**Variable genomic patterns of hybridization in two independent hybrid zones of damselflies**

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**ABSTRACT**

Hybrid zones with multiple independent contact regions between the same species allow to determine the relative importance of intrinsic and extrinsic factors in the evolution of hybrid zones and thus, parallelism in hybridization outcomes. In this study, we take advantage of two hybrid regions between the damselfly species *Ischnura elegans* and *I. graellsii* in Spain to measure: i) the extent of parallelism across geographic hybridization replicates, and what factors (intrinsic and extrinsic) drive that variation; and ii) if hybridization has an impact on the ability of species to expand their ranges. RAD sequencing was used to generate 5,702 SNPs to quantify population diversity and population differentiation, and a subset of 381 species-specific SNPs to analyze genotypic composition (individual ancestry and the proportion of individuals in different hybrid classes Our individual ancestry results showed on-going hybridization and introgression with different admixture-class distributions between hybrid regions and between populations explained by i) species proportions, ii) time elapsed since colonization, and iii) asymmetric and reinforced prezygotic barriers and Batson Dobzhansky and Müller (BDM) hybrid incompatibilities, and indicated a role of hybridization as a facilitator of species range expansions. Our study highlights the value of studying complex hybrid zones to gain insights into microevolutionary processes.

**INTRODUCTION**

In recent years, many insect species have experienced pronounced changes in their distribution, including several documented cases of range expansions due to anthropogenic pressures and ongoing climate change (González-Tokman et al., 2020; Sánchez-Guillén et al., 2016). Such expansions can cause an increase in sympatry between species, thereby facilitating novel interactions and hybridization among expanding species (Arce-Valdés & Sánchez-Guillén, 2022; Sánchez-Guillén et al., 2016). A common signature of range expansion is decreasing genetic diversity at the range limit, due to frequent founder effects and genetic drift (Slatkin & Excoffier, 2012). However, such loss of diversity can be counteracted by hybridization and introgression in situations where the expanding species comes in contact with a formerly allopatric sister taxa (Rieseberg et al., 2007). This process is of increasing interest because it can help to elucidate the role of hybridization as a facilitator of species range expansions (Pfennig et al., 2016).

Hybridization outcomes are manifold and include transfer of genetic material between species (introgression) potentially facilitating their adaptive evolution, fusion of species, genetic swamping of one species by another, eliciting reinforcement of reproductive isolation between incompletely isolated species, and the origin of new species (Seehausen, 2004). Understanding evolutionary outcomes of hybridization is of increasing interest for gaining general insights into species dynamics and conservation in response to environmental change (Abbott et al., 2013). Evidence is accumulating that both the extent and pattern of hybridization have important conservation implications due to their direct impact on biodiversity as a whole, and because this process can sometimes lead to the replacement of one of the hybridizing taxa (Abbott et al., 2013; Seehausen, 2004; Todesco et al., 2016). The likelihood of these outcomes ishighly dependent on intrinsic factors, such as the extent of reproductive isolation, as well as extrinsic factors, because hybridfitness may be dependent on the environment (Coyne & Orr, 2004). Complex hybrid zones in which species have multiple contact zones, such as mosaic or mottled hybrid zones, allow to test the consistency of hybridization outcomes (Abbott et al., 2013; Harrison & Larson, 2016). Thus, when the range of two closely related species is large and overlapping, and if this overlap results in hybridization, then hybridization at several spatial scales can provide insights into parallel patterns of hybridization in different demographic and ecological contexts (Abbott et al., 2013). Several empirical studies, mainly from plants (Buerkle & Rieseberg, 2001; Haselhorst & Buerkle, 2013; Sweigart et al., 2007) and fishes (Aboim et al., 2010; Mandeville et al., 2017; Nolte et al., 2009), but also from mammals (Good et al., 2008), amphibians (Vines et al., 2003) and insects (Gompert et al., 2014), have shown that the outcomes of hybridization varies among hybrid zones of the same species pairs while only few studies have shown consistency in the pattern, e.g., in plants (Buerkle & Rieseberg, 2001) and insects (Larson et al., 2014).

Odonata are a group that is heavily affected by increasing temperatures and many species are changing their distributions (Hassall et al., 2007; Hassall & Thompson, 2008; Hickling et al., 2005; Lancaster et al., 2016; Ott, 2010; Sánchez-Guillén et al., 2023). This is the case of the damselfly *Ischnura elegans* that has expanded its distribution towards northern (Sweden) and southern (Spain) latitudes (Dudaniec et al., 2018; Sánchez-Guillén et al., 2011). In Spain, *I. elegans* overlaps its distribution with *I. graellsii* which is a closely related species that share many morphological, genetic, and phenotypic traits, including preference traits for habitats (Sánchez-Guillén et al., 2011). The sympatric distribution of these species is patchy, with populations of one species, the other species, or both species at different proportions (Sánchez-Guillén et al., 2011).

These species have formed two hybrid zones in Spain that differ in the timing of secondary contact by around one hundred years. The north-center and Mediterranean hybrid zone *I. elegans* expands from north-east to inland and along the Mediterranean coast. The north-west hybrid zone was detected only along the coast (Ocharan-Larrondo, 1987). The north-central and Mediterranean hybrid zone is an expanding and larger one that is in contact with the allopatric distribution of both species, while the north-west hybrid zone is smaller and isolated from the north-central and Mediterranean zone and the allopatric distribution of *I. elegans* (Sánchez-Guillén et al., 2011).In both hybrid zones *I. elegans* has modified its environmental niche making it more similar to the *I. graellsii* niche (Wellenreuther et al., 2018). The adaptation of *I. elegans* to the Spanish thermal regime, which was investigated near its expansion front towards the south of Spain, has been facilitated by plasticity and associated epigenetic mechanisms (Swaegers et al., 2022a, 2023). Hybridization and introgression is high in these hybrid zones; 55-60% of the morphological *I. elegans* individuals were genetically introgressed or backcrossed with pure *I. elegans*, and 10-30% were F1 or F2 hybrids (Sánchez-Guillén et al., 2011; Wellenreuther et al., 2018), although this high prevalence of admixed individuals could have been overestimated by the use of a small number of microsatellites (cf. Miralles et al., 2023). Recently, a study using genome-wide SNPs detected restricted introgression on the sex chromosome compared to autosomes in these two species (Swaegers et al., 2022b), but the bidirectionality of hybridization and introgression in these hybrid zones has not yet been investigated in a detailed spatial and temporal context.

In the present study we take advantage of these two hybrid zones in Spain, which provide a rare opportunity to compare the dynamics of two independent and differently aged hybrid zones, and to test the role of hybridization in facilitating range expansions. We used Restriction site–Associated DNA (RAD) sequencing and a recent *I. elegans* reference genome assembly (Chauhan et al., 2021) to identify genome-wide SNPs in individuals from two regions, one from each hybrid zone, as well as from eight allopatric populations from both species in Spain and adjacent countries. First, we used species-specific SNPs to analyze genotypic compositions and introgression. We investigated whether intrinsic factors such as reproductive barriers, and extrinsic factors such as species proportions, colonization/recolonization events and time elapsed since secondary contact shape genotypic composition and introgression, and we discuss these results in light of parallelism at regional and local scales, or the lack thereof. Second, we used the full set of SNPs to compare the degree of genetic diversity and genetic differentiation in both hybrid zones and adjacent allopatric zones to evaluate the potential role of hybridization in facilitating range expansions.

We have made the following predictions for a younger-central hybrid region and an older-west hybrid region from the north-central and Mediterranean hybrid zone and the north-west hybrid zone, respectively. Our first prediction is that genotypic composition and patterns of introgression vary at both the regional and local scales, but this variation will be larger at local than at regional scale due fragmented habitat use throughout the sympatric distribution. Our second prediction is that the proportion of F1-F2 hybrids and backcrosses, and the rate of introgression, will be highest in mixed populations of both species, specifically when species proportions are biased toward *I. elegans*, because hybridization occurs only in the cross direction formed by *I. elegans* males and *I. graellsii* females (Arce-Valdés et al., 2023; Sánchez-Guillén et al., 2012). Our third prediction is that the proportion of F1-F2 hybrids and backcrosses, and rates of introgression will be highest in the older and geographically isolated west-hybrid region due to: i) the asymmetric reinforcement and the purge of Bateson, Dobzhansky and Müller incompatibilities detected in this hybrid region (Arce-Valdés et al., 2023); and ii) to its geographic isolation, i.e., its lack of gene flow with both, sympatric and allopatric *I. elegans* populations. Thus, we predict to detect a decrease of interspecific genetic differentiation between *I. elegans* and *I. graellsii* from sympatry because of the homogenizing effect of the interspecific gene flow, and this reduction will be more pronounced in the older-west hybrid region than in the younger-central hybrid region (Mayr, 1963; Wang et al., 2020). Our fourth prediction is that hybridization has facilitated the range expansion of *I. elegans* in Spain, and therefore that we will not detect a loss of intraspecific genetic diversity. Associated with this, we predict that we will also not detect an increase in intraspecific genetic differentiation in the sympatric *I. elegans,* due to genetic drift during the range expansion (Slatkin & Excoffier, 2012), since hybridization and introgression have counteracted them (Rieseberg et al., 2007).

**2. MATERIAL AND METHODS**

**2.1 The two hybrid zones**

*Ischnura elegans* extends from Greece to Sweden while *I. graellsii* is an Iberian-Maghreb endemic species with a more restricted range in western North Africa and the Iberian Peninsula (Askew, 1989). Both species overlap in their distribution in Spain, forming two geographically separated hybrid zones.

The north-central and Mediterranean hybrid zone is close to the allopatric distribution of *I. elegans* and has expanded towards central and south Spain (Fig. 1). Nowadays, this hybrid zone extendsto 30 provinces that have been colonized over the last 120 years: in 8 provinces *I. elegans* is present since at least 1900, in 10 since 1980, and in 12 since 2000; Fig 1A). We focused our study on one dispersal front of *I. elegans* from the north-central and Mediterranean zone, including three provinces in which *I. elegans* has been present since 2008, and which we call the younger-central hybrid region.

## The north-west hybrid zone is geographically isolated from both, the north-central and Mediterranean hybrid zone and the allopatric distribution of *I. elegans*. This zone is small and includes only two provinces in which *I. elegans* is present and with a similar distribution since 1980. We focused our study to one province of the north-west hybrid zone of *I. elegans* that we named older-west hybrid region, because *I. elegans* is present in this region since 1980.

## 2.2 Population samplings for genomic analyses

From the younger-central hybrid region we sampled seven populations (Arreo, Cañas, Mateo, Perdiguero, Valbornedo, Valpierre, and Villar) and from the older-west hybrid region we sampled four populations (Doniños, Laxe, Louro and Xuño). We included populations in which: 1) *I. elegans* was the dominant species; 2) *I. graellsii* was the dominant species, and 3) both species were present at different proportions (details in Table S1). Additionally, we sampled five populations from the allopatric distribution of *I. elegans* and three populations from the allopatric distribution of *I. graellsii* (Fig. 1B; Table S1). These allopatric populations were selected to cover a large part of their latitudinal distribution, with the aim of including a large and representative amount of the total genetic variation.

Sampling for genomic analyses were done between the 2011-2015. Sampled individuals could morphologically only be assigned to either *I. elegans* or to *I. graellsii* but not assigned a hybrid status as this requires molecular data. Morphological assignment was based on caudal appendages (only males), thorax color (only young females) and prothoracic tubercle (males and females; Monetti et al., 2002). Although species assignment is less reliable in females than males, females were also included in the genomic analysis to prevent underestimation of admixture proportions due to the Haldane’s rule (Swaegers et al., 2022b), and we have significant experience with the morphology of these species. For populations that were assigned *a priori* morphologically to *I. elegans* and *I. graellsii*, we included samples of both species and aimed to keep the species proportion observed in the field at each site (Tables S1-S2). Additionally, we included one population from north-east Spain (Menorca, in the Balearic Islands) to test the replacement of *I. graellsii* by *I. elegans* in the Balearic Islands (results are given in supplementary Figure S7). Samples were stored in 100% ethanol until DNA extraction.

During the years 2007-2018 (for the younger-central hybrid region) and 2000-2018 (for the older-west hybrid region) we conducted a revision of the presence of both species (species proportions) using males. Additionally, we compilated published data of presence/absence of these species in previous years (1987-2003; Table S3). Populations were visited between June and July during sunny days, and sampling was done with entomological nets.

## 2.3 Genome-wide SNP markers

## 2.3.1 DNA extraction and RAD library preparation

Genomic DNA from head and thorax tissues of 187 individuals from the 20 populations (6-10 individuals per site; Table S1) was extracted with Qiagen DNeasy Blood & Tissue Kit. Extracted DNA was quantified using Nanodrop and visually controlled for DNA degradation using a 1% agarose gel. Five single-digest RAD DNA libraries were processed according to the protocol implemented by Etter et al. (2011) and modified by Dudaniec et al. (2018). All samples, plus five sample replicates, were distributed across the five separately prepared RAD libraries with 40 unique barcodes used per library (sourced from Metabion). Each library was paired-end sequenced (2\*100 bp) on a separate lane of an Illumina HiSeq 2500 at SNP&SEQ Technology Platform at Uppsala University, yielding 180 million read pairs per lane (i.e. per library).

**2.3.2. Quality checking and SNP calling**

Libraries were processed using pipelines within STACKS v2.2 (Catchen et al., 2013; Rochette et al., 2019). Raw reads were demultiplexed with process\_radtags and PCR clones were identified and discarded with clone\_filter using default parameters. Sequence reads were aligned to the *I. elegans* draft genome assembly (Chauhan et al., 2021) using BOWTIE2 v.2.3 (mismatch allowance per seed alignment of 1, maximum mismatch penalty of 6 and minimum of 2, maximum fragment length of 1000 bp and minimum of 100 bp; Langmead & Salzberg, 2012). We used the ref\_map pipeline to detect SNPs using default parameters. Only SNPs with a minor allele frequency of >0.05 and a maximum observed heterozygosity of 0.7 were retained. Moreover, the locus had to occur in 80% of the individuals in a population and in 18 of the 20 populations to be included in the final SNP set. SNP markers were filtered to include only a single random SNP on each RAD tag to create a data set without closely linked loci (using the **--***write\_single\_snp* option in STACKS). Finally, by using the *I. elegans* reference genome (Chauhan et al., 2021) SNPs were filtered to include only those located on autosomal scaffolds. Exploratory analyses of population structure revealed possible hybridization in two of the *I. graellsii* samples from Seyhouse (Algeria); probably with a third *Ischnura* species (Figure S4). These two samples were removed from further analyses leaving the final total sample size of 185 (Table S1).

**2.3.3. Identification of species-specific loci**

Diagnostic species-specific loci are powerful markers for assigning later generation hybrids and detecting introgressed alleles in population genetic studies (Hohenlohe et al., 2011). To provide a list of species-specific markers, alternatively fixed SNPs between the parental species from the allopatric distribution (n=43 for *I. elegans* and n=25 for *I. graellsii*) were identified using VCFtools v0.1.16 (Danecek et al., 2011). SNPs for each of the two allopatric zones that had only one allele (*--max-maf* 0) were selected, and then, shared loci between the two allopatric zones were found using the *intersect( )* function of R (R Core Team, 2020). Following that, we applied to those loci the Hardy-Weinberg test implemented in VCFtools (*--hardy*), and removed loci fixed for the same allele in the two species (HE=0). The remaining 381 SNPs (out of the 5,702 SNPs) were considered as the species-specific markers set.

**2.4 Genotypic composition and introgression**

**2.4.1 Assignment to hybrid classes: Hybrid Index (HI) and Heterozygosity (HET)**

We used the R package INTROGRESS v1.2.3 (Gompert & Buerkle, 2010) to calculate individual introgression coefficients; hybrid index (HI-values) and individual heterozygosity (HET-values), and used both of them to classify individuals into different hybrid classes (Jordan et al., 2018). INTROGRESS was used with the subset of 381 species-specific SNPs, as the assignment to hybrid classes can be inexact when using nondiagnostic markers (Buerkle, 2005). INTROGRESS when using species-specific allele SNPs, calculates the hybrid index as the proportion of alleles inherited from one species, and the heterozygosity, as the proportion of alleles that are heterozygous, ranging from 0 (pure species) to 1 (F1 hybrids) because of pure species are 100% homozygous, while F1 hybrids are 100% heterozygous (Gompert & Buerkle, 2010). Thus, the HI-value gives the proportion of alleles inherited from one species, in this case *I. elegans* (e.g., 1.00=100% *I. elegans*, and 0.00 =100% *I. graellsii*,alleles), whereas HET-values, which range from 0.00 to 1.00 (0.00=all sites are homozygous, 1.00=all sites are heterozygous), indicate the timing of the hybridization event. First generation hybrids (F1 individuals) are expected to be heterozygous at all species-specific SNPs, while later-generation hybrids and backcrosses will have lower heterozygosity levels, and the HI-values of F1 and F2 individuals will be close to 0.5, while backcrosses will have a HI-value below (or up to) 0.5 (Fitzpatrick, 2012). However, due to model uncertainty (Mandeville et al., 2017) we were conservative and considered pure *I. elegans* or pure *I. graellsii* individuals with HI<5%, and introgressed *I. elegans* or introgressed *I. graellsii* individuals with HI=5-10%. Consistently, criteria for F1 and F2 hybrid classes were relaxed. Thus, we classified individuals into eight parental and hybrid classes (cf. Milne & Abbott, 2008; Walsh et al., 2015): (i) pure *I. elegans* (HI=0.95-1.000; HET≤0.08), (ii) pure *I. graellsii* (HI=0.000-0.05; HET≤0.08), (iii) introgressed-*elegans* (HI=0.900-0.950; HET≤0.16), (iv) introgressed-*graellsii* (HI=0.05-0.100; HET≤0.16), (v) backcross-*elegans* (HI=0.601-0.899; HET=0.118-0.449), (vi) backcross-*graellsii* (HI=0.101-0.399; HET=0.118-0.449), (vii) relaxed F1 hybrids (HI=0.400-0.600; HET≥0.700), and (viii) relaxed F2 hybrids (HI=0.400-0.600; HET=0.450-0.69).

To investigate whether the hybrid regions have similar proportions of hybrid classes, the observed hybrid-class distributions of individuals from central and west hybrid regions were used to estimate the predicted distribution of individuals in admixture-classes using a contingency table (assuming random distribution) and then compared to the observed admixture-class distribution in both hybrid zones. Z-tests with Yates’s corrections for small sample sizes were used to test for differences in the proportions of each hybrid class category.

The analyzed populations from both hybrid regions were assigned to three qualitative measures that reflects their genotypic compositions. This classification depends on the frequency distribution of the different hybrid classes: 1) introgressed hybridization pattern, when distribution spam from introgressed to pure individuals; 2) unimodal hybridization pattern, when the distribution spans a range of admixture and backcrosses towards one or toward both parental species; 3) bimodal hybridization pattern, when the distribution is deviated to the two parental genotypes, and few hybrids (F1 and F2 hybrids) are present (Jiggins & Mallet, 2000).

**2.5. Structure of the hybrid regions**

**2.5.1. Structure and principal component analyses**

ADMIXTURE v1.3.0 (Alexander & Lange, 2011) was run using the set of 5,702 SNPs and all samples to evaluate the genetic structure among populations. ADMIXTURE was run using the “unsupervised model” (without using sampling locality as a prior). With this model setting, ADMIXTURE evaluates *Q-*values to ancestral genetic clusters (K) in different runs, ranging from one (K=1) to the number of sampled populations + 1 (in our case K=21), and gives an optimal K as the one achieving the lowest cross-validation (cv) error. Furthermore, ADMIXTURE was also run using the subset of 381 SNPs (“unsupervised model”) and all populations to compare the genetic structure among populations suggested by the set of species-specific SNPs to the structure suggested by the complete set of SNPs. ADMIXTURE was run using K values (1 to 15), the reason for this was the reduction in the number of populations due to the use of all allopatric samples as a single fixed population for both species. We also tested the “supervised model” using allopatric localities as reference samples for both full 5,702 SNPs and 381 diagnostic SNPs datasets.

To visualize patterns of genetic differentiation among individuals and populations, we used principal component analyses (PCA) in the R package SNPRelate v1.6.4, function *snpgdsPCA( )* (Zheng et al., 2012), using the set of 5,702 SNPs and all samples. This was used to determine whether individuals from the allopatric zones and the two hybrid zones correspond to different genetic clusters.

**2.6 Hybridization as a facilitator of species range expansions**

**2.6.1 Genetic diversity and Hardy Weinberg equilibrium**

To test whether potential hybridization and introgression resulted in increased genetic diversity in the Spanish hybrid zones, we compared different genetic diversity estimates for *I. elegans* and *I. graellsii* from the allopatric and the sympatric distribution in each locality, for all individuals before excluding the hybrids (F1 and F2 hybrids) and after excluding hybrids. We calculated number of alleles (A), allelic richness (Ar), observed heterozygosity (HO), expected heterozygosity (HE), and nucleotide diversity (π) using the 5,702 SNPs set. Genetic diversity was measured at both population and region levels. Na, and Ar were estimated using the HIERFSTAT package v0.04-22 (Goudet, 2005) as implemented in R. Allelic richness was rarefacted for a minimum of eight alleles (or four diploid samples), which was the lowest sample size used for Hardy-Weinberg equilibrium testing. Meanwhile Ho and HE were calculated using PLINK v1.90b6.12 (Purcell et al., 2007), andπ and the percentage of SNPs with missing data (NA) with VCFtools. Kruskal-Wallis tests and posthoc paired Wilcoxon tests were used to compare the levels of each diversity estimate between regions (younger-central, older-west hybrid regions, and allopatry).

Populational and regional tests for Hardy-Weinberg disequilibrium per locus were estimated using the 5,702 SNPs using PLINKs mid-p modifier (Graffelman & Moreno, 2013). Then we estimated the ratios of SNPs at HW disequilibrium (*p* < 0.05) to the total number of genotyped SNPs.

**2.6.2 Genetic differentiation between species**

Overall and pairwise differentiation was assessed using the complete set of 5,702 SNPs by calculating FST (Weir & Cockerham, 1984) using 10,000 bootstraps with the R package StAMPPv1.6.1 (Pembleton et al., 2013). To test whether hybridization and introgression resulted in reduced differentiation between species in sympatry, we first compared overall interspecific genetic differentiation between *I. elegans* and *I. graellsii* from allopatry with the overall genetic differentiation between *I. elegans* and *I. graellsii* from the younger-central and the older-west hybrid regions (including all individuals minus the F1 and F2 hybrids). Second, pairwise intraspecific genetic differentiation among populations was calculated for *I. elegans* and *I. graellsii*, for all individuals before excluding the hybrids, and for all individuals minus the F1 and F2 hybrids.

**3. RESULTS**

**3.1 Characterization of the studied hybrid regions**

Both studied hybrid regions showed patchy distributions, with a mixture of populations containing only one of the species, or both species at different proportions (Fig. 1 and Table S2). In the younger-central hybrid region species proportions were unstable in time and highly differentiated among populations (Fig. 1C; Tables S2-S3), while in the older-west hybrid region species proportions and distribution range were comparably more stable in time but also highly differentiated among populations (Fig. 1D; Tables S2- S3).

**3.3 Complete set of SNPs and species-specific SNPs**

A total of 5,702 SNPs were detected after stringent filtering and of those 2,127 (37.3%) were polymorphic in *I. elegans* but invariable in *I. graellsii*, and 1,711 (30.0%) were polymorphic in *I. graellsii* but invariable in *I. elegans*. Of the remaining, 1,421 (24.9%) were polymorphic in both species, 62 (1.1%) fixed for the same allele in allopatric *I. elegans* and *I. graellsii,* and 381 (6.7%) species-specific, i.e., alternatively fixed between *I. elegans* and *I. graellsii* individuals from the allopatric populations (Table S5). Note that our set of species-specific alleles, which was determined by comparing 43 allopatric *I. elegans* and 25 allopatric *I. graellsii* individuals, might not represent entirely fixed alleles, but represent alleles with highly skewed frequencies between our species groups (Fitzpatrick, 2012; Jordan et al., 2018).

**3.4 Individual introgression coefficients, heterozygosity, and assignment to hybrid classes**

Introgression analyses using the set of species-specific SNPs were used to classify individuals from both hybrid regions into seven hybrid classes (pure *I. elegans*, pure *I. graellsii*, introgressed *I. elegans*, introgressed *I. graellsii*, backcross to *I. elegans*, backcross to *I. graellsii*, and F1 and F2 hybrids; Table S6). Samples that were *a priori* morphologically identified as *I. elegans* were classified in two classes: pure *I. elegans* and introgressed *I. elegans*, while samples *a priori* morphologically identified as *I. graellsii* were classified into five classes: pure *I. graellsii*, introgressed *I. graellsii*, back to *I. graellsii*, F1 hybrids and back to *I. elegans* (Table S6).

In both hybrid regions we detected on-going hybridization and introgression. In the younger-central hybrid region, pure *I. elegans* and pure *I. graellsii* represented the larger proportion of individuals (89.7%, n= 68). Admixed individuals included F1 hybrids, *I. graellsii* backcrosses, and introgressed *I. graellsii*, a pattern consistent with unidirectional introgression toward *I. graellsii* (Table S6; Fig. 2A)*.* In contrast, in the older-west hybrid region pure *I. elegans* and pure *I. graellsii*, although represented a larger proportion of individuals (65%, n=40) this was lower than in the younger-central hybrid region. Moreover, the proportion of admixed individuals included F1 hybrids, *I. graellsii* backcrosses, *I. elegans* backcrosses, introgressed *I. graellsii* and introgressed *I. elegans*, a pattern consistent with a bidirectional introgression (Table S6; Fig. 2B). We detected significant differences in the frequencies of hybrid classes between the older-west hybrid region and the younger-central hybrid region (Χ2(6)=15.824, p=0.0147). Z-tests per hybrid class identified statistically significant differences in the frequencies of introgressed *I. elegans* between both regions (Z=4.536, p=0.0331).

When hybrid class proportions were analyzed by population, we found introgression in five populations (Perdiguero, Valpierre, Louro, Laxe and Xuño) and ongoing hybridization (F1 and F2 hybrids and backcrosses) in five populations (Cañas, Perdiguero, Villar, Arreo and Louro; Fig. 3). Two hybridization patterns were detected in the populations showing on-going hybridization: 1) a bimodal pattern in five populations (Arreo, Cañas, Perdiguero, Valbornedo, and Villar); and 2) a flat unimodal pattern consistent with a hybrid swarm, in one population (Louro; Fig. 3).

**3.5 Population genetic analysis of the studied hybrid regions**

**3.5.1 Structure and principal component analyses**

Genetic structure analyses were done using the whole set of 5,702 SNP with ADMIXTURE, without and with supervision (i.e., using the allopatric samples as ancestry references; Supplementary Figure S7). Analyses without supervision indicated that the most likely number of ancestral populations (i.e., the K with the lowest cv error) was K=2 (Supplementary Figure S8), but it should be noted that the cv errors for K=2 and K=3 were similar. For K=2, the two genetic clusters corresponded very well to *I. graellsii* and *I. elegans*, respectively, and for K=3 the three genetic clusters corresponded to (i) *I. graellsii* , (ii) *I. elegans* from allopatry and the younger-central hybrid region, and (iii) *I. elegans* from the older-west hybrid region. For both K=2 and K=3, many samples with admixed ancestry were present (Supplementary Figure S7).

PCA allowed us to cluster *I. elegans*, *I. graellsii* and hybrids from allopatry and from both hybrid regions. The first axis of the PCA explained 38% of the total variation and clearly separated *I. elegans* and *I.* *graellsii* individuals from allopatric populations, while the second axis explained 2% of the total variation and separated *I. elegans* from younger-central, older-west hybrid regions and allopatry (Supplementary Figure S9). Consistent with the ADMIXTURE results, many individuals from both hybrid regions appeared in the same PCA quadrant as those occupied by the pure species from the allopatric distribution, while hybrids occupied intermediate positions of the first axis.

**3.5.2 Genetic diversity and Hardy Weinberg equilibrium**

Hybridization and introgression resulted in an increase or the maintenance of genetic diversity (π, A, Ar, Ho and HE) in the younger-central and the older-west hybrid regions, which was estimated with the whole set of 5,702 SNPs (Figure 4 and Supplementary Tables S10-S12).

When the genetic diversity parameters were compared between *I. elegans* from allopatry and *I. elegans* from sympatry in both hybrid regions (excluding F1 and F2 hybrids), significant differences in four estimates (Ar, Ho, HE and π) were detected for the older-west hybrid region (all estimates were higher in the west hybrid region; Fig. 4 and Tables S10-S12). We also found significantly higher Ar, Ho, HE and π in the older-west hybrid region compared to the younger-central hybrid region (Fig. 4 and Tables S10-S12). When comparing *I. elegans* from allopatry and *I. elegans* from sympatry in each of the hybrid regions not excluding F1 and F2 hybrids, all estimates of genetic diversity (Na, Ar, Ho, HE and π) were significantly higher for the west hybrid region than for *I. elegans* from allopatry; and only Ho was significantly higher than for *I. elegans* from the central hybrid region (Fig. 4 and Tables S10-S12).

When the genetic diversity parameters were compared between *I. graellsii* from allopatry and *I. graellsii* from sympatry in both hybrid regions, we only detected significant differences in Ar between allopatric samples and *I. graellsii* from the younger-central hybrid region when including hybrids (Fig. 4 and Tables S10-12).

No significant differences between regions were detected in the ratio of SNPs at HW disequilibrium per population (Tables S11-S12).

**3.5.3 Genetic differentiation between and within species in allopatry and sympatry**

Consistent with the finding that hybridization, admixture, and introgression results in reduced interspecific differentiation in sympatry, we found that the overall genetic differentiation between *I. elegans* and *I. graellsii* (excluding F1 and F2 hybrids) was lower in the sympatric (FST=0.683) than in the allopatric distribution (FST=0.725). When the overall genetic differentiation was compared between the studied hybrid regions, we found that the overall genetic differentiation between *I. elegans* and *I. graellsii* (excluding F1 and F2 hybrids) was lower in the older-west (FST=0.625) than in the younger-central hybrid region (FST=0.731).

Pairwise intraspecific population differentiation in *I. elegans* from allopatry ranged from FST=0.002-0.044 (8 out of the 10 pairwise FST values were significant at *p*<0.05/10), from the older-west hybrid region (excluding F1 and F2 hybrids) ranged from FST=0.012-0.100 (3 out of the 3 pairwise FST values were significant at *p*<0.05/3), from the younger-central hybrid region (excluding F1 and F2 hybrids) ranged from FST=0.002-0.013 (3 out of 10 pairwise FST values were significant at *p*<0.05/10; Table S13). Pairwise intraspecific population differentiation in *I. graellsii* from allopatry ranged from FST=0.008-0.068 (2 out of the 3 pairwise FST values were significant at *p*<0.05/3), from the older-west hybrid region FST=0.079 (1 out of the 1 pairwise FST values was significant at *p*<0.05), and from the younger-central hybrid region ranged from FST=0-0.036 (5 out of the 15 pairwise FST values were significant at *p*<0.05/15; Table S14).

Some populations were highly genetically distinct from many populations. Specifically, the *I. elegans* Doniños population from the older-west hybrid region showed comparatively large and statistically significant genetic differences with several other populations (Table S13). Similarly, the *I. elegans* Leuven population and the *I. graellsii* Seyhouse population from the allopatric distributions showed comparatively large and statistically significant genetic differences with several other populations (Tables S13-S14).

**DISCUSSION**

In this study, we characterized two mottled hybrid zones of *I. elegans* and *I. graellsii* in Spain. One hybrid zone was large and is still undergoing expansion and is situated in the north-central and Mediterranean part of Spain, and another small zone that was generally more stable and situated at the north-west of Spain. Thus, we focused our study on two evolutionary independent and differently aged hybrid regions. We provided evidence for on-going hybridization and introgression, and, as we predicted, we detected variable genomic patterns of hybridization between hybrid regions and populations, which were in part explained by 1) species proportions, 2) recolonization events, 3) time elapsed since colonization, and 4) the strength of reproductive barriers. We also provided evidence for a role of hybridization as a facilitator of species range expansions.

***Characterization of the Spanish sympatric*** ***distribution: two mottled and independent hybrid zones***

The hybrid zones were of different aged, in the north-central and Mediterranean hybrid zone *I. elegans* was detected from the early 1900s while in the north-west hybrid zone was detected from 1980s. Both hybrid zones showed patchy sympatric distributions, with a mix of populations containing only one species, and populations of both species at different proportions (Sánchez-Guillén et al., 2005, 2011). *Ischnura elegans* has adapted to the Spanish thermal regime through (evolved) plasticity and associated epigenetic mechanisms (Swaegers et al., 2022a, 2023). In fact, the occupied niche by *I. elegans* in Spain is like that of *I. graellsii* (Wellenreuther et al., 2018). *Ischnura elegans* is a species that disperses readily to new locations, and this dispersion is influenced by its reproductive ecology (e.g., females disperse longer distance than males) although much more by landscape topology and connectivity (Conrad et al., 2002). The north-west hybrid zone is geographically isolated from the north-central and Mediterranean hybrid zone and from the allopatric distribution of *I. elegans*, therefore, the arrival of *I. elegans* to this hybrid zone could have been passive due to wind, a fairly common type of dispersal in this genus. For instance, *I. hastata* has been able to colonize the Galapagos Archipelago and the Caribbean islands (Cordero-Rivera et al., 2023). Meanwhile, arrival and dispersion of *I. elegans* in the north-central and Mediterranean zone was probably facilitated by the increasing presence, during the 20th century, of suitable habitat such as small ponds to satisfy agricultural demands (Duarte et al., 2014; Jlassi et al., 2016). In both hybrid zones, the patchy sympatric distribution is probably a consequence of the colonization routes that *I. elegans* has been taken along rivers and its affluents.

Genetic structure analyses of the two studied hybrid regions confirmed the independent evolution of the hybrid zones since ADMIXTURE analysis identified three genetic clusters, one corresponding to *I. graellsii*, one corresponding to *I. elegans* from allopatry and the younger-central hybrid region, and one corresponding to *I. elegans* from the older-west hybrid region. Consistent with this finding, our PCA analysis separated *I. elegans* from the younger-central hybrid region and allopatry to those from the older-west hybrid region. The independent evolution or origin of hybrid regions was also detected in two Iberian endemic cyprinid fish (Aboim et al., 2010).

***Factors shaping genotypic composition and introgression***

In line with our predictions we also detected variable genomic patterns of hybridization and introgression between both hybrid regions. On-going hybridization was greater in the older-west hybrid region than in the younger-central hybrid region. The patterns of introgression also varied greatly between hybrid regions: introgression was unidirectional toward *I. graellsii* in the younger-central hybrid region whileit was bidirectional (toward both species) in the older-west hybrid region. Differential outcomes of hybridization may partly be explained by the strength and direction of reproductive barriers (Lepais et al., 2009; Mandeville et al., 2017; Vines et al., 2003) and therefore by the time since sympatry (Kronforst et al., 2007; Lemmon & Juenger, 2017; Liao et al., 2019). A bimodal distribution of the genotypic classes (mainly parental genotypes and few hybrids) is thought to be the consequence of strong but incomplete reproductive isolation (Harrison & Bogdanowicz, 1997), while weak reproductive isolation results in a unimodal distribution on the genotypic classes (the distribution spans a range of admixture and backcrosses; Szymura & Barton, 1991). When reproductive barriers are weak, and hybrids have some kind of advantage, the unimodal distribution consist largely of a ‘hybrid swarm’. On the other hand, when reproductive barriers are asymmetrical between reciprocal crosses, the distribution of the genotypic classes spans a range of admixture and backcrosses to only one of the parental species (Gompert et al., 2017; Jiggins & Mallet, 2000).

In the younger-central hybrid region, the distribution of the genotypic classes was consistent with a bimodal pattern, while in the older-west hybrid region, the distribution of the genotypic classes was consistent with a flat unimodal pattern “hybrid swarm”. In both hybrid regions, three out of the five studied populations with only one species (*I. elegans* or *I. graellsii*) showed different levels of introgression or backcrosses, indicating that although only one species was detected in the sampling year, both species were probably present in previous years. The difference in the distribution of the genotypic classes and introgression between both hybrid regions can be explained by the asymmetric reinforcement of the prezygotic barriers and the weakening of the postzygotic barriers detected in the older-west hybrid region (Arce-Valdés et al., 2023). In detail, in the older-west hybrid region, the asymmetrical reinforcement of the prezygotic isolation between *I. graellsii* males and *I. elegans* females prevents 98% of the hybridization in this cross direction, while the interspecific gene flow has purged BDM incompatibilities weakening postzygotic barriers in the opposite cross direction, i.e., between *I. elegans* males and *I. graellsii* females (Arce-Valdés et al., 2023). Although reinforcement of reproductive isolation has not yet been tested in the younger-central hybrid region, the strength of the prezygotic barriers was similar in strength and direction to that detected in the older-west hybrid region (unpublished data), and consistently with the reinforcement of the RI between *I. graellsii* males and *I. elegans* females, in both hybrid regions it has recently been detected a reproductive character displacement of the traits responsible of the mechanical isolation in *I. graellsii* males and *I. elegans* females (Ballén-Guapacha, in preparation). The above evidence supports the presence of reinforcement also in the younger-central hybrid region, although its intensity would be lower than in the older-west hybrid region, due to its proximity to the allopatric distribution of *I. elegans* which facilitates the introduction of non-admixed/introgressed individuals, and due to the most recent sympatry of *I. graellsii*, and thus less time/opportunity for purging BDM incompatibilities. In *Drosophila*, for instance, reinforcement can act rapidly, and within only five generations RI can reach 100% (Koopman, 1950; Matute, 2010).

In both hybrid regions local populations showed pronounced variation in species composition and in the degree of hybridization and introgression, which suggests that the local dynamics is even more distinct than the regional dynamics in this system (Fig. 2). The proximity to the introduction or source locality could affect genotypic composition and introgression (Fitzpatrick et al., 2010; Lepais et al., 2009), as the rate and directionality of introgression can be influenced by the species’ relative abundance and therefore by gene flow from the native to the non-native species. A non-native colonising species is usually rare and matings with the native species are likely, as was detected in other studied organisms (Fitzpatrick et al., 2010; Lepais et al., 2009; Quilodrán et al., 2019). Introgressed genes can reach high frequency in the non-native species by a rapid demographic growth, resulting in asymmetric introgression of neutral genes (Currat et al., 2008). Consistent with this finding, we detected greater admixture in populations with both species, specifically when species proportions were biased toward *I. elegans*. This may be because of hybridization occurring only in the cross direction formed by *I. elegans* males and *I. graellsii* females (Arce-Valdés et al., 2023; Sánchez-Guillén et al., 2011). Hybrid matings will be more probable when the rarer species contributes to hybridization with the female (in our hybrid system, when the rarer species is *I. graellsii*). This is because half of the mature males of the rare species, but only a very small minority of females, are never seen in copula (Rivera & Abad, 1999). This is especially true in populations experiencing recolonization events, since *I. elegans* can colonize an empty locality before or at the same time than *I. graellsii*, thus species proportion can bias to *I. elegans* facilitating hybrid matings between the common *I. elegans* males and the rarer *I. graellsii* females. In fact, local dynamics are commonly dominated by periodic dry-ups followed by recolonizations by one or both species because water is used for irrigation of neighbouring wine crops, or by management of invasive species (mainly American crab). Moreover, if a locality with a new environmental condition is recolonized, recolonization not only facilitates hybrid matings, but also, may favour transgressive hybrids adapted to the new environments (Stelkens et al., 2014). This could be the case of the north-western population, Louro, that became extinct due to water salination by seawater in 2009, and has since then become recolonized by both species, forming a hybrid swarm.

***Hybridization as a facilitator of species range expansions***

Consistently with our prediction, we found that hybridization has facilitated the range expansion of *I. elegans* in both hybrid regions in Spain. A common signature of range expansions is a reduced genetic diversity due to founder effects (Slatkin & Excoffier, 2012). However, hybridization and introgression with a locally adapted resident species during the range expansion process can be a counterforce that increases overall genetic diversity and/or specific alleles (Behm et al., 2010; Mehner et al., 2010; Pfennig et al., 2016; Rieseberg et al., 2007). Introgressive hybridization during the range expansion of *I. elegans* in Spain can be a source of new alleles to recently expanded populations in both hybrid zones. It appears that the range expansion has coincided with the hybridization of *I. elegans* in Spain, and this has not only prevented the loss of genetic diversity in both hybrid zones, but also increased diversity in the north-west hybrid zone. Dudaniec et al. (2018) investigated neutral and adaptive molecular signatures along the northward range expansion axis of *I. elegans* in where no other *Ischnura* spp. occur. Interestingly, also no decrease in genetic diversity along this axis was found; and observed heterozygosity was similar between core and range-expanded populations, even without hybridization opportunities. Another study investigating the genetic consequences of northern range expansion in damselflies did, however, report small genetic diversity losses in recently established populations (Swaegers et al., 2015). Due to introgression, *I. elegans* and *I. graellsii* populations showed slightly lower overall interspecific genetic differentiation in sympatry (FST=0.683) than in allopatry (FST=0.725), similar to patterns detected in other taxa (Anderson & Hubricht, 1938; Fu et al., 2020). Demographic processes associated with range expansions, such as repeated bottlenecks and genetic drift can also increase the level of differentiation between species (Freedman et al., 2010; Wang et al., 2014). This may have contributed to the high and significantly observed pairwise genetic differentiation between *I. elegans* and *I. graellsii* in the north-west hybrid zone respect to the north-central and the allopatric zones.

***Conclusions***

Studies examining the evolutionary outcomes of hybridization in complex hybrid regions such as mottled hybrid regions are extremely useful to test the consistency of hybridization outcomes and to disentangle the influence of intrinsic and extrinsic factors. We found that on-going hybridization and introgression were in part explained by extrinsic factors, such as species proportions, recolonization events and the time elapsed since colonization, and by intrinsic factors such as the strength and direction of reproductive isolation. We also detected that hybridization has facilitated the range expansion of *I. elegans* in both hybrid regions in Spain.

**ACKNOWLEDGEMENTS**

We are very grateful to Adolfo Cordero Rivera, who kindly allowed us to use his laboratory and material for the rearing experiments, and to Zalandrana Odonatology group who kindly help us with samplings and permissions in north-central Spain. We thank the following colleagues for kindly helping with collecting/sending samples: Adolfo Cordero Rivera, Iñaki Mezquita, Mario García-París, Bernat Garriós, Pere Luque, Xoaquín Baixeras, Francisco Cano, Jean Pierre Boudot, Jürgen Ott, Cedrick Vanappelghem, Philippe Lambret, and Phill Watts. We are grateful to Janet Nolasco Soto and Emmanuel Villafán de la Torre for technical support. Sequencing was performed by BGI (Hongkong) and the SNP&SEQ Technology Platform in Uppsala, which is part of the National Genomics Infrastructure (NGI) Sweden and Science for Life Laboratory, supported by the Swedish Research Council and the Knut and Alice Wallenberg Foundation. Bioinformatics analyses were performed on resources provided by the Swedish National Infrastructure for Computing (SNIC) at Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX) and the Huitzilin 2.0 HPC system at the Instituto de Ecología A.C (INECOL). The research was funded by the Karl-Tryggers Foundation (to RAS-G and MW), the Kungl. Vetenskapsakademien (BS2015-0001 to RAS-G), the Royal Physiological Society in Lund (the Nilsson-Ehle Foundation, 36118 to RAS-G, and 37369 to MW), the Swedish Research Council (621-2016-689 to BH) the Marie Curie Intraeuropean Fellowship (to JS, RAS-G, MW and BH), and from Mexican CONACYT (282922 to RAS-G).

**AUTHORS’ CONTRIBUTIONS**

RAS-G, MW and BH conceived the study idea. RAS-G, LRA-V and JS performed analyses. RAS-G, MW and BH wrote the first draft which was then edited by all co-authors.

**DATA ACCESSIBILITY**

Raw sequencing data files were uploaded to the NCBI Sequence Read Archive (XXXXX). Filtered VCF input file as well as all the scripts for the full pipeline analysis were deposited on OSF at:

<https://osf.io/5kg87/?view_only=438667bce73d41ecab7137a65c625ded>

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**Figure Legends**

**Figure 1.** Sympatric distribution of *I. elegans* and *I. graellsii*. **A)** Geographic distribution of *I. elegans* in Spain over time, from 1888-1979 (8 provinces, dark-grey), 1980-1999 (13 provinces, medium grey) and 2000-actuality (12 provinces, light grey). White color indicates only *I. graellsii* populations (15 provinces). **B)** Map of the Spanish hybrid zones: north-central and Mediterranean hybrid zone in blue, and north-west hybrid zone in orange, indicating sampled populations. Blue circles denote allopatric *I. elegans*, yellow circles denote allopatric *I. graellsii*. Inside of the Spanish hybrid zone, blue, yellow, and orange triangles denote *I. elegans* dominant, *I. graellsii* dominant or both species respectively. Orange range indicates the north-west hybrid zone and blue range indicates the north-central and Mediterranean hybrid zone. **C)** Zoom of the studied younger-central hybrid region from the north-central and Mediterranean hybrid zone. Blue, yellow, and orange marks denote *I. elegans* dominant, *I. graellsii* dominant or both species present populations. Triangles denote populations sampled for genomic analyses and circles field observations. **D)** Zoom of the studied older-west hybrid region from the north-west hybrid zone. Blue, yellow, and orange marks denote *I. elegans* dominant, *I. graellsii* dominant or both species present populations. Triangles denote populations sampled for genomic analyses and circles field observations.

**Figure 2.** INTROGRESS estimates of ancestry proportion (HI) and heterospecific ancestry (HET) by using the 381 fixed SNPs for *I. elegans*, *I. graellsii* and hybrids from **A**) younger-central hybrid region and **B**) older-west hybrid region. Brown boxes delimit ranges for hybrid categories. F1 and F2 hybrids (orange) are found in the apex of the triangle. Backcrosses to *I. elegans* or *I. graellsii* (orange) are found at intermediary levels of interspecific heterozygosity. Introgressed *I. elegans* or *I. graellsii* (brown) are found between introgressed and pure samples. Pure *I. elegans* (blue) and pure *I. graellsii* (yellow) are found opposite ends of the hybrid indexes values.

**Figure 3**. INTROGRESS individual **(***I. elegans* in blue, *I. graellsii* in yellow) and class proportionsestimates of ancestry proportion in **A**) central hybrid populations, and **B)** west hybrid populations. Populations were assigned to three qualitative measures depending on the frequency distribution of the different hybrid classes: 1) introgressed hybridization pattern, when distribution spans from introgressed to pure individuals; 2) unimodal hybridization pattern, when the distribution spans a range of admixture and backcrosses towards one or toward both parental species; 3) bimodal hybridization pattern, when the distribution is deviated to the two parental genotypes, and few hybrids (F1 and F2 hybrids) are present (Jiggins & Mallet, 2000).

**Figure 4.** Genetic diversity violin plots by regions.Violins widths represents the relative frequency of SNPs at each observed genetic diversity value, i.e., wider violin areas show higher number of SNPs at that measurement. Nucleotide diversity (π) observed (Ho) and expected heterozygosity (He) were estimated using all 5,702 SNPs. Horizontal lines at each violin represents the median, and the 0.05 and 0.95 quantiles. Points show the average value. Blue = *I. elegans* without hybrids; yellow = *I. graellsii* without hybrids; orange = *I. elegans* or *I. graellsii* including F1 and F2 hybrids.