Article

Another Chapter in the History of the European Invasion by the Western Conifer Seed Bug, *Leptoglossus occidentalis*: The Iberian Peninsula

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Abstract: The Western conifer seed bug, *Leptoglossus occidentalis*, is native to North America and has already been considered a significant pest in several European countries since its first observation in Italy in 1999. In Spain and Portugal, it was recorded for the first time in 2003 and 2010, respectively, and its impact on Stone Pine (*Pinus pinea*) is of major concern. Before developing control measures for this insect pest, it is paramount to clarify its spatiotemporal dynamics of invasion. Therefore, in this study, we aimed to (a) characterise the genetic structure and diversity and (b) invasion pathways of *L. occidentalis* populations in the Iberian Peninsula. To do so, specimens of *L. occidentalis* were collected at fourteen sites widely distributed within the Iberian Peninsula. We used mtDNA sequences of Cytochrome b and eleven microsatellite markers to characterise the genetic diversity and the population structure in the Iberian Peninsula. Our genetic results combined with the observational dates strongly support a stratified expansion of *L. occidentalis* invasion in the Iberian Peninsula proceeding from multiple introductions, including at least one in Barcelona, one in Valencia, and one in the west coast or in the Southeastern region.

Keywords: invasion routes; population genetics; seed feeder; stone pine

1. Introduction

Non-native insect species are often cited as drivers of biodiversity loss and other ecological impacts with the potential to severely change the dynamics and function of the invaded communities [1–5]. Additionally, non-native species are responsible for significant economic losses [6]. However, through an unprecedented and rather uncontrolled expansion of worldwide trade and tourism, humans are increasingly facilitating species transportation outside their native range [7], and in some cases, within a novel range (e.g., [8–10]).

The Western conifer seed bug, *Leptoglossus occidentalis* Heidemann, 1910 (Hemiptera: Coreidae), originates from western North America. It was first spotted in Europe in

Italy in 1999 [11,12] but was rapidly recorded from almost the entire continent in the following decade [13]. In its native range, this polyphagous seed feeder is capable of damaging the seeds of developing and mature cones from a number of coniferous species, especially in the genera *Pinus*, *Pseudotsuga*, *Picea*, *Larix*, and *Abies* [14]. During its invasion of Europe, *L. occidentalis* expanded its host range to native conifer species belonging to the same genera [15], especially the stone pine, *Pinus pinea*, which has thus rapidly been colonized by this pest all over the pine's Mediterranean basin distribution, from Portugal to Lebanon [16–21], and in North Africa [22]. Besides its ecological and landscape values, the stone pine has an important economic value due to its edible seeds that can reach retail prices of around 100 EUR per Kg [23]. The vast majority of the world's producing countries are located in the Mediterranean Basin (e.g., Italy, Turkey, Lebanon, and Tunisia; [24]), with almost 70% of the plantations of *P. pinea* in the world located in the Iberian Peninsula (Portugal, and Spain), making the western Mediterranean region the major producer of this valuable seed product [25]. Therefore, it is not surprising that most of the studies targeting this pest in the Mediterranean Basin are focused on *P. pinea*, highlighting its economic damage [16,17,21,26–28]. Identifying source populations and clarifying invasion pathways would increase our understanding of how this non-native species spread within the novel range, which is needed to complement management strategies [29].

Inferring invasion routes and source populations of non-native species is a key first stage for deciphering their population dynamics and ecological characteristics in the new environment. Historical and observational data on the spread of invasive populations, when complemented with genetic data, can lead to important insights into the sources, routes, and mechanisms involved in the invasion [30]. Thus, indirect methods, such as molecular markers, have proven to be relevant in helping to decipher the invasion routes of many species [10,31–34]. However, tracing invasion routes of the Western conifer seed bug is a complex task, primarily due to its high flight capacity, which allows a rapid range of expansion [35]. Furthermore, the complexity of tracing its invasion routes increases as adults aggregate during the autumn in human-made structures or in forest refuges, such as under the bark of coniferous trees [35]. This behaviour can favour the mixing of individuals from different populations, which may lead to a genetic homogenisation of the populations making it difficult to reconstruct the history of the invasion [36].

A recent genetic study of the invasion routes of *L. occidentalis* in Europe [13] showed that it was a two-step process. Populations from the native western North America were introduced to the eastern part of North America, from which multiple, independent introductions to Europe occurred, acting as a bridgehead (i.e., an invasive population serving as a source for others; [31]). These introductions, combined with the translocation of individuals within the European continent through human-mediated transportations as hitchhikers, were also suggested to have favoured the insect's rapid colonization [13]. The specific biological traits of this species, such as the strong flight capacity of the adults, its voltinism, and its polyphagous habits, probably contributed to accelerating the spread. Regarding the Iberian Peninsula, Lesieur et al. [13] presented scenarios for two separate introduction events to Spain: the first one in Barcelona, most probably coming directly from eastern North America, and the second one in Valencia, most likely resulting from an admixture event between an invasive European population from northern Italy and an eastern North American population. Nevertheless, only two populations from the Iberian Peninsula were included in the study. The invasion scenario of the entire region still needs to be updated, considering the high economic value of the main attacked host, *P. pinea*, in this region. Deciphering the origin of the Iberian populations from the first European sites recorded in Spain (i.e., Barcelona and Valencia), as well as the invasion routes in the Iberian Peninsula, may contribute not only to finding susceptible points of entry of this pest but also in understanding its population dynamics, leading to a more efficient monitoring and control strategy in the future. Furthermore, assessing the invasion pathways of such species will help identify the factors driving invasions in order to develop appropriate management strategies to avoid further introductions and the spread of non-native species [10,37,38].

In this study, we aimed to understand the spatial and temporal invasion patterns of *L. occidentalis* in the Iberian Peninsula. Using mitochondrial and microsatellite markers, we analysed samples of the Western conifer seed bug from the Iberian Peninsula coupled with a set of selected populations issued by Lesieur et al. [13]. By adding these populations to our dataset, we intended to assess whether (i) more than two introduction events happened in the Iberian Peninsula and (ii) whether the Western conifer seed bug spread within the Peninsula through local diffusion, long-dispersal, or both.

2. Methods

2.1. Sampling and DNA Extractions

Samples were collected from 14 sites distributed in the Iberian Peninsula between 2011 and 2015 (Table 1). The population dynamics of this species are highly variable, leading to high population densities in one year and very meagre densities in the following year. In fact, it was not possible to capture many individuals in the south of Portugal (Faro) or even in the south of Spain (Huelva, Cadiz) due to constant low population densities in the field during the years of collection. The insects were hand-picked from the tree canopy by a maximum of three adult individuals per tree in order to avoid inbreeding. They were immediately put into individual vials with absolute ethanol and stored at −21 °C until DNA extraction.

Table 1. Sampling information and genetic diversity (mtDNA and microsatellite markers) of native and invasive populations of the Western conifer seed bug, *Leptoglossus occidentalis*, based on the current study and Lesieur et al. [13].

Origin	Region	Latitude	Longitude	Year of Collection	Host	mtDNA				Microsatellites			
						N	Haplotype	Hd	π	N	He	Ho	AR	PAR
Eastern America	Montreal, Canada	45.562	−73.563	2011		5	H20 (1); H51 (3); H27 (1)	0.700	0.004	14	0.540	0.519	2.88	0.636
	Pittston, USA	44.222	−69.756	2011		7	H20 (5); H51 (2)	0.536	0.002	25	0.568	0.524	2.88	0.909
Europe	Yvoy-le-Marron, France	47.632	1.854	2012		5	H5(2); H20 (1); H51 (2)	0.800	0.003	31	0.623	0.508	2.96	0.091
	Lavercantière, France	44.637	1.318	2011		7	H5(2); H23 (1); H51 (4)	0.667	0.003	30	0.606	0.547	2.91	0.182
	Serre-Ponçon, France	44.523	6.332	2011		5	H20 (2); H51 (3)	0.600	0.003	29	0.571	0.468	2.89	0.000
	Alessandria, Italy	44.897	8.406	2011		5	H20 (3); H23 (2)	0.600	0.003	26	0.603	0.531	2.91	0.182
Iberian Peninsula	**Barcelona, Spain** *	**41.520**	**1.687**	**2012**		**5**	H23 (1); H51 (4)	**0.400**	**0.002**	**20**	**0.491**	**0.486**	**2.48**	**0.091**
	Valencia, Spain *	**39.447**	**−0.463**	**2012**		**6**	H20 (4); H51 (2)	**0.533**	**0.002**	**20**	**0.487**	**0.385**	**2.51**	**0.000**
	Almeria, Spain	37.712	−2.172	2012		8	H20 (8)	0	0	14	0.386	0.392	2.08	0.000
	Burgos, Spain	42.672	−3.417	2015	*P. pinaster*	3	H20 (1); H51 (2)	0.667	0.003	25	0.576	0.475	2.88	0000
	Galicia, Spain	42.364	−8.622	2015	*P. pinaster*	4	H5 (1); H23 (1); H51 (2)	0.833	0.004	30	0.607	0.528	2.96	0.000
	Lleida, Spain	42.637	1.247	2013		5	H20 (2); H23 (1); H51 (2)	0.800	0.003	20	0.603	0.468	2.97	0.000
	Salamanca, Spain	40.451	−6.19	2015	*P. pinea*	2	H5 (1); H20 (1)	1.000	0.003					
	Segovia, Spain	40.902	−4.007	2012		11	H5 (2); H20 (6); H51 (3)	0.733	0.003	14	0.564	0.410	2.84	0.000
	Soria, Spain	41.536	0.023	2013		3	H20 (2); H51 (1)	0.667	0.003	12	0.564	0.410	2.55	0.182
	Valladolid, Spain	41.304	−5.233	2015	*P. pinea*	6	H20 (5); H23 (1)	0.333	0.001	14	0.555	0.413	2.77	0.000
	Murcia, Spain	37.826	−1.016	2013		3	H20 (3)	0	0					
	Islas Cies, Spain	**42.227**	**−8.905**	**2012**		**2**	**H20 (2)**	**0**	**0**					
	Huelva, Spain	37.211	−6.563	2012		1	H20 (1)	-	-					
	Aveiro, Portugal	40.674	−8.727	2015	*P. pinaster*	3	H20 (2); H51 (1)	0.667	0.003	23	0.596	0.437	2.96	0.000
	Coruche, Portugal	38.961	−8.527	2015	*P. pinea*	5 + **3**	H5 (1); H20 (3 + **2**); H51 (1 + **1**)	0.700	0.003	27	0.615	0.506	3.02	0.182
	Faro, Portugal	37.236	−7.922	2015	*P. pinaster*	4	H51 (4)	0	0	4[+]	-	-	-	-

For each sampling location, we have indicated the region, site, host species, sample size (N), haplotype frequency (Hd), nucleotide frequency (π), mean expected heterozygosity (He), mean observed heterozygosity (Ho), allelic richness adjusted to a minimum sample size of six (AR), and private allelic richness (PAR) estimated after a rarefaction procedure. Bold corresponds to samples added from Lesieur et al. [13]. * Invasive populations shown to have been introduced directly from eastern North America [13]. [+]Used only for STRUCTURE analysis.

DNA was extracted from one or two legs of the adults using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The DNA was eluted in 200 μL of AE buffer. The numbers of individuals per population used for sequencing (mtDNA) and genotyping (microsatellites) are presented in Table 1. Briefly, to infer the genetic diversity and invasion routes of *L. occidentalis* populations in the Iberian Peninsula, the sequences data obtained by Lesieur et al. [13] were downloaded from GenBank (accession numbers: MG251986; MG252001; MG252004; MG252032), and added to the dataset for joint analyses (Table 1—populations in bold typeface). Moreover, we included eight populations from Lesieur et al. [13] in our microsatellite dataset: two populations from eastern North America (Montreal and Pittston) which were suggested to be the putative sources of the European invasion, four populations of the European-invaded areas (Yvoy-le-Marron, Lavercantière and Serre-Ponçon from France, and Alessandria from Italy), and two populations from the Iberian Peninsula which were suggested to have been introduced from eastern North America (Barcelona and Valencia).

2.2. mtDNA Sequencing and Analyses

We amplified a fraction of the cytochrome b for 60 individuals by a polymerase chain reaction (PCR) using the PCR reaction mix and procedure described in Lesieur et al. [13]. PCR products were analysed by gel electrophoresis in a 1.5% agarose gel to check for successful amplification. All PCR products were purified with the NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany) and were sequenced with the primer pair CP1 [39] and CB2 [40]. Sequencing was performed using the Big Dye Terminator Cycle Sequencing Kit (v. 3.0, Applied Biosystems, Foster City, CA, USA) and carried out on an ABI Prism 3500 Genetic Analyzer (Applied Biosystems). All sequences were obtained in the forward and reverse directions. Forward and reverse sequences were assembled and checked manually using CodonCode Aligner V.3.7.1 (CodonCode Corporation, Centerville, MA, USA). Following assembly, the sequences were aligned using CLUSTAL W [41] implemented in BioEdit 7.05. All sequences were truncated to the same length (662 bp) and did not present any insertion or deletion. The absence of a stop codon was checked with MEGA v.6 [42].

The number of haplotypes, haplotype diversity, and nucleotide diversity was obtained using DNAsp 5.0 [43]. Statistical parsimony networks with a 95% confidence level were computed with TCS v. 1.21 [44]. Haplotype distribution and frequency in the invaded range were projected onto a map of Europe using ArcGis v. 10.8 (ESRI, Redlands, CA, USA). Finally, to evaluate the variation in genetic diversity at different hierarchical levels and between different groups of populations, we used the analysis of molecular variation (AMOVA; [45]). We conducted two analyses for which we grouped the populations according to (i) their geographic proximity or (ii) the year of first observation for our populations of interest. All AMOVAs were performed with Arlequin v 3.5 [46] and tested with 50,000 permutations.

2.3. Microsatellite Genotyping and Analyses

A total of 179 individuals from the Iberian Peninsula were genotyped at the 11 microsatellite loci developed for *L. occidentalis* by Lesieur et al. [47]. PCR amplifications were conducted following the protocol described by Lesieur et al. [47]. The obtained PCR products were run in an ABI 3500 Genetic Analyzer using the size standard GeneScan-600 LIZ (Applied Biosystems). The program GeneMapper v. 4.1 (Applied Biosystems) was used to score the alleles. To be able to compare the two datasets, we used samples already analysed by Lesieur et al. [13] as controls in our genotyping procedure.

First, we assessed the genetic diversity and relationship between populations. Linkage disequilibrium was tested in each population for all pairs of loci with 10,000 permutations using ARLEQUIN 3.0 [48]. A significant deviation from the Hardy–Weinberg equilibrium was tested using GENALEX 6.41 [49] for each locus and population, with 1000 permutation steps and 100,000 steps in the Markov chain. Sequential Bonferroni corrections [50] for

multiple comparisons were applied to both tests. Allelic richness (AR) and frequencies, as well as observed and expected heterozygosity (Ho and He), were calculated for each locus and the population using GENALEX 6.41 [49]. The richness of the private allele (PAR) was calculated in HP-RARE using the rarefaction procedure [51], which compensated for the increase in the likelihood of sampling rare alleles as the sample size increased. FreeNA [52] was used to estimate the null alleles (NA) for each locus and in each population using the expectation-maximisation algorithm and to calculate pairwise F_{ST} values using the null allele correction. The correlation between geographic distances and pairwise F_{ST} values was tested with a linear regression model with the function *lm* from the stats package implemented in R software [53], both for the full invaded range and only the Iberian region.

To assess the population genetic structure, a discriminant analysis of the principal component (DAPC) [54] was computed using the adegenet package [55] with the entire dataset and then considering only the Iberian populations. Then, a population-based neighbour-joining (NJ) tree was reconstructed using ape [56] and hierfstat [57] packages. In a second step, we used a Bayesian clustering approach implemented in the program STRUCTURE 2.3.1 [58] to estimate the number of genetic clusters represented in the dataset and, in this way, attempted to disentangle the genetic structure over the sampled area. We used 200,000 burn-in steps followed by 1,000,000 MCMC simulation steps with the number of clusters (K) varying from 1 to 10 and a model allowing admixture in all runs. To ensure the consistency of the results, 20 independent runs for each value of K were performed. The optimal number of clusters (K) represented by the data was determined with the method described in Evanno et al. [59], implemented in STRUCTURE HARVESTER [60]. The modal result among runs was checked for each K by using the Greedy algorithm in Clumpp [61] with the online pipeline CLUMPAK [62]. The graphical display of the genetic structure was visualised with Distruct v.1.1 [63].
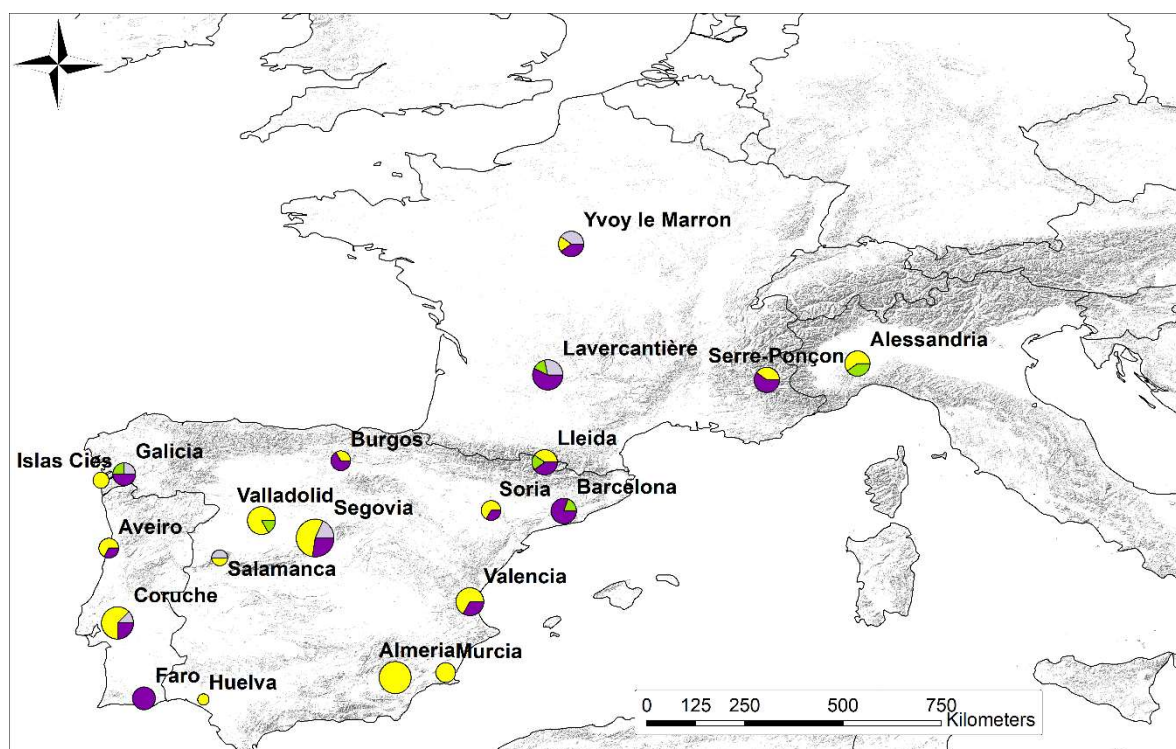
3. Results

3.1. mtDNA

In total, 60 individuals of the Iberian Peninsula from 13 localities were successfully sequenced, while 48 sequences were downloaded from GeneBank. The whole dataset corresponded to 16 localities in the Iberian Peninsula: one locality from Italy, three localities from France, and two localities in eastern North America. Overall, four haplotypes were detected in the Iberian Peninsula (Figure 1). In the whole dataset, the most frequent haplotypes were H20 and H51 (51% and 35% of the individuals, respectively). However, H20 was the most frequent among the Iberian populations (57%), whereas H51 presented the highest frequency in the rest of the European populations studied (41%). The fifth haplotype (H27) was found only in one individual from Montreal, eastern North America (Table 1). The overall haplotype and nucleotide (π) diversity were estimated at 0.613 and 0.0026, respectively, with the four regions, eastern North America, France, Italy, and the Iberian Peninsula, presenting similar mean values. Five populations from the Iberian Peninsula had a haplotype diversity of zero.

The AMOVA results did not support a clear genetic structure within the invasive populations (Supplementary Material Table S1). The largest amount of genetic differentiation was found within populations for both analyses (78% and 80%, respectively). Genetic differences among the groups (9% and 5%, respectively) and among populations within groups (13% and 15%, respectively) accounted for a smaller percentage of genetic variation.

Figure 1. Geographic distribution for *Leptoglossus occidentalis* populations of the mitochondrial haplotypes in Iberian Peninsula, France and Italy, based on the results of the current study and Lesieur et al. [13]. The different colours represent the haplotypes identified by Lesieur et al. [13] with circle size being proportional to the number of individuals. Known years of first observation: 1999: Alessandria; 2003: Barcelona; 2006: Serre-Ponçon, Lavercantière; 2008: Yvoy-le-Marron, Segovia, Almeria; 2009: Valencia, Burgos; 2010: Galicia, Coruche.

3.2. Microsatellite Data

In total, we genotyped 11 microsatellite loci and 183 sampled individuals collected from 10 Iberian localities for this study and included 195 individuals from eight localities genotyped by Lesieur et al. [13] (Table 1). The overall genotyping success was high (96.85%), with a mean of 10.64 alleles per locus. Linkage disequilibrium analysis revealed five cases of significant disequilibrium in the 605 pairwise tests, but a given pair of loci was never in a significant linkage disequilibrium more than two times. Thus, the 11 microsatellite markers were considered independent. The average proportion of null alleles for each locus was below 5% except for four loci, Lep04, Lep05, Lep31, and Lep36, which had a mean estimated proportion of null alleles above 8%. All populations were in the Hardy–Weinberg equilibrium (HWE) for all loci after the Bonferroni correction for multiple comparisons, except in eight out of 99 combinations of population x locus (Lep04 in Lleida, Galiza, Aveiro and Coruche; Lep36 in Segovia and Aveiro; MSLO07 in Lleida, and MSLO15 in Coruche). However, in global tests across all loci, we did not find populations deviating significantly from HWE. Therefore, the following analyses were performed with and without the loci Lep04 and Lep36 to ensure more robust and unbiased results. As the results were similar in both cases, only the results with the entire dataset are presented.

Overall, populations from the Iberian Peninsula presented the lowest and the highest allelic richness values in Almeria (2.08) and in Coruche (3.02), respectively (Table 1). However, the highest private allelic richness values were found in eastern North American populations (0.636 and 0.909) and the lowest (0.00) in nine populations of the invaded range. The observed heterozygosity (Ho) ranged from 0.385 to 0.528, and the expected heterozygosity (He) from 0.386 to 0.615. The lowest pairwise F_{ST} value (0.001) was found between Segovia and Burgos populations, and the highest (0.273) between Almeria and

Montreal populations (Table 2). Among the Iberian Peninsula, all populations showed high F_{ST} values, with the Montreal population (0.089–0.273). Almeria was the most differentiated population presenting high F_{ST} values with all populations except Soria and Valladolid. Barcelona presented high F_{ST} values with all Iberian Peninsula populations, ranging from 0.081 to 0.254. Finally, we did not find any correlation between pairwise F_{ST} and geographic distances for the European invasive populations (Supplementary Material Figure S1).

Table 2. Estimates of pairwise F_{ST} for *Leptoglossus occidentalis* populations.

Populations	E. America Montreal	Pittston	Italy Alessandria	France Yvoy-le-Marron	Laverc.	Serre-Ponçon	Spain Barcelona	Lleida	Soria	Valencia	Almeria	Burgos	Valladolid	Segovia	Galicia	Portugal Aveiro
Montreal	-															
Pittston	0.039	-														
Alessandria	0.072	0.034	-													
Yvoy-le-Marron	0.076	0.035	0.040	-												
Lavercantière	0.079	0.030	0.034	0.004	-											
Serre-Ponçon	0.073	0.029	0.036	0.014	0.018	-										
Barcelona	0.089	0.061	0.100	0.104	0.086	0.070	-									
Lleida	0.130	0.050	0.072	0.029	0.044	0.039	0.081	-								
Soria	0.155	0.076	0.109	0.076	0.104	0.063	0.151	0.066	-							
Valencia	0.128	0.035	0.075	0.090	0.094	0.091	0.141	0.096	0.090	-						
Almeria	0.273	0.164	0.203	0.178	0.205	0.180	0.254	0.147	0.070	0.149	-					
Burgos	0.111	0.038	0.057	0.020	0.035	0.032	0.112	0.022	0.059	0.046	0.130	-				
Valladolid	0.167	0.100	0.112	0.066	0.094	0.079	0.171	0.048	0.028	0.112	0.091	0.043	-			
Segovia	0.124	0.042	0.085	0.033	0.050	0.037	0.123	0.016	0.048	0.063	0.121	0.001	0.025	-		
Galicia	0.120	0.041	0.061	0.019	0.030	0.024	0.113	0.020	0.052	0.068	0.135	0.005	0.044	0.011	-	
Aveiro	0.106	0.039	0.044	0.020	0.032	0.025	0.117	0.025	0.054	0.062	0.140	0.009	0.056	0.013	0.003	-
Coruche	0.125	0.051	0.050	0.027	0.040	0.036	0.128	0.023	0.060	0.084	0.136	0.026	0.047	0.030	0.023	0.010

The DAPC showed that the four geographical groups (i.e., eastern North America, Italy, France, and the Iberian Peninsula) were genetically close, with some individuals from the four different groups overlapping (Figure 2). When considering only the Iberian Peninsula populations, Barcelona and Almeria clustered alone, whereas most of the individuals from the other populations overlapped (Supplementary Material Figure S2). The phylogenetic tree of populations was partly congruent with the DAPC results, with Almeria differentiating from all other populations, Barcelona differentiating from the Iberian Population but grouping with the eastern North American populations, and the French populations forming a monophyletic clade (Supplementary Material Figure S3).

All individuals were analysed using STRUCTURE software to decipher the populations' structure further. The results were consistent with the previous analyses. Evanno's method highlighted three main potential genetic clusters, with a smaller distinctive peak at K = 8 (Supplementary Material Figure S4). For K = 2, all the Iberian populations but Barcelona clustered together, whereas the French populations showed signs of admixture (Figure 3; Supplementary Material Figure S5B). For K = 3, two of the clusters were more defined than the third one. The eastern North American populations clustered together with Alessandria and Barcelona, and the populations of Almeria, Valencia, Soria, and Valladolid grouped together. Populations belonging to the third cluster were less clear but included the French populations and western Iberia (Supplementary Material Figure S5A). At K = 8, Barcelona, Almeria, and Montreal were clustering alone.

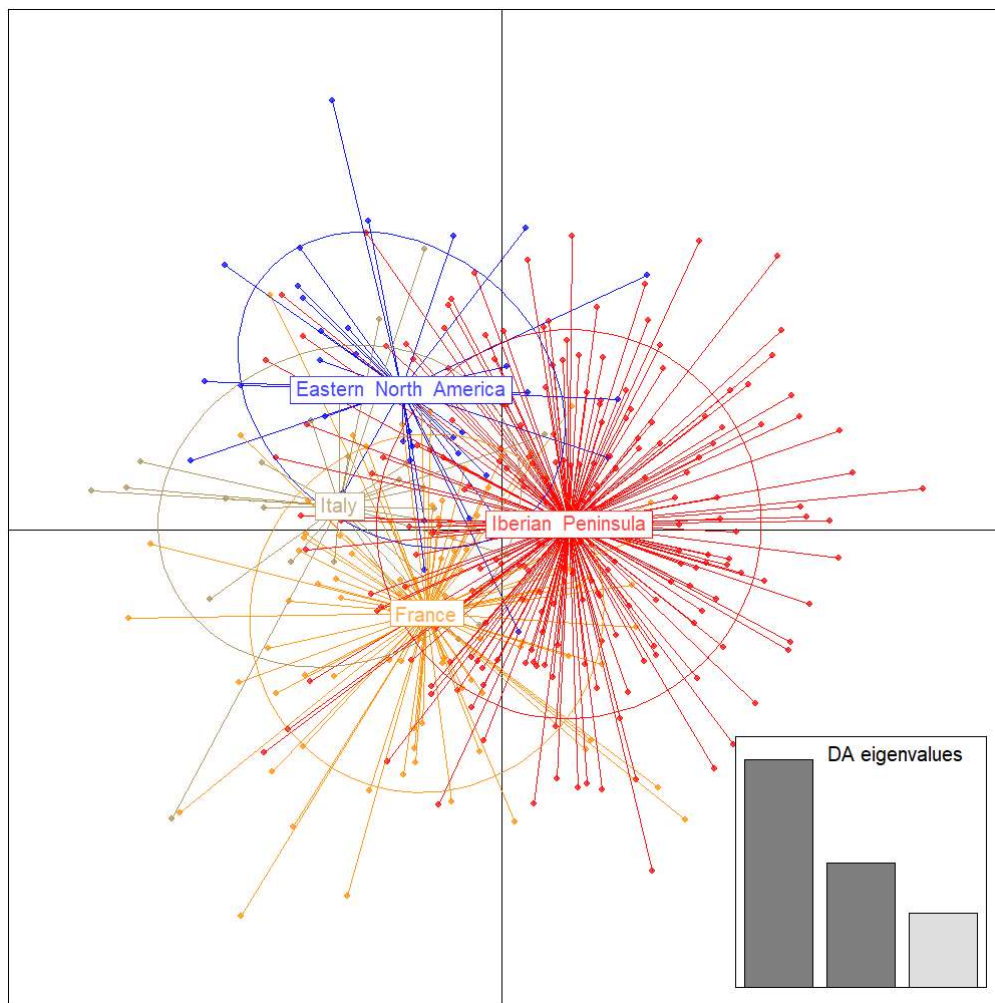**Figure 2.** Discriminant analysis of principal component (DAPC) for *Leptoglossus occidentalis* populations.
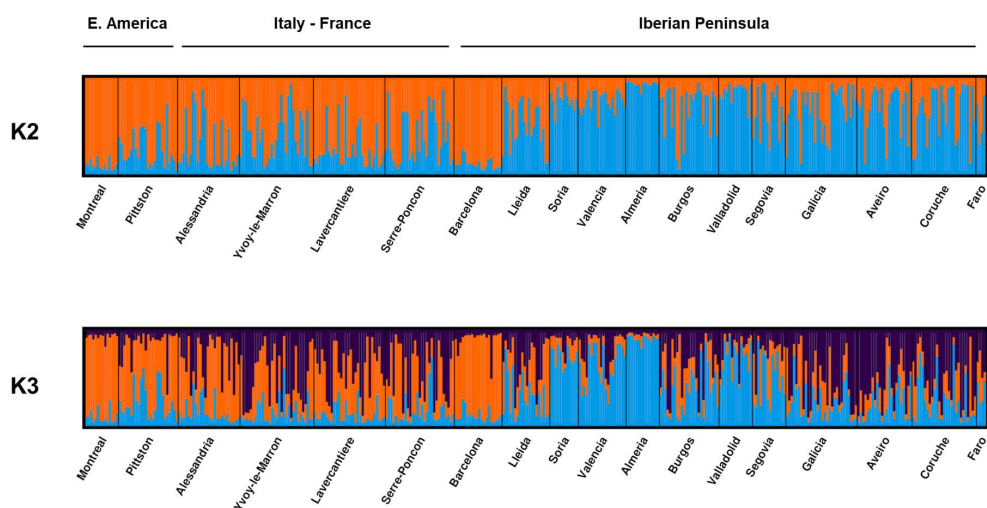

Figure 3. Graphical representation of *Leptoglossus occidentalis* population genetic structure estimated using a Bayesian clustering approach implemented in STRUCTURE for K = 2, the best K was determined by the Evanno method, and K = 3. The populations from eastern North America (E. America), Italy, France, Barcelona, and Valencia in Spain were incorporated into the dataset from Lesieur et al. [13]. Each individual is represented by a vertical line, and each colour represents a particular genetic cluster.

4. Discussion

By combining occurrence records with mtDNA and microsatellite data, we propose a scenario for the invasion dynamics of *L. occidentalis* in the Iberian Peninsula. The important genetic diversity found within the region tends to indicate multiple introductions. Based on the genetic analysis of a very limited number of Spanish populations (Barcelona and Valencia), Lesieur et al. [13] suggested the occurrence of two independent introductions in the Iberian Peninsula. Our results, relying on a much larger sampling in this region, suggest, at least, a third introduction in the south or west of the Peninsula and a mix of human-mediated and natural dispersal in the spread of the Western conifer seed bug in the Iberian Peninsula.

4.1. Genetic Diversity and Structure of the Iberian Populations

The observation of the identical haplotypes in Iberia and in the rest of Europe suggests that no novel introduction occurred in the Iberian Peninsula directly from western North America: the native region of *L. occidentalis*. However, all the haplotypes found previously in Europe and in eastern North America [13] were also observed in the Iberian Peninsula, together with a high nucleotide diversity and allelic richness showing no loss of genetic diversity in this region. The populations of non-native species are traditionally thought to experience a significant reduction in genetic diversity relative to their source populations due to founder effects and the arrival of only a few individuals during the introduction stage of the invasion [64]. However, more and more studies using neutral molecular markers are showing that such species do not necessarily present a significant loss of genetic diversity, often resulting from multiple introductions in the novel range or a single introduction of a large number of individuals [10,65–69].

The studied invasive populations of *L. occidentalis* revealed a genetic structure that could be partly explained by geographic proximity. Populations from eastern North America, Italy, France, and Barcelona (i.e., the northeastern part of the Iberian Peninsula) clustered together, whereas the rest of the Iberian Peninsula was divided into two other genetic clusters weakly linked to geography. One cluster was formed by all populations from the west coast (Aveiro, Coruche, Galicia, Faro), characterised by low F_{ST} values and high allelic richness suggesting the existence of gene flow between these populations. The other Iberian cluster was split into two groups which were not so clearly separated geographically and presented signs of admixtures. The first group had a more southernly distribution which included the southern districts of Almeria and Valencia but also Valladolid in the centre and Soria in the east. The other group had a more North and East distribution comprising Burgos and Lleida but also the central district of Segovia. Barcelona and Lleida, which are only a little over 150 km apart, did not clearly group together despite Lleida showing signs of admixture, and Barcelona formed its own cluster in the Iberian Peninsula. Lesieur et al. [13] considered the strong differentiation of the Barcelona population from the other European populations analysed to be the result of an independent introduction from eastern North America. Among the Iberian populations here analysed, Barcelona still forms a robust differentiated cluster which indicates possible spatial isolation of this population as suggested by the lower suitability of the Ebro Valley [70] and the barrier of the Pyrenees mountains on the North. The fact that the first record of *L. occidentalis* in Barcelona was in 2003, before all the other Iberian regions (between 2008 and 2010), suggests that this isolation has been maintained over time. Finally, the population of Almeria in Spain was an exception to the average genetic diversity of the Peninsula as it presented very low values. All individuals had the same haplotype (H20), and we found the lowest values of heterozygosity, allelic richness, and highest F_{ST} values in microsatellite loci. This pattern suggests the occurrence of a genetic bottleneck linked to a solid demographic decrease. This decline could be explained by either the introduction of very few individuals through human activities or environmental conditions that reduced the population to a few individuals. Indeed, southern Iberia presented low suitability for *L. occidentalis* survival due to high temperatures in climatic niche models [70] and low insect densities. In light of our

results, we cannot, however, exclude the possibility that the low genetic diversity observed in the results of a strong selection is due to the environmental conditions that have led individuals to be very well adapted to the southern climate.

4.2. Routes of Invasion in the Iberian Peninsula

If we only consider the dates of the first records, the species was first spotted in Barcelona in 2003 [71], while all the other first records in the Iberian Peninsula were grouped between 2008 and 2010 [72–76]. Such a difference between the first Iberian occurrence and most of the other detections without intermediate occurrences raises doubts on the hypothesis of a continuous geographic dispersal from the source population of Barcelona. Furthermore, our genetic data gave arguments to refute this hypothesis.

Our microsatellite analyses detected three defined clusters, including Barcelona, in the Iberian Peninsula with the existence of admixture but without any isolation by distances. This, together with the different first observational dates and the absence of a genetic diversity point in the occurrence of multiple introductions in the Iberian Peninsula, corresponds with at least a likely third introduction into the region. Multiple introductions are a common phenomenon in the history of the invasion of many non-native species [66,77–83] and were already proven to have occurred in Europe for the Western conifer seed bug [13]. In fact, the populations from Barcelona and Valencia were shown to likely result from two independent introductions in Iberia with divergent source populations [13]. The strong genetic differentiation between Barcelona and all the other Iberian populations tended to show that this population had been isolated from the rest of the Peninsula and that the colonization of Iberia could not result from a natural expansion from Barcelona. Furthermore, a natural expansion would mean a loss in genetic diversity [29,64] in the other Iberian populations, which was not detected in both the mtDNA and microsatellite data but also in the gene flow between close populations.

The second identified introductory point, Valencia (in 2008), populations are genetically closer to the other southern and central Spanish ones (Almeria, Valladolid, Soria). Thus, these latter populations could have resulted either from the local dispersal of the insect or from the introduction of individuals from the Valencia region. Historical data revealed that the first records of *L. occidentalis* occurred at the same time (2008) in localities of the South (Almeria) and centre (Segovia) of the Peninsula, which is distant by more than 400 km. Which population corresponds to the source population is difficult to assess. However, relying on observational dates, *L. occidentalis* was likely transported within the central region (south and centre) thanks to human activities. The strong genetic bottleneck observed in Almeria suggests that the insect was also likely introduced through human activities, even though we cannot rule out the possibility of natural dispersal. Adults present a good flight capacity [84] estimated at 20 km.day-1 maximum in laboratory studies [35]. However, this insect usually flies at great distances only at specific times, such as in the autumnal aggregation when searching for winter shelter. This period probably offered a too-narrow window for *L. occidentalis* populations to invade the whole of Iberia (approximately 1000 × 800 km) by natural and continuous dispersal.

The third genetic cluster identified in the Iberian Peninsula, the western coast, sheds light on a likely third introduction originating either from eastern North America, from the rest of invaded Europe, from within the Iberian Peninsula, or a mix of them. The populations of Coruche, Aveiro, Galiza, and Burgos had low F_{ST} between them. These populations presented a high genetic diversity both at the mtDNA and microsatellite level, with Coruche presenting the highest values in our entire dataset. Moreover, the occurrence of the haplotype H5 in the western Iberian Peninsula supports this hypothesis. The haplotype has been found only in Europe so far, with only one previous record from the central part of the Iberian Peninsula [13], whereas we found it in four different populations, including three from the coastal region. Moreover, given that the first occurrence records in the coastal region were only one to two years later than those in the North of Iberia, and the southern part, respectively, a natural expansion from these regions would be very

unlikely. However, the relatively low values of F_{ST} and the joint clade of west Iberia with France for K = 3 in the whole dataset could also suggest that France is the origin of the source population.

4.3. Cone Trade Market and the Invasion

Leptoglossus occidentalis has frequently been spotted on cones of stone pine, *Pinus pinea* [16,17,20,21]. World market demand for *P. pinea* nut kernels moves several hundred million euros annually [85,86]. The Iberian Peninsula produces nearly 70% of the world's traded nuts [23]. Cones are collected from December to April and are then sold to processing plants to be opened and commercialised. Sightings of adult insect populations in pinecone processing industrial units are very frequent (A. Farinha personal observation). A high volume of cones is transported between the leading producer countries, including Portugal, Spain, Italy, and Turkey, to be processed and sold [87]. Therefore, it is fairly probable that the insects may have been transported as hitchhikers by these intensive translocations, justifying both the rapid rate of invasion in the Peninsula and the high genetic diversity of Iberian invasive populations. In addition, the pine timber trade, as well as the Christmas tree trade, cannot be discarded as another source of hitchhiking for *L. occidentalis* [88]. Multiple introductions of individuals from genetically divergent populations result in high levels of intraspecific hybridisation (i.e., admixture), which can confer survival advantages [89] and the greater adaptation of the admixed individuals.

Human activities, namely the intensive cone trade and other commercial exchanges between European countries, may be the prominent factor responsible for long-distance *L. occidentalis* dispersal in the Iberian Peninsula. Stratified dispersal is probably affecting this pest population dynamics, increasing its capacity to disperse and ultimately leading to high levels of admixture, which, in turn, are likely to increase the probability of adaptation to new environments, potentially increasing the invasiveness and economic impact of this pest.

5. Conclusions

Our study provides evidence of multiple introduction events of the Western conifer seed bug in the Iberian Peninsula, which were likely favoured by human activities. This genetic evidence combined with first record dates suggests that stratified dispersal mechanisms, characterised by the insect's and human-mediated dispersal, better explain the history of *L. occidentalis* invasion in this region. Indeed, the intensive trade of cones of *P. pinea* in Iberia and the high capacity of this insect to hitchhike is probably leading to human-mediated dispersal. Besides the two independent introductions already suggested by Lesieur et al. [13], our results suggest at least a third introduction in the west of the Iberian Peninsula. These multiple introductions and translocations are the main obstacle to pest control and future management strategies. In addition, ecological studies of *L. occidentalis* populations, namely the climatic suitability, host preferences, and natural barriers, are crucial to understanding what can determine the range expansion of this pest. An accurate prediction and consequent management of the invasion dynamics of this species is only possible after a strong knowledge of its population dynamics.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data upon request from authors.

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