Mealybug species from Chilean agricultural landscapes and main factors influencing the genetic structure of *Pseudococcus viburni*

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The present study aimed to characterize the distribution of mealybug species along Chilean agro-ecosystems and to determine the relative impact of host plant, management strategy, geography and micro-environment on shaping the distribution and genetic structure of the obscure mealybug *Pseudococcus viburni*. An extensive survey was completed using DNA barcoding methods to identify Chilean mealybugs to the species level. Moreover, a fine-scale study of *Ps. viburni* genetic diversity and population structure was carried out, genotyping 529 *Ps. viburni* individuals with 21 microsatellite markers. Samples from 16 localities were analyzed using Bayesian and spatially-explicit methods and the genetic dataset was confronted to host-plant, management and environmental data. Chilean crops were found to be infested by *Ps. viburni*, *Pseudococcus meridionalis*, *Pseudococcus longispinus* and *Planococcus citri*, with *Ps. viburni* and *Ps. meridionalis* showing contrasting distribution and host-plant preference patterns. *Ps. viburni* samples presented low genetic diversity levels but high genetic differentiation. While no significant genetic variance could be assigned to host-plant or management strategy, climate and geography were found to correlate significantly with genetic differentiation levels. The genetic characterization of *Ps. viburni* within Chile will contribute to future studies tracing back the origin and improving the management of this worldwide invader.

Scale insects are worldwide-distributed agricultural pests that cause major economic losses of billions of dollars every year\(^1,2\), either through direct impact on crops or quarantine export restrictions\(^3,4\). Commonly known as mealybugs, scale insects belonging to the family Pseudococcidae are particularly difficult to manage\(^5,6\). Their small size and cryptic habits render them very inconspicuous so that they easily escape phytosanitary inspections. For example, the obscure mealybug *Pseudococcus viburni* (Signoret) has expanded across the globe and is now present in over 60 countries\(^7\). Chemical control of pseudococcids is often ineffective due to their concealed nature and patchy distributions\(^8,9\). As a consequence, many management programs rely on classical biological control and especially the use of encyrtid parasitoids\(^6,9\). The response to particular biological control agents varies depending on the mealybug species\(^10\), so characterizing the diversity and distribution of mealybugs becomes important for both fundamental research as well as in applied pest management.

The spatial distribution of agro-ecosystems is a key factor determining the species composition and population genetic structure of agricultural pests\(^11,12\). Chilean highly structured agricultural landscape represents an excellent case study, with 83% of fruit orchards being found in a 700 km long stretch of land

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ABI 3700 sequencer (Applied Biosystems) and 500 LIZ GeneScan™ size standard. Allelic profiles were microsatellite markers developed by Correa et al. identified as (see results section) and kept for further analysis using the 21 polymorphic

viduals from those populations. Individual PCRs were performed in a total volume of 10 μL. PCR products were separated by electrophoresis using an obtained for each individual using the Genemarker™ v1.75 software (SoftGenetics LLC).

′GAGAGTTMAASAGTACGTGAAAC-3′ extracted from at least five individuals per locality using the DNeasy extraction kit (QIAGEN, Hilden, Germany), and the conserved 28S ribosomal marker was PCR-amplified using the primers S3690 (5′-GAGAGTTMAASAGTACGTGAAAC-3′) and A4394 (5′-TCGGARGAACCGC-TACTA-3′)21. PCR reactions were performed in a total reaction volume of 25 μL and following the protocol of Abd-Rabou et al.22. The PCR mix was composed of 12.50 μL 2X Tmix (QIAGEN, Hilden, Germany), 0.125 μL of each primer (initial concentration of 100 μM) and 10.25 μL of ultrapure water. PCR conditions were: initial denaturation at 95°C for 15 min; 35 cycles of denaturation at 95°C for 30 s, hybridization at 58°C for 90 s, elongation for 60 s; final extension at 72°C for 10 min. PCR products were then analyzed on a QIAxcel Advanced System (QIAGEN) and sent for sequencing to Genoscreen (Lille, France) or Beckman Genomics (Takeley, United Kingdom). PCR products were sequenced on both strands and consensus sequences and alignments were created manually with Bioedit version 7.0.13.

Microsatellite Diversity Analyses. Out of the initial 38 sampling sites, a total of 16 samples were identified as Ps. viburni (see results section) and kept for further analysis using the 21 polymorphic microsatellite markers developed by Correa et al.24. Genotyping was carried out in a total of 529 individuals from those Ps. viburni populations. Individual PCRs were performed in a total volume of 10 μL, with cycling conditions as in Correa et al.24. PCR products were separated by electrophoresis using an ABI 3700 sequencer (Applied Biosystems) and 500 LIZ GeneScan™ size standard. Allelic profiles were obtained for each individual using the Genemarker™ v1.75 software (SoftGenetics LLC).

For each sampling locality, mean number of alleles, observed (H_o) and expected (H_e) heterozygosity were computed using the software GeneClass v225. Allelic richness was estimated standardizing sample size with the R package standArich (available online at http://www.ccmar.ualg.pt/maree/software.php?soft=standArich). Deviations from Hardy-Weinberg Equilibrium (HWE) were tested for each locus and sampling locality using the software Genepop v4.2.26. Per locus p-values were corrected for multiple testing within each population using the false discovery rate (FDR) procedure27. The presence of null alleles was evaluated with the FreeNA software, and 10,000 bootstrap replicates were used to test for significance28. Genetic signals of recent bottlenecks affecting Ps. viburni were analyzed using the software BOTTLENECK v.1.2.0225.
Genetic connectivity of *Pseudococcus viburni* populations. Pairwise FST estimates$^{30}$ and Fisher’s exact probability tests for genotypic differentiation$^{31}$ were performed using the software Genepop v4.2$^{26}$. Levels of genetic differentiation between sample sites were also estimated using the $D_{ST}$ statistic, which includes a multiplicative partitioning of diversity, based on the effective number of alleles rather than on the expected heterozygosity$^{32}$. Pairwise $D_{ST}$ statistic estimates and their significance using 1,000 bootstrap replicates were obtained with the pair.pops.Dest function in the DEMETICS package in R (http://www.r-project.org/). Sampling localities were grouped with the neighbor-joining algorithm and the pairwise genetic chord distance$^{33}$ as implemented in the software Populations v1.2.30 (http://bioinformatics.org/populations/).

Table 1. Mealybug populations sampled along Chile and molecular identification using the 28S gene region.
Genetic clusters were then inferred by minimizing deviation from HWE and linkage disequilibrium (LD) with the Bayesian clustering approach implemented in STRUCTURE. Parameters were estimated assuming an admixture model with correlated allele frequencies and no prior location information. We carried out 20 replicate runs for each value of the number of clusters (K), set between 1 and 16 (i.e. the number of sampling sites). Each run consisted of a burn-in period of 200,000 Markov chain Monte Carlo (MCMC) iterations, followed by 1,000,000 MCMC iterations. The highest level of genetic structure was inferred through the ΔK approach as implemented in STRUCTURE Harvester. Finally, a discriminant analysis of principal components (DAPC) was used to identify clusters of genetically-related genotypes. DAPC (like the sPCA method presented below) differs from the Bayesian method in STRUCTURE by not assuming HWE or linkage equilibriu

Spatial genetic structure analyses. Isolation by distance (IBD) was tested by performing a linear regression of the genetic distances (FST or DST) and the geographical distances (Km) and obtaining the Pearson's correlation coefficient. Matrices of genetic distance and geographical distance were subjected to a Mantel test with 10,000 permutations in Arlequin v.3.5. A spatially-explicit multivariate method (sPCA: spatial analysis of principal components) was used to further explore the spatial patterns of genetic variability between sites. Spatial connectivity networks were built using the sampling location data and three common algorithms (Delaunay triangulation, relative neighbor and sphere of influence). In order to select for the network that better fits the genetic data, the corrected Akaike Information Criteria (AICc) was computed for each graph using the orthoAIC function of the spacemakerR package. The connectivity graph with the highest AICc was used to test for the presence of spatial autocorrelation against the null hypothesis that allele frequencies are distributed at random. Spatial correlation can be either positive or negative, resulting in principal components with positive or negative eigenvalues. Principal components with significant positive eigenvalues indicate that sites are genetically more similar to their neighbors than expected by chance (global structures), while those with significant negative eigenvalues highlight local differentiation among neighboring sites. Spatial autocorrelation (Moran's I) was tested for each component using the permutation procedure implemented in the sdepp package and 1,000 permutations.

Non-genetic factors influencing population structure. Several analyses of molecular variance (AMOVA) were performed in order to evaluate the importance of non-genetic categorical factors on the distribution of genetic diversity. In particular, sample sites were grouped according to (i) administrative region (AT, CQ, VL, MT, OH or ML), (ii) type of agricultural management (conventional or organic production), and (iii) host plant (grape, apple, plum or pear). The significance for the AMOVAs was obtained through non-parametric permutation procedures (with 20,000 permutations) as implemented in Arlequin v.3.5. To examine whether non-genetic environmental factors may influence genetic differentiation levels in Ps. viburni, temperature and precipitation variables were recovered to 1-km spatial resolution from WorldClim version 1.4. The climate variables included temperature and precipitation parameters, namely: i) annual mean temperature, ii) maximum temperature of the warmest month, iii) minimum temperature of the coldest month, iv) annual precipitation and v) precipitation seasonality (Coefficient of Variation). A Spearman’s rank correlation test was then carried out between pairwise genetic distances (FST or DST) and the Euclidean distance between climate variables for each pair of sampling sites. Spearman’s correlation measures association based on the rank differences between two vectors and indicates how well a monotonic function describes their relationship.

Results

Molecular identification of mealybug species. The present survey of scale insects infesting Chilean crops identified four mealybug species (Planococcus citri, Pseudococcus longispinus, Pseudococcus meridionalis and Pseudococcus viburni). Ps. viburni was the most widespread pest, being found on vineyards, apple trees, plum trees or pear trees in six out of seven administrative regions sampled: Atacama (AT), Coquimbo (CQ), Valparaíso (VL), Metropolitana (MT), O’Higgins (OH) and Maule (ML) (Table 1; Fig. 1). Ps. longispinus was also present in several regions and dominated apple fields south of Maule and in Biobío. Finally, Pl. citri and Ps. meridionalis were only found sporadically on vineyards or orange trees (Fig. 1).

Microsatellite Diversity Analyses. Ps. viburni samples showed an overall mean number of alleles of 3.06, with values ranging from 2.29 (ML1) to 3.62 (VL3). The overall mean allelic richness was 2.97 (N = 20 genotypes), with MT2 presenting the highest (3.52) and AT1 the lowest (2.20) values (Table 2). After correction by the FDR procedure, seven sites still showed significant HWE deviations (Table 2). Frequency of null alleles for each microsatellite marker ranged from 0.002 (PV071) to 0.133 (PV060). Nevertheless, the presence of null alleles did not affect global FST estimates, and similar values were obtained using the raw dataset (FST = 0.126) or the corrected dataset (FST = 0.125). Similarly, pairwise FST values did not vary after FREENA correction for the presence of null alleles.
Genetic connectivity of *Pseudococcus viburni* populations. Pairwise FST values ranged from 0.011 (between ML2 and ML3) to 0.350 (between AT1 and ML3) (Table 3). All pairs of *Ps. viburni* samples showed significant differentiation levels after Fisher’s exact tests (P < 0.05). The results obtained using the distance-based neighbor-joining approach showed a clear support for the separation of AT1 and CQ1 from the remaining samples (Fig. 2a). Despite most bootstrap values were low, a strong support was found for some groups sites, such as VL1-VL2, ML2-ML3, MT1-MT2 and VL3-VL4. Structure Harvester selected K = 3 as the best representative number of clusters based on likelihood and ΔK (Ln = −16457; ΔK = 443.35). The first cluster included the two Northernmost sites (AT1 and CQ1), a second cluster was composed of samples from the Aconcagua (VL1 and VL2), Cachapoal (OH1), Colchagua (OH2 and OH3) and Curicó (ML1) valleys; and a third cluster was formed by the Casablanca (VL3 and VL4), Maipo (MT1 and MT2), Colchagua (OH5) and Curicó (ML2 and ML3) valleys (Figs 1, 2b). Finally, the DAPC method also support the presence of a cluster including AT1 and CQ1 samples, but indicated large overlapping between the samples assigned to the second (blue) and third (green) clusters given by STRUCTURE (Fig. 2c).

Spatial genetic structure analyses. AMOVA results showed that a significant 7.73% of the total genetic variability can be explained by administrative regions (FCT = 0.074; P < 0.0001). A significant positive correlation was found between geographical distance and either FST (r² = 0.79; P < 0.0001) or DST values (r² = 0.75; P < 0.0001) when considering all the 16 sample sites, indicating the presence of IBD. When the IBD test was performed without the two most distant localities (AT1 and CQ1), a
weaker but still significant pattern was also observed both using FST ($r^2 = 0.068; P = 0.028$) or DST values ($r^2 = 0.121; P = 0.004$).

According to the corrected AIC criterion, the best connectivity graph for explaining the Ps. viburni allele frequency data was obtained from the Sphere of Influence (SoI) algorithm ($AICc = −42.356$; non-zero links $= 34$), which showed a lower AICc value than both the Delaunay triangulation ($AICc = −41.325$;
of Pseudococcidae 4. Indeed, host-plant preference patterns are in agreement with previous studies on the biology and systematics and was more abundant in Apple orchards south of that region. These different spatial distribution and Metropolitana) and became less frequent south of O’Higgins, while

the difficulties of morphology-based identification of mealybugs. The main infesting Chilean crops. Therefore, previous reports of results establish, thanks to DNA barcoding methods, the presence of both populations from Chile. 

Non-spatial factors influencing population structure. No significant genetic variance could be assigned to differences among organic and conventional management strategies (FCT = −0.0006; p-value = 0.402) or among host plants (FCT = −0.015; p-value = 0.587) (Table 4). As for the effect of climatic environmental factors, Spearman’s rank test indicated a strong correlation between genetic distances and differences in maximum temperature of the warmest month (ρ = 0.615; p-value < 0.001), minimum temperature of the coldest month (ρ = 0.645; p-value < 0.001), annual precipitation (ρ = 0.523; p-value < 0.001) and precipitation seasonality (ρ = 0.537; p-value < 0.001). However, the correlation between genetic distances and annual mean temperature was much smaller and non-significant after FDR correction (ρ = 0.239; p-value = 0.008).

Discussion
The distribution of insect pest species across agricultural ecosystems can be shaped by multiple factors, ranging from specific associations with the cultivated plant, environmental gradients or the use of pesticides. The study of these multiple factors, together with an accurate discrimination between mealybug species, are determinant for an adequate adjustment of management strategies. Previous morphology-based surveys of Chilean mealybugs have found Ps. viburni, Ps. meridionalis, Ps. cribata, Ps. longispinus and Pl. ficus, but the only molecular study performed up to date could not recover the last two species20. Our results establish, thanks to DNA barcoding methods, the presence of both Ps. longispinus and Pl. citri infesting Chilean crops. Therefore, previous reports of Pl. ficus could correspond in fact to Pl. citri, given the difficulties of morphology-based identification of mealybugs. The main Pseudococcus species infesting Chilean fruit crops was the obscure mealybug Ps. viburni, followed by Ps. longispinus and Ps. meridionalis. Both Ps. viburni and Ps. longispinus were recovered from vineyards and fruit orchards. Nevertheless, while Ps. viburni occurred mainly in vineyards around the central regions of Chile (Valparaíso and Metropolitana) and became less frequent south of O’Higgins, Ps. longispinus showed an opposite pattern and was more abundant in Apple orchards south of that region. These different spatial distribution and host-plant preference patterns are in agreement with previous studies on the biology and systematics of Pseudococcidae4. Indeed, Ps. viburni belongs to the grape mealybug (Ps. maritimus) species complex, while the long-tailed mealybug (Ps. longispinus) and related species are commonly found on fruit orchards (e.g. avocado43,44 and pear45).

The host preference observed for different species did not extend to different populations within Ps. viburni, since no significant genetic variance could be assigned to host plant or management strategy. This lack of genetic structuring by host indicates that Ps. viburni populations are able to infest fruit orchards in an opportunistic way despite their preference for vines4. Rather than host plant or management strategy, our results highlight the importance of geography and environmental factors in shaping the intra-species population genetic structure of the obscure mealybug. The integrative analyses of micro-climatic variables and genetic markers show that the severity of climate (i.e. differences in the extremes of temperature
or precipitation seasonality) is significantly correlated with genetic differentiation levels. Meteorological changes or edaphic factors have been previously proposed to modify the physiology of the plant and alter its resistance to scale insect attack\(^{46,47}\), but this is the first study to provide evidence on the importance of environmental factors on shaping scale insects distribution and genetic structure.

Climate differences might correlate with geographical distances, so that spurious genetic-climate correlations could result from an isolation by distance pattern. AMOVA, IBD and sPCA analyses all support the presence of reduced connectivity between \(P. viburni\) samples related to their spatial distribution. The fact that IBD remained significant even after excluding the two most distant populations indicates that genetic differentiation is also important at a local scale, where climatic differences are minimal. Therefore, geography cannot be discarded as a key factor shaping the population genetic structure of the obscure mealybug. In a recent study, the maritime pine bast scale (\(Matsuococcus feytaudi\)) was found to be highly structured geographically, and the authors relate this fact to the limited dispersal capacity of the insect and the patchy distribution of the obligate host\(^{48}\). Mealybug species are known to present low dispersal abilities, so long distance movements could only be driven by human activities and agricultural practices\(^4\).

Figure 3. Spatial genetic structure of \(P. viburni\) populations from Chile. (A) Different connectivity networks tested; (B) The two main global components of the sPCA analysis, showing that the populations from the Valparaíso (VL3-VL4) and Metropolitana (MT1-MT2) regions have larger negative scores (white squares) than locations on the other clusters; (C) Plot of the two components with the largest negative values, showing that local changes are significant in the northern samples and within the O’Higgins area (both white and black squares present within clusters). Figure created using the software CorelDRAW X6.
The peculiar shape of Chile, being only 177 km wide in average but 4300 km long, together with the low mobility of mealybugs, could well explain the observed geography-driven genetic structure of Ps. viburni. Significant results ($p < 0.05$) after 20,000 permutations are indicated in bold.

Table 4. Global Analyses of Molecular Variance (AMOVA) as a weighted average over loci carried out to compare the effect of categorical factors on the genetic structure of Pseudococcus viburni. Significant results ($p < 0.05$) after 20,000 permutations are indicated in bold.

| Source of Variation | df  | SS    | Variance components | % variation | F statistics |
|---------------------|-----|-------|---------------------|-------------|-------------|
| Among groups        | 5   | 350.639 | 0.305               | 7.37        | $F_{CT} = 0.074$ |
| Among populations within groups | 10  | 210.896 | 0.263               | 6.36        | $F_{SC} = 0.069$ |
| Within populations  | 1042| 3717.699| 3.568               | 86.28       |             |
| Among groups        | 1   | 38.407  | $-0.002$            | $-0.06$     | $F_{CT} = -0.001$ |
| Among populations within groups | 14  | 523.128 | 0.515               | 12.63       | $F_{SC} = 0.126$ |
| Within populations  | 1042| 3717.699| 3.568               | 87.43       |             |
| Among groups        | 3   | 75.003  | $-0.058$            | $-1.44$     | $F_{CT} = -0.015$ |
| Among populations within groups | 12  | 486.531 | 0.535               | 13.23       | $F_{SC} = 0.131$ |
| Within populations  | 1042| 3717.699| 3.568               | 88.21       |             |

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Author Contributions
T.Z., T.M. and F.P. conceived the ideas and led the writing; M.C., E.L. and F.P. analyzed the data; M.C., D.C. and A.A. collected the data. M.C. prepared Figures 1–3. All authors reviewed the manuscript.

Additional Information
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