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# From divergence to contact: demographic history and genomic context shape introgression across independent damselfly hybrid zones

**Miguel Stand-Pérez**
Instituto de Ecología
**Luís Rodrigo Arce-Valdés**
INECOL https://orcid.org/0000-0001-6445-7534
**Jesús Ordaz-Morales**
Instituto de Ecología, A.C. https://orcid.org/0000-0002-7678-4986
**Janne Swaegers**
University of Leuven https://orcid.org/0000-0003-1952-3170
**Jesús Ramsés Chávez-Ríos**
UNAM
**Carla Gutierrez-Rodriguez**
Instituto de Ecología, A.C. https://orcid.org/0000-0002-7142-4164
**Enrique Ibarra-Laclette**
INECOL - Instituto de Ecología, A.C.
**Bengt Hansson**
Lund University https://orcid.org/0000-0001-6694-8169
**Fernanda Baena-Díaz**
Instituto de Ecología (INECOL)
**Rosa Sánchez Guillén**
rosa.sanchez@inecol.mx

INECOL https://orcid.org/0000-0001-6024-8321

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# Abstract

Hybridization outcomes often vary across space and time, yet the relative roles of demographic history and chromosomal differences in shaping introgression remain unclear. Here, we investigate three replicated hybrid zones between the damselflies Ischnura elegans and Ischnura graellsii across Spain to test whether genomic introgression patterns are repeatable across independently formed zones. Using genome-wide data, we combined demographic modeling, genomic cline, and functional annotation of introgressed loci. Demographic inference supported three independent secondary contact events of different ages: The South-east zone forming first (~207 years ago), followed by the North-west (~73.5 years) and North-central (~33 years) hybrid zones. Despite similar cline steepness across autosomes, asymmetric gene flow from I. graellsii into I. elegans was observed, with a low overlap of introgressed loci between zones. These loci were mainly associated with broad physiological and regulatory functions. In contrast, the X chromosome showed steeper clines, suggesting strong intrinsic genomic constraints. Together, demographic history explains geographic heterogeneity in introgression, whereas chromosome architecture imposes consistent constraints. These findings highlight how replicated hybrid zones can disentangle contingent versus repeatable genomic responses during early stages of speciation with gene flow.


# INTRODUCTION

Hybridization is widely recognized as a dynamic evolutionary process capable of generating diverse outcomes ranging from genetic swamping, species replacement, reinforcement, hybrid speciation, stable hybrid zones, or adaptive introgression (Abbott et al. 2013). However, these outcomes are rarely uniform, even within the same pair of species (Harrison & Larson 2016). Growing evidence shows that the consequences of hybridization depend not only on intrinsic factors such as genetic divergence, mutations, sex determination system, but also on the demographic and environmental context in which secondary contact occurs (Peñalba et al. 2024). Understanding why similar species interactions lead to divergent genomic and evolutionary trajectories remains a central challenge in evolutionary biology.

One key source of variation arises from differences in demographic history and the timing of secondary contact. Hybrid zones formed at different times may experience contrasting reproductive barriers, effective population sizes, genetic incompatibilities, and ecological conditions, all of which influence the strength and direction of introgression (Harrison & Larson 2014, 2016). Distributional range shifts driven by climate change or post-glacial warming have repeatedly generated scenarios in which closely related taxa come into contact under heterogeneous demographic contexts, creating natural laboratories for studying speciation with gene flow (Taylor et al. 2015). Yet most studies focus on single hybrid zones, limiting our ability to disentangle repeatable genomic responses from historically contingent outcomes.

In addition to demographic processes, genomic architecture plays an important role in shaping introgression patterns. Sex chromosomes and some genomic regions associated with reproductive isolation often show reduced permeability to gene flow (Dobzhansky 1936; Fraïsse & Sachdeva 2021),

suggesting that intrinsic genetic barriers can impose predictable constraints across hybrid systems. At the same time, widespread variation in autosomal introgression among populations indicates that introgression patterns are not solely determined by species-specific differences but emerge from interactions between genome architecture and local demographic or ecological conditions (Abbott et al. 2016). This dual role of intrinsic genomic constraints and extrinsic historical context raises the issue of the extent to which introgression patterns are repeatable across independently formed hybrid zones within the same species pair.

Comparisons among multiple hybrid zones provide a powerful framework to investigate this issue. Spatially replicated zones differing in age, demographic context, and ecological conditions allow us to evaluate whether hybridization produces consistent genomic signatures or whether outcomes diverge depending on historical contingencies (e.g. Harrison & Larson 2016; Vines et al. 2003). Such systems align with the mosaic hybrid zone concept, in which geographically structured variation in selection, demography, and environmental context generates heterogeneous genomic landscapes of introgression (Rand & Harrison 1989). Importantly, examining replicated contact zones within a single species pair offers a unique opportunity to separate lineage-specific genomic effects from broader demographic processes (Harrison & Larson 2016). However, most previous evidence for repeatable patterns of introgression has been derived from interspecific comparisons, either across different species pairs or among independent hybrid systems (Edelman et al. 2019; Fontaine et al. 2015; Gompert et al. 2012; Martin et al. 2013; Taylor & Larson 2019). This highlights the need for systems that allow such patterns to be evaluated within a single species pair across multiple hybrid zones.

A particularly useful framework for quantifying these patterns is the genomic cline theory (Barton 1979), which can describe how introgression rates may vary across loci and between zones. Unlike traditional clines based on geography, genomic clines use hybrid indices or admixture proportions as independent variables (Szymura & Barton 1991). This approach is especially powerful for detecting outlier loci whose introgression patterns deviates significantly from the genome-wide neutral expectation (Bailey 2022; Fitzpatrick 2013). Thus, the rate and pattern of introgression at a given locus can reveal whether it is linked to reduced hybrid fitness or, conversely, to adaptive or heterotic advantages (Gompert & Buerkle 2011).

Our study system focuses on two closely related damselfly species that provide a rare opportunity to investigate recent divergence, range expansion, and hybridization in real time. *Ischnura elegans* is a widespread Euro-Siberian species that has undergone rapid southward range expansion, whereas its sister species *I. graellsii* has a much narrower distribution, being largely restricted to the Iberian Peninsula and northern Africa (Ocharan-Larrondo 1987). Over the last decades, and with increasing speed in recent years, *I. elegans* has expanded into the range of *I. graellsii* in Spain (Monetti et al. 2002; Wellenreuther et al. 2018; Sánchez-Guillén et al. 2011b) generating several geographically structured hybrid zones. These hybrid zones differ markedly in their ecological and geographic context, the strength and asymmetry of reproductive barriers, patterns of introgression, and evolutionary outcomes. The South-east (SE) hybrid zone, located along the Mediterranean coast, represents the oldest

documented zone and is characterized by a progressive shift in population composition, whereby *I. elegans* individuals have largely replaced *I. graellsii* individuals, together with the persistence in *I. elegans* populations of alleles introgressed from *I. graellsii* (Sánchez-Guillén et al. 2011b; Wellenreuther et al. 2018). By contrast, the North-west (NW) hybrid zone which is geographically isolated from both allopatric *I. elegans* populations and other hybrid zones, exhibits extensive bidirectional introgression, reduced interspecific genetic differentiation, and reinforcement acting primarily on mechanical barriers (Arce-Valdés et al. 2025; Ballén-Guapacha et al. 2024; Sánchez-Guillén et al. 2012). Finally, the North-central (NC) hybrid zone is larger and currently expanding, showing predominantly unidirectional introgression from *I. graellsii* into *I. elegans*, together with stronger postmating isolation involving both mechanical and fertility barriers (Sánchez-Guillén et al. 2012).

Across the three hybrid zones, genomic analyses reveal consistently reduced introgression on the X chromosome and an underrepresentation of males among admixed individuals, consistent with Haldane's rule in an X0 sex-determination system (Swaegers et al. 2022b). Together, these hybrid zones constitute a powerful natural laboratory for examining the repeatability and contingency of hybridization outcomes, the interaction between range expansion and reinforcement, and the chromosomal differences in reproductive isolation during the earliest stages of speciation. *Beyond their role as a model for hybridization and reinforcement, recent studies have shown that the southward expansion of I. elegans has been accompanied by rapid ecological and physiological responses to warmer climatic conditions, including convergence in thermal tolerance and life-history traits toward the native sister species I. graellsii* (Swaegers et al. 2022a). *These findings highlight the colonization of warmer environments by I. elegans is reshaping the ecological context in which secondary contact and hybridization occur.*

Despite numerous studies conducted in this hybrid system, the origins of each hybrid zone remain unresolved as does the dual role that demographic conditions and chromosome differences may play in shaping the evolutionary consequences of hybridization. For this reason, this study aims to investigate the demographic history and patterns of genomic introgression between *I. elegans* and *I. graellsii* across three hybrid zones in Spain. Specifically, we aim to (i) reconstruct the demographic history of this hybrid system by estimating the timing of the cessation of gene flow between species (origin of the allopatric distribution of the species), the formation of subsequent hybrid zones, and key demographic parameters. Given historical records of the southward expansion of *I. elegans*, we expect hybrid zones to have originated through independent and temporally staggered secondary contact events associated with this range expansion (Monetti et al. 2002; Wellenreuther et al. 2018). (ii) Model genomic clines within each hybrid zone to characterize patterns of genetic introgression. Based on previous evidence of asymmetric introgression and differences in reproductive barriers among zones, we predict that genomic clines will differ in the direction and magnitude of introgression among hybrid zones (Sánchez-Guillén et al. 2011b, 2012; Swaegers et al. 2022b; Arce-Valdés et al. 2025). (iii) Investigate the functional roles of loci exhibiting excess introgression between parental species across hybrid zones. We predict that introgressed loci will be associated with broad cellular and regulatory processes rather than functions directly related to reproductive isolation (Horta et al. 2025).

# MATERIAL AND METHODS

### Sampling design, RAD-seq data processing and SNP calling

We used genomic RAD-sequencing data of *I. elegans* and *I. graellsii* previously published by Swaegers et al. (2022b) which provide dense genome-wide coverage across allopatric populations and multiple hybrid zones differing in their evolutionary dynamics. These data comprise 262 individuals from 26 populations sampled between 2005 and 2015, including the allopatric distribution of *I. graellsii* in Spain, Portugal, and the Maghreb in northern Africa (n = 51), six populations from the allopatric distribution of *I. elegans* ranging from France and the United Kingdom to central Europe (n = 52), five populations from the North-west (NW) hybrid zone (n = 49), seven populations from the North-central (NC) hybrid zone (n = 68), and four populations from the South-east (SE) hybrid zone (n = 42; Figure 1).

We assembled these sequences following the Stacks v2.2 pipeline (Catchen et al. 2013; Rochette et al. 2019). First, we demultiplexed and quality-filtered raw RAD-seq reads with *process_radtags*, removed PCR duplicates using *clone_filter* with default parameters, and aligned the cleaned reads to the most recent *I. elegans* reference genome (Price et al. 2022) using the BWA-MEM algorithm implemented in BWA v0.7.17 (Li & Durbin 2009). We processed the resulting BAM files with *gstacks* and called variants using *populations* with the *–ordered-export* flag, filtering variants to retain a minimum minor allele frequency of 0.02 across all individuals. Using VCFtools v0.1.16 (Danecek et al. 2011), we further filtered the resulting VCF file to include only bi-allelic SNPs, recovering a total of 2,177,590 markers.

Following O'Leary et al. (2018), we tested several filtering schemes to retain an optimal number of high-quality SNPs and samples (Supplementary File 1). We selected the scheme retaining genotypes with a minimum sequencing depth of 5, loci with a maximum of 10% missing data, and samples with a maximum of 15% missing data, which resulted in 10,093 SNPs. We pruned markers for linkage disequilibrium using Plink v1.90 (Purcell et al. 2007) with the *--indep-pairwise* function, applying a sliding window of 50 SNPs and a step size of 5 SNPs. Pairwise linkage disequilibrium was estimated among SNPs within each window, and one random SNP was retained when the genotypic correlation coefficient ($r^2$) exceeded 0.5. We then used allopatric samples as references for a preliminary supervised Admixture v1.3.0 analysis (Alexander & Lange 2011) and classified individuals from sympatric populations as putative pure-species samples when their Q values were ≥ 0.999 for either parental species. We tested Hardy–Weinberg disequilibrium across the full distribution of both species using these pure-species samples in VCFtools and removed SNPs that showed significant deviation from Hardy–Weinberg equilibrium in both species simultaneously ($p < 0.05$). Finally, for subsequent analyses, we divided the full SNP dataset into autosomal (6,922 SNPs) and X-linked (236 SNPs) subsets, both comprising 237 samples (Supplementary Table 1, Figure 1).

### Demographic history inference

To investigate the origins of the three hybrid zones and reconstruct the demographic history of both species in Spain, we tested alternative scenarios of secondary contact and range expansion that could

account for the contrasting evolutionary outcomes observed among hybrid zones. We employed the software DIYABC-RF (Collin et al. 2021) following best-practice recommendations for demographic inference using genomic data (Marchi et al. 2021). This framework combines Approximate Bayesian Computation with a Random Forest algorithm to compare observed genetic data against simulated datasets, enabling the identification of the most likely demographic scenarios. In addition, the use of machine learning enhances both the accuracy and robustness of model selection and parameter estimation, particularly in complex evolutionary histories involving divergence, gene flow, and admixture (Collin et al. 2021). This integrative approach is especially well-suited to disentangle the demographic processes underlying hybrid zone formations **in systems characterized by recent divergence, ongoing gene flow, and spatially structured secondary contact (Csilléry et al. 2010;** Collin et al. 2021**)**, as in *I. elegans* and *I. graellsii*.

Using the autosomal dataset (6,922 SNPs), we compared eight alternative demographic scenarios that differed mainly in whether the hybrid zones had a single origin or originated independently or alternatively resulted from sequential colonization events involving already established hybrid zones. These scenarios would reflect alternative hypotheses about the temporal and spatial dynamics of secondary contact following the recent southward expansion of *I. elegans* (details in Supplementary Figure 1).

For each scenario, we performed 3,000 coalescent simulations and evaluated the fit between simulated and observed data using a principal component analysis of all summary statistics included in the software. We selected the best scenario via random forest classification using 1,000 trees, which allowed for accurate discrimination among competing models despite the high dimensionality of the data. Then, we did another 40,000 coalescent simulations under the best-supported scenario to estimate the following demographic parameters: the timing of the cessation of gene flow between species (assuming 0.67 generations per year, i.e., 1.5 years per generation, according to Corbet et al. (2006)), effective population sizes, and admixture rates. To determine whether this number of simulations was adequate, we implemented a random forest regression approach to estimate error metrics, comparing results from the 40,000 simulations with those from a randomly selected subset of 12,000 simulations. As no meaningful differences in error metrics were detected between the two simulation datasets, we considered that the results obtained with 40,000 simulations, using a random forest analysis composed of 1,000 trees and 30% of these simulations as out-of-bag testing samples, were sufficiently robust.

**Genomic cline analyses**

To describe patterns of introgression between multiple loci in each hybrid zone, we performed genomic clines analyses based on genotype frequencies along a genome-wide admixture gradient. This approach allows the identification of loci showing excessive or restricted gene flow, thereby linking zone-specific demographic histories with heterogeneity in genomic introgression (Gompert & Buerkle 2011). Genomic cline analyses were performed for both autosomal (6,922 SNPs) and X-linked (236 SNPs) datasets.

However, the SE hybrid zone was excluded from these analyses since only introgressed *I. elegans* individuals, as identified from SNP genomic data, were included from this region, precluding the estimation of genomic clines based on both parental and admixed individuals. Before the analyses, we applied additional filtering criteria tailored to genomic cline analyses. We filtered markers using the allopatric parental populations of *I. elegans* and *I. graellsii* as references, retaining only SNPs with less than 20% missing data in each parental group, an allele frequency difference greater than 0.10 between species and a minor allele frequency above 0.20. These criteria were applied to enrich the dataset for loci showing contrasting allele frequencies between the allopatric reference groups. Genomic clines were then modeled using the R package gghybrid (Bailey 2022).

First, we estimated hybrid indices using a single Markov chain Monte Carlo (MCMC) run with 3,000 iterations and a burn-in of 1,000 iterations. Hybrid indices were then used to fit genomic clines through a second MCMC with 5,000 iterations and a burn-in of 2,000 iterations. In both analyses, MCMC stability was assessed using real-time trace plots. Finally, statistically significant differences in cline steepness and center between NW and NC hybrid zones were assessed using a paired Wilcoxon signed-rank test (Wilcoxon 1945) in both autosomes and X-linked loci. On the other hand, statistically significant differences in cline steepness between autosomes and X-linked loci for both the NW and NC hybrid zones were assessed using a Wilcoxon rank-sum test (Wilcoxon 1945).

**Functional annotation of introgressed loci**

To explore the heterogeneity of introgression across the genome and assess whether similar genomic regions are involved across hybrid zones, we classified outlier SNPs based on their cline parameters (Bailey 2022). Specifically, SNPs with cline steepness values lower than one were classified as showing excessive gene flow, whereas SNPs with higher values were classified as showing restricted introgression. Additionally, based on cline center estimates, SNPs with center values greater than 0.5 were interpreted as carrying *I. graellsii* alleles in an *I. elegans* genomic background, whereas values lower than 0.5 indicated *I. elegans* alleles in an *I. graellsii* background. Loci were considered outliers only when these patterns were statistically significant ($p < 0.05$; Bailey 2022). We used Fisher's exact test (Fisher 1922) to compare the frequencies of each outlier class between the NW and NC hybrid zones. Finally, we functionally annotated outlier SNPs showing excessive introgression to characterize the biological process potentially associated with these loci. Because our objective was to infer the potential biological roles of introgressed variants, we focused on Gene Ontology (GO) annotations within the Biological Process category, which provides a functional framework for interpreting how annotated genes may contribute to organismal responses. For this, we mapped outlier SNPs to the GFF annotation file of the *I. elegans* reference genome (Price et al. 2022) and identified annotated coding sequence (CDS), mRNA, or exon features that directly contained the genomic position of each outlier SNP. Given the reduced representation nature of RAD-seq data, only the SNP positions themselves were considered, and surrounding genomic regions were not evaluated. For each feature, we retrieved the corresponding protein sequences from GenBank using NCBI E-utilities (Sayers 2022). When outlier SNPs were located within features associated with multiple homologs, isoforms, or protein variants, we randomly selected a

single representative protein sequence per SNP. Protein sequences were then analyzed using InterProScan v.5 (Jones et al. 2014) to identify conserved protein domains based on integrated protein signature databases. GO terms were assigned to each sequence according to the functional annotations associated with the detected InterPro entries.

# RESULTS

### Demographic history inference

We compared eight alternative demographic scenarios describing the formation of the SE, NW, and NC hybrid zones, considering either independent admixture events or sequential formation mediated by migration among hybrid zones (Supplementary Figure 1). Using 3,000 simulations per scenario, all proposed models were consistent with the observed dataset based on PCA analysis of summary statistics (Supplementary Figure 2). Among the tested models, Scenario 1, describing the hybrid zones as independently formed admixture events, with the SE hybrid zone forming first, followed by the NW, and most recently the NC hybrid zone, received the highest support according to random forest votes (517 votes, posterior probability = 0.456; Figure 2; Table 1). Under this scenario, the SE hybrid zone showed a low genetic contribution from *I. graellsii* (4.5%). Admixture proportions were more balanced in the NW (*I. graellsii* = 54.3%, *I. elegans* = 45.7%), and in the NC (*I. graellsii* = 53.5%, *I. elegans* = 46.5%; Figure 2).

A demographic expansion preceding the cessation of gene flow was inferred around 6,306 generations ago (90% CI: 9,728–2,258), corresponding to around 9,459 years ago (90% CI: 14,592–3,387; assuming 0.67 generations per year, i.e., 1.5 years per generation, Corbet et al. (2006)). During this period, effective population size increased from 881 at the onset of the expansion to present-day allopatric population sizes estimated for *I. elegans* (6,130) and *I. graellsii* (6,904; Figure 2; Table 2). The cessation of gene flow between *I. elegans* and *I. graellsii* subsequently estimated at approximately 3,570 generations ago (90% CI: 7,118–1,585; Figure 2; Table 2), corresponding to 5,355 years ago (90% CI: 10,677–2,378). More recently, parameter estimates indicate that the SE hybrid zone was established approximately 138 generations ago (90% CI: 329–56; Figure 2; Table 2), corresponding to 207 years ago (90% CI: 494–84). The NW hybrid zone formed more recently, around 49 generations ago (90% CI: 154–13; Figure 2; Table 2), equivalent to 73.5 years ago (90% CI: 231–19.5), followed by the NC hybrid zone, which formed approximately 22 generations ago (90% CI: 72–11; Figure 2; Table 2), corresponding to 33 years ago (90% CI: 108–16.5). Finally, effective population sizes estimated for the SE (Ne = 6,014) and NW (Ne = 6,592) hybrid zones were similar, whereas the NC showed a smaller effective population size (Ne = 4,048; Table 2).

### Genomic cline analyses

#### *Autosomal genomic clines*

For the autosomal SNP dataset, genomic cline analyses revealed no significant differences in cline steepness between the NW (median = 2.636) and NC (median = 2.64; Figure 3a) hybrid zones (paired Wilcoxon signed-rank test: V = 882,637; p = 0.379; Figure 3a; Supplementary Figure 3). In contrast, the genomic cline centers differed significantly between hybrid zones (paired Wilcoxon signed-rank test: V = 712,398; p < 0.001). The median cline center was 0.48 in the NW hybrid zone and 0.59 in the NC hybrid zone (Figure 3b).

Outlier analyses identified differences in the number of autosomal loci with excessive introgression patterns between hybrid zones. A similar number of autosomal SNPs showing excessive gene flow were detected in the NC (n = 48) compared to the NW hybrid zone (n = 42; Supplementary Figure 4a). Of these, four outlier SNPs were shared between both hybrid zones. Conversely, four SNPs associated with restricted gene flow were detected in NW (Supplementary Figure 3a), and one in the NC hybrid zone (Supplementary Figure 3b).

Directional introgression analyses identified asymmetries between hybrid zones. We detected 133 SNPs originating from *I. elegans* introgressed into the genome of *I. graellsii* in the NW and 154 in the NC hybrid zone, with 43 SNPs shared between both hybrid zones (Supplementary Figure 4b). Notably, a substantially higher number of SNPs showed introgression in the opposite direction. Specifically, 304 SNPs from *I. graellsii* were detected in the genome of *I. elegans* in NW and 442 in NC hybrid zone, of which 163 were shared between the two hybrid zones (Supplementary Figure 4c).

After merging both types of outliers, including SNPs showing excessive gene flow and those exhibiting bidirectional or unidirectional introgression, we detected a greater number of SNPs with excessive bidirectional introgression in the NC and NW hybrid zones (20 unique to NC, 27 unique to NW, and two shared; Figure 4a). Similarly, numerous SNPs showed unidirectional introgression from *I. graellsii* into *I. elegans* (21 unique to NC, 8 unique to NW, and one shared; Figure 4b). In contrast, far fewer SNPs showed introgression in the opposite direction, from *I. elegans* into *I. graellsii* (three unique to NC, two unique to NW, and one shared; Figure 4c).

***X-linked genomic clines***

For the X-linked SNP dataset, genomic cline steepness (Supplementary Figure 5) differed between hybrid zones (paired Wilcoxon signed-rank test: V = 2,106; p = 0.03). Slopes were steeper in the NC (median = 4.57) than in the NW hybrid zone (median = 4.12; Supplementary Figure 6a). Furthermore, the genomic cline center median was shifted toward *I. elegans* in NC (median = 0.67) and toward *I. graellsii* in NW hybrid zone (median = 0.40; Supplementary Figure 6b), with the difference between zones being statistically significant (paired Wilcoxon signed-rank test: V = 1,429; p = 0.003). Furthermore, no X-linked SNPs were identified as excessive gene flow in NW and only one in NC hybrid zone.

Finally, when comparing cline steepness between autosomal and X-linked SNPs, we found that X-linked loci exhibited significantly steeper clines than autosomal loci in both hybrid zones. In the NW hybrid zone, the median steepness was 2.63 for autosomal SNPs and 4.12 for X-linked SNPs (Wilcoxon rank-

sum test: W = 74,482; p < 0.001). Similarly, in the NC hybrid zone, median steepness was 2.64 for autosomal loci and 4.57 for X-linked loci (Wilcoxon rank-sum test: W = 63,932; p < 0.001). This pattern suggests a stronger restriction to introgression on the sex chromosome relative to the autosomes.

**Functional annotation of introgressed loci**

Among the 85 autosomal SNPs showing excess introgression between parental species (Figure 4), we identified 19 protein sequences associated with 30 GO terms within the biological process category (Supplementary Table 2). Most annotated loci were associated with broad cellular and regulatory functions. The first group of GO terms comprised processes related to nucleotide biosynthesis and metabolism, signal transduction, transmembrane transport, DNA repair, protein phosphorylation, and proteolysis. The second group included processes associated with cytoskeletal organization, transcriptional regulation, RNA processing, extracellular matrix organization, multicellular development, and myofibril assembly. Although the specific annotated loci differed between hybrid zones, similar classes of biological processes were represented in both regions (Supplementary Table 2). Transcriptional regulation and transmembrane transport were detected in both hybrid zones, whereas signaling pathways, cytoskeletal organization, extracellular matrix organization, and developmental processes were more frequently represented in the NW hybrid zone, and DNA repair, RNA processing, and biosynthetic pathways were more common in the NC hybrid zone.

# DISCUSSION

Why hybridization leads to divergent outcomes remains a central question in evolutionary biology. Our results show that recent divergence, spatially replicated but temporally distinct secondary contact, and contrasting permeability to gene flow between X-linked and autosomal loci jointly shape the demographic trajectories and introgression patterns observed across the three hybrid zones between *I. elegans* and *I. graellsii* in Spain. Consistent with our predictions, hybrid zones originated independently and at different times, genomic clines varied in direction and magnitude among zones, and loci showing excess introgression are associated with cellular, metabolic, and regulatory processes.

***Demographic history during the early stages of speciation***

Post-glacial climatic fluctuations likely shaped the evolutionary history of *I. elegans* and *I. graellsii*. Phylogenetic estimates place the onset of divergence between these species within the last ~100,000 years (Sánchez-Guillén et al. 2020; Blow et al. 2021), with our demographic analyses indicating a more recent cessation of gene flow between 2,378 and 10,677 years ago, suggesting a prolonged history of interaction following initial divergence within the Holocene. This timing coincides with climatic warming following the Last Glacial Maximum (~21,000 years ago), which reshaped species distributions across Europe (Hewitt 2000). Given that the Iberian Peninsula served as a significant glacial refugium (Gómez & Lunt 2007; Hewitt 1999), the common ancestor of *I. elegans* and *I. graellsii* likely persisted there throughout much of the last ~100,000 years. Following post-glacial climatic warming, part of this ancestral lineage may have expanded northwards beyond the Pyrenees while populations remaining in

the Iberian Peninsula maintained gene flow with the expanding northern populations. Our demographic analyses suggest that the cessation of gene flow between the Iberian and northern lineages occurred much later, between approximately 2,378 and 10,677 years ago, marking the final stage of speciation and giving rise to the distributions of *I. graellsii*, restricted to the Iberian Peninsula and North Africa, and *I. elegans*, distributed beyond the Pyrenees. Following the cessation of gene flow, the strong dispersal ability and ecological flexibility of *I. elegans* likely facilitated rapid expansion and local adaptation to cold freshwater systems in northern Europe (Dudaniec et al. 2018; Lancaster et al. 2015). Such post-glacial divergence coupled with rapid range expansion is increasingly recognized as a recurrent pattern in Odonata, including *Ischnura*, *Calopteryx*, and *Enallagma*, where high dispersal ability and ecological flexibility promote shallow genetic divergences, demographic expansions, and secondary contact among closely related lineages (Turgeon & McPeek 2002; Wellenreuther & Sánchez-Guillén 2016). Similar post-glacial divergence and expansion scenarios have been documented across a wide range of taxa such as insects (Capblancq et al. 2015, 2019; Mas-Peinado et al. 2022; Schmitt et al. 2006), fishes (Schluter & McPhail 1992; Volckaert et al. 2002), and birds (Ellegren et al. 2012; Sætre & Sæther 2010; Warmuth et al. 2021), indicating that recent divergence followed by secondary contact is a recurrent outcome of post-glacial range dynamics.

Demographic modeling indicates that secondary contact occurred independently across hybrid zones under distinct temporal and demographic contexts, providing a framework to interpret heterogeneous introgression patterns. DIYABC-RF estimates suggest the earliest contact occurred in the SE hybrid zone ~207 years ago, consistent with the first records of *I. elegans* in Spain (Ocharan-Larrondo 1987) and with large effective population sizes and long-term asymmetric introgression (Sánchez-Guillén et al. 2011b). In contrast, the NW (~74 years ago) and NC (~33 years ago) zones represent more recent contacts with initially balanced admixture but divergent introgression trajectories between them —bidirectional in NW and unidirectional in NC (Swaegers et al. 2022b), facilitated by rapid adaptations of *I. elegans* to the warm and human-modified agricultural ponds in the Iberian Peninsula (Swaegers et al. 2022a; Wellenreuther et al. 2018). Together, these temporally structured histories support a mosaic hybrid zone framework (Rand & Harrison 1989), where variation in the timing and demographic context of secondary contact shapes genomic heterogeneity, introgression patterns, and the evolution of reproductive isolation across zones (Arce-Valdés et al. 2025; Ballén-Guapacha et al. 2024).

***Heterogeneous introgression across recently formed hybrid zones***

Our results indicate that chromosomal differences strongly influence patterns of gene flow across the genome. Autosomal loci show widespread excess introgression, but only a small fraction of these loci is shared among hybrid zones, suggesting that autosomal gene flow is shaped by local demographic and ecological conditions rather than intrinsic species differences. In contrast, X-linked loci showed significantly steeper clines than autosomal loci in both hybrid zones, indicating stronger restriction of introgression across the sex chromosome. This pattern previously reported in this system (Swaegers et al. 2022b), supports the view that sex chromosomes often act as stable barriers to gene flow (Horta

et al. 2025; Presgraves 2018), although demographic history may further modulate their permeability to introgression.

The shift in cline centers toward *I. elegans* in the younger NC hybrid zone suggests that the direction of introgression is not determined solely by the time elapsed since secondary contact. Previous work in this system has shown that reproductive barriers change asymmetrically from allopatry to sympatry, with reinforcement strengthening mechanical isolation in the *I. graellsii* male x *I. elegans* female cross, while gene flow relaxes postzygotic barriers in the reciprocal direction (Arce-Valdés et al. 2025; Ordaz-Morales et al. 2026). This reduction in postzygotic isolation may also be consistent with the progressive purging of Bateson-Dobzhansky-Muller incompatibilities through repeated hybridization, allowing greater autosomal introgression while maintaining stronger constraints on the X chromosome, where incompatibilities are expected to accumulate. Thus, the younger NC hybrid zone may exhibit a stronger directional bias in introgression because asymmetric reproductive isolation favors gene flow in one cross direction. By contrast, the older NW hybrid zone, characterized by weaker postzygotic barriers and a longer hybridization history, may have accumulated more bidirectional gene flow. An additional mechanism that may contribute to the observed asymmetry is hybridization load, whereby alleles from populations with larger effective population sizes may be favored over those from recently bottlenecked populations (Schumer et al. 2018). Because the Iberian populations of *I. elegans* originated from a recent range expansion, effective population sizes at the colonization front may have experienced reduced effective population sizes relative to those inferred for allopatric populations in our demographic analyses. Under this scenario, introgression from larger and more genetically diverse populations could be selectively favored. This interpretation is consistent with the effects of genetic drift and demographic bottlenecks previously documented in this system, including their morphological (Ballén-Guapacha et al. 2024), adaptive (Sánchez-Guillén et al. 2011a; Wellenreuther et al. 2011), and genomic (Swaegers et al. 2022b) consequences.

Beyond these genome-wide introgression patterns, our findings reinforce previous evidence that the NC and NW hybrid zones have followed contrasting evolutionary trajectories, differing in reproductive barrier strength (Arce-Valdés et al. 2025; Ordaz-Morales et al. 2026) and reproductive character displacement (Ballén-Guapacha et al. 2024). The results are consistent with a growing body of literature showing that genomic permeability to introgression can vary geographically within the same species pair (Harrison & Larson 2014; Taylor et al. 2015). In our system, such heterogeneity may arise from differences in effective population size, the strength of reproductive barriers at the onset of secondary contact, and the time since initial hybridization. Variation in these factors may generate markedly different genomic outcomes across geographically replicated hybrid zones (Ravinet et al. 2017).

These findings reinforce the view that hybrid zones are dynamic systems that evolve through time rather than representing static endpoints of species interactions. The recent divergence of these species, likely associated with post-glacial processes, and their subsequent secondary contact and rapid range expansion in the Iberian Peninsula under contemporary climate warming (Sánchez-Guillén et al. 2011b) make this system particularly informative for understanding speciation with gene flow under natural

conditions. Consistent with patterns documented in other hybrid model systems, such as *Heliconius* butterflies (Edelman et al. 2019; Martin et al. 2013), *Bombina* toads (Fijarczyk et al. 2011), *Ficedula* flycatchers (Ellegren et al. 2012), and house mice (Macholán et al. 2011), the contrasting genomic patterns observed among hybrid zones of different ages in *I. elegans* and *I. graellsii* illustrate how introgression dynamics and speciation trajectories can shift throughout the demographic history of recently diverged species.

***Functional implications of introgressed loci***

The functional annotations of loci showing excess introgression suggest that gene flow between *I. elegans* and *I. graellsii* may involve genes associated with broad cellular, metabolic, and regulatory processes rather than functions directly linked to reproduction. Several annotated biological process terms were related to signal transduction, protein phosphorylation, nucleotide biosynthesis, transcriptional regulation, transmembrane transport, DNA repair, RNA processing, and cytoskeletal organization. Although these annotations do not demonstrate adaptive introgression by themselves, they are consistent with the idea that introgressed variants may contribute to general physiological performance in heterogeneous environments (Edelman & Mallet 2021). This interpretation is consistent with previous work in *I. elegans* showing that responses to environmental gradients, warming, and anthropogenic stressors often involve metabolic, developmental, transport, and regulatory pathways rather than reproductive functions alone (Dudaniec et al. 2018; Swaegers et al. 2020; Wos et al. 2023, 2024).

In the Iberian Peninsula, where *I. elegans* has expanded into warmer environments already occupied by *I. graellsii*, introgression at loci involved in such broadly acting processes could potentially facilitate physiological adjustment to local conditions. For example, terms related to nucleotide metabolism, signaling pathways, transmembrane transport, and DNA repair may be relevant to physiological maintenance and cellular responses to environmental stress (Monaghan et al. 2009; Wos et al. 2024), while cytoskeletal organization, RNA processing and transcriptional regulation may reflect variation in development and gene regulatory processes, consistent with previous evidence showing that thermal adaptation in *I. elegans* involves differences in gene expression and pathways related to cytoskeletal function (Swaegers et al. 2020). Such variation could contribute to phenotypic responses during range expansion into novel environments. Importantly, these patterns should be interpreted as functional associations rather than evidence of enrichment or direct adaptation, but they are compatible with previous evidence that *I. elegans* can respond rapidly to climatic and environmental gradients through genes involved in general physiological and developmental pathways. Future analyses using whole-genome sequencing, denser marker coverage, environmental association tests, and functional enrichment approaches will be necessary to determine whether these introgressed loci are truly involved in local adaptation to Iberian environments or simply reflect neutral introgression at genes with general cellular roles.

# Conclusions

Our results show that hybridization between *I. elegans* and *I. graellsii* reflects has generated a heterogeneous introgression landscape across independently formed hybrid zones with distinct demographic histories. Variation in the timing of secondary contact, admixture proportions, and effective population sizes was associated with strongly zone-specific introgression dynamics, whereas chromosome architecture imposed consistent constraints: autosomes remained comparatively permeable to gene flow, while the X chromosome acted as a stable barrier. Functional annotation of loci showing excess introgression further indicated that introgressed variants were primarily associated with broad cellular, metabolic, and regulatory processes rather than functions directly linked to reproduction. These findings suggest that demographic context drives geographic variation in introgression outcomes, while intrinsic chromosome architecture and the functional characteristics of introgressed loci may promote partly predictable evolutionary responses during the early stages of speciation with gene flow.

# Declarations

### Data Archiving

All scripts used in this study are available in the OSF repository https://osf.io/hmz7k/overview?view_only=931486f31f0b4b69a025bf2407aa45da and raw genomic data are available in the NCBI Short Read Archive (SRA) Database under SRA accession PRJNA850104.

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# Tables

**Table 1.** Random forest vote counts for the eight demographic scenarios tested in the DIYABC-RF analysis. Scenario 1 received the highest number of votes, indicating the strongest support among all models based on the simulated datasets.

| Scenario | Votes |
|---|---|
| Scenario 1* | 517 |
| Scenario 2 | 372 |
| Scenario 3 | 6 |
| Scenario 4 | 21 |
| Scenario 5 | 25 |
| Scenario 6 | 22 |
| Scenario 7 | 28 |
| Scenario 8 | 9 |

**Table 2.** Predicted parameter estimates from the DIYABC-RF analysis under the selected demographic scenario 1. Parameters include t: times of species divergence and hybrid zone formation, Ne: effective population sizes, r: admixture proportions representing the genetic contribution of *I. graellsii* (with the complement corresponding to *I. elegans*).

| Parameter | Median | Lower CI | Upper CI |
|---|---|---|---|
| t ancestral | 6,306 | 2,258 | 9,728 |
| t divergence | 3,570 | 1,585 | 7,118 |
| t South-east | 138 | 56 | 329 |
| t North-west | 49 | 13 | 154 |
| t North-central | 22 | 11 | 72 |
| Ne ancestral | 881 | 126 | 9,415 |
| Ne allopatric *I. elegans* | 6,130 | 2,258 | 9,573 |
| Ne allopatric *I. graellsii* | 6,904 | 3,070 | 9,683 |
| Ne South-east | 6,014 | 1,830 | 9,597 |
| Ne North-west | 6,592 | 2,236 | 9,319 |
| Ne North-central | 4,048 | 459 | 9,183 |
| r South-east | 0.045 | 0.013 | 0.106 |
| r North-west | 0.543 | 0.414 | 0.688 |
| r North-central | 0.535 | 0.398 | 0.673 |

# Figures

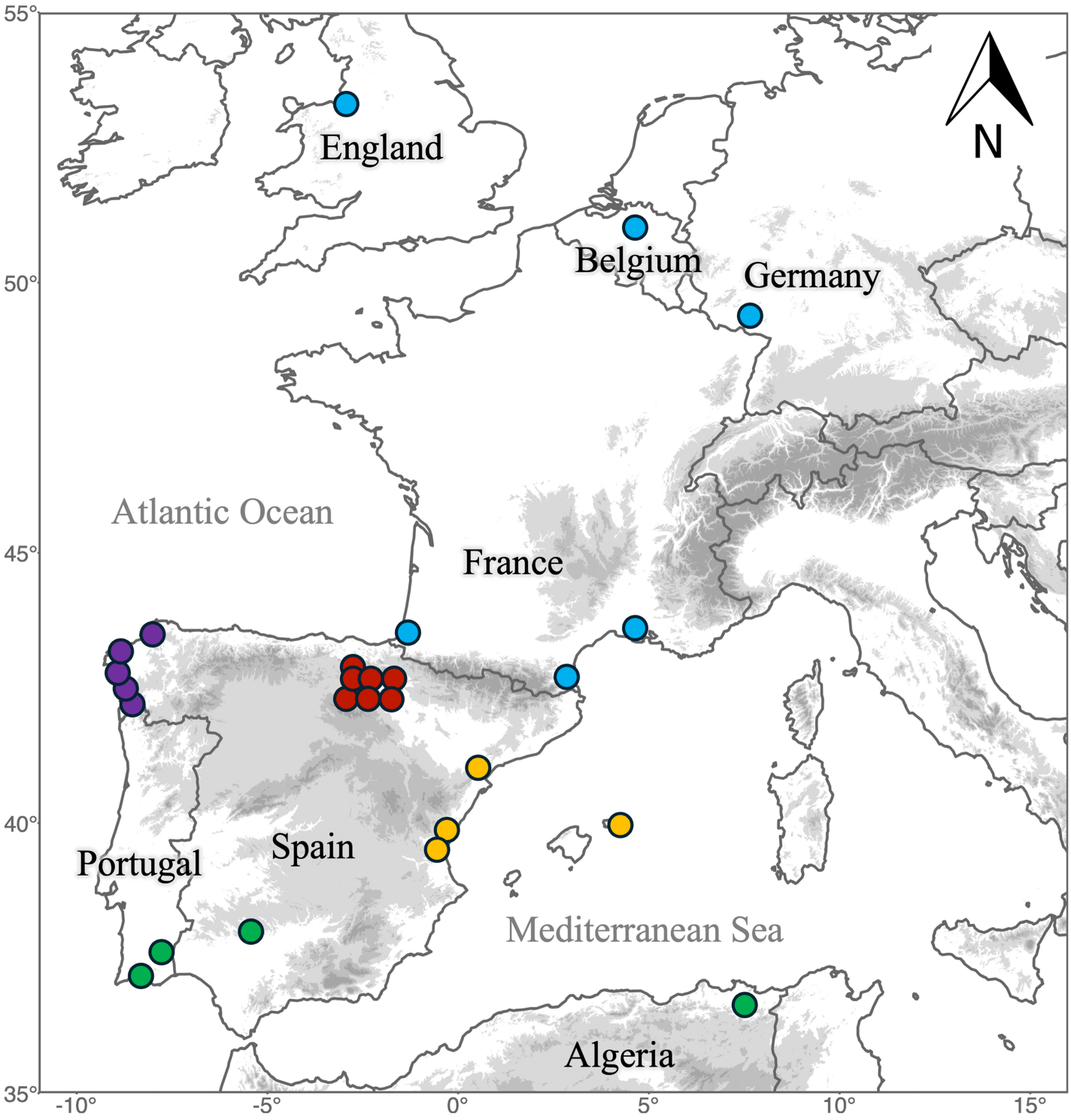



**Figure 1**

Geographic distribution of allopatric and hybrid sampling localities of *I. elegans*and *I. graellsii* across Europe and North Africa. Colors indicate population type: Green,allopatric *I. graellsii*; blue,allopatric *I. elegans*; yellow,South-east hybrid zone; purple,North-west hybrid zone; and red, North-central hybrid zone.

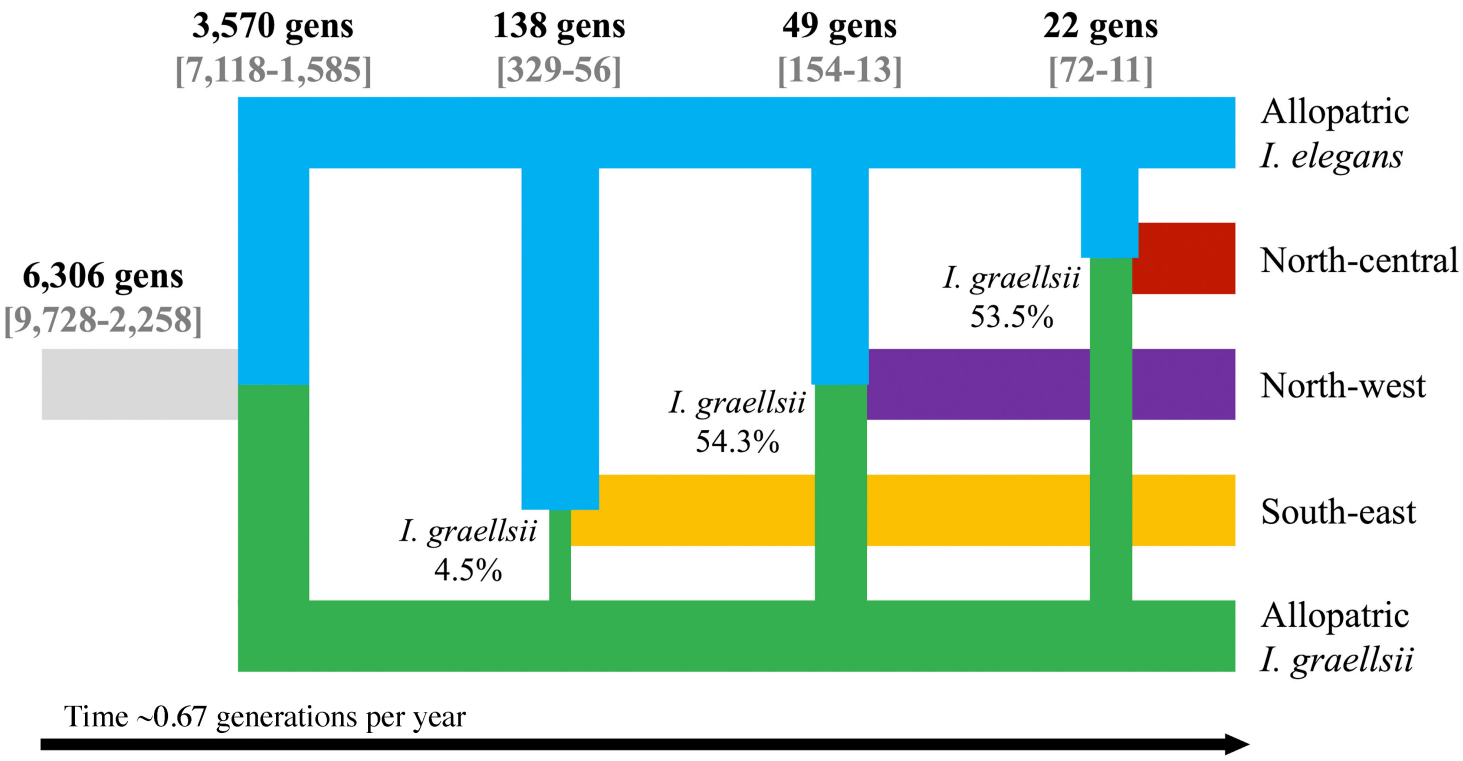



**Figure 2**

Best-supported demographic scenario inferred using DIYABC-RF for the formation of the three hybrid zones.Time is shown in generations before present, with the earliest time point representing a demographic expansion preceding the cessation of gene flow between lineages. The model supports independent origins for each hybrid zone, with the South-east hybrid zone forming first, followed by the North-west hybrid zone and then the North-central hybrid zone. Admixture proportions represent the genetic contribution of *I. graellsii* to each hybrid zone, with the remaining proportion corresponding to *I. elegans*.

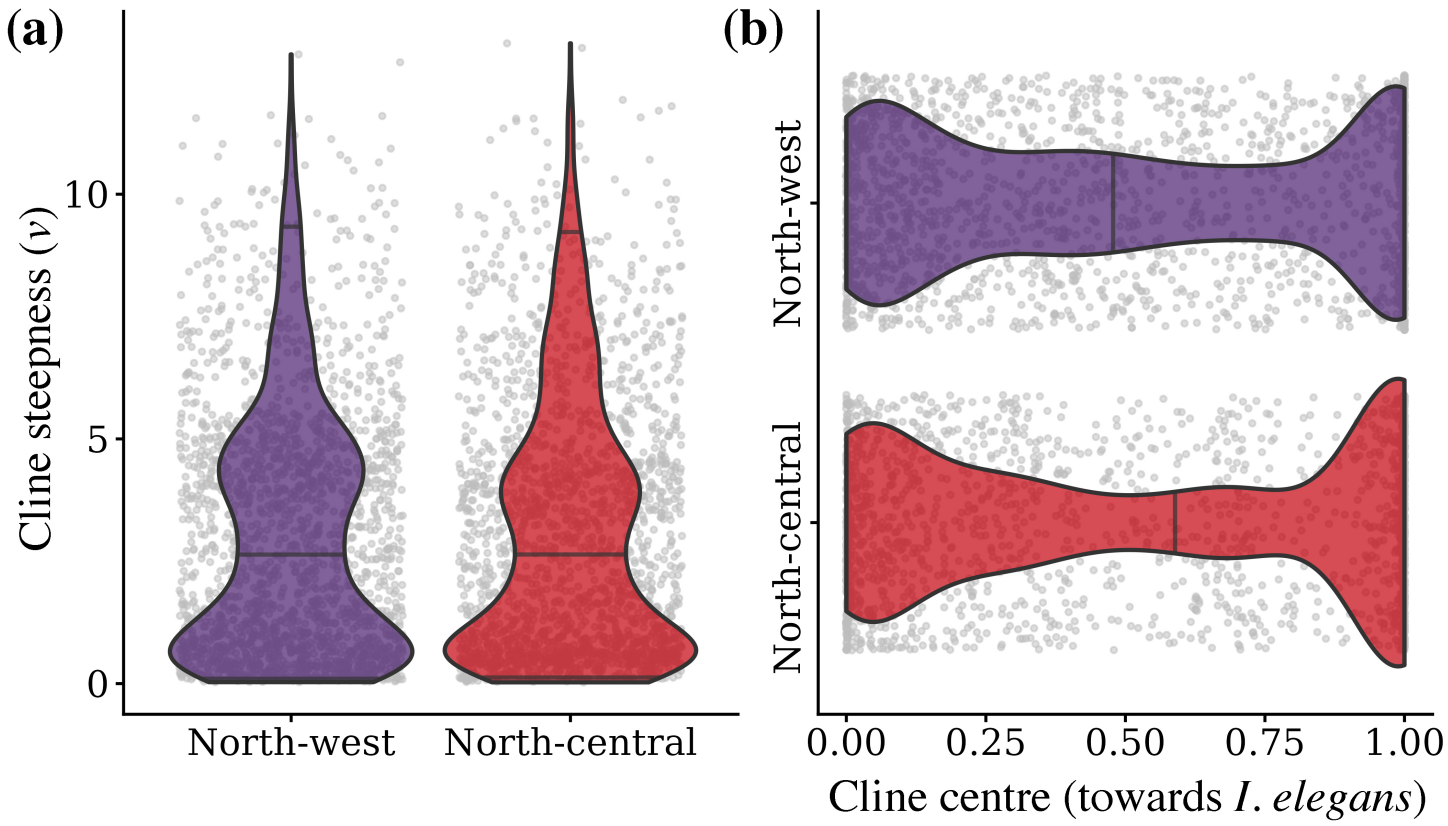



**Figure 3**

Genomic cline variation across hybrid zones in *I. elegans* and *I. graellsii*.Violin plots of cline steepness (a) and cline center (b) for the North-west and North-central hybrid zones based on the autosomal SNPdataset. Lines within each violin indicate the median and the 0.025 and 0.975 quantiles, and individual SNPs are shown in grey dots. Cline steepness reflects the strength of deviation from genome-wide neutral expectations of introgression, with higher values indicating steeper transitions across the admixture gradient. Cline center represents the position of allele frequency change along the hybrid index, indicating the direction of introgression between parental genomic backgrounds.

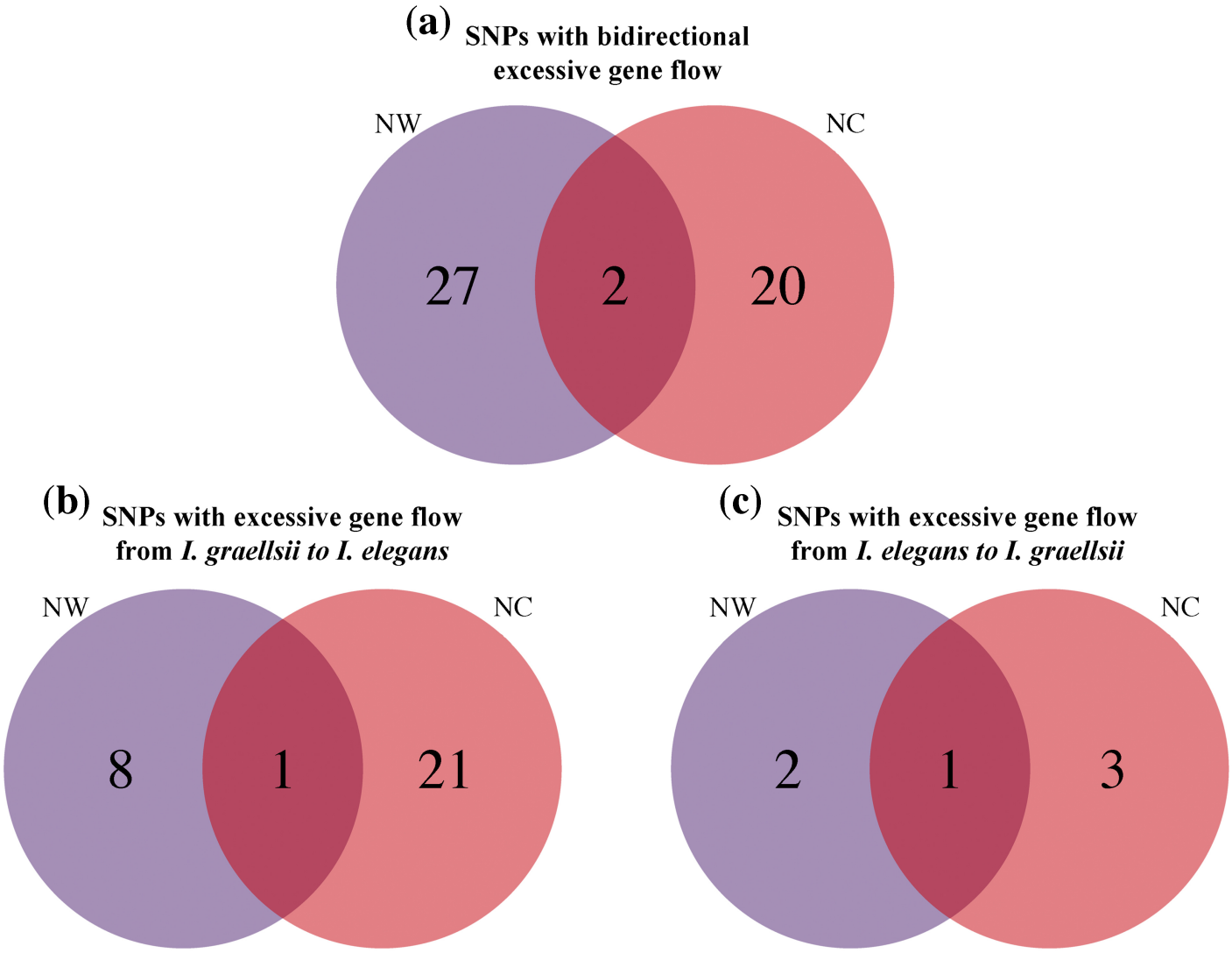



**Figure 4**

Asymmetric genomic introgression between *I. elegans*and *I. graellsii*. Outlier SNPs showing excessive bidirectional introgression (a) and excessive directional introgression between parental species, with (b) introgression of *I. graellsii* alleles into the *I. elegans* genomic background, and (c) introgression of *I. elegans* alleles into the *I. graellsii* genomic background.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- DemographySupplementaryMaterial.pdf
- DemographySupplementaryFile1.html