has since gained additional supporters (e.g. Lande, 1982; Rice, 1996; Gavrilets, 2000; Boake, 2005). According to this view, increased sexual selection (and hence sexual conflict) facilitates the evolution of diverse male reproductive strategies that are in turn counteracted by female counterstrategies, thus providing elevated opportunities for speciation as more evolutionary avenues of male–female interaction evolve (Gage *et al.*, 2002). Some recent studies of closely related species support such a role for sexual behaviour in species divergence (Price, 1998; Gray & Cade, 2000; Boake, 2005; Mendelson & Shaw, 2005). However, carefully controlled studies have been less clear, with some providing support (Arnqvist *et al.*, 2000; Martin & Hosken, 2003), whereas others failed to find an association (Gage *et al.*, 2002).

Dragonflies and damselflies (Odonata) are a group of animals where adaptation can rapidly be caused by sexual selection (Misof, 2002; McPeek & Gavrilets, 2006; Svensson *et al.*, 2006). Sexual selection appears to promote speciation in dragonflies (Misof, 2002), and in damselflies, there is an evidence that sexual selection may be an important component of speciation. However, species within the radiation of North American *Enallagma* damselflies differentiated primarily in characters important to interspecific mate recognition, that is, the male cerci and the female mesostigmal plates, and this divergence proceeded in the absence of significant niche diversification (Brown *et al.*, 2000; McPeek & Brown, 2000; McPeek *et al.*, 2008a). In addition to damselflies being an interesting radiation driven by sexual and natural selection, they are also optimal study objects because reproductive barriers can be estimated with the accuracy under both laboratory (Sánchez-Guillén *et al.*, 2012) and natural conditions (McPeek *et al.*, 2008a; Wellenreuther *et al.*, 2010b; Sánchez-Guillén *et al.*, 2012). Damselflies allow reproductive barriers to be studied beyond the F_1-hybrids (Sánchez-Guillén *et al.*, 2012) that are typically being used to estimate post-zygotic effects in many species (Edmands, 2002), because they can be reared and crossed with relative ease in captivity (Van Gossum *et al.*, 2003; Sánchez-Guillén *et al.*, 2005). However, despite the suitability to study reproductive barriers in odonates, empirical estimates of the isolation–divergence relationship have only rarely been attempted (Tierney, 1996; Sánchez-Guillén *et al.*, 2012).

Here, we explore the relationship between reproductive isolation and genetic divergence between eight damselfly genera by reviewing existing data on reproductive isolation and conducting extensive field and laboratory studies. By doing so, we fill an important taxonomic gap in the understanding of how closely reproductive isolation is linked to genetic divergence in this ancient insect group. Based on the assumption that different degrees of genetic distances between species pairs should positively correlate with the completion of the speciation process, (i) we measure the strength of reproductive isolation between eight damselfly genera (*Calopteryx*, *Coenagrion*, *Enallagma*, *Erythromma*, *Ischnura*, *Lestes*, *Pyrrhosoma* and *Sympecma*) using both field and laboratory approaches, and (ii) we estimate the genetic divergence below which isolating barriers are insufficient to prevent hybrid formation. Lastly, iii) we test our estimates in the field on two *Ischnura* sister species pairs predicted to hybridize based on their genetic distances by evaluating the realized degree of hybridization in a natural setting.

Materials and methods

Study genera and literature review

Damselfly species in the genus *Ischnura* lack precopulatory courtship behaviour. Instead, males actively search for females and will initiate mating by grasping the female by the prothorax with their anal appendages, thereby forming the 'tandem position'. In this position, the male and female are joined, but the genitalia are not engaged. If the female is willing to mate, she will bend her abdomen so that mating organs of both sexes come into contact forming the copulatory 'wheel position' (Corbet, 1999). Copulation can be impeded by the mismatch of the male anal appendages with the mesostigmal plates located on the female pronotum, hampering the tandem position, or by a mismatch of the male and female mating organs, hampering copulation (Sánchez-Guillén *et al.*, 2012). Copulation stage in odonates is divided into three behavioural phases: i) sperm removal of previous mating males' sperm stored in the female sperm storage organs, ii) insemination and iii) male mate guarding (Miller & Miller, 1981).

The contribution of sexual and natural selection and hybridization to reproductive isolation in the eight studied damselfly genera has been investigated in most cases. Intragenera hybridization in the wild does not occur in *Erythromma, Pyrrhosoma* and *Sympecma,* because species within these genera rarely coexist (Sánchez-Guillén, unpublished data). In the damselfly genus *Lestes*, a group in which many species co-occur in sympatry, and with very similar ecological requirements, interspecific tandems are common in the European species of this genus (accounts for 70% of the observed interspecific interactions); however, mating events (12.5% of the observed interspecific interactions) are rare (Sánchez-Guillén, unpublished data). For species within the genus *Coenagrion,* an evidence of

putative hybrids comes from morphological examinations, but molecular analyses failed to support this (Lowe *et al.*, 2008). The genus *Calopteryx* shows large differences in secondary sexual wing traits (Svensson *et al.*, 2007) and strong sexual selection for wing phenotype (Waage, 1975). Ecological work on the European *Calopteryx* species *C. splendens* and *C. virgo* found only minor interspecific niche differences (Svensson, 2012; Wellenreuther *et al.*, 2012), and molecular work on the occidental European species of *Calopteryx* genus showed that species show little genetic differentiation (Maibach, 1985). Despite the lack of large niche partitioning in *Calopteryx* spp., hybridization rates are very low (Mullen & Andrés, 2007; Tynkkynen *et al.*, 2008). Similarly, in the genus *Enallagma,* premating barriers appear to evolve independently of niche diversification (McPeek & Brown, 2000; McPeek *et al.*, 2008a), despite up to 12 species co-occurring in the same body of water (McPeek & Brown, 2000). The genus *Enallagma* also shows random mating among congeneric species, and even if mechanic isolation is important in several species pairs, it is not complete in all, suggesting past asymmetric hybridization (Turgeon *et al.*, 2005). Finally, species within the genus *Ischnura* co-occur sympatrically over large parts of their ranges and are morphologically and ecologically similar, and in some cases, extensive hybridization occurs (Johnson, 1975; Leong & Hafernik, 1992; Monetti *et al.*, 2002; Sánchez-Guillén *et al.*, 2011).

We conducted a literature review to characterize the contribution of different reproductive barriers at the interspecific, intergeneric and interfamily level (Table 1). Our data set consists of 31 sexual interactions between sympatric and allopatric species pairs of 30 species (Table 1). We grouped the data into seven potential reproductive barriers (description in Table 1), following detailed descriptions for 19 pre- and post-zygotic reproductive barriers in ischnurids (Sánchez-Guillén *et al.*, 2012). Coyne & Orr (1989) categorized post-zygotic isolation as a discrete variable that assumed values from zero (both sexes viable and fertile) to one (both sexes sterile or inviable). In our study, we assigned a selected value of reproductive isolation for each reproductive barrier, which ranged from zero (when fertile hybrids were detected) to seven (when sexual interaction, but no physical contact was detected) and was divided by seven. This gives an index of reproductive isolation ranging from 0 (no isolation) to 1 (complete isolation) (see Table 1 for a complete description of reproductive barriers and values).

Measures of correlation between reproductive isolation and genetic distances

We selected two mitochondrial genes, cytochrome oxidase II (COII) and cytochrome b (CYTB), and one nuclear gene, the ribosomal subunits of 18S–28S (18S–28S) based on their diverse evolutionary rates (Fritz *et al.*, 1994). Supplemental Table S1 shows accession numbers of the sequences downloaded from GenBank. Additionally, the DNA of *I. asiatica*, *I. elegans*, *I. elegans ebneri*, *I. fountaineae*, *I. genei*, *I. graellsii*, *I. pumilio*, *I. saharensis* and *I. senegalensis* (between 3 and 9 samples/species, n total = 50 samples) was extracted from the head using a standard phenol/chloroform–isoamyl alcohol extraction protocol (Sambrook *et al.*, 1989). Samples were amplified by PCR for part of COII, CYTB and 18–28S. Amplifications were carried out using universal primers: 673 bp of the COII with the primers TL2-J-3037 and C2-N-3494, and C2-J-3400 and TK-N-3785 (Simon *et al.*, 1994), 457 bp of the CYTB with the primers CB-J-10933 and TS1-N-11683 (Simon *et al.*, 1994) and approximately 700 bp (depending on the length of the sequence in each species) of the nuclear gene 18S–28S with the primers LITS and H28S (Samraoui *et al.*, 2002). DNA amplifications were carried out in 10 μL, and amplification conditions were as follows: 1–2 ng of DNA (2 μL), 5.0 μL of 2X Ready Mix^{TM} PCR Master Mix (1.5 mM $MgCl_2$), 1μL of 10×BSA, 0.3μL of $MgCl_2$ (50 mM), 1.1 μL of distilled water and 0.3 μL of each primer (10 pmol) in a 'GeneAmp PCR system 2700' thermocycler (Applied Biosystems). The PCR program had an initial cycle of 95 °C for 3 min, followed by the annealing temperature for 1 min, with an elongation period at 72 °C for 45 s, followed by 34 cycles at 95 °C for 30 s, with annealing for 45 s, and an elongation phase at 72 °C for 45s, and a final extension phase at 72 °C for 10 min. Bidirectional sequencing reactions were conducted using the Bigdye^{TM} terminator cycle sequencing kit (Applied Biosystems) using the automatic sequencer ABI3100. Forward and reverse sequences were edited in Codon Code Aligned (CodonCode, Dedham, MA, USA), and consensus sequences were aligned with ClustalX (Thompson *et al.*, 1997) implemented in Mega, version 5 (Tamura *et al.*, 2011) (GenBank accession numbers: KC430114-KC430232).

Kimura 2-parameter genetic distances (Kimura, 1980) were calculated between 17 taxa (330 bp, 36 sequences) for mtDNA COII, 13 taxa (317 bp, 51 sequences) for mtDNA CYTB and between 16 taxa (485 bp, 53 sequences) for nDNA 18S–28S. All samples of a species were clustered in the same group, and the genetic distances were estimated between groups. Rate of variation among sites was modelled with a gamma distribution (shape parameter = 1) with Mega, version 5 (Tamura *et al.*, 2011). To maximize the use of available data, yet account for phylogenetic nonindependence of species pairs, we generated a reduced set of phylogenetically 'corrected' species pairs (following Coyne & Orr, 1989, 1997; Yukilevich, 2012). We used nested averaging to reduce all pairwise comparisons across each internal phylogenetic node to a single comparison (which applies to both reproductive isolation

Table 1 Summary of sexual interactions between species pairs.

Species (♂)	Species (♀)	Geographical region	Prezygotic interactions (pre- and post-mating)				Post-zygotic interactions			Ref
			1 (1)	2 (0.84)	3 (0.67)	4 (0.50)	5 (0.33)	6 (0.17)	7 (0)	
Ischnura pumilio	*Ischnura elegans*	Sympatric	+	+	–					(Miller & Fincke, 2004)
Ischnura barberi	*Ischnura ramburii*	Sympatric	+	+	+	+	–			(Deviche, 2010)
Ischnura pumilio	*Ischnura graellsii*	Sympatric	+	+	+	+	+	–		(Cordero, 1989)
Ischnura damula	*Ischnura demorsa*	Sympatric	+	+	+	+	+	–		(Johnson, 1975)
Ischnura demorsa	*Ischnura damula*	Sympatric	+	+	+	+	+	–		(Johnson, 1975)
Ischnura graellsii	*Ischnura elegans*	Sympatric	+	+	+	+	+	+	+	(Sánchez-Guillén *et al.*, 2012)
Ischnura elegans	*Ischnura graellsii*	Sympatric	+	+	+	+	+	+	+	(Sánchez-Guillén *et al.*, 2012)
Ischnura elegans	*Ischnura genei*	Sympatric	+	+	+	+	+	+	+	(Sánchez-Guillén unpublished data)
Ischnura genei	*Ischnura elegans*	Sympatric	+	+	+	+	+	+	+	(Sánchez-Guillén unpublished data)
Ischnura graellsii	*Ischnura saharensis*	Sympatric	+	+	+	+	+	+	+	(Sánchez-Guillén unpublished data)
Ischnura saharensis	*Ischnura graellsii*	Sympatric	+	+	+	+	+	+	+	(Sánchez-Guillén unpublished data)
Ischnura graellsii	*Ischnura genei*	Allopatric	+	+	+	+	+	+	?	(Sánchez-Guillén unpublished data)
Ischnura genei	*Ischnura graellsii*	Allopatric	+	+	+	+	+	+	?	(Sánchez-Guillén unpublished data)
Ischnura e. ebneri	*Ischnura fountaineae*	Sympatric	?	?	?	?	?	+	?	(Corbet, 1980)
Ischnura gemina	*Ischnura denticollis*	Sympatric	+	+	+	+	+	+	+	(Tierney, 1996)
Ischnura denticollis	*Ischnura gemina*	Sympatric	+	+	+	+	+	+	+	(Tierney, 1996)
Enallagma carunculatum	*Ischnura cervula*	Sympatric	+	–						(Miller & Fincke, 2004)
Ischnura pumilio	*Platycnemis pennipes*	Sympatric	+	+	–					(Kunz, 2005)
Enallagma cyathigerum	*Ischnura denticollis*	Sympatric	+	+	–					(Miller & Fincke, 2004)
Ischnura elegans	*Coenagrion pulchellum*	Sympatric	+		+	–				(Bick & Bick, 1981)
Ischnura elegans	*Erythromma lindenii*	Sympatric	+	+	+	–				(Miller & Fincke, 2004)
Ischnura elegans	*Erythromma najas*	Sympatric	+	+	+	–				(Miller & Fincke, 2004)
Ischnura perparva	*Enallagma anna*	Sympatric	+	+	+	–				(Bick & Bick, 1981)
Pyrrhosoma nymphula	*Ischnura elegans*	Sympatric	+	+	+	–				(Miller & Fincke, 2004)
Enallagma carunculatum	*Ischnura perparva*	Sympatric	+	+	+	–				(Miller & Fincke, 2004)
Ischnura elegans	*Coenagrion puella*	Sympatric	+	+	+	+	?			(Utzeri & Belfiore, 1990)
Ischnura elegans	*Enallagma cyathigerum*	Sympatric	+	+	+	+	?			(Bick & Bick, 1981)
Ischnura elegans	*Pyrrhosoma nymphula*	Sympatric	+	+	+	+	?			(Miller & Fincke, 2004)
Ischnura pumilio	*Coenagrion puella*	Sympatric	+	+	+	+	?			(Miller & Fincke, 2004)
Ischnura pumilio	*Enallagma cyathigerum*	Sympatric	+	+	+	+	?			(Miller & Fincke, 2004)
Pyrrhosoma nymphula	*Ischnura pumilio*	Sympatric	+	+	+	+	?			(Garner, 2003)
Enallagma hageni	*Ischnura cervula*	Sympatric	+	+	+	–				(Bick & Bick, 1981)
Ischnura elegans	*Lestes sponsa*	Sympatric	+	+	+	–				(Miller & Fincke, 2004)
Ischnura elegans	*Lestes viridis*	Sympatric	+	+	+	–				(Miller & Fincke, 2004)
Ischnura erratica	*Lestes disjunctus*	Sympatric	+	+	+	–				(Miller & Fincke, 2004)
Ischnura elegans	*Calopteryx splendens*	Sympatric	+	+	+	+	?			(Seggewise, 2008)
Ischnura elegans	*Sympecma fusca*	Sympatric	+	+	+	+	?			(Seggewise, 2008)

Data were grouped into seven categories based on the temporal order of reproduction barriers in odonates: [(1) sexual interaction (i.e. both species interact without physical contact); (2) tandem acceptance (i.e. both species have physical contact, and male attempts the tandem); (3) tandem occurrence (i.e. tandem is formed)], post-mating, prezygotic [(4) 'wheel position' mating (i.e. mating takes place)], and post-mating, post-zygotic [(5) oviposition (i.e. egg laying), (6) hybrid viability (i.e. embryos developing), (7) hybrid fertility (hybrid males and females produce fertile or partially fertile F_2 hybrids) (see Sánchez-Guillén *et al.*, 2012). The sign + denotes that the interaction was detected (in the literature), and the sign – indicates that the interaction was impeded by incompatibility, and the sign ? denotes the lack of information. The sign – denotes that the field was left blank. We assigned an index of reproductive isolation to each interaction (1–7), which ranged from zero (when fertile/partial fertile hybrids are produced) to one (when there is no physical contact) and was divided by seven (categorical values in brackets).

and genetic distances). Reciprocal crosses were averaged. Three neighbour-joining trees were generated (Fig. S1), one for each gene: COII (n = 17 taxa; n = 51 samples), CYTB (n = 15 taxa; n = 58 samples), 18S–28S (n = 17 taxa; n = 55 samples) (Fig. S1), using MEGA, version 5 (Tamura *et al.*, 2011). Neighbour-joining trees were used to conduct phylogenetic corrections where the confidence probability (multiplied by 100) of each interior branch length was estimated using a bootstrap test (1000 replicates). The 'corrected' data set was subsequently reduced from 20 to 13 comparisons for COII, 14–8 comparisons for CYTB and 21–13 comparisons for 18S–28S.

To estimate the evolutionary rate of reproductive isolation, we used the nonparametric Spearman rank correlation between reproductive isolation and genetic distances of the phylogenetically 'corrected' species pairs. Additionally, to evaluate whether the mean genetic distance observed for two categories of reproductive isolation (premating and post-mating, prezygotic isolation and post-mating, post-zygotic isolation) are significantly different from each other, we used Mann–Whitney U tests (corrected for multiple comparisons using Bonferroni procedure). Finally, we theoretically predict that species pairs that have Kimura 2-parameter genetic distances similar to or below species pairs forming hybrids are prone to undergo hybridization themselves. The threshold hybridization range was calculated based on the genetic distances (mean±SE) between all species pairs that are forming hybrids in the wild.

Measures of hybridization in the field

Ischnura elegans, *I. genei*, *I. graellsii* and *I. saharensis* occur in the Mediterranean basin. *Ischnura elegans* and *I. graellsii* overlap in northern and eastern Spain where they face unidirectional introgressive hybridization (Sánchez-Guillén *et al.*, 2011), *I. elegans* and *I. genei* partially overlap in Tyrrhenian Islands, and *I. graellsii* and *I. saharensis* occur sympatrically in Maghreb. These three pairs of species can be induced to hybridize in the laboratory (Sánchez-Guillén unpublished data). We examined the presence of hybrids in two populations of *I. genei* where *I. elegans* appear with low frequency (Foxi and Coghinas), in one population of *I. graellsii* (Saïdia), which is parapatric with *I. saharensis*, and in one population of *I. saharensis* (Berkane), which is sympatric with *I. graellsii*.

Between 1999 and 2009, we sampled 16 allopatric, parapatric and sympatric populations from Europe and northern Africa (Fig. S2, see Table S2 for sampling locations). A minimum of 20 adult males per population were sampled. Captured individuals were stored in 100% ethanol until DNA extraction. DNA extractions were carried out from the head using the phenol/chloroform–isoamyl alcohol protocol. Genotypes were assayed (following Sánchez-Guillén *et al.*, 2011) at five microsatellite loci because of the difficulty to successfully cross-amplify some of the microsatellite markers developed for *I. elegans* (Wellenreuther *et al.*, 2010a) in the four sister species. Fragment size determination and allelic designations were carried out in GeneMapper 3.0 (Applied Biosystems). The final sample size included 247 individuals from 16 populations (Table S3).

Measures of genetic diversity, namely expected heterozygosity, observed heterozygosity, number of alleles and the allelic richness, were calculated using FSTAT, version 2.9.3.2 (Goudet, 1995; Table S3). All populations were in Hardy–Weinberg equilibrium. We used PCA-GEN (Goudet, 1995) for a principal component analysis (PCA) to capture the highest variation in the genetic dissimilarity among species. Based on the PCA results, we used the Bayesian statistical framework STRUCTURE, version 2.3.3 (Pritchard & Stephens, 2000), to determine which individuals from sympatric populations of *I. elegans* and *I. genei,* and *I. graellsii* and *I. saharensis* can be classified as hybrids, similar to previous work on *I. elegans* and *I.* graellsii (Sánchez-Guillén *et al.*, 2011). We applied the 'admixture model' with 'independent allele frequencies', a 'burn-in' period of 20 000 replicates and a sampling period of 100 000 MCMC replicates. The number of genetic clusters (K) was 1 to n + 1 populations, and we performed 10 iterations for each cluster. Thus, we generated multiple posterior probability values (log-likelihood (lnL) values) for each K, and the most likely K was evaluated by the ΔK method (Evanno *et al.*, 2005). After that, we used admixture analyses in STRUCTURE to assign individuals of *I. saharensis* from North Morocco to two clusters, one representing *I. graellsii* and the other representing *I. saharensis*. The same analysis was carried out to assign *I. genei* individuals from Sicily to the two clusters, one representing *I. elegans* and the other representing *I. genei*. We used 'prior population information' because it facilitates the clustering process of the reference individuals and allows calculating admixture proportions (and ± 90% credible regions) of each individual. Additionally, we used 'population flag' option to exclude Sardinian *I. genei* populations (Coghinas and Foxi) and North African *I. graellsii* (Saïdia) and *I. saharensis* (Berkane) populations as reference individuals from each respective analysis. The analysis was run for 100 000 MCMC replicates, after an initial burn-in period of 20 000 replicates, using 'independent allele frequencies' for five iterations.

To generate simulated genotypes of hybrids and backcrosses, we used HYBRID-LAB (Nielsen *et al.* 2006) using the genotypes of 25 individuals of *I. graellsii* and 26 individuals of *I. saharensis*, and 25 individuals of *I. elegans* and 25 individuals of *I. genei*, all of which were collected from allopatric populations as initial genotypes. We generated 25 genotypes of the following crosses: first-generation hybrid (F_1; i.e. $sp_1 \times sp_2$),

second-generation hybrid (F_2; i.e. $F_1 \times F_1$), first backcross with sp_1 (1BC; i.e. $F_1 \times sp_1$), first backcross with sp_2 (1BC; $F_1 \times sp_2$), second backcross with sp_1 (2BC; $1BC \times sp_2$), sp_2 (2BC; $1BC \times sp_2$). Admixture proportions (± 90% credible intervals) of artificial hybrids were evaluated with STRUCTURE to infer levels of introgression in sympatric populations by comparing admixture proportion for artificial hybrids and backcrosses with admixture proportion in sympatric populations.

Results

Reproductive isolation

Reproductive barriers between 16 *Ischnura* species pairs belonged to two categories: premating isolation ($n = 1$) and post-mating, post-zygotic isolation ($n = 15$) (see Table 1). However, reproductive barriers between species from different genera (*Enallagma*, *Pyrrhosoma*, *Coenagrion* and *Erythromma*) belonging to the family 'Coenagrionidae' mainly belonged to premating isolation ($n = 10$) and only six to post-mating, prezygotic isolation (Table 1). Barriers between species from different families [*Lestidae* (*Lestes* and *Sympecma*) and *Calopterygidae* (*Calopteryx*)] belonged to premating isolation ($n = 3$) and post-mating, prezygotic isolation ($n = 2$) (Table 1).

The relationship between genetic distance and reproductive isolation

Pairwise genetic distances between damselfly genera ranged from −0.09% to 28.00% for mtDNA COII, −0.01–18.13% for mtDNA CYTB and 0.00–72.18% for nDNA 18S–28S (Table S2). Without phylogenetic correction, we detected a significant positive correlation between genetic distance and reproductive isolation, and the results were similar for the three genes analysed. For COII, the correlation was $r = 0.837$ (range −0.09–22.27%, $n = 20$, $P < 0.0001$), for CYTB $r = 0.652$ (range −0.01–17.90%, $n = 14$, $P = 0.013$) and for 18S–28S $r = 0.651$ (range 0.00–65.83%, $n = 21$, $P = 0.002$, Fig. 1). Species pairs which formed hybrids (forthcoming genetic distance values separated by ± denote mean±SD) (0.004 ± 0.008, $n = 7$ for COII), (0.008 ± 0.018, $n = 8$ for CYTB) and (0.003 ± 0.004,

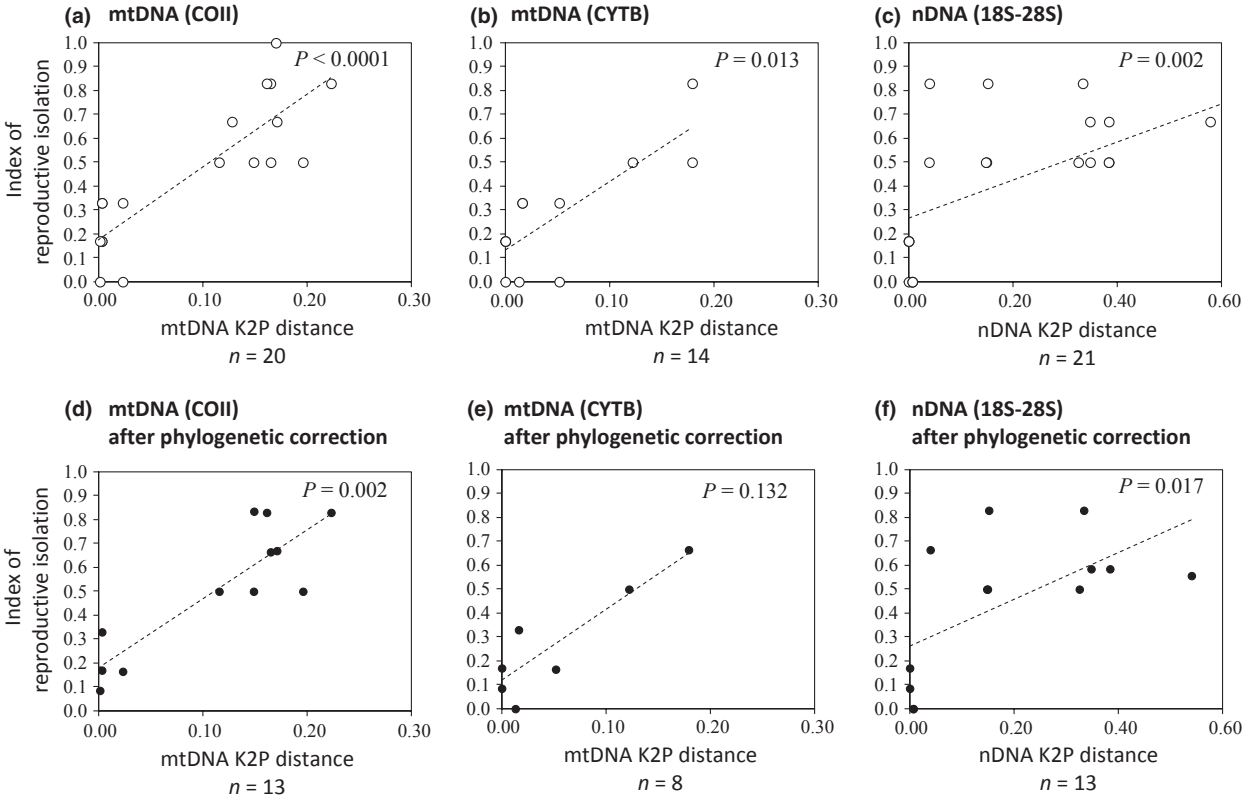

Fig. 1 Relationship between reproductive isolation and genetic distance Kimura 2-parameters (K2P): before phylogenetic corrections: mtDNA (COII) (a), mtDNA (CYTB) (b) and nDNA (18S–28S) (c); after phylogenetic corrections: mtDNA (COII) (d), mtDNA (CYTB) (e) and nDNA (18S–28S) (f). Solid black lines represent the tendency line.

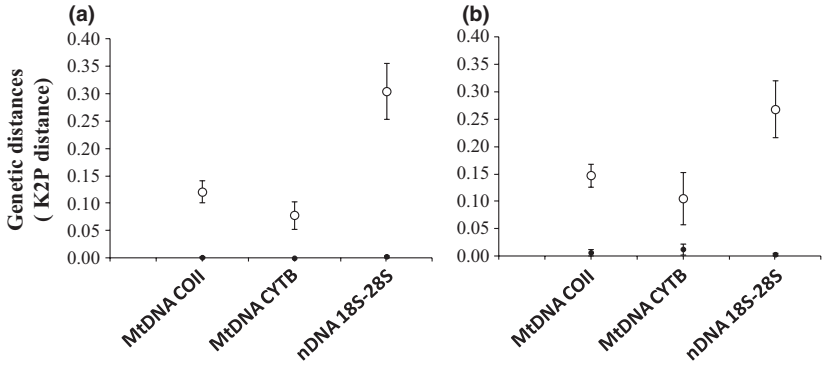

Fig. 2 Genetic distance (Mean ± ES) Kimura 2-parameters (K2P) before and after phylogenetic corrections for pair of species forming (black dots) and not forming hybrids (white dots).

n = 7 for 18S–28S) were less genetically divergent than those not forming hybrids (0.0129 ± 0.073, n = 13 for COII), (0.0094 ± 0.076, n = 6 for CYTB) and (0.305 ± 0.183, n = 14 for 18S–28S, Fig. 2) (COII sequences: Mann–Whitney U-test=2.5, P = 0.001; CYTB sequences: Mann–Whitney U-test=2.5, P = 0.006; and 18S–28S sequences: Mann–Whitney U-test=0, $P < 0.0001$).

After phylogenetic correction, we only detected a significant positive correlation in COII and 18S–28S, but not in CYTB. For COII, the correlation was r = 0.781 (range −0.09–22.27%, n = 13, P = 0.002), and for 18S–28S, r = 0.658 (range 0.00–53.96%, n = 13, P = 0.017, Fig. 1). For CYTB, however, the null hypothesis of no correlation could not be rejected (r = 0.599; range −0.01–17.89%, n = 8, P = 0.132, Fig. 1), although a trend of a positive association was visible (Fig. 1). Species pairs which formed hybrids (0.007 ± 0.011, n = 4 for COII), (0.013 ± 0.022, n = 5 for CYTB) and (0.003 ± 0.004, n = 4 for 18S–28S, Fig. 2) were less genetically divergent than those not forming hybrids (0.148 ± 0.062, n = 9 for COII), (0.106 ± 0.083, n = 3 for CYTB) and (0.268 ± 0.156, n = 9 for 18S–28S, Fig. 2) (COII sequences: Mann–Whitney U-test=1.00, P = 0.011; and 18S–28S sequences: Mann–Whitney U-test=0.0, P = 0.007), except when using CYTB sequences (Mann–Whitney U-test=1.00, P = 0.074).

Data from COII and 18S–28S showed that species pairs with genetic distances below a threshold of 0.0067 ± 0.011 (range −0.43–1.78%) for COII and 0.0033 ± 0.004 (range −0.052–0.713%) for 18S–28S are susceptible to hybridize and produce hybrids.

Levels of genetic distance and reproductive isolation in the field

Pairwise genetic distances between *I. elegans* and *I. genei* (0.32% for COII and 0.00% for 18S–28S, Table S2) and *I. graellsii* and *I. saharensis* (−0.09% for COII and 0.70% for 18S–28S, Table S2) overlapped with the estimated thresholds of genetic divergence. Both the PCA and Bayesian statistical framework (Fig. 3) confirm this finding, supporting the presence of hybrids between *I. elegans* and *I. genei* and *I. graellsii* and *I. saharensis*. The three significant PCA axes accounted for 74.28% of the variation in the data. The first two PC axes (58.11% variation, Fig. 3a) showed a clear species cluster, but no location cluster: the first PC axis separated allopatric populations of *I. saharensis*, *I. genei* and *I. elegans*, whereas the second PC axis separated allopatric populations of *I. graellsii* from all allopatric populations of the remaining species. The parapatric population (Saïdia) of *I. graellsii* was clustered with the allopatric population. However, the parapatric of *I. genei* (Foxi and Coghinas-*I. genei*) and the sympatric population of *I. saharensis* (Berkane) were clustered intermediate between *I. elegans* and *I. genei*, and *I. graellsii* and *I. saharensis*, respectively, indicating contemporary hybridization in these populations.

Although the PCA revealed a clear separation of the four allopatric species clusters in addition to an intermediate sympatric cluster, the ΔK method suggested only three clusters as the most likely population structure (Fig. 3b): the first and second clusters corresponded to *I. graellsii* and *I. elegans*, whereas the third was best represented by *I. genei* and *I. saharensis*.

Assignment tests gave strong support for hybridization in sympatric populations. In assignment tests using genotype information of *I. graellsii* and *I. saharensis* (Fig. 3c), the majority of allopatric *I. graellsii* (52 of 53) and *I. saharensis* (27 of 31) were assigned with > 90% certainty to their species cluster (Fig. 3c). In sharp contrast to this, the majority of parapatric *I. graellsii* (47.5–70.6%) and sympatric *I. saharensis* samples (37.5–72.1%) were classified as intermediate between both species clusters. In both populations, the percentage of credible regions represented not only first-generation (F_1; i.e. *I. graellsii*× *I. saharensis*) or second-generation hybrids (F_2; i.e. F_1 hybrids × F_1 hybrids), but also first and successive backcrosses (see Fig. 3c). Similarly, in assignment tests using genotype information of *I. elegans* and *I. genei*, 94 of the 100 allopatric *I. elegans* samples and 29 of the 32 allopatric *I. genei* samples were assigned with at least 90% certainty to each of the two

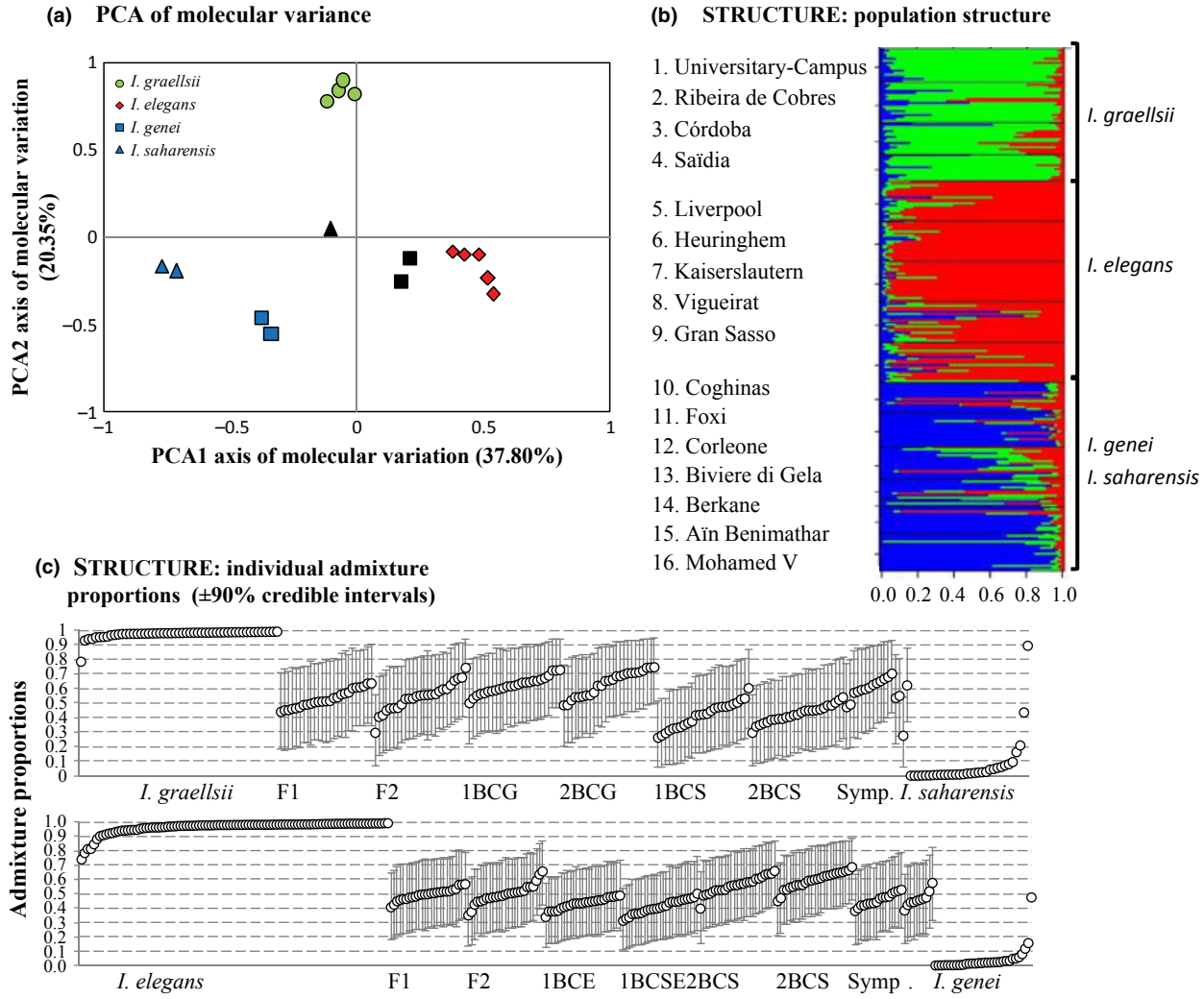

Fig. 3 (a) Principal component analysis of allopatric (grey symbols) and sympatric (black symbols) *I. elegans*, *I. genei*, *I. graellsii* and *I. saharensis* populations. First axis 37.80% (F_{ST} = 0.057, P = 0.23), second 20.31% (F_{ST} = 0.031, P = 0.12) and third 16.13% (F_{ST} = 0.024, P = 0.09). The first and second axes represent the first two factorial components. (b) Population structure of *I. elegans*, *I. genei*, *I. graellsii* and *I. saharensis* based on STRUCTURE for K = 3. Individuals are represented by single vertical lines broken into two segments, proportional to their respective membership in the two genetic clusters. (c) Individual Bayesian assignment probabilities for K = 2. Assignment proportions for *I. graellsii* and *I. saharensis* were calculated using three allopatric *I. graellsii* and two allopatric *I. genei* populations (first-generation F_1; i.e. *I. graellsii*× *I. saharensis*, second-generation hybrid F_2; i.e. F_1 hybrids × F_1 hybrids and backcross with *I. graellsii* 1BCG and 2BCG; i.e. F_1 hybrids or successive × *I. graellsii*) and with *I. saharensis* 1BCS and 2BCS; i.e. F_1 hybrids or successive × *I. saharensis*), as well as one parapatric *I. graellsii* (Saïdia) and one sympatric *I. saharensis* population (Berkane). Assignment proportions for *I. elegans* and *I. genei* were calculated using five allopatric *I. elegans* and two allopatric *I. genei* populations (first-generation F_1; i.e. *I. elegans*×*I. genei*, second-generation hybrid F_2; i.e. F_1 hybrids × F_1 hybrids and backcrosses with *I. elegans* 1BCE and 2BCE; i.e. F_1 hybrids or successive × *I. elegans* and backcrosses with *I. genei* 1BCG and 2BCG; i.e. F_1 hybrids or successive × *I. genei*), as well as two parapatric *I. genei* populations (Coghinas and Foxi).

species clusters (Fig. 3c), whereas both *I. genei* populations from the parapatric region were intermediate between the two clusters (38.4–58.0% assignment to *I. elegans*) corresponding to either first- or second-generation hybrids and successive backcrosses with *I. elegans* and also *I. genei* (Fig. 3c).

Discussion

A taxonomically broad evaluation of the relationship between reproductive isolation and genetic divergence is essential for elucidating general mechanisms in the speciation process. A large body of work has accumu-

lated supporting a positive correlation between reproductive isolation and genetic divergence, for instance in *Drosophila*, butterflies, toads, birds and angiosperms (Coyne & Orr, 1997; Sasa *et al.*, 1998; Presgraves, 2002; Price & Bouvier, 2002; Moyle *et al.*, 2004), although some exceptions to this have been found (Lessios & Cunningham, 1990; Edmands, 2002; Scopece *et al.*, 2007). Despite the solid number of studies investigating this topic, notable taxonomic gaps exist. Odonates make an important contribution to our general understanding of whether the relationship holds across diverse groups, because they represent the most ancient winged insects order and also a group where sexual selection had a large effect on the diversification process (McPeek & Brown, 2000; Svensson, 2012), with sometimes little evidence of niche diversification between closely related species (Stoks *et al.*, 2005; Wellenreuther *et al.*, 2012). Here, we directly test for an isolation–divergence correlation in 30 species pairs of damselflies and report a positive association across a wide range of values. Furthermore, our data support the usefulness of this correlation to predict hybridization.

Evidence that natural selection is involved in the origin of species is strong, for example, as seen in the rapid diversification evident in adaptive radiations (Schluter, 2000), or in the strong association between ecological divergence and reproductive isolation in many species pairs (Funk *et al.*, 2006). Artificial selection experiments mimicking natural selection also commonly produce reproductive isolation as a correlated response (Rice & Hostert, 1993). The link between reproductive isolation and genetic divergence in radiations driven by sexual selection and conflict is less well known. Strong sexual selection and conflict might lead to the rapid evolution of reproductive isolation, whereas overall levels of neutral genetic divergence might evolve less quickly. Some support for this idea comes from studies showing that signatures of speciation by sexual selection can be detected in insects, frogs, fish and birds despite low genetic divergence (see Panhius *et al.*, 2001). In our study, we found that only one of the 16 interactions between *Ischnura* taxa was prevented before zygote formation, whereas both pre- and post-zygotic barriers were found to significantly reduce gene flow in other damselfly taxa (Table 1). Levels of genetic divergence were low even in fully reproductively isolated ischnurids [K2P D = 0.0067 ± 0.011 STD (COII) and K2P D = 0.0033 ± 0.004 STD (18S–28S)]. The low overall genetic divergence between congeneric species despite often high levels of isolation is consistent with the idea that sexual selection can be a powerful force in the development of mating barriers in this group.

The Mediterranean ischnurids *I. elegans, I. genei, I. graellsii* and *I. saharensis* all show low interspecific genetic divergence of < 1%, and similar distances between species were also detected with allozymes (Carchini *et al.*, 1994; Neis' D = 0.00–0.352%), although population structure analyses revealed good species barriers (Fig. 3). These four *Ischnura* species are ecologically and morphologically similar, but males can unambiguously be identified by reproductive structures; in particular, the morphology of the prothorax and anal appendages shows clear species-specific structures (Dijkstra & Levington, 2006). Antagonistic mating interactions and sexual conflict are likely to be involved as drivers of speciation in odonates (Svensson, 2012), and a possible outcome of these interactions can be the rapid divergence of male genitalia (Eberhard 2004), as has been shown in odonates (Cordero-Rivera *et al.*, 2004), but also in other animal groups such as seed beetles (Cayetano *et al.*, 2011). Mismatch in the anatomy of anal appendages causes complete or near-complete isolation in odonates (Robertson & Paterson, 1982; McPeek *et al.*, 2008b; Sánchez-Guillén *et al.*, 2012). For example, in an exhaustive study on 19 isolating barriers between *I. graellsii* and *I. elegans*, mechanical isolation was the most important barrier (Sánchez-Guillén *et al.*, 2012). The same study found that sexual selection was much weaker between the aforementioned species (Sánchez-Guillén *et al.*, 2012), which is typical for odonate species showing little divergence in colour traits between the sexes (Sánchez-Guillén *et al.*, 2012). In contrast, hybrid formation is almost completely prevented between congeneric *Calopteryx* and *Mnais* species (Hayashi *et al.*, 2004; Tynkkynen *et al.*, 2008) through strong sexual selection for wing phenotypes (Svensson *et al.*, 2004). Thus, it appears that strong sexual selection on secondary sexual traits is a more potent mechanism to prevent hybrid formation in odonates than mechanical isolation.

In damselflies, both nuclear and mitochondrial estimates of genetic divergence were good predictors of reproductive isolation (Fig. 1), and the usefulness of this correlation was corroborated by our ability to predict hybridization. Specifically, the PCA (Fig. 3) suggested the presence of intermediate populations between *I. elegans* and *I. genei* and between *I. graellsii* and *I. saharensis* in parapatry or sympatry, and the admixture analyses revealed a pattern of hybridization and introgression consistent with the cross-directions detected under laboratory conditions. Consistent with our observation, Mallet (2007) found a negative correlation between mtDNA divergence and the number of hybrids found in wild *Heliconius* species. Likewise, in a ring species complex of lizards, overall genetic divergence was a good predictor of the complete cessation of genetic interactions (Pereira *et al.*, 2011). The results from this study thus point towards a positive correlation between the degree of divergence and reproductive isolation, consistent with the majority of work that has been done on other taxa so far. This suggests that there may be a general pattern in the acquisition of reproductive isolation in animals, which can be useful when forecasting which pairs of species may become vulnerable

to hybridize upon contact. One area for which this is particularly relevant is environmental change. It is known that range shifts induced by environmental change can affect the equilibrium between hybrid formation and selection acting against unfit hybrid production (Taylor *et al.*, 2006). This may lead to the loss of a species. In fact, local extinction of *I. graellsii* has been detected in the north of Spain, where *I. elegans* has recently arrived, and introgressive hybridization displaces *I. graellsii* (Sánchez-Guillén *et al.*, 2005, 2011, 2012, 2013). The high predictive ability of these measures indicates suitability in conservation to predict the risk of hybridization between species due to environmental-driven secondary contact.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Phylogenetic relations by neighbour-joining distance tress.

Figure S2 Map showing sampled populations.

Table S1 Accession numbers for the three genes and species and abbreviated names used in the phylogenetic analyses.

Table S2 Species, sampling locality, ecology and year, latitude and longitude, sample size for molecular analysis (N), observed (H_O) and expected heterozygosity (H_E), number of alleles and allelic richness (R_A).

Table S3 Pairwise genetic distances (%) (Kimura 2-parameter) between ischnurids and seven damselfly genera (*Calopteryx*, *Coenagrion*, *Enallagma*, *Erythromma*, *Lestes*, *Pyrrhosoma* and *Sympecma*) for mtDNA COII (330 bp, 36 sequences, 17 taxa), mtDNA CYTB (317 bp, 51 sequences, 13 taxa) and nDNA 18S-28S (485 bp, 53 sequences, 16 taxa).

Data deposited at Dryad: doi:10.5061/dryad.vn110

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