Variable genomic patterns of hybridization in an expanding hybrid zone of damselflies

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Abstract

The outcome of hybridization is of major interest in evolutionary and conservation biology. Here, we investigate (i) the genomic signal of the hybridization dynamics, (ii) the strength of reproductive barriers preventing copulation in heterospecific and hybrid crosses, and (iii) the population dynamics (stability of species proportions) of the two damselfly species Ischnura elegans and I. graellsii in two differently aged Spanish hybrid regions. RAD sequencing in these hybrid regions and in allopatric populations was used to generate 5,702 SNPs to quantify population diversity and population differentiation, and a subset of 381 species-specific SNPs to analyze individual ancestry and the proportion of individuals in different hybrid classes. Our individual ancestry results showed the presence of F1 and F2 hybrids, in line with on-going hybridization and bidirectional backcrossing in both hybrid regions, with almost complete absence of genetically pure I. elegans and I. graellsii. Different admixture-class distributions were in part explained by 1) different mean strength of reproductive barriers in the hybrid regions, with stronger barrier in the older hybrid region, 2) local dynamics (continuous recolonization events), 3) proximity to introduction site, and 4) time elapsed since colonization. Consistent with theoretical expectations, introgression maintained (in the younger hybrid region) or increased genetic diversity (in the older hybrid region), and reduced genetic differentiation between local populations in both hybrid regions. Whether this will facilitate the ongoing range expansion of I. elegans in Spain is an interesting avenue for future research.

INTRODUCTION

In recent years, many insect species have experienced pronounced changes in their distribution, including several documented cases of range expansions (González-Tokman et al., 2020; 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 a 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 as many regions are facing changes due to anthropogenic pressures and ongoing climate change, which can lead to novel interactions and hybridization among expanding species (Sánchez-Guillén et al., 2016). 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). Thus, 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).

Hybridization outcomes are manifold and include transfer of genetic material between species (potentially facilitating their adaptive evolution), fusion of species, genetic swamping of one species by another, elicit reinforcement of reproductive isolation between incompletely isolated species, and the origin of new species (Seehausen, 2004). The likelihood of these outcomes is highly dependent on intrinsic factors, such as the extent of reproductive isolation, as well as extrinsic factors, because hybrid fitness may be dependent on the environment (Coyne, 2004). The study of multiple contact regions of the same species pair allows to test the consistency of hybridization outcomes (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, Mason, & Willis, 2007) and fishes (Aboim, Mavárez, Bernatchez, & Coelho, 2010; Mandeville et al., 2017; Nolte, Gompert, & Buerkle, 2009), but also from mammals (Good, Handel, & Nachman, 2008), amphibians (Vines et al., 2003) and insects (Gompert et al., 2014), have shown that the outcomes of hybridization varies among hybrid regions 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, White, Ross, & Harrison, 2014). Detecting parallelism in the prevailing genomic signatures allows gaining insights into the process of hybridization itself, for example, if species have genomic regions that introgress more readily than others.

Odonata are a group that is heavily affected by increasing temperatures and many species are changing their distributions (Hassall & Thompson, 2008; Hassall, Thompson, & French, 2007; Hickling, Hill, & Thomas, 2005; Lancaster et al., 2016; Ott, 2010). Within odonates, the damselfly genus *Ischnura* is extremely species rich (around 70 species) (Dijkstra & Kalkman, 2012; Sánchez-Guillén et al., 2020) and includes many closely related and recently diverged species that co-occur in partial sympatry over parts of their range (Sánchez-Guillén, Muñoz, Rodríguez-Tapia, Arroyo, & Córdoba-Aguilar, 2013; Wellenreuther & Sánchez-Guillén, 2015). This genus has also other interesting properties such as high local abundances and wide-ranging distribution patterns, and it is highly enabled for field identification and sampling, making this a suitable group to explore adaptive introgression caused by environmental change. Indeed, there are several examples of extensive hybridization between species within the *Ischnura* genus, for example, between *I. denticollis* and the endangered *I. gemina* in the San Francisco Bay (Leong & Hafernik, 1992; Sánchez-Guillén et al., 2014), and in Europe between *I. graellsii*and *I. saharensis,* and between *I. genei* and *I. elegans* (Sánchez-Guillén, Córdoba-Aguilar, Cordero-Rivera, & Wellenreuther, 2014).

However, the best documented and explored case of hybridization in*Ischnura* is between *I. elegans* and *I. graellsii* in Spain (Monetti, Sánchez-Guillén, & Cordero Rivera, 2002; Sánchez-Guillén, van Gossum, & Cordero-Rivera, 2005; Sánchez-Guillén, Wellenreuther, & Cordero-Rivera, 2012; Wellenreuther et al., 2018).*Ischnura elegans* and *I. graellsii* are among the most frequently studied damselfly species within the order Odonata, and as such have become eco-evolutionary model species that have been studied extensively for hybridization and reproductive isolation (Monetti*et al.* , 2002; Sánchez-Guillén *et al.* , 2005, 2011, 2012, 2014b; Wellenreuther & Sánchez-Guillén, 2016; Wellenreuther *et al.* , 2018). *Ischnura elegans* and *I. graellsii* are closely related and share many morphological, genetic and phenotypic traits (Sánchez-Guillén et al., 2011), including preference traits for habitats, and they occupy similar ecological niche space in Spain (Wellenreuther et al., 2018). *Ischnura elegans* has dramatically expanded its distribution in Spain during last 40 years (Sánchez-Guillén et al., 2011), where its distributional range nowadays overlaps almost completely with *I. graellsii* (Fig. 1A-B), which is endemic to Spain and Maghreb in north-western Africa. The Mediterranean coast represents the oldest hybrid region with the firsts records of *I. elegans* in the late 19$^{\text{th}}$ century (Ocharan, 1987), with increasing records over the country from the early 1980s and onwards (Ocharan, 1987), and a fast expansion from 2000 to the present (Fig. 1B). A previous molecular study with microsatellites

estimated that in the Spanish hybrid populations 55-60% of the *I. elegans* individuals were introgressed or backcrossed, and that from 10-30% were F_1 or F_2 hybrids (Sánchez-Guillén et al., 2011). In the Spanish hybrid region, *I. elegans* has replaced *I. graellsii* from the coast and modified its original environmental niche to become more similar to the *I. graellsii* niche (Wellenreuther et al., 2018).

In the present study, we focus on two hybrid regions in Spain, one in north-west Spain (Galicia) and one in north-central Spain (La Rioja, Navarra and Avila). 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 the two hybrid regions as well as from eight allopatric populations from both species in Spain and adjacent countries. First, we used species-specific SNPs to analyze the fine-scale individual ancestry and proportion of individuals in different hybrid classes (ranging from pure individuals to F_1 and F_2 hybrids). With this analysis we were particularly interested in confirming the occurrence of hybrids and backcrosses, and in evaluating the local and regional scales of hybridization dynamics. We used data on the strength of reproductive barriers and colonization history in an aim to explain the present-day hybridization patterns. Second, we used the full set of SNPs to compare the degree genetic variation and genetic differentiation in the Spanish hybrid regions and adjacent allopatric regions. With these analyses we were interested in evaluating whether hybridization and introgression have increased the level of genetic variation of individuals in the different hybrid classes. We discuss our results in light of parallelism in hybridization and introgression within and between regions, or the lack thereof, and in relation to previous data on biased mating success among *I. elegans* and *I. graellsii* . This work is a step towards understanding the potential role of hybridization and introgression in facilitating range expansions.

2. MATERIAL AND METHODS

2.1. Populations sampling

2.1.1 Allopatric populations

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). We sampled individuals from five populations from the allopatric distribution of *I. elegans* and in three populations from the allopatric distribution of *I. graellsii* (Fig. 1A; Table 1). 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.

2.1.2 Sympatric populations

During fieldwork in the years 2014-2018 in north-central and north-west Spain, we conducted a revision of the distribution data of both species along the Iberian Peninsula from 2011 (reviewed in Boudot et al., 2009). From two hybrid regions, north-central Spain (Arreo, Cañas, Mateo, Perdiguero, Valbornedo, Valpierre, and Villar) and north-west Spain (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. Additionally, available information of presence/absence from previous years (1987-2003) was also included. Populations were visited between June and July during sunny days, and sampling was done with entomological nets. Immature and mature males were included when estimating the number of individuals in each species (species proportion). 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 S4).

For the genomic work, a minimum of 20 adult males were collected during the flight season between 2008-2015 using hand nets and stored in 100% ethanol until DNA extraction. Because phenotypic hybrid assignment is only possible through detailed morphological analyses of male caudal appendages and prothoracic tubercle (see Monetti et al., 2002), all individuals in this study (of which a majority were females) were*a priori* morphologically assigned to either *I. elegans* or*I. graellsii* based on their thorax colour and prothoracic

tubercle morphology. For populations with both species (*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 (Table 1). Previous data and our results allow us to discuss the present results in a time- and space-frame context.

2.2. Reproductive isolation

Mating incompatibility between *I. graellsii* males and *I. elegans* and hybrid females is known in populations from the north-west hybrid region (Sánchez-Guillén, Wellenreuther, & Cordero-Rivera, 2011), but not its potential temporal and spatial variation. Thus, the strength of the reproductive barriers involved in preventing copulation in north-central was used to test temporal and/or spatial variation. Similar data from north-west populations are available from an unpublished study (Arce-Valdés, Ballén-Guapacha, Chávez-Rios & Sánchez-Guillén, under review).

For reproductive isolation analysis, last instar larvae of approximately 200 *I. elegans* and 200 *I. graellsii* individuals, both from north-central Spain (Mateo, Valbornedo and Villar), were sampled in June-July of 2016-2017. Larvae were transported to the laboratory and maintained until adulthood (for details about larval rearing methodology see Sánchez-Guillén et al., 2005; Van Gossum, Sánchez-Guillén & Cordero-Rivera, 2003). Mechanical isolation to reach the copulation was estimated by measuring incompatibility between the male cerci and female prothorax (males attempt to grasp females) and the incompatibility between male-female genitalia (both genitalia come into contact), in heterospecific crosses of *I. elegans* with *I. graellsii*females, and *I. graellsii* males with *I. elegans*females. Additionally, hybrids in the laboratory (from crosses between*I. elegans* and *I. graellsii*) were used to produce backcrosses to measure mechanical isolation in both directions (hybrid males with *I. elegans* females and *I. elegans* females with hybrid females; and hybrid males with *I. graellsii* females and*I. graellsii* males with hybrid females).

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 1) 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 in Etter et al. (2011) and modified in 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, Hohenlohe, Bassham, Amores, & Cresko, 2013; Catchen, Amores, Hohenlohe, Cresko, & Postlethwait, 2011). 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 (see Supplementary Figure S1). These two samples were removed from further analyses leaving the final total sample size of 185 (Table 1).

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, Amish, Catchen, Allendorf, & Luikart, 2011). To provide a list of species-specific markers, alternatively fixed SNPs between the parental species from the allopatric distribution (n=43 *I. elegans* and n=25 *I. graellsii*) were identified using VCFtools v0.1.16 (Danecek et al., 2011). SNPs for each of the two allopatric regions that had only one allele (*–max-maf* 0) were selected, and then, shared loci between the two allopatric regions were found using the *intersect( )* function of R (R Core Team, 2016). 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 (H_E=0). The remaining 381 SNPs (out of the 5,702 SNPs) were considered as the species-specific markers set.

2.4 Hybridization analyses

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

We used the R package INTROGRESS v1.2.3 (Gompert & Alex 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 (c.f. 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 (F_1 hybrids) because of pure species are 100% homozygous, while F_1 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 (F_1 individuals) are expected to be heterozygous at all species-specific SNPs, while later-generation hybrids and backcrosses will have a lower heterozygosity levels, and the HI-values of F_1 and F_2 individuals will be close to 0.5, while backcrosses will have a HI-value below (or up to) 0.5 (Fitzpatrick, 2012). However, because of the lack of pure *I. elegans* and pure *I. graellsii* in the three hybrid regions [only 3 out of the 102 individuals were pure *I. elegans* (2) or pure *I. graellsii* (1)], criteria for F_1 and F_2 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=1.000; HET[?]0.000), (ii) pure *I. graellsii* (HI=0.000; HET[?]0.000), (iii) introgressed-*elegans* (HI=0.900-0.999; HET[?]0.118), (iv) introgressed-*graellsii* (HI=0.001-0.100; HET[?]0.118), (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 F_1 hybrids (HI=0.400-0.600; HET[?]0.600), and (viii) relaxed F_2 hybrids (HI=0.400-0.600; HET=0.450-0.599).

To investigate whether the hybrid regions have similar proportions of hybrid classes, the observed hybrid-class distributions of individuals from north-central and north-west (north-east was not included in the statistical analyses due to small sample sizes) 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 regions. Z-tests with Yates's corrections for small sample sizes were used to test for differences in the proportions of each hybrid class category between north-west and north-central hybrid regions.

The twelve analyzed populations from the Spanish hybrid zone were assigned to three qualitative measures that reflects their genotypic compositions. This classification depends on the frequency distribution of the different hybrid classes: 1) unimodal hybridization pattern, when the distribution spans a range of admixture and backcrosses towards both parental species; 2) bimodal hybridization pattern, when the distribution is deviated to the two parental or parental-like genotypes, and few hybrids (F_1 and F_2 hybrids) are present

(Jiggins & Mallet, 2000); and 3) introgressed hybridization pattern when the distribution was deviated to one parental-like genotype.

2.4 Population genetic analysis of the Spanish hybrid region

2.4.1. 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 localities due to the use of all allopatric samples as a single fixed population for both species.

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 regions and the two hybrid regions correspond to different genetic clusters.

2.4.2 Genetic diversity and Hardy Weinberg equilibrium

To test whether potential hybridization and introgression resulted in increased genetic diversity in the Spanish hybrid regions, 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 (F_1 and F_2 hybrids) and after excluding hybrids. We calculated number of alleles (A), allelic richness (Ar), observed heterozygosity (H_O), expected heterozygosity (H_E), and nucleotide diversity (π) using the 5,702 SNPs set. Genetic diversity was measured at both population and regional 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 H_E were calculated using PLINK v1.90b6.12 (Purcell et al., 2007), and π 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 (north-west and north-central hybrid regions, and allopatry).

Population 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, the ratios of SNPs at HW disequilibrium ($p < 0.05$) to the total number of genotyped SNPs, as well as average p -values per population were calculated.

2.4.3. Genetic differentiation between species

Overall and pairwise differentiation was assessed using the complete set of 5,702 SNPs by calculating F_{ST} (Weir and Cockerham 1984) using 10,000 bootstraps with the R package StAMPP v1.6.1 (Pembleton, Cogan, & Forster, 2013). To test whether hybridization and introgression resulted in reduced differentiation between species in sympatry, we first compared overall genetic differentiation between*I. elegans* and *I. graellsii* from allopatry with the overall genetic differentiation between *I. elegans* and *I. graellsii* from the Spanish hybrid region (including all individuals minus the F_1 and F_2 hybrids). Second, pairwise genetic differentiation among localities was calculated for*I. elegans* and *I. graellsii* , for all individuals before excluding the hybrids, and for all individuals minus the F_1 and F_2 hybrids.

3. RESULTS

3.1 Local dynamics

Ischnura elegans has increased its presence in central Spain over the last ten years. Currently, it is present in 32 provinces (in 8 provinces since at least 1900, in 21 since 1980, and in 32 since 2000; see Fig 1B), and although it is not yet a resident in 15 southern provinces, observations of *I. elegans* in the field are increasing also in these provinces.

The twelve populations from the Spanish hybrid region were sampled at least four times over a period of 4-10 years (north-central hybrid region) and a period of 4-5 years (north-west hybrid region). Data from this study, and available data from previous studies were compiled as documented in (Supplementary Table S2). In the north-central hybrid region, studied localities were frequently subjected to desiccation (and therefore recolonization) because of water is used for irrigation of neighbouring wine crops, or by management of invasive species (mainly American crab). Cañas, Perdiguero and Villar all periodically dry up, and a recolonization from nearby populations of both species is regularly happening. In fact, Cañas and Villar were emptied and recolonized a couple of years before we sampled them. During the studied 4 years, at least two recolonization events were recorded. In this region, species proportions were unstable, and the seven studied localities were sympatric (at different species proportions) at the end of the study in 2018 (see Table 2). However, although studied localities in the north-central hybrid region were also exposed to recolonization events, but in this case, by natural desiccation (evaporation) or by brought brackish water event (lagoon retro dune), species proportions were stable in the studied period (Table 2).

3.2 Reproductive isolation

In both hybrid regions, i.e., north-central and north-western Spain, we detected strong and asymmetric mechanical reproductive isolation. In north-central Spain, mechanical and tactile barriers between *I. elegans* males and *I. graellsii* females impeded 65% of the matings, while between *I. graellsii* males and *I. elegans* females 76% of matings were impeded. When those barriers were estimated in *I. elegans*backcrosses, i.e., between *I. elegans* males and hybrid females, barriers impeded 33% of the matings, while between hybrid males and*I. elegans* females, barriers impeded 75% of matings. More isolation was detected in *I. graellsii* backcrosses, between*I. graellsii* males with hybrid females 100% of the copulations were prevented, but we find no isolation between hybrid males and*I. graellsii* females (0%) (Figure 2A). The strength of mechanical and tactile barriers in heterospecific crosses and *I. elegans* and *I. graellsii* backcrosses in north-western Spain are given in Figure 2B [data from (Arce-Valdés, Ballén-Guapacha, Chávez-Rios, & Sánchez-Guillén, under review; Sánchez-Guillén et al., 2012)].

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 (Supplementary Table S3). 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 (see Fitzpatrick, 2012; Jordan et al., 2017).

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 in the Spanish hybrid regions into eight hybrid classes (pure *I. elegans* , pure *I. graellsii* , introgressed*I. elegans* , introgressed *I. graellsii* , backcross to*I. elegans* , backcross to *I. graellsii* , $F_1$and F_2 hybrids) (Table 3; Fig. 2C-D). Samples that were*a priori* morphologically identified as *I. elegans* were classified in three classes: pure *I. elegans* , introgressed*I. elegans* and *I. elegans* backcrosses. Introgressed*I. elegans* represented the larger proportion of individuals (59 out of 65) in both hybrid regions, while only four *I. elegans*backcrosses were found (all in the north-west hybrid region) and only two pure *I. elegans* (one in north-central and one in north-west; Table 3; Fig. 2C-D). Samples *a priori* morphologically identified as *I. graellsii* were classified into five classes: pure *I. graellsii* , introgressed *I. graellsii* , back to *I. graellsii* , F_1 and F_2 hybrids. Introgressed *I. graellsii* was

the category that included the larger proportion of individuals (37 out of 52) in both hybrid regions [note morphologically *I. graellsii* were not present in the Menorca from the north-east hybrid region], followed by nine F_1 and F_2 hybrids, five *I. graellsii* backcrosses in both hybrid regions and one pure *I. graellsii* in north-central Spain (Table 3; Fig. 2C-D).

To investigate whether the north-west and north-central hybrid regions had a similar hybridization pattern, hybrid class proportions were compared with Z tests using Yates's corrections for small sample sizes. We found that both regions showed similar hybrid class proportions for all hybrid classes except for the class *I. elegans* introgressed (that showed a non-significant trend; $?^2$=3.438, df=1,p =0.063) and for *I. elegans* backcrossed ($?^2$=6.181, df=1, p =0.013), where the north-west hybrid region had higher proportions. Additionally, when hybrid class proportions were analyzed by population, we found extensive introgression in most populations and ongoing hybridization (F_1 and F_2 hybrids) in four localities (Canas, Perdiguero, Villar and Louro) (Fig. 3A). Three hybridization patterns were detected in the twelve populations: 1) a bimodal pattern in five populations (Arreo, Canas, Perdiguero, Valbornedo, and Villar); 2) an introgressed pattern in five populations (Mateo, Valpierre, Doninos, Laxe and Xuno); and a unimodal pattern in one population (Louro) (Fig. 3B).

3.5 Population genetic analysis of the Spanish hybrid region

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 S4). 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 S5), 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 a third cluster was found among *I. elegans*populations from the north-west hybrid region. For both K=2 and K=3, many samples with admixed ancestry were present (Supplementary Figure S4).

PCA allowed us to cluster *I. elegans* , *I. graellsii* and hybrids from allopatry and from the three hybrid regions. The first axis of the PCA explained 39% of the total variation and clearly separated*I. elegans* and *I. graellsii* individuals from allopatric localities, while the second axis explained 2% of the total variation and separated some of the individuals from north-central and north-west hybrid regions (Supplementary Figure S6). Consistent with the ADMIXTURE results, many individuals from the three 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 of genetic diversity (π, A, Ar, Ho and H_E) in the hybrid regions, which was estimated with the whole set of 5,702 SNPs (Figure 4 and Supplementary Tables S7- S9). When the genetic diversity parameters were compared between *I. elegans* from allopatry and *I. elegans*from sympatry in each of the hybrid regions excluding $F_1$and F_2 hybrid individuals, significant differences in all five estimates (π, Na, Ar, Ho and H_E) were detected for the north-west hybrid region (all estimates were higher in the north-west hybrid region; Figure 4 and Supplementary Tables S7- S9). We also found significantly higher Ar, Ho and H_E in the north-west hybrid region compared to the north-central hybrid region (Figure 4 and Supplementary Tables S7-S9). Similarly, when comparing*I. elegans* from allopatry and *I. elegans* from sympatry in each of the hybrid regions not excluding F_1 and F_2 hybrids, all estimates of genetic diversity (π, A, Ar, Ho and H_E) were significantly higher for the north-west hybrid region than for *I. elegans* from sympatry and than for *I. elegans* from the north-central hybrid region (Figure 4 and Supplementary Tables S7- S9). No significant differences between regions were detected in the ratio of SNPs at HW disequilibrium per population ($p<0.05$; see Supplementary Tables S8-S9).

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* was lower in the sympatric (excluding F_1 and F_2 hybrids) (F_{ST}=0.691) than in the allopatric distribution (F_{ST}=0.725).

Pairwise intraspecific population differentiation in *I. elegans* from allopatry ranged from F_{ST}=0.002-0.245 (8 out of the 10 pairwise F_{ST} values were significant at the$p$ <0.05/10), from the north-west hybrid region (excluding F_1 and F_2 hybrids) ranged from F_{ST}=0.012-0.100 (3 out of the 3 pairwise F_{ST} values were significant at the$p$ <0.05/3), from the north-central hybrid region (excluding F_1 and F_2 hybrids) ranged from F_{ST}=0.004-0.013 (1 out of the 10 pairwise F_{ST} values was significant at the$p$ <0.05/10) (Supplementary Table S10). Pairwise intraspecific population differentiation in *I. graellsii* from allopatry ranged from F_{ST}=0.008-0.068 (2 out of the 3 pairwise F_{ST} values were significant at the$p$ <0.05/3), from the north-west hybrid region F_{ST}=0.042 (1 out of the 1 pairwise F_{ST}values was significant at the p <0.05), and from the north-central hybrid region ranged from F_{ST}=0-0.014 (0 out of the 15 pairwise F_{ST} values was significant at the$p$ <0.05/15) (Supplementary Table S11).

Some populations were highly genetically distinct from many populations. Specifically, the *I. elegans* Doniños population from the north-west hybrid region showed comparatively large and statistically significant genetic differences with several other populations (Supplementary Tables S10-S11). 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 (Supplementary Tables S10-S11).

DISCUSSION

Proximity to the introduction site and local dynamics

Our admixture analysis with the set of species-specific loci showed bidirectional introgressive hybridization between *I. elegans* and*I. graellsii* . Individuals from both hybrid regions, north-west and north-central Spain, mainly belonged to the introgressed *I. elegans* and introgressed *I. graellsii* classes, and less frequently to the backcross to *I. elegans* , backcross to *I. graellsii* , F_1 and F_2 hybrid classes. Almost no pure *I. elegans* or pure *I. graellsii* were found in the hybrid regions.

When comparing the distribution of parental and hybrid classes between north-west and north-central hybrid regions, all classes were at similar frequency proportions except for the introgressed *I. elegans* class, which was significantly higher in the north-west hybrid region. The proximity to the introduction or source locality could affect hybridization outcomes (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, Nussberger, Montoya-Burgos, & Currat, 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, Ruedi, Petit, & Excoffier, 2008). The north-central hybrid region is closer to the allopatric distribution of *I. elegans* and disconnected to the*I. elegans* populations from the north-west hybrid region. In fact, the number of populations dominated by *I. elegans* , and the abundance of *I. elegans* in populations with both species in the north-western hybrid region is lower than in the north-central hybrid region which is closer to the allopatric distribution of *I. elegans* (Boudot et al., 2009; Sanchez-Guillen, Van Gossum, & Cordero Rivera, 2005; Sánchez-Guillén et al., 2011)

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. 2A-B). In four out of the six study populations where both species co-occur at different numbers (Cañas, Louro, Perdiguero and Villar), nine individuals morphologically classified *I. graellsii* were genetically assigned to F_1 or F_2 hybrid classes. This is consistent with observations in the laboratory that F_1 hybrids are morphologically similar to *I. graellsii* (Sanchez-Guillen et al., 2005),

and independent of the reciprocal cross direction (*I. elegans* male mated with *I. graellsii* or hybrid female, or *I. graellsii* male mated with*I. elegans* or hybrid female) that has produced the F_1 or the F_2 hybrid (RAS-G personal observation). Local dynamics was dominated by periodic dry-ups followed by recolonizations by one or both species. For instance, Cañas and Villar (north-central populations) were emptied and recolonized a couple of years before we sampled them. Similarly, the north-western population Louro became extinct due to salobrisation by seawater in 2009, and was recolonized by both species, forming a hybrid population with high levels of admixture. In Arreo and Valbornedo we did not detect F_1 or F_2 hybrids, even though both species are present. Even the six studied populations with only one of the genotypical-parental species [Doniños, Laxe and Menorca (only*I. elegans*), and Mateo, Valpierre and Xuño (only *I. graellsii*)] showed different levels of introgression or back crosses, indicating that although only one species was detected in the sampling years both species were possibly present in the population in the years before. This can be the case of Xuño that had both species during 2003-2006 and was naturally dried in 2007 and after that recolonized by*I. graellsii* , which explains the observed levels of admixture.

Strength of the prezygotic reproductive barriers

Differential outcomes of hybridization may partly be explained by the strength of the prezygotic reproductive barriers (Lepais et al., 2009; Mandeville et al., 2017; Vines et al., 2003) which varies across species (Good et al., 2008; Sánchez-Guillén et al., 2014) and in hybridizing species, with the time since sympatry (Kronforst, Young, & Gilbert, 2007; Lemmon & Juenger, 2017; Liao et al., 2019). The frequency distribution of hybrid classes in a hybrid zone may reflect the strength and direction of reproductive barriers. A bimodal distribution of the genotypical-like classes (parental or introgressed individuals) are thought to be the consequence of strong but incomplete reproductive isolation (e.g., Harrison & Bogdanowicz, 1997), while lower levels of prezygotic isolation resulting in a unimodal distribution on the genotypic classes (the distribution spans a range of admixture and backcrosses toward both parents) (e.g., Szymura & Barton, 1991) (Gompert, Mandeville, & Buerkle, 2017; Jiggins & Mallet, 2000).

The high prevalence of admixed individuals in north-central Spain can be explained by its level of reproductive isolation as was, for instance, detected in Catostomus fish species (Mandeville et al., 2017) and intertidal snails (Littorinids) (Stankowski et al., 2020). In north-central Spain, 70% of the heterospecific matings between phenotypically *I. elegans* and *I. graellsii* were prevented, while the backcrosses to *I. elegans* and the backcrosses to*I. graellsii* were less and similarly prevented (54% and 50%, respectively). In fact, from the north-central hybrid region, five populations (Arreo, Cañas, Perdiguero, Valbornedo and Villar) show a bimodal distribution (introgressed individuals with *I. elegans*and *I. graellsii*) and Mateo and Valpierre presented an introgressed distribution (introgressed individuals with *I. graellsii*). However in the north-west hybrid region, heterospecific matings and backcrosses with *I. elegans* were similar and strongly prevented (80% and 79% respectively), while only 46% of the backcrosses with *I. graellsii* were prevented. Arce-Valdés*et al.* (under review) found evidence of an incipient strengthening of the prezygotic isolation in this hybrid region. From the north-west hybrid region, three populations (Doniños, Laxe, Xuño) presented an introgressed distribution towards *I. elegans*(Doniños, Laxe) and towards *I. graellsii* (Xuño), and one population (Louro) presented a unimodal pattern. Differences in the admixture-class distributions between the Spanish hybrid regions (*I. elegans*class was significantly higher in the north-west hybrid region) can be explained by the reinforcement of the strength of the reproductive isolation between *I. elegans* and *I. graellsii* in the north-west hybrid region.

Range expansion and time elapsed since the colonization

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, Ives, & Boughman, 2010; Mehner et al., 2010; Pfennig, Kelly, & Pierce, 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 the Spanish hybrid region. 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 ge-

netic diversity in both hybrid regions, but also increased diversity in the north-west hybrid region. Further, consistent with theoretical expectations, hybridization also resulted in reduced interspecific differentiation in sympatry, i.e., genetic differentiation between*I. elegans* and *I. graellsii* was lower in the sympatric than in the allopatric distribution. 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 genetic differentiation in sympatry (mean=0.691) than in allopatry (mean=0.725), similar to patterns detected in other taxa (e.g., Anderson & Hubricht, 1938; Fu, Lu, Fu, & Wang, 2020). Demographic processes associated with range expansions, such as repeated bottlenecks and genetic drift can also increase the level of differentiation between species (Freedman, Thomassen, Buermann, & Smith, 2010; Wang, Abbott, Ingvarsson, & Liu, 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 region respect to the north-central and the allopatric regions. In summary, the higher level of genetic divergence and genetic differentiation detected in the north-west hybrid region can be explained by local demographic processes (bottlenecks and genetic drift) and the time since the colonization.

Conclusions

Studies examining the evolutionary outcomes of hybridization along space and time are necessary to understand whether hybridization outcomes represent general patterns or are species and context specific. In this study, we provide evidence for on-going hybridization and bidirectional backcrosses, with different admixture-class distributions in different damselfly hybrid regions which may in part be explained by 1) proximity to introduction site, 2) local dynamics, 3) the strength of reproductive barriers, and 4) the time elapsed since colonization. We also provide evidence of increased genetic diversity and reduced genetic differentiation in a range expansion when opportunities for hybridization occur.

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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

All data files, as well as the scripts for the full pipeline analyses are deposited on OSF at:

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

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Table 1. Species, sampling locality and country, sample size for molecular analysis (N) indicates the number of morphologically identified samples (*I. elegans* -like (I. e) and*I. graellsii* -like (I. g), ecology, sampling year,

latitude/longitude.

Region
Allopatry
North-central hybrid region
Norh-west hybrid region
North-east Spain (Menorca)

Two samples removed after SNP calling due to possible hybridization with a third unknown species. $^{+}$ ADMIXTURE analy

Table 2. Local dynamics. Hybrid region, population locality, first (older) data of the species at the population, year when the population was colonized by a second species (*I. elegans* in the case of previously *I. graellsii*populations, or *I. graellsii* in the case of previously *I. elegans* populations), recolonization of the population because of desiccation by management of invasive species or natural causes, current dominant species, molecular population data.

Hybrid Region	Locality	Oldest data	Colonization	Recolonization	Current dominant species	Molecular data
North-central Spain	Mateo	2015, only *I. graellsii*	2016, *I. graellsii*, occasionally *I. elegans*	2017 (dried), both species	2018, *I. graellsii*, occasionally *I. elegans*	2015
	Valpierre	2008, only *I. graellsii*	2016, *I. graellsii,* occasionally *I. elegans*		2017-2018, *I. graellsii,* occasionally *I. elegans*	2015
	Villar	2008, both species			2015-2018, both species	2015
	Arreo	2008, both species			2008-2015, both species	2008
	Perdiguero	2008, both species			2015-2018, both species	2015

Hybrid Region	Locality	Oldest data	Colonization	Recolonization	Current dominant species	Molecular data
	Las Cañas	2007, both species		2012 (dried), both species	2015-2017, both species	2015
	Valbornedo	2008, *I. elegans*			2015-2018, both species	2015
North-west Spain	Doniños	1987, only *I. elegans*			1987-2021, *I. elegans* occasionally *I. graellsii*	2014
	Laxe	2000, only *I. elegans*		2000 (dried), *I. elegans*	2001-2021, *I. elegans, occasionally I. graellsii*	2014
	Xuño	2001, only *I. graellsii*	2003*, I. graellsii*, occasionally *I. elegans*		2004-2006, both species 2007-2021, only *I. graellsii*	2014
	Louro	1980, both species		2010 (brackish water), *I. graellsii,* occasionally *I. elegans*	2013-2021, *I. graellsii,* occasionally *I. elegans*	2013

Note: Cañas in 2012 and Mateo in 2017, were desiccated by management of invasive species (mainly American crab) and by use of water for irrigation, respectively. Laxe in 2000 was naturally desiccated (evaporation), and Louro (retro dune lagoon) in 2010 suffered a brackish water event.

Table 3. Distribution of individuals in eight ranges of HI/HET resulting from Introgressed. North-central Spain included 68 samples (33*I. elegans* -like and 35 *I. graellsii* -like) and north-west included 40 samples (21 *I. elegans* -like and 19 *I. graellsii* -like. All hybrids (F_1 and F_2) were *a priori,* morphologically identified as *I. graellsii,*which is consistent with the morphology of the F_1 and F_2 hybrids from the laboratory (Monetti et al., 2002; R. A. Sánchez-Guillén et al., 2005) which usually resemble *I. graellsii* individuals.

Classes	Hybrid Index (HI)	Heterozygosity (HET)	Allopatry	Spanish hybrid region	Spanish hybrid region
				north-central	**north-west**
Morphologically *I. elegans*	Morphologically *I. elegans*	Morphologically *I. elegans*	Morphologically *I. elegans*	Morphologically *I. elegans*	Morphologically *I. elegans*
Pure *I. elegans*	1.000	[?]0.000	43 (100%)	1 (3.04%)	0 (0.00%)
Introgressed *I. elegans*	0.900-0.999	¡0.118	-	32 (96.96%)	19 (82.60%)
Back to *I. elegans*	0.601-0.899	0.118-0.449	-	0 (0.00%)	4 (17.40%)
F_2 hybrids	0.400-0.600	0.450-0.599	-	0 (0.00%)	0 (0.00%)
F_1 hybrids	0.400-0.600	[?]0.600	-	0 (0.00%)	0 (0.00%)

Classes	**Hybrid Index (HI)**	**Heterozygosity (HET)**	**Allopatry**	**Spanish hybrid region**	**Spanish hybrid region**
Morphologically *I. graellsii*	Morphologically *I. graellsii*	Morphologically *I. graellsii*	Morphologically *I. graellsii*	Morphologically *I. graellsii*	Morphologically *I. graellsii*
Pure *I. graellsii*	0.000	[?]0.000	25 (100%)	1 (2.86%)	0 (0.00%)
Introgressed *I. graellsii*	0.001-0.100	¡0.118	-	27 (77.14%)	10 (58.82%)
Back to *I. graellsii*	0.101-0.399	0.118-0.449	-	3 (8.57%)	2 (11.76%)
F_2 hybrids	0.400-0.600	0.450-0.599	-	0 (0.00%)	2 (11.76%)
F_1 hybrids	0.400-0.600	[?]0.600	-	4 (11.43%)	3 (17.65%)

Figure Legends

Figure 1 . Studied hybrid region. **A)** Map of the 20 sampled populations. Dark blue circles denote allopatric *I. elegans* , yellow circles denote allopatric *I. graellsii* . Inside of the Spanish hybrid region, dark blue, yellow and orange circles denote *I. elegans* dominant, *I. graellsii* dominant or both species, respectively (1 indicates north-west populations, 2 indicates north-central populations and 3 the Balearic population). **B)**Zoom of Spain, showing the geographic distribution of *I. elegans* over time, from 1888-1979 (8 provinces, dark-grey), 1980-1999 (13 provinces, medium grey) and 2000-actuality (12 provinces, light grey). White colour indicates only*I. graellsii* populations (15 provinces).

Figure 2. Strength of mechanical barriers to reach the copula in **A**) north-central hybrid region (colonized by *I. elegans* at least 14 years ago), and **B)** north-western hybrid region (colonized by *I. elegans* at least 41 years ago). INTROGRESS estimates of ancestry proportion (HI) and heterospecific ancestry (HET) by using the 381 fixed SNPs for *I. elegans* , *I. graellsii* and hybrids from**C**) north-central hybrid region and **D**) north-western hybrid region. F_1 and F_2 hybrids (orange) occupy the apex of the triangle. Backcrosses to *I. elegans* introgressed *I. elegans* and pure *I. elegans*(light blue). Backcrosses to *I. graellsii* , introgressed *I. graellsii* and pure *I. graellsii* (light blue).

Figure 3 . INTROGRESS individual and class proportions estimates of ancestry proportion in **A**) north-central hybrid populations, and **B)** north-west hybrid populations. Populations were assigned to three qualitative measures depending on the frequency distribution of the different hybrid classes: unimodal hybridization pattern, when the distribution spans a range of admixture and backcrosses towards both parental species; bimodal hybridization pattern, when the distribution is deviated to the two parental or parental-like genotypes, and few hybrids (F_1 and F_2 hybrids); and introgressed hybridization pattern, when the distribution was deviated to one parental-like genotype.

Figure 4. Heterozygosity violin plot by population. **A**) *I. elegans* without hybrids, **B**) *I. elegans* with hybrids, **C**)*I. graellsii* with hybrids, **D**) *I. graellsii*without hybrids. Violin width represents the relative frequency of SNPs at each observed heterozygosity value, i.e., wider violin areas show higher number of SNPs at that heterozygosity. The line and the circle at each violin represents the median and the average H_O, while the triangle points at the average H_E. Finally, the value above each violin shows the percentages of SNPs that showed statistical differences between H_O and H_E ($p < 0.05$) to the total number of non-missing-data SNPs.