Isolation barriers and genetic divergence in non-territorial *Argia* damselflies

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Isolation barriers work at different instances during the mating process in odonate insects. In territorial damselflies, heterospecific interactions are mainly precluded by sexual (visual) isolation, while in non-territorial damselflies, heterospecific interactions are mostly precluded by mechanical isolation and sexual (tactile) isolation. In this study we investigated the strength of three premating barriers (visual, mechanical and tactile), genetic divergence and degree of sympatry (on their entire distribution) between four non-territorial *Argia* damselflies (*A. anceps*, *A. extranea*, *A. oenea* and *A. tezpi*). Our results are explained in the light of learned mating preferences and Kaneshiro's hypothesis. We detected a strong reproductive isolation between all pairs of species by the joint action of the three studied barriers [visual (90.6%), mechanical (8.7%) and tactile (0.7%)]. Sexual (visual) isolation was the most important barrier, perhaps driven by learning mating preferences. One of the studied species, *A. extranea*, which is the most derived of the studied species, showed a highly asymmetric isolation in reciprocal crosses, which is consistent with Kaneshiro's hypothesis. Moreover, we detected a negligible ecological niche differentiation between the studied species (70% of shared distribution). Our results suggest that sexual (visual) selection may be an important force driving speciation in non-territorial species. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2017, **120**, 804–817.

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INTRODUCTION

A central question in evolutionary biology concerns the accumulation of reproductive barriers during speciation. One of the greatest contributions to the modern synthesis was the concept of reproductive isolation mechanisms (barriers) by Dobzhansky (1937). Reproductive barriers can prevent gene flow before (premating barriers) or after mating (postmating barriers), and before (prezygotic) or after (postzygotic) fertilization (Dobzhansky, 1937; Coyne & Orr, 2004). In relation to this, one long-standing information gap concerns the order at which the reproductive barriers accumulate at the initial steps of diversification, and the intensity with which these barriers prevent genetic exchange. Numerous studies in as diverse taxa as plants, insects, fishes, birds or mammals (for references see Sánchez-Guillén *et al.*, 2014a) have been done to address the importance of pre- and postzygotic barriers in the accumulation of reproductive isolation. Moreover, some of these studies (e.g. angiosperms, Moyle, Olson & Tiffin, 2004; frogs, Sasa, Chippindale & Johnson, 1998; birds, Price & Bouvier, 2002; butterflies, Presgraves, 2002; and damselflies, Sánchez-Guillén *et al.*, 2014a) are in line with the 'speciation clock' phenomenon, i.e. the idea that reproductive isolation is a by-product of

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gradual genetic divergence. However, an increased sexual selection can provide more opportunities for speciation (Gage *et al.*, 2002) and thus can accelerate the accumulation of reproductive isolation (see Darwin, 1871).

Sexual selection is a key driver of divergence, speciation, and radiation in odonate insects (Wellenreuther & Sánchez-Guillén, 2016). Visual, olfactory and tactile signals are involved in both intraspecific and interspecific recognition (sexual isolation) in these insects (Wellenreuther & Sánchez-Guillén, 2016). Intraspecific sexual selection *via* learned mating preferences has been recently found to be an important process in three different odonates genera (*Calopteryx*, *Enallagma* and *Ischnura*, Fincke, Fargevieille & Schultz, 2007; Svensson *et al.*, 2007; Sánchez-Guillén *et al.*, 2013). However, the role of learning in the interspecific sexual isolation context comes only from calopterygid damselflies, whose males learn to discriminate against heterospecific mates following courtship interactions (Waage, 1979; Wellenreuther, Tynkkynen & Svensson, 2010). Learning in these species could occur during such interactions as males have the time to 'inspect' and differentiate individual females. Thus, one key issue here is whether learning can still occur in species where individuals do not engage in such premating interactions (which is the case of non-territorial species and without courtship), which is pivotal to establish if such mechanism is widespread during sexual isolation in odonates.

Argia is a good model genus to investigate the role of learning ability in sexual isolation in non-territorial odonates and without courtship. This monophyletic genus is one of the most specious Nearctic genus from the New World (Caesar & Wenzel, 2009), with around 120 described species (Garrison, 1994). For instance, up to six *Argia* species have been found in sympatry (e.g. Central Mexico; Peralta-Vázquez, 2009). Additionally, *Argia* is a non-territorial genus which lacks courtship behaviour (Bick & Bick, 1982) and whose populations occur usually at high densities. Importantly, two classical studies of reproductive isolation in damselflies (Paulson, 1974; Robertson & Paterson, 1982) have highlighted the importance of both visual and tactile signals in sexual isolation in *Argia* species.

The aim of this study was to test the role of learning in interspecific sexual isolation and the 'speciation clock' phenomenon in odonates. To this end, we investigated the strength of visual, mechanical and tactile isolation between four pairs of closely related species of the non-territorial genus *Argia* (Fig. 1) in one community with high density for the four species, but with different species proportions. First, to establish a basis for isolation barriers, we estimated the strength of sexual isolation in terms of visual, mechanical and tactile isolation. Second, we linked the strength of such isolation with the presumed genetic divergence among all four species by using a previously sequenced ribosomal gene (see Caesar & Wenzel, 2009). This link is fundamental to see whether isolation is a consequence of interspecific genetic differences. Third, based on previous findings that showed the importance of the degree of sympatry (Wellenreuther *et al.*, 2010) in the intensity of the sexual isolation, we tested the correlation between degree of sympatry and reproductive isolation strength. For this, we measured the degree of sympatry between the four studied species in their complete distribution, by using modern techniques of distributional modelling.

METHODS

STUDY SPECIES AND POPULATIONS

We used four sympatric *Argia* species (*Argia anceps*, *A. extranea*, *A. oenea* and *A. tezpi;* Fig. 1) which are common in Mexico, and usually show overlapping distributions (all authors', unpubl. data). *A. anceps* males are completely blue (thorax and abdomen) with slightly amber wings, while females are dark brown and blue (Fig. 1A). *A. extranea* males show violaceous-blue thorax coloration and prominent ventrolateral black streaks on the 8th–10th abdominal segments. *A. extranea* females have light-blue thorax coloration and almost complete light-brown abdominal coloration, except for the 8th and 9th abdominal segments which are blue (Fig. 1B). *A. oenea* males have red eyes, and blue thorax and abdomen with black stripes, while females have brown thorax and abdomen (Fig. 1C). Males of *A. tezpi* have a solid metallic thorax, while females have light dark brown thorax and abdomen (Fig. 1D; see Garrison, Ellenrieder & Louton, 2009 for further descriptions). Our four studied species are related within the genus, and vary in relative abundance in our study site, Tetlama river (18°46′05″N, 99°14′17″W), in Xochitepec, Mexico. To obtain actual measures of population density of each species, we counted adults of the four species in separate days at the beginning and at the end of the experiment of reproductive isolation, which started at early November and ended at late December of 2012. Density measures were estimated as the number of adults captured per minute, on a 100 m transect on the river. Such countings were done at noon, when adult activity was at its highest level (all authors', unpubl. data).

Figure 1. Males and females of all *Argia* species studied: *A. anceps* in A, *A. extranea* in B, *A. oenea* in C and *A. tezpi* in D. Pictures were courtesy of Keith D . P. Wilson (A), and Erland Refling Nielsen (B and C). Photo D taken by A ngela Nava-B olaños.

REPRODUCTIVE ISOLATION: SEXUAL (VISUAL AND TACTILE) AND MECHANICAL BARRIERS

Reproduction in non-territorial odonate species without courtship, such as *Argia* damselflies, starts when a male finds a female and attempts to mate (*attempt to tandem*) without any behavioural input from her. To achieve copulation, the male must grasp the female with his anal appendages by holding her mesostigmal plates (*tandem*) and after that, the female must accept the mating by bending her abdomen, thus allowing the contact between male's and female's primary genitalia (reaching the so-called *wheel position*). Based on the reproductive biology of odonates, copulation can be firstly precluded by the lack of sexual attraction of the male by the female, so that males do *not attempt the tandem* (visual sexual isolation). If the male is sexually attracted by the female and she attempts to form the tandem, the *tandem* position can be impeded by the incompatibility between male cerci and female mesostigmal plates (mechanical isolation). Thirdly, if the *tandem*

is formed, *wheel position* can be prevented by tactile isolation because of male's inappropriate stimulation of the female, so that the female does not bend her abdomen (tactile sexual isolation) to form the *wheel position*, and if the female accept to bend her abdomen, by mechanical incompatibility between male and female primary genitalia (mechanical isolation) (see Wellenreuther & Sánchez-Guillén, 2016).

To avoid handling the focal organisms (males) we manipulated the females. Only sexually mature females showing bright body colours and rigid body structure (Corbet, 1999) were used for the reproductive isolation experiment, which was done on windless and sunny days, from 10:00 to 15:00 h, in the field. However, we could not control whether females were virgin or not, as there was no way to have tracked their mating history. Alive female models of each species were tied with a fine thread to allow them to move freely (see Cordero, 1999; Cordero-Rivera & Sánchez-Guillén, 2007, for similar methodologies). These models were presented repeatedly to

males of the four species, to record male responses. Females were presented on average four times for species (this is, some females were presented as less as 1 time, but as many as 12 times). Females were presented to males as these were found along the river. It is highly likely that we did not 'use' the same male as we moved away several meters to find another new male. Only those females that were in good physical condition were considered. Male responses were categorized as: (1) *no sexual response*, which included males that did not have sexual response but show responses like: from faces off and/ or approaches without physical contact, to flies away or moves around the perch; (2) *attempt of tandem*, which was when the male approached the female and *attempted the tandem* by curving his abdomen; (3) *tandem*, which was when the male grasped the female with his anal appendages; and (4) *mating*, which was when female does genital contact with the male. However, in the case of conspecific couples, we did not let them to reach *wheel position*, to prevent harming the female models that would occur when disconnecting a mating pair. Those males that did not show any type of response to female models were not considered in the analysis. This methodology allows deducing mating formation, based on the premise that males reached copulation in 95% of tandem events among conspecifics (Cordero-Rivera & Sánchez-Guillén, 2007). In total, 147 (*A. anceps*), 54 (*A. extranea*), 211 (*A. oenea*) and 192 (*A. tezpi*) male responses were recorded.

Because of each reproductive step is relative to the previous one, the percentages of responses were estimated as following: *attempt to tandem* was estimated respect to the total number of presented females, and *tandem* was estimated respect to the total number of *attempts to tandem*. The absolute contribution of both reproductive barriers [sexual (visual and tactile) and mechanical)] were estimated following a previously published methodology (see Sánchez-Guillén, Wellenreuther & Cordero-Rivera, 2012; Sánchez-Guillén *et al.*, 2014a). Visual (sexual) reproductive isolation was estimated as (RI sexual) = 1 − (*tandem attempts* that a heterospecific female was presented)/*tandem attempts* that a conspecific female was presented). Mechanical reproductive isolation was estimated as (RI mechanical) = 1 − (*tandems/attempts to tandem* in heterospecific interactions)/(*tandems/attempts to tandem* in conspecific interactions). Tactile (sexual) isolation (RI tactile) = 1 − (*copula/tandem* in heterospecific interactions)/(*copula/tandem* in conspecific interactions). All indexes ranged from −1 to 1 with negative values indicating higher success in heterospecific than conspecific combinations. In order to test whether the reproductive isolation indexes (sexual and mechanical recognition) are statistically significant we used a chi-square test.

Alternatively, we also computed cumulative link mixed models (CLMM; (Fox, 2002; Agresti & Maria, 2011) to test significance of the strength of the studied reproductive barriers (sexual, mechanical and tactile). CLMM link cumulative probabilities to linear predictors and thus can be used for ordinal data with mixed effects. In our experiment, we have three cumulative probabilities (*attempt to tandem*, *tandem* and *copula*), and mixed effects because females were involved in more than one presentation, thus data are not independent from each other. We assigned 0 to no sexual responses, 1 to *attempts tandem* and 2 to *tandems*. We did not observe any copulation, so this response was not included in the analyses. We constructed one model for each studied species and male's responses were evaluated. CLMM were fitted with the clmm2 function in package ordinal (Christensen, 2015). Statistical analyses were done using R version 3.0.2 (R Core Development Team 2012) software. Maximum likelihood estimates of the parameters are provided using the Laplace approximation to estimate the likelihood function (Supporting Information, Table S2). The *P*-values for the location coefficients provided are based on *Wald* statistics, and a more accurate test was provided by likelihood ratio tests, obtained with deviance analyses.

CORRELATION BETWEEN REPRODUCTIVE ISOLATION AND THE GENETIC DIVERGENCE AND DEGREE OF SYMPATRY

The phylogenetic relationship between the North American *Argia* species, including our four studied *Argia* species have been resolved by Caesar & Wenzel (2009) using the mtDNA gene 16S. Genetic distances between each pair of species have been estimated using the best evolutionary model, which was inferred, by using MEGA V. 6. The evolutionary model Tamura 3-parameters considers differences between transitions and transversions, and provides equal substitution rates among sites. The correlation between the strength of sexual and mechanical isolation and genetic divergence and degree of sympatry were estimated by using the Pearson's correlation.

SPECIES DISTRIBUTION: OVERLAPPING RANGES AND ESTIMATION OF GEOGRAPHICAL SYMPATRY

Two parameters were used to measure the potential and observed sympatry. To measure the former, ecological niche models for each pair of species were used, and the potential overlapping range (between each pair of species) was estimated. To measure observed sympatry, frequency counts of each species

in each locality were done, and thus, the total degree of observed sympatry was derived from the relation between the number of sympatric localities vs. the total number of localities in which each species was found.

Our study area included North and Central America and, covering land between the latitudes 53.00–0.00N, and the longitudes −130.00 to −55.00W. As bioclimatic variables, we used WorldClim 1.4 (www.worldclim.org) data set (Hijmans *et al.*, 2005) at 0.041666669 cell size. To establish a set of uncorrelated climatic variables, we intersected the variables with target group points, and with 10 000 points randomly selected in the extension of the study area (*M*), and with special extent delineating *M* for each particular species, we eliminated some variables with an exploratory data analysis and a Pearson correlation analysis (i.e. any value > 0.7). After this, the final data set included mean diurnal range (bio 02), temperature seasonality (bio 04), mean temperature of warmest quarter (bio 10), precipitation of wettest month (bio 13), precipitation of driest month (bio 14), and precipitation seasonality (bio 15).

Presences of the four species were compiled from literature records, confirmed records in Odonata Central (http://www.odonatacentral.org), CONABIO records (www.conabio.gob.mx), and GBIF (www.gbif.org) (accessed October 2014) records which were carefully checked for geographic accuracy. Some records are registered to county, which are rather heterogeneous in size, ranging from small and climatically homogeneous in the US Great Plains, to large and heterogeneous at the US Pacific coast. To avoid inaccurate geo-referencing, only county records from the Great Plain distributions were used. The final data set included 372 unique presences, 68 for *A. anceps*, 114 for *A. extranea*, 112 for *A. oenea* and 78 for *A. tezpi*.

Species distribution models were generated with Maxent 3.3.3k (Phillips, Anderson & Schapire, 2006), to choose the best background. Models were constructed setting several parameters to default ('Auto features', convergence = 10–5, maximum number of iterations = 500, background = 10 000). However, we used random seed (with a test percentage of 30), 10 replicates, removed duplicate records, ran bootstrap replicated type, and no extrapolation and no clamping. All this to find which combination of settings and variables generated the best outcomes (highest area under the curve, or AUC) while minimizing the number of model parameters, as well as producing 'closed', bell-shaped response curves which guaranteed model calibration (Elith, Michael & Steven, 2010). The best background was 10 000 points randomly selected in the extension of the study area. Final models were built with BIOMOD (Biodiversity

Modelling) package in R software. This package is a platform for predicting species' distribution, including the ability to model the distribution using various techniques and test patterns. We trained models using four widely used algorithms (Maxent, RF, GBM and MARS). From individual models obtained with these different algorithms, we generated a 'consensus model' where the final model indicated the degree of overlap between models. This was done to reduce biases and limitations of use only individual models. The final validation of models was performed with TSS (True Skill Statistics), average net rate of successful prediction for sites of presence and absence (Liu, White & Newell, 2009), ranging from −1 to 1, where the more positive values indicate a higher degree of accuracy and discrimination model (Allouche, Tsoar & Kadmon, 2006).

A total of 52 models were generated, and whose performance was assessed by mean of the AUC and TSS statistics, while minimizing the number of model parameters, and the best presence/absence models using the '10 percentile-training presence' are presented. The degree of sympatry between each pair of species was estimated in terms of the observed sympatry (the proportion of locations in which one of the studied species coexisted with at least another of the studied species). Additionally, potential sympatry was estimated as the proportion of potential locations in which one of the studied species overlaps with the potential distribution of at least another of the studied species.

RESULTS

STRENGTH OF THE REPRODUCTIVE ISOLATION

Male responses, to conspecific and heterospecific females are shown in Table 1, Figure 2. Because of the high but not complete sexual isolation (Table 2), *tandem* position was reached in three out of the 12 possible heterospecific combinations (Table 1, Fig. 2) but copulation was strongly precluded by sexual (tactile) isolation, i.e. in any case the female bent her abdomen to form the *wheel position* (Table 1).

Sexual (visual) isolation of *A. anceps* males was stronger with *A. oenea* (0.91) and *A. tezpi* (0.73) than with *A. extranea* (0.55) (Table 1, Fig. 2). Although mechanical isolation was complete (1.00) with *A. extranea* females, preventing completely the gene flow, it was not with *A. oenea* (−0.38) and *A. tezpi* (0.54) as it allowed the formation of heterospecific *tandems* (Table 1, Fig. 2). In the case of *A. extranea* males, sexual (visual) isolation was complete (1.00) (Table 1, Fig. 2, Supporting Information, Tables S1, S2) with the three heterospecific females, and thus, mechanical and tactile isolation could not be

Table 1. Male responses to conspecific and heterospecific female presentations and indexes of reproductive isolation (visual, mechanical, tactile and total)

Species combination	Type	N	Attempt to tandem (%)	Tandem (%)	Copula	RI Visual	RI Mechanical	RI Tactile	RI Total
♂ A. anceps – ♀ A. anceps	Conspecific	32	11 (34.34)	8 (72.7)	–	–	–	–	–
♂ A. extranea – ♀ A. extranea	Conspecific	11	3 (27.3)	1 (33.3)	–	–	–	–	–
♂ A. oenea – ♀ A. oenea	Conspecific	66	32 (48.5)	12 (37.5)	–	–	–	–	–
♂ A. tezpi – ♀ A. tezpi	Conspecific	71	23 (32.4)	10 (43.5)	–	–	–	–	–
♂ A. anceps – ♀ A. extranea	Heterospecific	20	5 (25.0)	0	0	0.545	1.000		1.000
♂ A. anceps – ♀ A. oenea	Heterospecific	53	1 (1.9)	1 (100.0)	0	0.909	−0.375	1.000	1.000
♂ A. anceps – ♀ A. tezpi	Heterospecific	42	3 (7.1)	1 (33.3)	0	0.727	0.542	1.000	1.000
♂ A. extranea – ♀ A. anceps	Heterospecific	13	0	0	0	1.000			1.000
♂ A. extranea – ♀ A. oenea	Heterospecific	22	0	0	0	1.000			1.000
♂ A. extranea – ♀ A. tezpi	Heterospecific	8	0	0	0	1.000			1.000
♂ A. oenea – ♀ A. anceps	Heterospecific	42	3 (7.1)	1 (33.3)	0	0.906	0.111	1.000	1.000
♂ A. oenea – ♀ A. extranea	Heterospecific	24	5 (20.8)	0	0	0.844	1.000		1.000
♂ A. oenea – ♀ A. tezpi	Heterospecific	79	7 (8.9)	0	0	0.781	1.000		1.000
♂ A. tezpi – ♀ A. anceps	Heterospecific	39	2 (5.1)	0	0	0.913	1.000		1.000
♂ A. tezpi – ♀ A. extranea	Heterospecific	29	5 (17.2)	0	0	0.783	1.000		1.000
♂ A. tezpi – ♀ A. oenea	Heterospecific	53	9 (17.0)	0	0	0.609	1.000		1.000

First and second column represent the species combination and type of interaction. N represent the total number of presentations in each species combination. Fourth column represent the total number and the percentage (in brackets) of males that sexually responded to the female model. Fifth column represents total number and percentage (in brackets) of males that reached the tandem. Sixth column represents total number and percentage (in brackets) of males that reached the copula (*wheel position*). Strength of the sexual (visual), mechanical, tactile and total isolation are given in the 8th–10th columns. Strength values ranged from zero (no isolation) to one (complete isolation), (–) denotes data not available.

measured because it was prevented by the previous reproductive stage (sexual isolation). Sexual (visual) isolation of A. oenea males was strong but not complete with any heterospecific female [A. tezpi = 0.78, A. anceps = 0.91 or A. extranea = 0.84; (Table 1, Fig. 2)]. In all cases, the degree of sexual isolation measured by behavioural response of sexual interest was significant (Supporting Information, Table S1). Moreover, mechanical isolation was only complete (1.00) with A. tezpi and A. extranea females, allowing heterospecific *tandems* with A. anceps (0.11) (Table 1, Fig. 2, Supporting Information, Table S2). Sexual (visual) isolation of A. tezpi males was strong but not complete with any of the three heterospecific *Argia* females [A. oenea = 0.61; A. tezpi = 0.91; and A. anceps = 0.78], while mechanical isolation was complete (1.00) with the three heterospecific females (Table 1, Fig. 2).

Sexual (tactile) isolation was strong, as none of the three females (two of A. tezpi, one of A. oenea and one of A. anceps) that formed a heterospecific *tandem* bent her abdomen to form the wheel position, i.e. none of them accepted to mate.

Our results by the CLMM model indicated that species recognition in terms of sexual isolation was greater between conspecifics than between heterospecific (Table 2, Supporting Information, Table S2, S3). In some heterospecific cases, these species could produce *tandem* (Supporting Information, Table S2). The likelihood ratio test testing the effect of male *sexual response* to females of different species is shown in Figure 2, and indicated the predicted probability by models for each behavioural response or indicators of sexual isolation (visual) and mechanical isolation (Supporting Information, Table S2). Although heterospecific recognition occurs, species recognition in total terms (species recognition and mechanical compatibility) was significantly higher in conspecific than in heterospecific combinations in three out of the four species: in A. anceps [deviance = 18.6   Pr(χ) = < 0.001], A. oenea [deviance = 54.5   Pr(χ) = < 0.001] and A. tezpi [deviance = 22.1   Pr(χ) = < 0.001; Table 2, Supporting Information, Table S3].

POPULATION DENSITY, SPECIES DISTRIBUTION AND DEGREE OF SYMPATRY

Species proportions in the studied community was highly dissimilar in the year of study: A. extranea
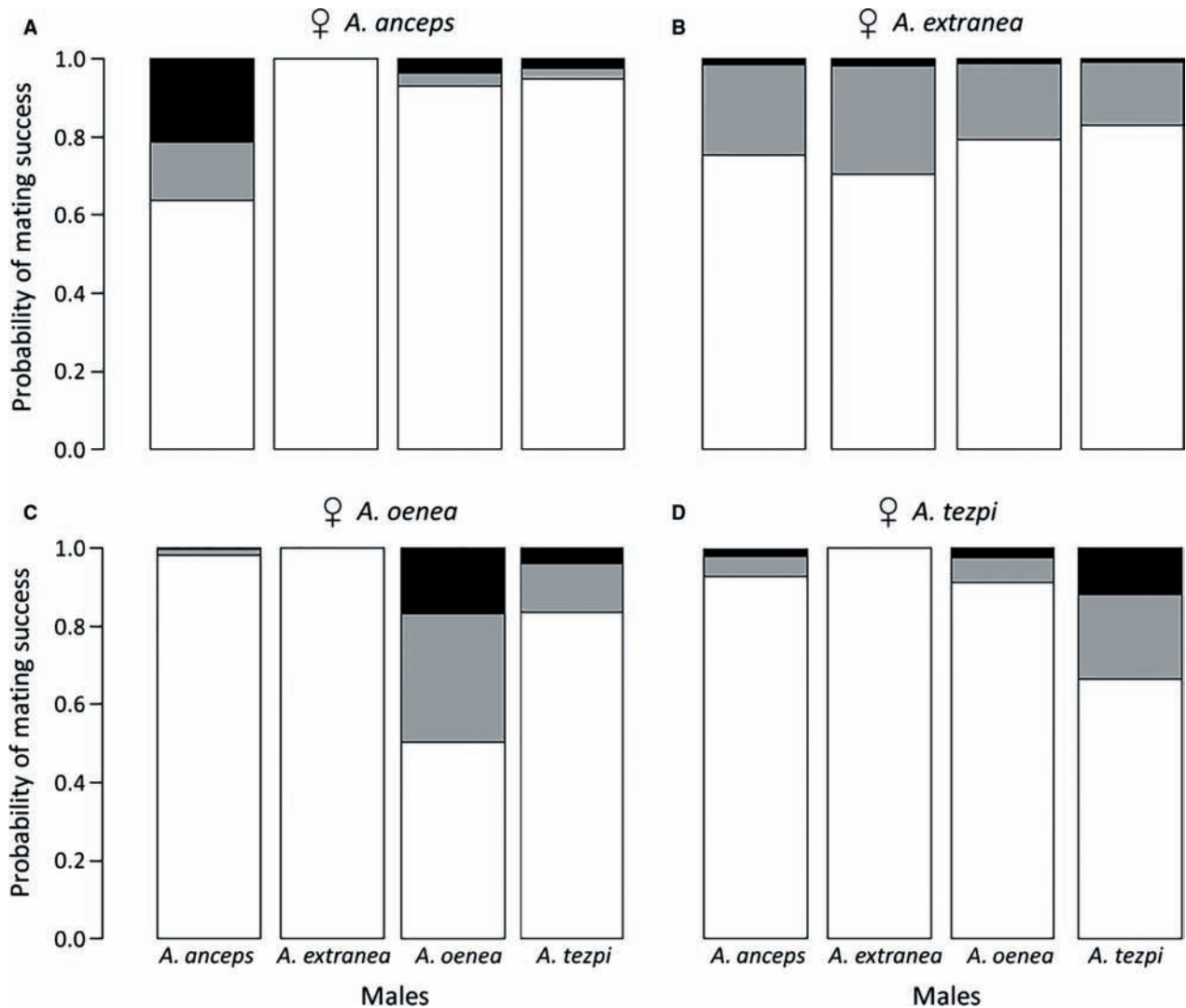
Figure 2. Maximum likelihood estimated by cumulative link mixed models for reproductive isolation. Intensity of the predicted values of reproductive isolation barriers estimated from the predicted values of the probability of male responses: not sexually responses (NSR) (white), *attempt of tandem* (A T) (grey), and *tandem* (T) (black) in conspecific vs. heterospecific combinations for the four studied species: (A) *A. anceps*; (B) *A. extranea*, (C) *A. oenea*; and (D) *A. tezpi* females.

was the less abundant species (7%) followed by *A. anceps* (26%), *A. oenea* (30%) and *A. tezpi*, which was the most abundant *Argia* damselfly species (37%).

Degree of sympatry and allopatry between each pair of species in terms of the observed sympatry (number of overlapped locations) and observed allopatry (number of non-overlapped locations) is shown in Table 3. Potential distributions were estimated by using niche models. Best models were those with the best performance levels, i.e. with the highest TSS values (*A. anceps* = 0.889, *A. extranea* = 0.88, *A. oenea* = 0.879 and *A. tezpi* = 0.897). Potential distributions in terms of potential sympatry (overlapped km^2) and observed

allopatry (non-overlapped km^2) are supplied in Table 3, Figure 3 and Supporting Information, Table S4. In summary, the four *Argia* studied species showed a wide potential range in North America and shared (between each other) more than 80% of their territory.

CORRELATION BETWEEN REPRODUCTIVE ISOLATION AND THE GENETIC DIVERGENCE AND DEGREE OF SYMPATRY

Figure 4A, B show phylogenetic relations between the four *Argia* studied species. Pairwise of genetic distances between the *Argia* damselflies ranged from

Table 2. Likelihood ratio test of the male's efficiency in conspecific vs. heterospecific female presentations

	Deviance	Pr (χ)
A. anceps	18.578	**< 0.001**
A. extranea	0.840	0.840
A. oenea	54.481	**< 0.001**
A. tezpi	22.091	**< 0.001**

Table shows deviance values and significance values ($P < 0.05$) in bold. The intensity of male responses, in terms of visual (sexual) recognition, was significantly higher in conspecific than heterospecific interactions for *A. anceps*, *A. oenea* and *A. tezpi*.

(4.60–7.76%) for the mtDNA 16S. *Argia anceps* showed the highest levels of genetic divergence with the other three species (5.75%, 7.20% and 7.76% with *A. extranea*, *A. oenea* and *A. tezpi*, respectively; Supporting Information, Table S5). The degree of genetic divergence between the other three pair of species: *A. extranea* and *A. oenea* (4.60%); *A. extranea* and *A. tezpi* (4.84%); and *A. oenea* and *A. tezpi* (5.99%) were much lower (Supporting Information, Table S5).

A significant negative correlation ($r = -0.678$, $P < 0.05$; Fig. 4C) between observed sympatry and sexual (visual) isolation was detected in the *Argia* studied species. However, we did not detect a significant correlation between genetic divergence and degree of sexual (visual) isolation ($r = -0.116$, $P = 0.72$; Fig. 4D). Mechanical isolation was not included in these correlational analyses because of this barrier was almost complete in nine out of the 12 species combinations.

DISCUSSION

In territorial damselflies and with courtship, heterospecific interactions are mainly precluded by visual sexual isolation, while in non-territorial damselflies and without courtship, heterospecific interactions are mostly precluded by mechanical or tactile sexual isolation (see Wellenreuther & Sánchez-Guillén, 2016). For example, in territorial calopterygids, male wing melanisation is used during intra- and interspecific recognition (Waage, 1979; Wellenreuther *et al.*, 2010). To date, in the studied non-territorial damselflies with learned mate preferences, such as *Ischnura* and *Enallagma* genera, intra- and interspecific recognition was mainly through mechanical (physical contact of primary and secondary genitalia) or sexual (tactile) barriers

Table 3. Observed and potential distribution ranges: sympatric and allopatric degrees

Species	Current (observed) distribution		Potential distribution	
	Absolute-sympatric (locations)	Relative-sympatry	Absolute-sympatry (km^2)	Relative-sympatry
A. anceps–all species	42	0.70	264 101	0.77
A. extranea–all species	53	0.54	328 864	0.86
A. oenea–all species	65	0.71	334 050	0.71
A. tezpi–all species	40	0.63	207 808	0.91
A. anceps–A. extranea	28	0.47	245 272	0.72
A. anceps–A. oenea	29	0.48	240 768	0.70
A. anceps–A. tezpi	19	0.32	170 253	0.50
A. extranea–A. anceps	28	0.28	245 272	0.64
A. extranea–A. oenea	34	0.34	299 225	0.78
A. extranea–A. tezpi	21	0.21	159 719	0.42
A. oenea–A. anceps	29	0.32	240 768	0.51
A. oenea–A. extranea	34	0.37	299 225	0.63
A. oenea–A. tezpi	35	0.38	200 736	0.42
A. tezpi–A. anceps	19	0.30	170 253	0.74
A. tezpi–A. extranea	21	0.33	159 719	0.70
A. tezpi–A. oenea	35	0.55	200 736	0.88

Table shows current distribution in terms of the absolute and relative number of sympatric locations. Potential distribution areas (km^2) were estimated by using ecological niche models; absolute values represent the extent in km^2 of sympatry areas, and the relative values represent the proportion of the area of sympatry. Data are given for each species respect to the other three species (*A. anceps–all species*, *A. extranea–all species*, *A. oenea–all species* and *A. tezpi–all species*) and for each pair of species combination.

(Robertson & Paterson, 1982; Fincke *et al.*, 1997; Sánchez-Guillén, Van Gossum & Cordero-Rivera, 2005; McPeek, Shen & Farid, 2009; Sánchez-Guillén *et al.*, 2012, 2014b). In our study, reproductive isolation was symmetrical in all species except *A. extranea* (which was the most derivative of the studied species). *Argia extranea* males showed complete visual isolation with the other three studied species, while *A. extranea* females were the most preferred females in heterospecific combinations, although they were completely isolated by the action of the mechanical barriers. Reproductive isolation was complete by the joint action of the three studied barriers (visual, mechanical and tactile). Sexual (visual) isolation was the most important barrier in all species, precluding the 90.6% of the matings. Although mechanical isolation was almost complete, only precluded the remaining 8.7% of the matings. Finally, isolation was very strong via female tactile recognition, precluding the last 0.7% of the matings, due to that in none of the three heterospecific *tandems*, females accepted the copula. However, since we recorded male responses only, future research should also focus on female responses.

A salient result of our study showed that *A. extranea* showed highly asymmetry in reciprocal crosses with the other three studied species. Males of *A. extranea* were completely isolated by sexual (visual) isolation, while *A. extranea* females were the most preferred females in heterospecific combinations, but were completely isolated by mechanical incompatibilities. Interestingly, in relation to the four studied species, *A. extranea* is the less abundant species in the community; the most derivate species (Fig. 4A, B); and shows the lowest degree of pairwise genetic divergence. Although *A. tezpi* males were partially attracted to the three heterospecific females, *tandems* were in all cases mechanically precluded, and thus *A. tezpi* males are also reproductively isolated to the other three species. Moreover, although *A. oenea* and *A. anceps* males are involved in heterospecific *tandems* with *A. anceps* and *A. tezpi* females in the case of *A. oenea*, and with *A. oenea* females in the case of *A. anceps*, they are also reproductively isolated by sexual (tactile) isolation, which was complete on the basis that none of the three females that formed a *tandem* (with a heterospecific male) bent her abdomen to form the *wheel position*, i.e. none of them accepted the copulation.

REPRODUCTIVE ISOLATION, GENETIC DIVERGENCE AND PHYLOGENETIC RELATIONS

Males of the four *Argia* species were discriminative and preferred to mate with their conspecific females, although only one species (*A. extranea*) was completely isolated to the other three *Argia* species by sexual (visual) isolation. Because of reproductive barriers act in a sequential way, the contribution of each barrier (to total reproductive isolation) depends on the contribution of previous barriers. For instance, if sexual isolation precludes 85% of matings between males of *A. tezpi* and females of *A. anceps*, although the absolute intensity of the mechanical isolation is complete (100%), it can only prevent the remaining 15% (relative contribution), being thus, less important precluding isolation than sexual isolation.

Our observations are in contrast with previous studies in other coenagrionid non-territorial damselflies such as *Ischnura* or *Enallagma* (Robertson & Paterson, 1982; Fincke *et al.*, 1997; Sánchez-Guillén *et al.*, 2005, 2012, 2014b; McPeek *et al.*, 2009), but are in line to what has been observed in the territorial damselfly genus *Calopteryx* (Waage, 1979; Wellenreuther *et al.*, 2010). Evidence from these previous studies suggested that in territorial taxa with courtship, sexual (visual) isolation is the most important reproductive barrier, while in non-territorial taxa, without courtship, mechanical isolation is the most important reproductive barrier. However, based on the positive correlation detected between genetic divergence and degree of the reproductive isolation in damselflies (Sánchez-Guillén *et al.*, 2014a), our results are not contradictory with previous observations, but also complementary. In our study, all four *Argia* species were more genetically divergent between each other (from 4.84% to 7.76%) than the previously studied *Ischnura* damselflies, whose genetic divergence was lower than 1% in all pairwise combinations (Monetti, Sánchez-Guillén & Rivera, 2002; Sánchez-Guillén *et al.*, 2012, 2014b). Thus, assuming the 'speciation clock' phenomenon, and based on evidences from previous studies with *Ischnura*, *Enallagma* and *Calopteryx* damselflies and our results with *Argia* species, our results suggest that mechanical barriers appeared earlier than the sexual (visual) barriers in the speciation process in odonates, but since mechanical barriers can only prevent the gene flow allowed by sexual isolation, their importance is lower in more divergent species, such as the case of the studied *Argia* species.

The relative strength of the isolating barriers depends on the direction of gene exchange (Arnold & Hodges, 1995; Tiffin, Olson & Moyle, 2001; Takami *et al.*, 2007), and in this context, asymmetries in premating (sexual and mechanical) barriers have been discussed intensively (e.g. Kaneshiro, 1980). The Kaneshiro (1980) hypothesis proposed that when one species is derived from another via a founder event, females from the ancestral species are more likely to reject males from the derived species which would
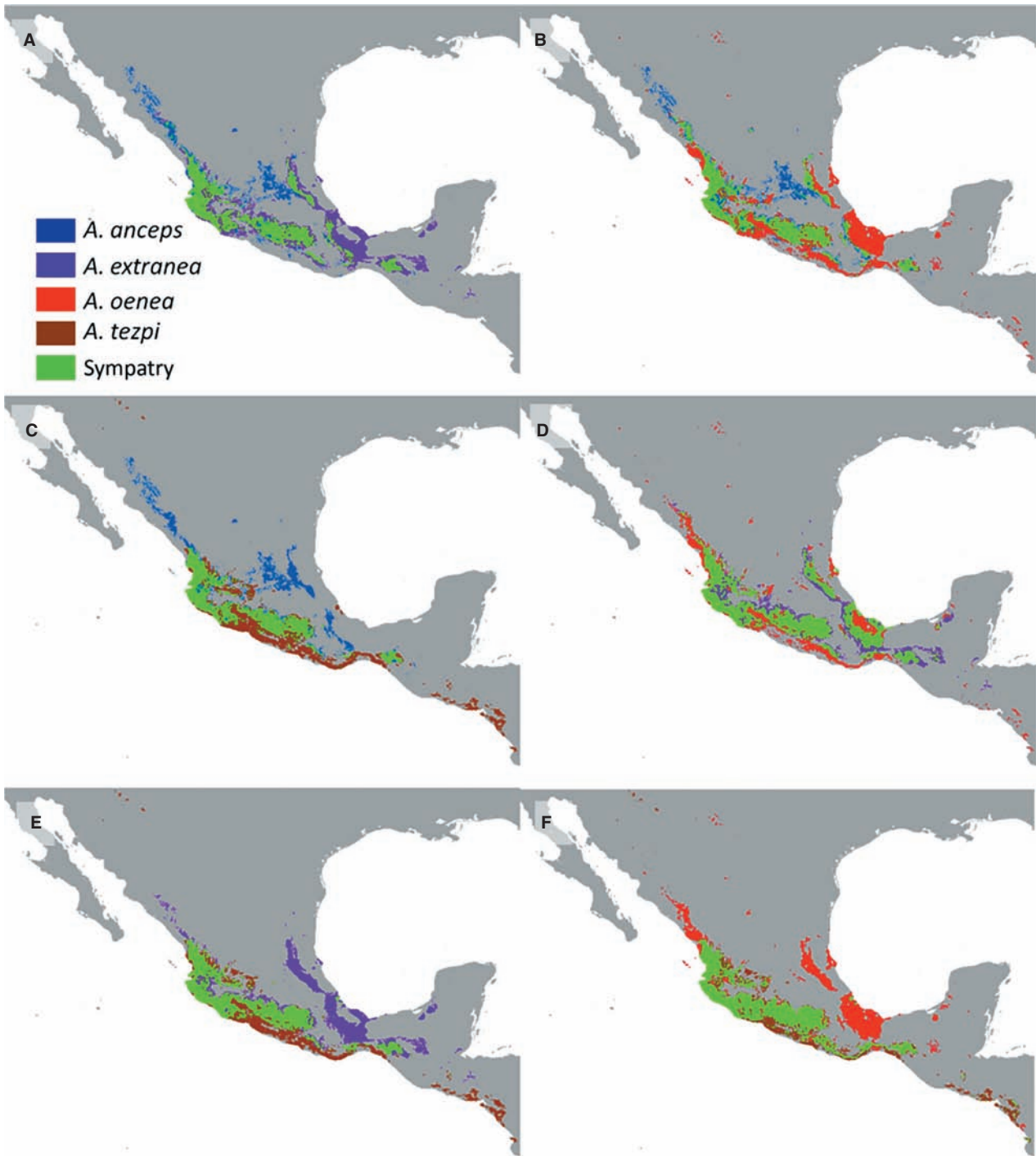
Figure 3. Degree of potential sympatry between the six pairs of species combinations. Overlapping areas between the four *Argia* studied species were predicted by using potential distributional models: green colour represents areas in which suitable conditions converge between each pair of species: (A) *A. anceps* and *A. extranea*; (B) *A. anceps* and *A. oenea*; (C) *A. anceps* and *A. tezpi*; (D) *A. extranea* and *A. oenea*; (E) *A. extranea* and *A. tezpi*; and (F) *A. oenea* and *A. tezpi*.

have changed more [especially due to the loss of behavioural (mating) traits] than males from the ancestral species. Several examples of the asymmetry in reproductive isolation come from the genus *Drosophila* (Watanabe & Kawanishi, 1979; Kaneshiro, 1988) as well as from salamanders
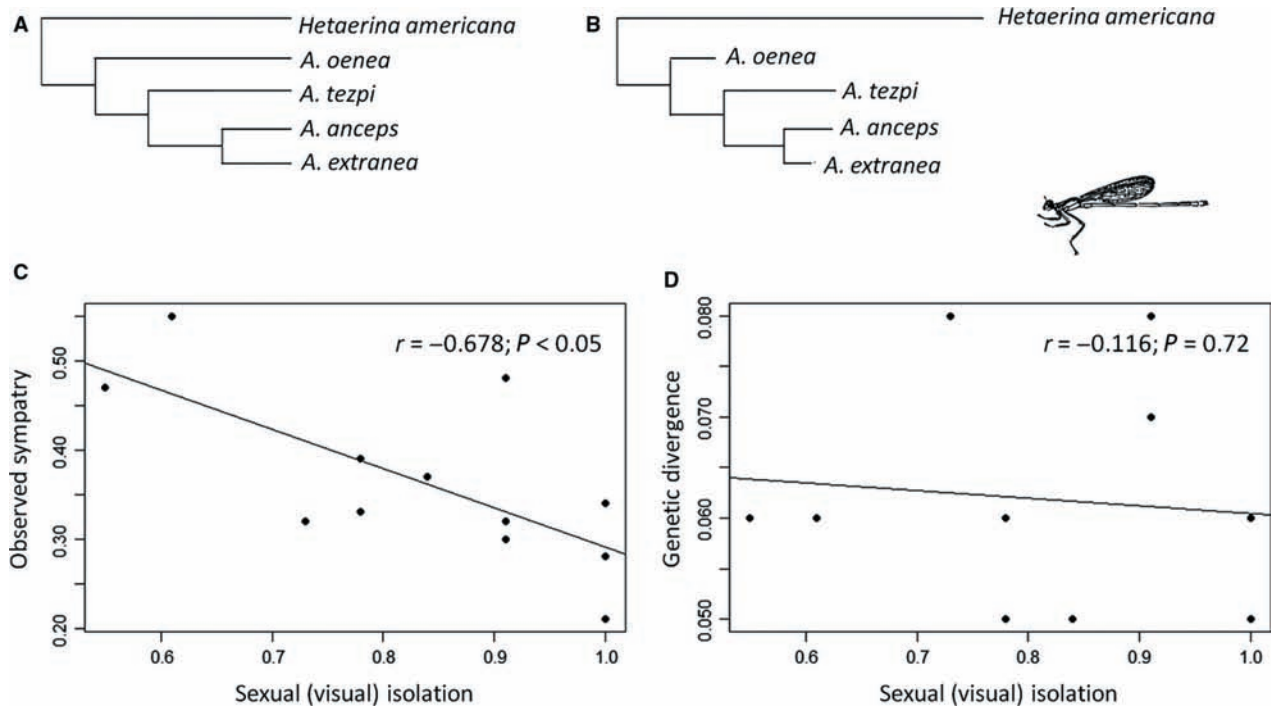
Figure 4. Evolutionary trees and correlation between genetic divergence and degree of sympatry with the strength of sexual (visual) isolation. A , strict consensus parsimonious tree from combined rDNA 16S. B , best maximum likelihood tree from the rDNA 16S. Both trees have been re-drawn from C aesar & Wenzel (2009), and indicate a simplified relative relationships. C , correlation between the degree of observed sympatry and the strength of sexual (visual) isolation ($r = -0.678$, $P < 0.05$). D , correlation between genetic divergence with the degree of sexual (visual) isolation ($r = -0.116$, $P < 0.72$).

(Arnold & Hodges, 1995), parasitic wasps (Bordenstein, Drapeau & Werren, 2000), snakes (Shine *et al.*, 2002) and more recently from *Ischnura* damselflies (Sánchez-Guillén *et al.*, 2012). Both, mechanical (tactile) and sexual (visual) isolation are part of the mechanisms of species recognition in odonates. According to the Kaneshiro (1980) hypothesis, sexual asymmetrical isolation involves preferential mating with females of the most derivative species by males of the ancestral species. The reciprocal asymmetrical isolation observed in *A. extranea* with the other *Argia* studied species, is a candidate species to test this Kaneshiro hypothesis.

REPRODUCTIVE ISOLATION AND DEGREE OF SYMPATRY AND THE ROLE OF LEARNING MATING PREFERENCES

Our findings are in line with the role of learning mating preferences in interspecific sexual isolation, which is consistent with a positive frequency dependent selection. Males learn to prefer the conspecific females, and this learning depends on the frequency of the heterospecific females in the community, i.e. the higher the density of the heterospecific female is in the community, the higher the degree of the

sexual (visual) isolation is in between these pair of species. Our study has also showed evidences of the role of learning in heterospecific sexual recognition in non-territorial and without courtship coenagrionid damselflies. Previous studies had supported its role in territorial odonates (see Waage, 1979; Wellenreuther *et al.*, 2010). Thus, sexual (visual) isolation can be driven by learning mate preferences in a non-territorial and without courtship genus (*Argia*), suggesting that visual recognition and learning ability may be widespread in odonates, including non-territorial species.

Our results showed that all four *Argia* species have a high degree of sympatry (their ecological characteristics have been preserved), sharing more than 70% of their potential distribution. We hypothesize that the observed finding in sexual (visual) isolation in the studied locality may be a general pattern in other sympatric localities of these species based on the high degree of sympatry between the four studied species (around 70%). It is possible that learning has allowed these four congeners to share the same habitats. Take for example the case of *A. oenea*. Although this species shares the fewest environmental characteristics, it still shows a high

degree of niche conservatism. One explanation for such patterns may lie on the speciation process in this genus. It is known that when speciation processes are driven by sexual selection, there is low niche differentiation. This may be the case for the four *Argia* species studied. Radiation *via* sexual selection is not related to resource exploitation, and thus reproductive isolation is not linked to the build-up of ecological niche diversification (Gittenberger, 1991; Rundell & Price, 2009). Other explanations for such radiation are those mediated by chromosomal architecture, gene duplication or ploidy levels, or through processes arising from mating male-female interactions, such as sexual conflict and learning (Gittenberger, 1991; Rundell & Price, 2009). The low interest in radiations *via* sexual selection have limited the number of studies that have dealt with the concept so that only a few examples exist (Mendelson & Shaw, 2005; Kozak & Wiens, 2006; Pereira & Wake, 2009; Wilke *et al.*, 2010).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Chi-squared tests of reproductive isolation (sexual and mechanical) between *Argia* species.

Table S2. Maximum likelihood provided by cumulative link mixed models.

Table S3. Cumulative link mixed models used to analyse the reproductive success measured in the different components of fitness.

Table S4. Potential distribution of *Argia* species.

Table S5. Estimates of evolutionary divergence between *Argia* species.