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¿La variación en la composición de bacterias endosimbióticas modifica la agresividad de un insecto plaga?: consecuencias para el manejo

Does the variation in the composition of endosymbiotic bacteria modify the aggressiveness of an insect pest?: consequences for management

Por/by

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Resumen general

Las asociaciones simbióticas entre distintos organismos e insectos son comunes en la naturaleza. Algunas de estas asociaciones pueden conferir una ventaja adaptativa para los hospederos. Uno de los casos mejor estudiados es la asociación entre los áfidos, un grupo de insectos fitófagos, que a menudo constituyen plagas agrícolas, y distintas bacterias endosimbióticas. Los áfidos se encuentran asociados a una bacteria primaria de carácter obligado, *Buchnera aphidicola*, la cual es vital para la sobrevivencia de los áfidos, ya que les aporta aquellos aminoácidos esenciales que los áfidos no son capaces de obtener a partir de su dieta basada en el floema de sus plantas hospederas. Por otro lado, también existe una serie de bacterias de carácter secundario o facultativo, las cuales, si bien no son esenciales, pueden producir importantes efectos en el fenotipo de los áfidos, incluyendo protección contra el parasitismo, tolerancia al estrés térmico, ampliación del rango de uso de plantas hospederas, entre otros, todos rasgos que favorecen que los áfidos sean una importante y difícil plaga de controlar.

Una importante plaga agrícola que produjo grandes pérdidas en la agricultura en los años 1970s en Chile es el áfido de cereales *Sitobion avenae.* Este áfido fue presumiblemente introducido desde Europa y la zona Mediterránea, y tuvo un importante éxito en su establecimiento en Chile. El control de *S. avenae* se logró gracias a la introducción de enemigos naturales tales como depredadores y parasitoides. Hasta ahora, se desconoce el papel que los endosimbiontes facultativos pueden tener sobre el éxito de esta plaga, y de cómo podrían modificar el fenotipo de estos áfidos. En la actualidad se asume que los endosimbiontes facultativos juegan un papel muy importante en la capacidad de éxito de las especies plaga cómo los áfidos, es por ello, que el estudio de éstos es necesario para poder comprender el éxito de plagas agrícolas en distintos ambientes, más aún, en aquellos lugares donde esta plaga ha sido introducida exitosamente como es el caso de Chile. El conocimiento del rol de los endosimbiontes sobre importantes rasgos de importancia agronómica como la protección contra el control biológico de áfidos o la tolerancia al stress térmico, podría ser usado para desarrollar nuevas estrategias de control. Esto parece relevante tanto para para la situación de nuestro país, dado que el control de *S. avenae* está sujeto al éxito del control biológico, como a nivel global, bajo un escenario de cambio climático.

Esta tesis se centró en el estudio de los endosimbiontes secundarios en *S. avenae*, de forma de encontrar patrones que podrían dar cuenta del rol putativo de éstos en el éxito de *S. avenae* como plaga invasiva. De esta forma, en el capítulo 2 se estudió la actual diversidad genética de *S. avenae* en Chile, el área invadida, y Francia, el área nativa, durante la primavera de 2017 y 2018. Este tipo de estudio permitió identificar aquellos genotipos más exitosos en ambos países y así poder posteriormente asociarlo a la presencia de sus endosimbiontes secundarios. Los resultados mostraron que la diversidad genética de *S. avenae* en Chile es muy baja comparado a su área

nativa; de hecho, diez genotipos representaron el 84% del total de la diversidad genética. Además, se encontró importante variación entre eso diez genotipos los años estudiados.

Dado que asociaciones específicas entre genotipos de *S. avenae* y especies de endosimbiontes secundarios podrían dar cuenta del rol putativo de éstos en el éxito invasivo de *S. avenae*; en el capítulo 3 se estudió la distribución espacial y temporal de los endosimbiontes secundarios en *S. avenae* tanto en Chile como en Francia, además se estudió la existencia de posibles asociaciones entre los genotipos más frecuentes de *S. avenae* y especies de endosimbiontes en ambos países. Si bien no se detectó una clara evidencia de variación espacial en la frecuencia de endosimbiontes secundarios, se encontró una importante variación temporal entre las temporadas estudiadas en Chile, con *Hamiltonella defensa* presente solo en uno de los años estudiados. Por otra parte, la presencia de *H. defensa* estuvo ligada a la presencia de determinados genotipos, los cuales en su mayoría no estuvieron presentes en la temporada 2017, dando cuanta de una posible asociación entre genotipos de *S. avenae* y determinadas especies de endosimbiontes, relación que no fue detectada en Francia, ya que los endosimbiontes secundarios estuvieron ampliamente distribuidos en la mayoría de los genotipos.

En el capítulo 4 se estudió la diversidad genética de los endosimbiontes secundarios de diferentes genotipos de *S. avenae*, incluyendo tantos genotipos frecuentes de Chile como de Francia. Además, se evaluó el uso de diversos tratamientos basados en antibióticos para la eliminación de las distintas cepas de bacterias detectadas y cómo la ausencia de estos endosimbiontes secundarios afectan los rasgos de historia de vida. Los resultados de este capítulo mostraron que los genotipos estudiados en Chile solo poseen una cepa de la bacteria *Regiella insecticola*, mientras que los genotipos franceses poseían las cuatro cepas detectadas. Por otra parte, ciertas cepas de bacterias fueron más susceptibles a los tratamientos con antibióticos, mientras que otras, principalmente la cepa de *R. insecticola* encontrada en los genotipos de Chile, lograron ser eliminadas a mayores concentraciones de antibióticos. Por otro lado, la eliminación de los endosimbiontes secundarios no tuvo un efecto sobre los rasgos de historia de vida en ninguno de los genotipos estudiados, demostrando que probablemente el efecto de las distintas cepas detectadas solo podría ser manifestados bajo condiciones específicas.

Finalmente, dado que ciertos endosimbiontes secundarios podrían otorgar protección contra el parasitismo y tolerancia térmica, en el capítulo 5 se estudió la variación temporal en la densidad de *S. avenae*, su tasa de parasitismo, la frecuencia de sus endosimbiontes secundarios y su relación a las temperaturas máximas registradas en dos zonas con diferentes temperaturas máximas durante dos temporadas. Los resultados mostraron una mayor densidad de áfidos en la zona con mayores temperaturas máximas, sin embargo, la tasa de parasitismo no mostró diferencias entre las zonas. Por otro lado, el efecto de las temperaturas máximas registradas dependió de la zona y temporada analizada. *Regiella insecticola* fue el endosimbionte más frecuente en amabas zonas y

temporadas, ya que estuvo presente en el 95% del total de individuos analizados, mostrando que aparentemente no tendría un rol en conferir protección contra parasitoides o tolerancia termal a *S. avenae* en el campo.

General abstract.

Symbiotic associations between different organisms and insects are common in nature. Some of these associations may confer an adaptive advantage for the hosts. One of the best-studied cases is the association between aphids, a group of phytophagous insects, which often constitute agricultural pests, and different endosymbiotic bacteria. Aphids are associated with a primary bacterium of an obligate nature, *Buchnera aphidicola*, which is vital for the survival of aphids since it provides them with those essential amino acids that aphids are not able to obtain from their diet based on the phloem of their host plants. On the other hand, there is also a series of secondary or facultative bacteria, which, although they are not essential, can produce significant effects on the aphid phenotype, including protection against parasitism, tolerance to heat stress, and widening the host plants range of their hosts, among others, all traits that make aphids an important and challenging pest to control.

An important agricultural pest that caused significant losses in agriculture in the 1970s in Chile is the cereal aphid *Sitobion avenae*. This aphid was presumably introduced from Europe and the Mediterranean zone and had significant success in Chile's establishment. The control of *S. avenae* was achieved thanks to the introduction of natural enemies such as predators and parasitoids. Until now, the role that facultative endosymbionts may have on this pest's success and how they could modify the phenotype of these aphids is unknown. Nowadays, it is assumed that facultative endosymbionts play a critical role in the success of pest species such as aphids, that is why the study of secondary endosymbionts is necessary to understand the success of agricultural pests in different environments, moreover, in those places where this pest has been successfully introduced, like Chile. The knowledge of the role of endosymbionts on important traits of agronomic importance, such as protection against the biological control of aphids or tolerance to heat stress, could be used to develop new control strategies. This last seems relevant for our country's situation, given that *S. avenae* is subject to the success of biological control and, at a global level, under the climate change scenario.

This thesis focused on the study of secondary endosymbionts in *S. avenae* to find patterns that could account for the putative role of secondary endosymbionts in the success of *S. avenae* as an invasive pest. Hence, in chapter 2, the current genetic diversity of *S. avenae* in Chile, the invaded area, and France, the native area, was studied during the springs of 2017 and 2018 in both countries later to associate it with the presence of secondary endosymbionts. The results show that the diversity of *S. avenae* in Chile is exceptionally low compared to its native area; in fact, ten

genotypes represented 84% of the total genetic diversity. Moreover, significant variation between years in the presence of a particular genotype was found.

Because specific associations between *S. avenae* genotypes and secondary endosymbiont species could account for the putative role of endosymbionts in the invasive success of *S. avenae*; In chapter 3, the spatial and temporal distribution of secondary endosymbionts in *S. avenae* from Chile and France was studied. Furthermore, possible associations between the most frequent genotypes of *S. avenae* and endosymbiont species in both countries were also examined. Although no clear evidence of spatial variation was detected in the frequency of secondary endosymbionts, a significant temporal variation was found between Chile's studied seasons, with *Hamiltonella defensa* present only in one of the years studied. On the other hand, *H. defensa* was linked to the presence of specific genotypes, most of which were not present in the 2017 season, indicating a possible association between certain genotypes of *S. avenae* and certain species of endosymbionts. This relationship was not detected in France since secondary endosymbionts were widely distributed in most genotypes.

In chapter 4, we studied the genetic diversity of the secondary endosymbionts of different genotypes of *S. avenae*, including the most frequent genotypes from Chile and France. Moreover, various antibiotic-based treatments to eliminate the different bacteria strains were tested to evaluate later how the absence of these secondary endosymbionts affected life-history traits on their hosts. This chapter's results showed that the genotypes studied in Chile only possess one strain of the bacterium *Regiella insecticola*, while the French genotypes possessed the four strains detected. On the other hand, certain bacteria strains were more susceptible to the antibiotic treatments, while others, mainly the R. *insecticola* strain found in the Chilean genotypes, were eliminated at higher concentrations of antibiotics. On the other hand, eliminating the secondary endosymbionts did not affect the life-history traits in any of the studied genotypes, showing that the effect of the different strains detected could only be manifested in the phenotype of aphids under specific conditions.

Because certain secondary endosymbionts could protect their hosts from parasitism and confer thermal tolerance, chapter 5 studied the temporal variation in the density of *S. avenae*, its parasitism rate, the frequency of its secondary endosymbionts, and its relationship to the maximum temperatures recorded in two zones that displayed different maximum temperatures during two consecutive seasons. The results showed a higher density of aphids in the zone with the higher maximum temperatures; however, the parasitism rate did not show differences between the zones. On the other hand, the effect of the maximum temperatures on aphid density and parasitism rate depended on the zone and season analyzed. *Regiella insecticola* was the most frequent endosymbiont in both zones and seasons since it was present in 95% of the total of individuals analyzed, showing that it apparently would not have a role conferring protection against parasitoids or thermal tolerance to *S. avenae* at field level.

Chapter 1

1. General Introduction

1.1 Functional symbiosis

The association between microorganisms and animals is ubiquitous, mainly because microorganisms' communities can benefit the hosts by improving the exploitation of resources or increasing resistance to environmental stress (Gauthier et al., 2015). The symbiosis is non-genetically inherited and can significantly impact the phenotypic plasticity of their hosts (Bonduriansky & Day, 2009), allowing populations of the host organism to cope more quickly with environmental changes through phenotypic changes.

A well-studied case of functional symbiotic association is that observed between aphids and a group of bacteria. Aphids are herbivorous insects that feed on their host plants' phloem and frequently constitute pests in the agroecosystems. Indeed, aphids are widely distributed worldwide, causing significant economic losses directly through feeding and indirectly through virus transmission to their host plants (Blackman & Eastop, 2000). All aphids harbor the bacterium *Buchnera aphidicola*, an essential endosymbiont for aphid survival. It provides nutrients that the aphid does not obtain from its diet, and for this reason, it has been classified as an obligate or primary endosymbiont (Douglas, 1998). Moreover, aphids can harbor one or more species of secondary endosymbiont bacteria, which, although they are not essential for aphid survival, can modify the aphid phenotype to the point of granting it advantages under certain stress conditions (Montllor, Maxmen & Purcell, 2002; Oliver et al., 2003, 2008; Burke, Fiehn & Moran, 2010).

The relationship between endosymbionts and aphids is phylogenetically ancient, reaching up to 150-200 million years in *Buchnera* (Moran et al., 1993; Clark et al., 2000). Primary endosymbionts reside intracellularly in specialized organs called primary bacteriocytes and are maternally inherited (Buchner, 1965; Baumann, 2005), while secondary endosymbionts can be transmitted vertically and horizontally (Oliver et al., 2010). In the pea aphid race complex, *Acyrthosiphon pisum*, aphid races specialized on different host plants show a different endosymbiotic composition, indicating a kind of co-speciation between symbionts and aphids (Peccoud et al., 2009). So far, eight facultative endosymbiotic bacterial taxa have been identified, five of them belong to the Gammaproteobacteria class (*Hamiltonella Defensa, Regiella insecticola, Serratia Symbiotica, Ricketsiella viridis,* and *Fukatsuia symbiotica*), two are Alphaproteobacteria (of the *Rickettsia* and *Wolbachia* genera), and one belongs to Mollicutes (*Spiroplasma sp.*) (Gauthier et al., 2015).

1.2 Can endosymbiotic bacteria explain the invasiveness of an aphid pest?

Among the most significant phenotypic consequences produced by endosymbiont bacteria are those that modify agronomically relevant traits, i.e., those that are related to the aggressiveness of

an aphid pest. For example, protection against natural enemies (such as parasitoid wasps and entomopathogenic fungi) (Oliver et al., 2003, 2008, 2010; Vorburger et al., 2010; Russell et al., 2013; Vorburger, 2014), host plant range expansion (Tsuchida, Koga & Fukatsu, 2004), and tolerance to heat stress (Montllor, Maxmen & Purcell, 2002; Russell & Moran, 2006), among others, all these traits modifiable by the composition of facultative endosymbionts. These effects give aphids a relative advantage against nature's adverse conditions, making them pests more challenging to control.

Protection against parasitoids would play a relevant role in pest management since the presence of certain bacteria could decrease the efficiency of biological control (Vorburger, 2014). In *A. pisum* this trait is mainly determined by the bacterium *H. defensa*, which protects aphids against parasitism through an immunity mechanism to the embryonic development of parasitoids (Oliver et al., 2003), while in the green peach aphid, *Myzus persicae*, this trait is conferred by the bacterium *R. insecticola* and in a lesser extent by *H. defensa* (Vorburger et al., 2010). On the other hand, resistance against fungal pathogens such *as Pandora neoaphidis* in the *A. pisum* complex has been linked to the bacteria *R. insecticola*, *Rickettsia*, *Rickettsiella*, and *Spiroplasma* (Ferrari et al., 2004; Scarborough, Ferrari & Godfray, 2005; Łukasik et al., 2013).

Besides the protective effect against natural enemies, some facultative endosymbionts such as *S. symbiotica* have also been reported conferring tolerance to thermal stress (Montllor et al., 2002). This feature acquires great relevance under a scenario of agriculture increasingly impacted by Climate Change (Feldhaar, 2011). In contrast to the benefits conferred by endosymbiotic bacteria by modifying the aphid phenotype, it has also been reported that carrying certain bacteria could involve some trade-offs. For example, some bacteria are detrimental to the biological performance of aphids under certain environmental conditions. Thus, for example, aphids infected with *H. defensa* bacteria have a better survival against the attack of parasitoids but show less defensive behavior against predators' attack (Dion et al., 2011; Vorburger, 2014). This last suggests that the global endosymbiotic composition of an aphid (i.e., the microbiome) can be highly environment-specific in terms of costs and benefits (Vorburger, 2014); therefore, the advantages provided by secondary endosymbionts could depend on the variation of the environment over time (within and between seasons) and space (aphid distribution range) (Ferrari & Vavre, 2011).

1.3 Endosymbiont’s Manipulation.

The selective elimination of endosymbionts using antibiotics such as ampicillin (McLean et al., 2011), and the transfection of bacteria from one host to another by micro-injections (Chen & Purcell, 1997; Oliver et al., 2003; Koga et al., 2007), have made possible to study the effect produced by each endosymbiont on the aphid phenotype in greater detail, given the difficulty for cultivating these bacteria outside their host (Baumann & Moran, 1997). The development of these techniques gains

relevance due to the possibility of manipulating the microbiome of organisms, either by selectively eliminating certain endosymbionts or transferring endosymbionts from one host to another, as these techniques allow altering the endosymbiotic composition of individuals without affecting the primary bacterium (*Buchnera*) and thus, evaluate the effect of different facultative endosymbionts on the aphid's phenotype.

1.4 Study system: the grain aphid.

The grain aphid, *Sitobion avenae*, is considered an important pest of cereals globally (Alkhedir et al., 2013). In Chile, populations of this introduced aphid have remained below the threshold of economic damage mainly after the introduction of parasitoid wasp in the 1970s, which act as biological control agents (Starý et al., 1993); among them, the parasitoid *Aphidius ervi* represents one of the best examples of biological control of *S. avenae* worldwide (Zepeda-Paulo et al., 2015). *Sitobion avenae* was presumably introduced accidentally from Europe and the Mediterranean zone (Blackman & Eastop, 2000). In Chile, populations of *S. avenae* have shown to be constituted by a few genotypes (> 80%) that are highly predominant in time and space and usually referred to as "superclones" (Vorburger, Lancaster & Sunnucks, 2003; Figueroa et al., 2005). Despite the low genotypic diversity found in populations of *S. avenae* in Chile (< 5%), these aphids are widely distributed throughout different agroclimatic zones and are colonizing various cereal crops and wild grasses (Figueroa et al., 2005).

The low genetic diversity but high invasiveness of *S. avenae* in Chile seems a paradox. However, aphids such as *S. avenae* display specific characteristics that favor the invasion in a new environment. On the one hand, the general mode of reproduction of aphids includes alternating a sexual reproduction phase at the beginning of autumn with several parthenogenetic asexual reproduction phases in spring and summer (Simon et al., 1999). However, in the case of introduced *S. avenae* populations, a predominant parthenogenetic reproduction of a few genotypes (superclones) has been observed throughout the year, which allows an exponential increase in their population sizes (Wilson, Sunnucks & Hales, 1999, 2003; Haack et al., 2000). On the other hand, it is known that parthenogenetic aphids can still generate variation through DNA mutations, chromosomal rearrangements, and rare interspecific hybridization events, which can increase the phenotypic variation on which natural selection may acts (Simon, Rispe & Sunnucks, 2002; Wilson, Sunnucks & Hales, 2003), as well as the variation in the efficiency in the use of some host plants (Sunnucks et al., 1998). Likewise, the asexual aphid lineages may undergo phenotypic changes that increase the opportunity for adaptive evolution (Wilson, Sunnucks & Hales, 2003). For example, superclones of *S. avenae* constitutively express plant toxin detoxifying enzymes at a low energy cost, which allows them to have more energy for parthenogenetic reproduction in unfavorable environments such as when they feed on cereals with high levels of chemical defenses (Castaneda et al., 2009; Castañeda, Figueroa & Nespolo, 2010). Likewise, clones of the cereal

aphid, *Rhopalosiphum padi*, and the pea aphid, *A. pisum*, show genetic co-variation between traits such as fecundity and production of winged individuals under different environmental conditions (Nespolo et al., 2009; Artacho et al., 2011), which favors their plasticity to invade new environments. Besides those mechanisms of phenotypic variation, it has been proposed that facultative endosymbiont bacteria can add even more variation (Oliver et al., 2010). In that case, the phenotypic alternatives being expressed by the same clonal lineage (or clone) seem to multiply, which would better explain parthenogenetic pests' success.

Overall, there is little empirical data about the impact of endosymbiont bacteria as a mediator of variation that increase the invasive potential of superclone aphids in Chile. However, a first record of the presence, frequency, and geographic distribution of facultative endosymbiont bacteria was documented for several aphid species in Chile, including *S. avenae* (Sepúlveda et al., 2017). In that study, evidence of the facultative endosymbionts *R. insecticola* and *H. defensa* was found. Interestingly, a significant geographic variation in the distribution of these endosymbionts between two contrasting agroclimatic zones was found. Indeed, samples of *S. avenae* collected on cereals in the south-central zone of Chile showed the bacterium *H. defensa* in high frequency (48%), while the frequency of *R. insecticola* was extremely low (~ 1%). In contrast, the oppositive trend was observed for Chile's central zone, where a high frequency of *R. insecticola* (67%) and a complete absence of *H. defensa* was reported. Those results have led to the proposition that endosymbiont bacteria would allow aphids to respond differentially to abiotic variables such as temperature.

1.5 The problem: How the success of *Sitobion avenae* is explained? The role of endosymbionts.

When aphids invade a new area, their populations suffer from the adverse effects of bottlenecks followed by genetic drift, which can limit the introduction's success by randomly decreasing the genetic diversity and reducing the adaptive evolutionary capacity of populations (Sakai et al., 2001). Thus, the aphid propagules have reduced genetic diversity and time for the appearance of new genetic variants that can quickly cope with the new environment's conditions. Symbiosis represents an essential source of phenotypic variation without genetic change (Bonduriansky & Day, 2009), which could play a key role during insect pests invasion. In *S. avenae*, the evident success of specific genotypes (superclones) leads to hypothesize whether part of that success may be due to certain endosymbionts playing a pivotal role in modifying certain traits allowing the wide distribution and high frequency of superclones in Chile. Traits that allow aphids to colonize different environments, such as thermal tolerance, would favor superclones to survive and reproduce in environments with high temperatures, which could also imply a serious threat to agriculture under the Climate Change scenario. This last is precisely the current situation in central Chile, where temperatures during the summer frequently exceed the maximum temperatures at which aphids of the *Sitobion* genus can reproduce (28°C in the laboratory) (INE, 2015; Turak et al., 1998).

Moreover, the protection given by symbionts against natural enemies, like parasitoids, could also explain the success of superclones.

On the other hand, the adverse effects faced by aphids during their introduction could also affect the endosymbiont composition, hence, selecting those bacteria that can confer an adaptative advantage to the new environment. However, the endosymbionts' role in certain genotypes' success, like the superclones, is also poorly understood in native populations of aphids, which makes the interpretation of their role in the invaded areas even more difficult. Comparing the pest status of *S. avenae* and their endosymbiont composition between a native and invaded area could bring some insights into the key factors that explain the success of *S. avenae* in Chile.

Therefore, the following questions arise: Are the superclones from Chile the same as the native area? Is the diversity of endosymbionts in Chile the same that those from the native area? Do superclone genotypes have different endosymbiont compositions compared to less frequent genotypes? What is the effect of different facultative endosymbionts on the biological fitness of *S. avenae*?

Hence, this Ph.D. thesis was aimed to evaluate whether the secondary endosymbiont composition can explain part of the success of *S. avenae* in Chile. This thesis is organized in a paper format, in which every chapter represents an objective of the thesis. Hence, a general introduction and general conclusions are provided. In chapter 2, the genetic diversity of *S. avenae* in Chile and its native area (France) was studied, identifying the current frequent genotypes (or superclones) from both countries. In chapter 3, we assessed to the spatial and temporal distribution of genotypes and their secondary endosymbionts in Chile and France. In chapter 4, the genetic diversity of *S. avenae* secondary endosymbionts was explored, and a protocol of secondary endosymbionts disinfection was set up and implemented to evaluate the effect of secondary endosymbionts on the aphid phenotype. Finally, in chapter 5 the effects of secondary endosymbionts involving protection to parasitism and tolerance to high temperatures were evaluated at the field level in Chile.

Keywords: *Sitobion avenae*, invasive pest, genetic diversity, secondary endosymbiont, superclone, thermal tolerance, parasitism rate.

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Hypothesis

Specific associations between genotypes of *Sitobion avenae* and certain endosymbionts species explain the prevalence of superclones in Chile.

Predictions

- Specific associations between superclone genotypes and certain species of endosymbionts are expected. These associations are found in both; an invaded country (Chile) and a native country (France).

- The removal of secondary endosymbionts should produce adverse effects on the fitness of *S. avenae*, measured as lower fecundity, body mass, and developmental time.

- Lower parasitism rates should be detected at high frequencies of secondary endosymbionts at the field level. Moreover, secondary endosymbionts should increase their frequency as the maximum temperatures increases in the field.

Objectives

- Assess the genetic diversity of *S. avenae* in Chile and France and determine each country's more frequent genotypes (superclones) (Chapter 2).

- Characterize the spatial and temporal distribution of secondary endosymbionts from *S. avenae* in Chile and France and their association with frequent MLGs (Chapter 3).

- Study the genetic diversity of different MLGs from *S. avenae* and implement an elimination of secondary endosymbionts protocol to assess the effect of secondary endosymbiont elimination in the intrinsic rate of increase, the body mass, and the developmental time (Chapter 4).

- Evaluate whether the aphid density, parasitism rate, and secondary endosymbiont frequency and composition are affected by the maximum temperature at field level in zones with different climates (Chapter 5).

2. Chapter 2

Title

The genetic diversity of invasive and native populations of *Sitobion avenae.*

Objective:

Assess the genetic diversity of *S. avenae* in Chile and France and determine each country's more frequent genotypes (superclones).

2.1 Abstract:

The grain aphid *Sitobion avenae* is a native insect pest from Europe and the Mediterranean zone. It was introduced to Chile in the late 1960s, rapidly spreading throughout all the Chilean territory and causing important cereal damage. Their genetic diversity was studied 20 years ago, showing a very low genetic diversity with the presence of a few highly prevalent genotypes; however, evolutionary processes and new invasions events may lead to the increase of genetic diversity, which could threaten the equilibrium reached by countries as Chile that depend on the biological control to maintain *S.avenae* under control. Therefore, we studied the spatial and temporal genetic diversity of *S. avenae* in different zones of an invaded (Chile) and native area (France) in the springs of 2017 and 2018. We further assessed the genetic relatedness of the multilocus genotypes (MLGs) from Chilean and French *S. avenae*, determining the phylogenetic relatedness among both countries. We found a lower genetic diversity in Chile (G/N= 0.123) than in France (G/N= 0.347); ten MLGs comprised over 80% of Chile's total genetic diversity. Furthermore, the most frequent Chilean MLGs were closely related among them, comprising four genetic groups. No genetic structuration between zones or years was detected in Chile. In contrast, small but significant structuration was detected between zones and years between sampled populations of the grain aphid in France, with significant isolation by distance. Contrarily to the genetic diversity, the frequency of Chilean MLGs changed dramatically throughout the studied years, showing an alternation in the presence of close phylogenetic-related MLGs. Our findings showed that the genetic diversity of *S. avenae* populations in Chile is extremely low as reported previously; however, we detected small changes in the microsatellite sequences that account for the temporal variation detected among the main MLGs.

Keywords: *Sitobion avenae*, grain aphid, multilocus genotype, introduced pest, native range, genetic diversity.

2.2 Introduction

Invasive species are one of the main threats to biodiversity, ecosystems, agriculture, and public health (Lee, 2002). Understanding the factors that facilitate invasive pests to succeed and spread in new environments is critical for food safety, and it has been the focus of fundamental studies on invasions dynamic. Higher genetic diversity is believed to facilitate biological invasions (Lockwood, Hoopes & Marchetti, 2013), because it makes population persistence and colonization easier (Ahlroth et al., 2003).

With about 5000 known species, aphids are among the most invasive insect pests affecting agricultural systems (Blackman & Eastop, 2000; Van Emden & Harrington, 2007). Aphids can rapidly colonize and spread in new environments mainly due to a) their reproduction mode, as they can alternate between sexual and asexual phases; b) the development of insecticide resistance; c) the development of defenses to plant chemistry and natural enemies, and d) their symbiosis with obligate and facultative bacteria that confer aphids with several relative advantages including protection to natural enemies and heat tolerance (Simon et al., 2018; Figueroa et al., 2018).

Population bottleneck and genetic drift during introduction events may reduce the genetic diversity in newly established invasive populations (Sakai et al., 2001), as reported in aphids (Figueroa et al., 2005; Zepeda-Paulo et al., 2010; Kim, Hoelmer & Lee, 2017). Furthermore, introduced populations of aphids show to lose their ability to reproduce sexually, turning into clonal populations (Figueroa et al., 2005; Harrison & Mondor, 2011; Simon et al., 2018; Figueroa et al., 2018). The lack of sexual reproduction may limit their genetic diversity and adaptive potential (Simon, Rispe & Sunnucks, 2002; Simon, Stoeckel & Tagu, 2010), besides producing some overrepresented genotypes in the introduced area (Figueroa et al., 2005; Harrison & Mondor, 2011).

Sitobion avenae is one of the main cereal pests worldwide (Alkhedir, Karlovsky & Vidal, 2013), causing damages to crops due to their mode of feeding (i.e., sap-feeding) and by transmitting plant viruses (e.g., the barley yellow dwarf virus (BYDV)) (Dedryver et al., 2005). In Chile, *S. avenae* is the main species attacking cereals (Starý, 1995). It was presumably introduced from Europe and the Mediterranean zone in the late 1960s producing important losses in cereal yields (Blackman & Eastop, 2000). As part of governmental biocontrol programs, several natural enemies species were introduced, including parasitoids and predators (Starý et al., 1993). The biocontrol program was so successful that it has allowed maintaining aphids under the threshold of economic damage without

new biological control introductions and reducing pesticide application. In Western Europe, presumable part of its native area, *S. avenae* displays the full array of reproductive strategies, including sexual, intermediate, and asexual lineages (Dedryver et al., 1998; Simon et al., 1999); however, in Chile, *S. avenae* have shown to perform only parthenogenetic reproduction (Figueroa et al., 2005). The parthenogenetic reproduction has produced that *S. avenae* have an exceptionally low genetic diversity (< 5%), with a few highly prevalent multilocus genotypes (MLGs), also called "superclones" (Figueroa et al., 2005).

Parthenogenetic reproduction can lead to the rapid accumulation of mutations (Pamilo, Nei & li, 1987), producing that invasive species could evolve in the new environment. The last report of *S. avenae*, including many individuals in an extensive Chile area, was performed almost 20 years ago (Figueroa et al., 2005), reporting four main superclones, widely distributed in all the cereal production areas, and explaining the 90% of the genetic diversity. As *S. avenae* caused disastrous consequences in the economy of cereals in Chile, it is crucial to continuously monitor their populations and evolutionary changes, in order to anticipate future evolutionary responses and facilitating pest management decision-making.

Therefore, genetic comparative studies of invasive species in an invaded and native ranges are necessary to improve the understanding of invasive species' success mechanisms, providing information on an invader's essential biological characteristics. These studies can also determine the genetic background of the invasive organism's founding population (Ross et al. 2003, 2007; Ross & Shoemaker 2008), the species' invasion pathway throughout its introduced range (Bonizzoni et al. 2004). This kind of surveys give data to predict the invasive organism's evolutive responses and their future distributions, which can also help to predict the incidence of new invasions (Sakai et al., 2001). Thus, this study was aimed to assess the genetic diversity of *S. avenae* in invaded (Chile) and native (France) areas during the springs of 2017 and 2018. We evaluated the phylogenetic relationship of multilocus genotypes (MLGs) in each country and between populations, also determining the geographic distribution of the genetic diversity and the spatial and temporal prevalence of the most frequent multilocus genotypes (MLGs) found in each country.

2.3 Materials and Methods

2.3.1 Aphid sampling

Aphid populations were sampled in in wheat and corn crops during the spring of 2017 and 2018 in Chile (September to December) and France (March to May) (Figure 1). A total of 69 populations were collected (25 from Chile and 44 from France). Chilean populations were sampled in Central Chile (zone A) and South-Central Chile (zone B) (Table 1), while French *S. avenae* populations

were obtained from the Northwest (NO), Northeast (NE), Southwest (SO), and Southeast (SE) in France. (Figure 1) (Table 2).

From each field, 5-25 adult wingless individuals of *S. avenae* were sampled, separated by at least 10 meters to limit the chances of sampling individuals belonging to the same parthenogenetic colony. Aphids were stored in Eppendorf tubes with 95% ethanol for further molecular biology analyses.

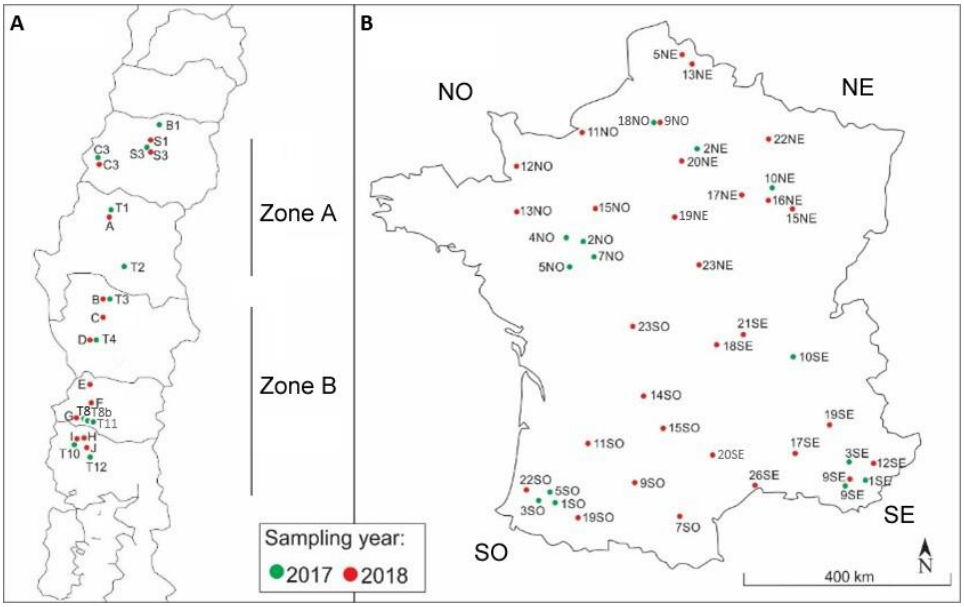

Figure 1. Sampling locations of *Sitobion avenae* populations in (A) Chile and (B) France. Different colors indicate the sampling year.

2.3.2 DNA extraction and microsatellite amplification

DNA was extracted from each individual aphid and genotyped. DNA extractions were carried out by the prepGEM DNA extraction Kit (ZYGEM™). For aphid genotyping, we used a Multiplex PCR kit (Correa et al. in prep., appendix 7.1) which allowed the amplification of seven microsatellites loci (Sm12, S3.43, S19, S5.L, Sm17, Sm10, and S3.R; Sunnucks et al., 1996; Wilson et al., 2004). Amplified PCR fragments were scored using GeneMarker v1.75 (SoftGenetics LLC).

2.3.4 Data analysis

2.3.4.1 Multilocus genotypes

To study the genetic diversity and structure of *S. avenae* populations, we used the multilocus genotypes (MLGs) approach, where each genotype is result of combining the alleles at all seven

microsatellites. The MLGs approach assumes individuals with the same genotype descent from a genetically identical ancestor (Loxdale, 2008). The software GenClone v2.0 was used to identify each MLG (Arnaud-Haond & Belkhir, 2007).

2.3.4.2 Bayesian population genetic analysis.

A Bayesian clustering approach was used to assign MLGs detected in each population to genetic clusters, as implemented in the software Structure v2.3.4 (Pritchard & Wen, 2002). This approach minimizes deviations from Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium (LD), determining the number of clusters that better represent data differentiation. The analysis was run using an admixture model of ancestry (i.e., each individual is represented by a fraction of its genome coming from some of the K hypothetical populations in the sample) with correlated allele frequencies. The number of clusters (K) were set from 1 to 15, and a total of 10 replicate runs were carried out for each value of K. Each run comprised 1,000,000 Markov chain Monte Carlo (MCMC) iterations after a burn-in period of 200,000 iterations. We used the ΔK approach implemented in the software Structure Harvester. Although this clustering algorithm assumes panmixia, the approach is still robust enough when some assumptions are violated due to the asexual reproduction mode of aphids. When multimodality was observed over structure runs, the most frequent clustering pattern for a given K-value was identified using the package CLUMPP, and the results were plotted using Distruct v1.1 (Rosenberg, 2004).

2.3.4.3 Genetic relatedness in Chilean and French populations.

To study the genetic relatedness among MLGs, a Neighbor-Joining (NJ) tree of MLGs was built for a) Chilean MLGs, b) French MLGs, and c) Chilean and French MLGs. Every NJ tree was constructed based on a distance matrix of allele shared distances (DAS), computed by the software Populations v1.2.32 (Langella, 2007). For graphical purposes, only those French MLGs observed in more than one copy (i.e., represented by >1 individual) were used (i.e., 70 MLGs).

2.3.4.4 Genetic diversity analyses.

Because clonal amplification of MLGs can affect data analysis and the interpretation of results (Sunnucks et al., 1997), the genetic diversity in populations was performed without repeated MLGs.

The genetic diversity was then calculated for each population using different indexes available in the software Arlequin v3.5.2.2 (Excoffier & Lischer, 2010) and Fstat v2.9.3.2 (Goudet, 2001). Hence, we assessed the clonal diversity (G/N), where G is the number of MLGs and N is the number of genotyped individuals, the ratio between unique (U) and multicopy MLGs (M), the number of alleles per locus (Na), the standardized allelic richness over loci for each population, the expected (H_e) and observed (H_o) heterozygosities, the linkage disequilibrium (LD), the inbreeding coefficients (F_{IS}), and departures from the Hardy-Weinberg Equilibrium (HWE).

2.3.4.5 Spatial and temporal variation of genetic diversity

To evaluate the degree of differentiation among populations, F_{ST} estimates (Weir & Cockerham, 1984) were calculated with the Arlequin v3.5.2.2 software (Excoffier & Lischer, 2010). To examine the component variance of genetic differentiation among populations, an analysis of molecular variance (AMOVA) (Excoffier et al., 2005) was also performed. Hence F_{ST} and AMOVA were performed to evaluate differentiation a) between countries and b) between years within countries.

To test whether the geographic distance is whether or not correlated to the genetic distance among populations, we performed a Mantel test for Chilean and French populations. For this purpose, we used the *Vegan* package (Oksanen et al., 2020) implemented in the R software v 3.6.1 (Team, 2019). Hence, the Pearson correlation method, with 10,000 permutations, between $F_{ST}/(1-F_{ST})$ and the distance (in kilometers) among the sampled populations was used for Chilean and French *S. avenae* populations.

Finally, the spatial and temporal differences considering the ten most frequent MLGs (MFMLG) between zones and years in Chile and France, was assessed by performing a Chi-square test implemented in R software (Team, 2019). Comparisons were performed using the package *vcd* package's association function (Meyer et al., 2017).

2.4. Results

2.4.1. Diversity of multilocus genotypes.

In total, 1,296 *S. avenae* individuals were genotyped, 456 from Chile and 838 from France. A total of 291 MLGs were found, with 56 MLGs in Chile (G/N= 0.123) and 235 in France (G/N= 0.347). The ten most frequent MLGs (MFMLG) from both countries are listed in Table 1. Hence, 10 MLGs represented 84% of all the genetic diversity in Chilean populations, while in France, the ten most abundant MLGs represented the 54% of the total genetic diversity. In Chile, 24 out of 56 MLGs were observed more than once, while in France, 70 out of 235 MLGs were found in >1 individual (Table 2).

Table 1. N° of copies and allele composition of the most frequent multilocus genotypes (MFMLG) of the grain aphid *Sitobion avenae* found in cereal crops in Chile and France.

Country	MLG	N° of copies	N° populations	Microsatellites						
				Sm12	S3.43	S19	S5.L	Sm17	Sm10	S3.R
Chile	233	90	13	139/139	185/193	134/136	221/223	201/213	167/170	338/338
	252	66	12	139/147	185/193	136/136	221/225	201/213	167/167	338/360
	234	47	15	139/139	185/193	134/136	221/223	201/213	167/170	338/360
	251	47	13	139/147	185/193	136/136	221/225	201/213	167/167	338/338
	166	38	10	133/133	185/193	134/134	221/225	201/213	170/170	338/360
	260	36	14	147/147	185/185	134/136	223/225	195/201	167/170	344/360
	181	23	8	153/153	185/185	136/136	221/223	195/197	170/170	338/338
	249	16	8	139/147	185/193	136/136	221/225	201/213	157/167	338/338
	165	14	10	133/133	185/193	134/134	221/225	201/213	170/170	338/338
	182	8	5	153/153	185/185	136/136	221/223	195/197	170/170	338/360
France	155	96	31	131/133	185/185	120/120	221/227	195/195	167/167	338/338
	111	81	29	119/131	185/185	136/136	221/223	211/213	167/167	344/360
	36	77	22	131/131	185/199	120/136	221/225	213/213	167/170	338/360
	122	51	24	119/119	165/185	000/000	223/223	195/213	167/167	338/338
	10	50	23	137/137	185/185	120/120	223/223	211/213	167/170	338/360
	219	31	7	139/139	179/207	140/140	221/225	203/207	154/167	338/338
	22	25	31	131/142	165/199	120/120	221/221	213/213	167/167	338/348
	205	16	11	139/151	185/185	120/120	225/225	195/205	167/167	338/356
	104	13	6	119/131	185/185	120/120	221/225	211/213	167/170	338/338
	192	13	6	139/142	185/185	136/136	225/225	205/211	167/167	338/360

2.4.2 Population structure

When the whole dataset is analyzed (i.e., populations from both countries), the Bayesian clustering analysis failed to provide a single robust value for K. The results revealed that the most probable number of genetic clusters was K = 2 (Ln P(K) = 3092.51, ΔK = 11.7) followed by 8 (Ln P(K) = 1067.52, ΔK = 10.7) (Figure 2). Interestingly, K=2 successfully subdivided the populations in a Chilean and a French group.

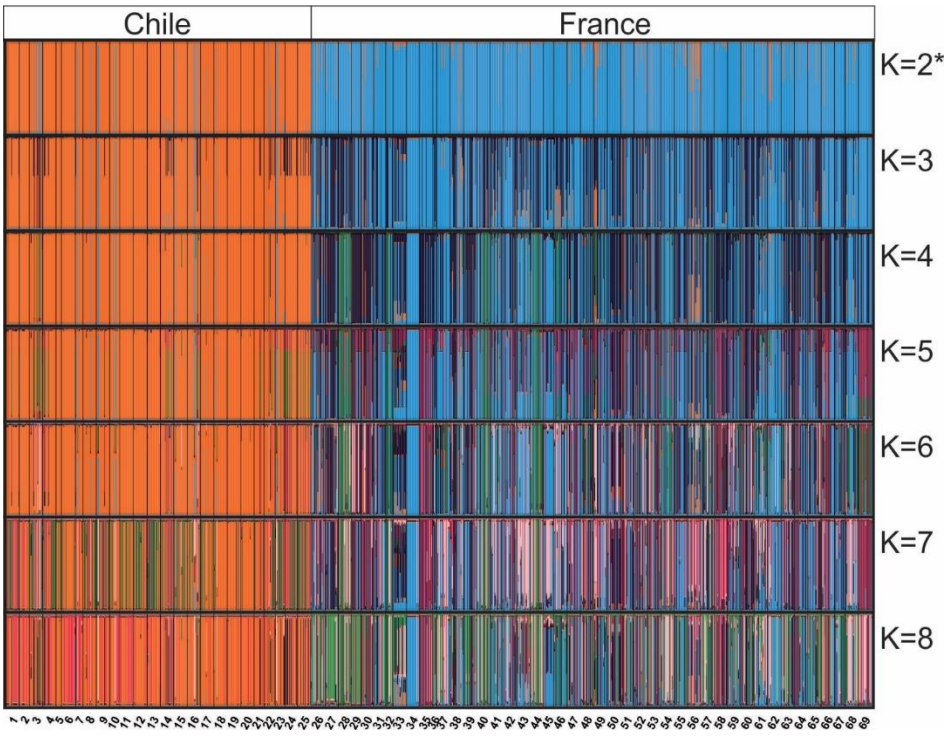

Figure 2. Estimated membership of each population to each cluster identified by the STRUCTURE Bayesian clustering method.

2.4.1 Phylogenetic relatedness

The NJ tree showed a close relationship among Chilean MLGs. Most MLGs were assigned to one of four "genetic groups". These groups comprised nearly 70% of the total MLGs and approximately 94% of the total individuals in Chile. MLGs from group 1 were the most abundant, containing five of the MFMLGs, and comprised up to 40% of individuals' total population (Figure 3A). In contrast, French MLGs seemed less related, with no evidence of "genetic groups"; however, some patterns can be distinguished, as the MLGs 155, 36, and 104 were "relatively" close genetically. These three MFMLG comprised barely 23% of the total French *S. avenae* population (Figure 3B).

When MLGs from Chilean and French populations were analyzed together, the NJ tree showed a close relatedness among Chilean and French MLGs. Main Chilean MLGs were grouped, evidencing closer genetic distance among them than with French MLGs; however, some others, including three of the MFMLGs, seemed more genetically close to French MLGs. The most prominent example is MLG 260, which is closely related to French MLG 257 (Figure 3C); indeed, all MLGs comprising group 3 seemed close to French MLGs, which was also observed in genetic group 4.

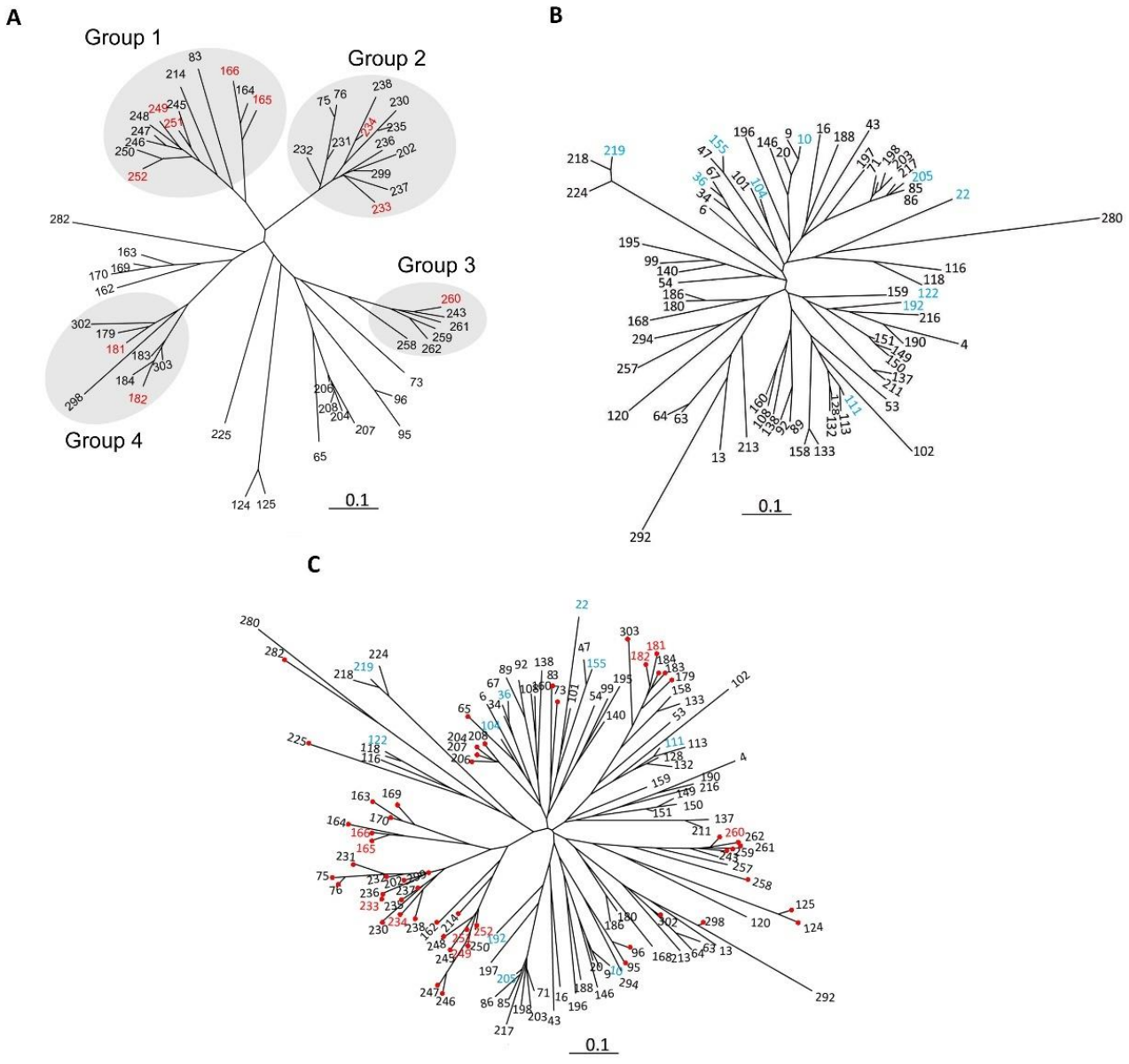

Figure 3. Neighbor-Joining tree of MLGs from Chile (A), France (B), and including MLGs from both countries (C). MFMLG from Chile and France are colored in red and blue, respectively. Red dots indicate Chilean MLGs.

2.4.2 Genetic diversity within populations

Overall, Chilean populations showed a lower genetic diversity than the French populations (Table 2). The average Na ranged from 2.14- 4.67 in Chile, while in France, it ranged from 2.50-6.71. The allelic richness (A) was also higher in French populations (1.960-2.860) than in Chile (1.980-2.670). Current linkage disequilibrium was detected for both countries; however, the maximum of linked loci detected in Chile (14/21) was lower than in France (21/21). Almost all Chilean populations showed negative F_{IS} values and heterozygote excess, except population 3, which showed a significant positive F_{IS}. The oppositive trend was observed in French populations, which showed significant positives F_{IS} values, and heterozygote deficit, in almost all populations (Table 2).

Table 2. Genetic diversity of Chilean and French populations of *S. avenae*. Number of individuals analyzed (N), total number of multilocus genotypes (G), clonal diversity (G/N), ratio of unique/multicopy genotypes (U/M), mean number of alleles (Na), allelic richness over loci (A), observed heterozygosity (Ho), expected heterozygosity (He), loci under disequilibrium out of possible tests (LD), inbreeding coefficient (F_{IS}) considering all clonal copies or one single copy per genotype.

															F_{IS}	
Population	**Sampling code**	**Host**	**Country**	**Zone**	**Year**	**N**	**G**	**G/N**	**U/M**	**Na**	**A**	**Ho**	**He**	**LD**	**One copy**	**All copies**
1	b1	Wheat	Chile	Zone A	2017	20	5	0,250	2/3	2.857	2.35	0,629	0.648	1/21	0.033	-0.239
2	C3	Wheat	Chile	Zone A	2017	15	5	0.333	2/3	2.571	2.23	0.686	0.619	3/21	-0.123	-0.311
3	S3	Wheat	Chile	Zone A	2017	20	10	0.500	7/3	4.571	2.67	0.614	0.717	14/21	0.150*	0.017
4	T1	Wheat	Chile	Zone A	2017	20	7	0.350	3/4	2.857	2.24	0.653	0.603	2/21	-0.091	-0.115
5	T10	Wheat	Chile	Zone B	2017	8	4	0.500	2/2	2.143	1.98	0.762	0.530	0/21	-0.561	-0.608
6	T11	Wheat	Chile	Zone B	2017	21	6	0.286	2/4	2.714	2.24	0.705	0.624	11/21	-0.147	-0.278
7	T12	Wheat	Chile	Zone B	2017	11	6	0.545	4/2	3.286	2.32	0.619	0.627	2/21	0.015	-0.119
8	T2	Wheat	Chile	Zone A	2017	20	6	0.300	3/3	3.0	2.32	0.700	0.639	3/21	-0.105	-0.372
9	T3	Wheat	Chile	Zone B	2017	20	5	0.250	1/4	3.143	2.37	0.700	0.633	0/21	-0.122	-0.366
10	T4	Wheat	Chile	Zone B	2017	15	5	0.333	1/4	3.0	2.35	0.729	0.650	2/21	-0.138	-0.268
11	T8	Wheat	Chile	Zone B	2017	20	3	0.150	0/3	2.286	2.09	0.679	0.592	3/21	-0.175	-0.28
12	T8b	Wheat	Chile	Zone B	2017	21	7	0.333	3/4	2.714	2.24	0.735	0.620	6/21	-0.203	-0.429
13	A	Wheat	Chile	Zone A	2018	20	11	0.550	7/4	3.286	2.21	0.571	0.577	7/21	0.01	-0.199
14	B	Wheat	Chile	Zone B	2018	20	8	0.400	2/6	3.429	2.24	0.571	0.573	5/21	0.002	-0.055
15	C	Wheat	Chile	Zone B	2018	20	7	0.350	3/4	3.0	2.18	0.643	0.568	2/21	-0.147	-0.342
16	D	Wheat	Chile	Zone B	2018	20	13	0.650	9/4	4.667	2.3	0.654	0.682	6/21	0.042	-0.047
17	E	Wheat	Chile	Zone B	2018	20	6	0.300	3/6	2.857	2.12	0.648	0.555	3/21	-0.188	-0.518
18	F	Wheat	Chile	Zone B	2018	20	7	0.350	5/2	3.167	2.09	0.655	0.612	1/21	-0.076	-0.467
19	G	Wheat	Chile	Zone B	2018	20	5	0.250	3/5	2.429	2.15	0.607	0.590	1/21	-0.028	-0.581
20	H	Wheat	Chile	Zone B	2018	20	5	0.250	2/5	2.429	2.04	0.600	0.543	2/21	-0.12	-0.453
21	I	Wheat	Chile	Zone B	2018	15	7	0.467	3/7	2.857	2.13	0.612	0.556	6/21	-0.111	-0.287
22	J	Wheat	Chile	Zone B	2018	17	8	0.471	5/3	3.429	2.35	0.595	0.630	12/21	0.062	-0.163
23	S1	Wheat	Chile	Zone A	2018	14	7	0.500	4/3	3.143	2.26	0.599	0.585	3/21	-0.024	-0.132
24	S3	Wheat	Chile	Zone A	2018	19	6	0.316	3/3	3.0	2.24	0.571	0.593	2/21	0.04	0.001
25	C3	Wheat	Chile	Zone A	2018	21	8	0.381	5/3	3.143	2.18	0.546	0.560	5/21	0.028	-0.064

Subtotal						**457**	**56**	**0.123**	**32/24**	**3.04**	**2.24**	**0.643**	**0.605**	**20/21**	**-0.079**	**-0.267**
26	10NE	Corn	France	NE	2017	20	11	0.550	6/5	4.571	2.51	0.545	0.656	1/21	0.175*	0.191*
27	10SE	Wheat	France	SE	2017	20	9	0.450	6/9	4.143	2.33	0.405	0.576	2/21	0.313*	0.195*
28	1SE	Wheat	France	SE	2017	20	14	0.700	12/14	6.286	2.78	0.546	0.744	20/21	0.275*	0.190*
29	1SO	Wheat	France	SO	2017	14	3	0.214	0/3	2.667	2.02	0.389	0.533	0/21	0.317*	0.148
30	2NE	Corn	France	NE	2017	19	13	0.684	11/2	5.0	2.45	0.442	0.619	5/21	0.296*	0.270*
31	2NO	Corn	France	NO	2017	18	11	0.611	6/5	4.0	2.39	0.547	0.629	2/21	0.137*	0.125*
32	3SE	Wheat	France	SE	2017	11	7	0.636	6/1	4.857	2.61	0.531	0.678	4/21	0.232*	0.191*
33	3SO	Corn	France	SO	2017	20	6	0.300	2/4	2.571	1.96	0.429	0.465	7/21	0.082	0.05
34	4NO	Corn	France	NO	2017	19	2	0.105	0/2	2.5	2.29	0.750	0.694	0/21	-0.125	-0.751
35	5NE	Corn	France	NE	2017	20	7	0.350	6/1	3.857	2.35	0.586	0.609	4/21	0.042	-0.113
36	5NO	Corn	France	NO	2017	6	6	1.000	6/0	3.571	2.27	0.457	0.566	1/21	0.21*	0.210*
37	5SO	Corn	France	SO	2017	20	8	0.400	3/5	4.0	2.49	0.497	0.672	6/21	0.275*	0.238*
38	7NO	Corn	France	NO	2017	20	16	0.800	13/3	5.286	2.55	0.500	0.669	15/21	0.259*	0.230*
39	9NO	Wheat	France	NO	2017	20	10	0.500	6/4	3.429	2.27	0.484	0.597	14/21	0.200*	0.106
40	9SE	Wheat	France	SE	2017	20	13	0.650	11/2	5.857	2.68	0.473	0.705	16/21	0.339*	0.264*
41	11NO	Wheat	France	NO	2018	20	14	0.700	12/2	6.0	2.64	0.421	0.694	20/21	0.404*	0.227*
42	11SO	Wheat	France	SO	2018	20	11	0.550	8/3	4.714	2.37	0.477	0.611	17/21	0.229*	0.113*
43	12NO	Wheat	France	NO	2018	20	15	0.750	13/2	5.429	2.42	0.459	0.633	19/21	0.284*	0.260*
44	12SE	Wheat	France	SE	2018	20	10	0.500	8/2	5.857	2.72	0.514	0.708	7/21	0.286*	0.224*
45	13NE	Wheat	France	NE	2018	15	5	0.333	0/5	3.2	1.99	0.600	0.644	0/21	0.077	-0.132
46	13NO	Wheat	France	NO	2018	20	18	0.900	16/2	6.714	2.86	0.555	0.764	21/21	0.280*	0.295*
47	14SO	Wheat	France	SO	2018	20	9	0.450	6/3	4.857	2.52	0.520	0.666	12/21	0.231*	0.127*
48	15NE	Wheat	France	NE	2018	20	15	0.750	13/2	6.714	2.66	0.588	0.696	15/21	0.160*	0.110*
49	15NO	Wheat	France	NO	2018	20	15	0.750	13/2	6.143	2.76	0.508	0.729	21/21	0.313*	0.235*
50	15SO	Wheat	France	SO	2018	20	8	0.400	3/5	3.714	2.3	0.523	0.588	1/21	0.119	0.160*
51	16NE	Wheat	France	NE	2018	20	13	0.650	9/4	5.0	2.43	0.478	0.616	5/21	0.231*	0.218*
52	17NE	Wheat	France	NE	2018	20	14	0.700	11/4	6.143	2.5	0.501	0.649	7/21	0.235*	0.174*
53	19NE	Wheat	France	NE	2018	20	12	0.600	7/5	4.571	2.48	0.481	0.652	5/21	0.273*	0.220*
54	17SE	Wheat	France	SE	2018	20	12	0.600	9/3	5.571	2.39	0.476	0.597	4/21	0.209*	0.209*
55	20SE	Wheat	France	SE	2018	20	8	0.400	5/3	3.429	2.28	0.589	0.596	1/21	0.013	-0.03
56	18SE	Wheat	France	SE	2018	20	11	0.550	6/5	4.571	2.45	0.613	0.617	6/21	0.007	-0.016
57	19SE	Wheat	France	SE	2018	20	17	0.850	15/2	5.857	2.56	0.519	0.664	10/21	0.224*	0.229*
58	19SO	Wheat	France	SO	2018	20	6	0.300	12/4	3.714	2.41	0.429	0.630	7/21	0.337*	0.231*
59	20NE	Wheat	France	NE	2018	20	15	0.750	12/3	5.429	2.31	0.452	0.583	3/21	0.230*	0.214*

60	21SE	Wheat	France	SE	2018	20	10	0.500	7/3	4.286	2.35	0.464	0.599	1/21	0.236*	0.181*
61	22NE	Wheat	France	NE	2018	20	19	0.950	18/1	5.571	2.38	0.460	0.611	4/21	0.252*	0.241*
62	22SO	Wheat	France	SO	2018	20	9	0.450	8/1	3.714	2.24	0.500	0.569	10/21	0.126	-0.218
63	23NE	Wheat	France	NE	2018	20	14	0.700	10/4	5.286	2.48	0.443	0.637	3/21	0.312*	0.288*
64	23SO	Wheat	France	SO	2018	20	9	0.450	5/4	3.714	2.29	0.514	0.601	4/21	0.154*	0.038
65	26SE	Wheat	France	SE	2018	20	12	0.600	7/5	4.857	2.53	0.568	0.658	2/21	0.143*	0.101*
66	7SO	Wheat	France	SO	2018	20	13	0.650	9/4	4.429	2.3	0.476	0.589	1/21	0.197*	0.196*
67	18NO	Wheat	France	NO	2018	16	12	0.750	9/3	4.429	2.32	0.429	0.585	12/21	0.277*	0.185*
68	9SE	Wheat	France	SE	2018	20	11	0.550	5/6	4.857	2.52	0.538	0.654	1/21	0.185*	0.153*
69	9SO	Wheat	France	SO	2018	20	9	0.450	5/4	3.429	2.04	0.476	0.479	6/21	0.006	-0.073
Subtotal						**838**	**235**	**0.280**	**116/119**	**4.65**	**2.419**	**0.503**	**0.63**	**21/21**	**0.206**	**0.129**

(*) indicates significant deviations from H-W equilibrium.

2.4.3 Spatial and temporal genetic differentiation among populations

F_{ST} analysis showed that Chilean and French populations are significantly differentiated (F_{ST}= 0.06615, P= < 0.001). Chilean zones showed no differentiation among them (F_{ST} = -0.00639, P= 0.73), which also agrees with the Mantel test (r= 0.030, P= 0.309), showing that genetic distance is not related to geographical distance for Chilean populations of *S. avenae* (Figure 4A). Contrarily, France showed small but significant differentiation among zones (Table 3). which is also supported by the Mantel test performed for the 44 populations (r= 0.20, P= 0.001) (Figure 4B). The analysis per year showed non-significant differences between years in Chile (F_{ST}=-0.00591, P= 0.56) and France (F_{ST}= 0.00073, P= 0.38).

Table 3. F_{ST} values performed for every zone in French populations.

	NO	NE	SO	SE
NO	-			
NE	0.01239*	-		
SO	-0.00132	0.02177*	-	
SE	0.00436	0.02232*	0.00571	-

(*) p-values < 0.05.

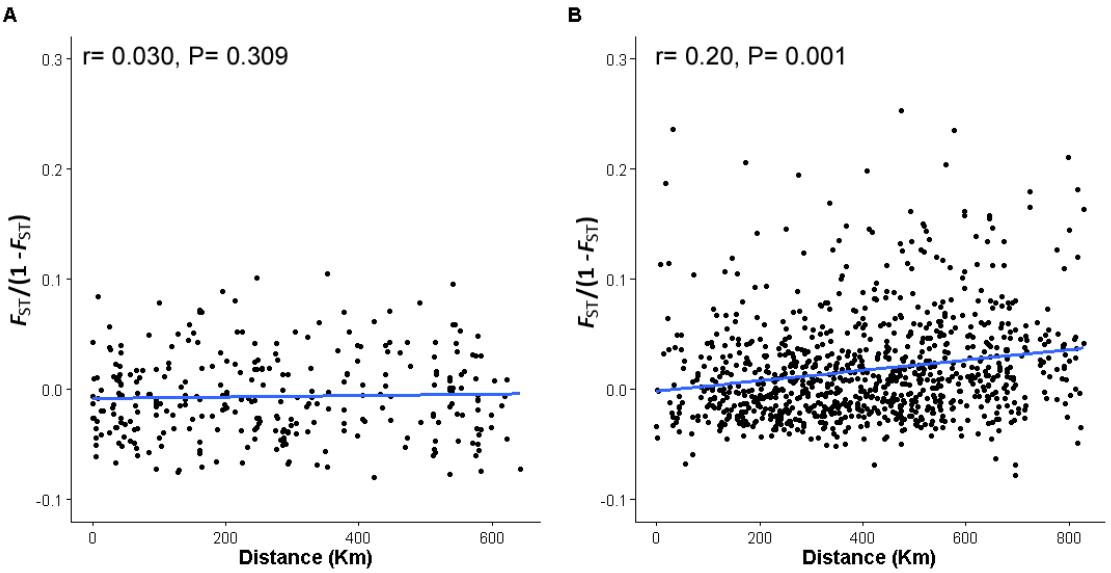

Figure 4. Isolation-by-distance for populations in (A) Chile and (B) France.

AMOVA results showed that the variance between countries was significant, but the variance within individuals explained 75.64 % of the total variance (Table 4). On the other hand, the year seemed not to explain the variance in Chilean *S. avenae* populations; indeed, as low genetic diversity was detected, there was no significant effect on any factor. The year seemed not to be explaining the French *S. avenae* variance either, but indeed, for the variation within individuals in years and within individuals.

Table 4. Analyses of the Molecular Variance (AMOVA) as a weighted average over loci were carried out to compare the effect of categorical factors on the *S. avenae* genetic structure.

	Source of variation	df	Sum of squares	Variance components	Percentage of variation	P-value
Country	Among countries	1	7.182	0.03628	6.52	<0.001
	Among individuals within countries	289	179.055	0.09930	17.84	<0.001
	Within individuals	291	122.500	0.42096	75.64	<0.001
Year (Chile)	Between years	1	0.574	-0.00259	0.15	0.921
	Among individuals within years	59	42.459	-0.13608	15.36	0.997
	Within individuals	61	60.500	0.99180	84.49	0.998
Year (France)	Between years	1	0.580	-0.00019	-0.04	0.828
	Among individuals within years	261	161.734	0.11782	23.49	<0.001
	Within individuals	263	101.000	0.38403	76.55	<0.001

The geographic distribution of the MFMLGs for Chile and France was also studied. The results showed significant differences between zones in Chile (χ^2= 62.76, df= 9, P= <0.001) and France, (χ^2= 210.29, df=27, P= < 0.001). In Chile, the differences were given by a higher frequency of the MLG 233 in zone B, while the oppositive was found in zone A (Figure 5A and Figure S1). In France, the MLG 36 was most

frequently found in NO than in NE and SE, while MLG 155 is more frequent in NE and SO than in NO and SE. MLG 192 was more frequent in SE, and MLG 219 was more frequent in SE and less in NE, NO, and SO (Figure 5C) (Figure S3).

Differences in the presence and frequency of almost all the MFMLGs between years were detected in Chile (χ^2= 339.99, df= 9, P= <0.001). For instance, MLG 166 was only present in 2017, while MLG 233, 251, 181, 249 and 165 were only present in 2018 (Figure 5C) (Table 5) (Figure S2). Small variations between years were observed in France MFMLGs (χ^2= 210.29, df= 9, P= < 0.001), with the exception of MLG 10 and 219 which were more frequently found in 2017 (Figure S4).

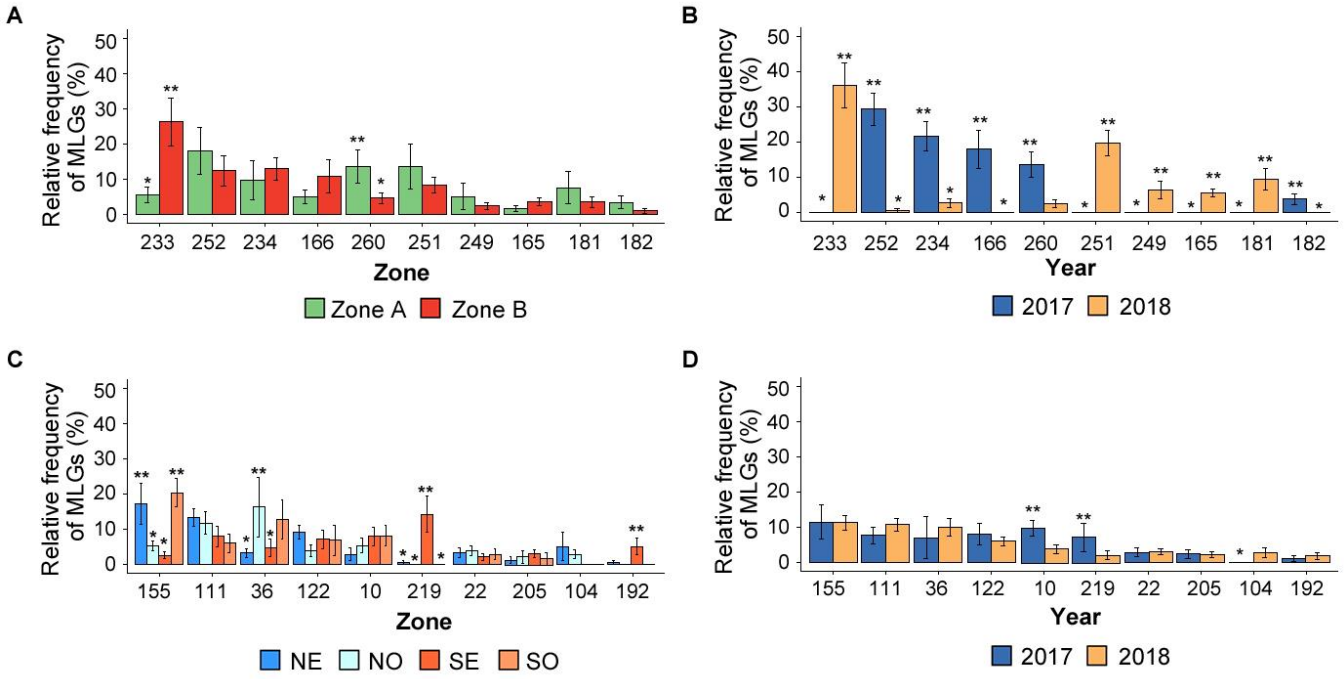

Figure 5. Relative frequency (Mean ± SE) of the MFMLGs in populations according to (A-C) the zone and the (B-D) year in Chile (A-B) and France (C-D). Asterisks indicate that Pearson residuals' values are lower than expected (*) or higher than expected (**) in the chi-square test.

2.5 Discussion

2.5.1 Genetic diversity of introduced and native populations of *S. avenae.*

Lower genetic diversity is expected in introduced populations due to genetic bottleneck and founder effect processes during invasive species' introduction (Sakai et al., 2001). Our results support the low genetic diversity of *S. avenae* previously found in Chile (Figueroa et al., 2005) and the lower genetic diversity of introduced aphids than their native area (Kim, Hoelmer & Lee, 2017). Moreover, the heterozygote excess found reflects the long-term asexual reproduction in Chilean populations of *S. avenae* (Balloux, Lehmann & De Meeûs, 2003). Previously, Figueroa et al. (2005) reported that four MLGs comprised nearly 90% of the total genetic diversity in *S. avenae* from Chile, constituting a 0.04 of genotypic diversity. Twenty years later, we found a higher genotypic diversity (G/N= 0.123), but still extremely low compared to the native area (G/N= 0.347). Ten MLGs (MFMLGs) comprised 84% of the total genetic diversity detected in Chilean populations; however, some of them showed to be closely genetically related, as they probably diverged from the same MLGs, which would reduce the total MFMLGs to only four genetic groups, a pattern that was not observed in France where evident genetic groups were detected. Invasive species may evolve after their introduction, mainly due to the action of selective agents in the new area; this is even more evident when organisms reproduce mainly by parthenogenesis since advantageous traits can be more rapidly spread in populations (Pamilo, Nei & li, 1987). Whether the Chilean MFMLGs possess any advantageous characteristic compared to other no frequent MLGs is not clear yet, but some previous studies can give some helpful insights. For instance, frequent Chilean MLGs can thrive with plants' secondary chemistry and be prevalent in various cereals, unlike less frequent MLGs, which showed better performance in particular cereals (Figueroa et al., 2004). This last is also supported by studies that indicate that Chilean superclones have flat energetic costs for non-induced detoxification enzymes on defended plants (Castañeda et al., 2010) and more plastic probing behavior on cereals (Barrios-Sanmartín, Figueroa & Ramírez, 2016), suggesting that common Chilean genotypes are more generalists in terms of the plant chemistry array. Selection may favor generalist or general-purpose clone organisms that can survive in all environments, which are featured by wide tolerance and low fitness variation (Lynch, 1984; Figueroa et al, 2018), especially when they arrive in a new environment since they must rapidly feed and spread. Hence, the ability to feeding on a broader range of cereals can be an evidence of selection favoring those clonal MLGs with the ability to colonize various cereals over those that may have a better performance in a particular cereal but cannot spread in others (Figueroa et al., 2004), altough this needs to be confirmed studying the performance of different genotypes.

On the other hand, our results are consistent with previous reports on the genetic diversity, heterozygote deficit, and high F_{IS} values reported in *S. avenae* in France (Vialatte et al., 2005; Dedryver et al., 2008).

Current deviations from HWE in French populations by positive F_{IS} and heterozygote deficit agree with the presence of sexual reproduction in French populations of *S. avenae* (Dedryver et al., 2008).

A few MLGs, although not as frequent as in Chile, were prevalent in all the country and the studied years. The MLG 155 was present in all zones studied in France. Previous studies have reported that French *S. avenae*, even when performing all the reproduction modes, tend to increase the sexual reproduction northward (Dedryver et al., 2008, 2009); however, we found significant positive F_{IS} values for all studied zones in France; even a lower F_{IS} average was found in SO, reflecting the occurrence of sexual reproduction of *S. avenae* in all French zones.

2.5.2 Multilocus genotypes relatedness.

Our results show similar results to those reported by Figueroa et al. (2005), where a few groups of genotypes contained almost all the genetic diversity for *S. avenae* in Chile. We found that most of the MFMLGs are closely related, where MLGs 233 and 234 are very similar examples. When Chilean and French MLGs were analyzed, most Chilean MLGs and MFMLG were grouped, meaning that they probably descend from the same MLG. Nevertheless, MLGs 180, 181, and 260 were more distant from the other MFMLGs; indeed, those MLGs and other lesser common MLGs were grouped with the French MLGs. Previously Figueroa et al. (2005) reported that S1 (the most frequent MLG found in their study) was identical to G6, a common MLG from France. Hence, it is probable that MLGs 180, 181, or 260 are the same S1 detected in that study, and due to mutations accumulated in the time at the microsatellite loci, they are currently more differentiated. Hence, that MLG S1 detected by Figueroa et al. (2005) was probably a recent introduction in the time when that study was performed as they shared the same alleles with the French G6. However, the possibility of subsequent invasions events after the study of Figueroa et a. (2005) should not be ruled out.

2.5.3 Spatial and temporal genetic diversity.

The lack of genetic structure between zones in Chile confirms the high dispersion ability of *S. avenae*. Contrarily, French populations showed a small but significant degree of genetic structuring among zones, and a significant correlation between the genetic and the geographic distances. Aphids can display various reproduction modes, including cyclical parthenogenesis and obligate parthenogenesis (Simon et al., 1999). Hence, sexual aphids can produce overwintering eggs that can survive to freezing temperatures during winter, as parthenogenetic aphids are shown to die with temperatures under -8°C in the field (Powell & Bale, 2004). In Chile, *S. avenae* mainly reproduce by parthenogenesis, while in France, the whole array of reproduction modes is present (Dedryver et al., 1998). Sexual aphids can spread to colder areas, while parthenogenetic aphids are restricted to areas with mild winter

temperatures. The restricted spread of parthenogenetic *S. avenae* to colder areas could explain the differences in the spatial genetic structure. Previously, a pattern of increased sexual reproduction northward was observed in French populations of *S. avenae* (Dedryver et al., 2008). Our data, however, showed significant positive values of F_{IS} in almost all French populations, suggesting the presence of sexual reproduction among all sampled zones; however, SO showed lower F_{IS} values than the other zones, which could partially support the previous findings in France.

On the other hand, no sign of temporal genetic structuring was detected in Chilean and French populations. A lack of structuring in Chile is expected, as very low genetic diversity was detected, with a few genetic groups that are highly prevalent in space and time. A small but significant effect of genetic variance of the individuals within years was detected in French populations. However, this was probably due to the more considerable sampling effort conducted during 2018, as more populations were sampled during that year, producing the higher G/N value detected in this year.

When the spatial and temporal prevalence of the MFMLGs was studied, we found significant differences between zones in Chile, mainly due to a higher frequency of the MLG 233 in zone B and a higher frequency of the MLG 260 in zone A. Moreover, the closely related MFMLGs showed temporal variation; hence, MLG 233 was absent in 2017, while MLG 234 showed its higher frequency; on the contrary, MLG 233 had a high frequency in 2018, while MLG 234 only was present in 6 individuals. The same pattern was observed with MLGs 252, 251, and 249; 165 and 166; and 181 and 182. This pattern was already reported by Figueroa et al. (2005), with some highly prevalent MLGs variating their presence among years. Genetic drift can cause changes in the frequencies of clones; however, the observed changes in the presence and frequency of MFMLGs can also result from an intense clonal selection. Whether these frequent and closely related MLGs (e.g., MLG 233 and 234) have any differential characteristic that accounts for the observed shifts in our results is unknown, and further studies, including more seasons sampled, are needed.

On the other hand, the French MLG 155 was the most frequent genotype in France and showed to be highly distributed among all zones and years; previously, Hack et al. 2000 already reported the high prevalence of two asexual genotypes in France. Our study supports their findings as probably MLG 155 is the same asexual genotype found in that study.

2.6 Conclusions

Invasive pests can display genetic diversity even in the absence of sexual reproduction, being able to evolve and develop new strategies to face the selective agents in heterogeneous environments. Furthermore, new invasions events are a permanent threat, which could unbalance any equilibrium

reached in countries where biological control successfully control aphid pests, as the case of Chile. Our results show that *S. avenae* still have low genetic diversity, as was found 20 years ago; nevertheless, some highly frequent MLGs have diverged from their ancestral founder and showed changes between years. However, these changes have occurred at microsatellite loci, which are known to be neutral; hence, whether the observed shifts between years are due to selection or the result of drift is unclear. Nevertheless, it is important to continuously monitor the genetic diversity of invasive species since it could bring interesting data on adaptation under asexual reproduction and detect new introduction events that could threaten Chilean agriculture again.

2.7 References

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3. Chapter 3

Tittle

Spatial and temporal secondary endosymbionts distribution in native and introduced populations of *Sitobion avenae.*

Objective:

Characterize the spatial and temporal distribution of secondary endosymbionts from *S. avenae* in Chile and France and their association with frequent MLGs.

3.1 Abstract

The role of secondary endosymbionts (SE) providing advantages during colonization to new environments in invasive species has been poorly studied. SE adds an additional source of phenotypic diversity to their hosts, which could help to cope the effects of genetic bottlenecks during the invasion process, representing an advantage for invasive species. In this work, we studied the spatial and temporal frequency of SE in the English grain aphid *Sitobion avenae*, sampled from an invaded (Chile) and native (France) areas. We looked for any association between the SEs with the most frequent genotypes of *S. avenae* from both countries, searching for associations that could give some insights into the putative role of SE on the success of *S. avenae* in Chile. We sampled a wide area from Chile and France during the springs of 2017 and 2018 and determined a) the diversity of SE species found in each country, b) the proportion of the SE found in the different climatic zones and seasons from both countries, and c) the association of certain SE species with the most frequent genotypes (MLGs) observed in both countries. We found a higher diversity of SE species in the native range. There was no evidence of differential geographic distribution of SE in any country, but significant differences between seasons for the presence of *Hamiltonella defensa* in Chile, which was completely absent in season 2017. Our results show valuable information of the SE dynamics in invasive and native areas, showing a strong association among certain MLGs of *S. avenae* with some SE species in an area where aphids have few signs of sexuality. Also, we report a significant effect this association on the population's dynamics of MLGs and SE at the field level.

Keywords: *Sitobion avenae*, endosymbionts, native range, introduced range, invasive pest.

3.2 Introduction

Symbiosis constitutes one of the most intimate associations between organisms, bringing individuals heritable traits of high selective advantage in specific environments (Margulis, 1976). In insect pests, symbiosis has shown to be particularly important. Aphids harbor a primary endosymbiont, *Buchnera aphidicola,* which resides in special cells called "bacteriocytes." This bacterium is essential for the survival of aphids, as it provides them with essential amino acids and vitamins absent from the plant phloem (Buchner, 1965). Also, aphids can harbor several secondary endosymbionts (SE), which are not essential for their survival, but they can have substantial impacts on the aphid phenotype (Oliver et al., 2010). Indeed, aphids harboring specific SE have shown to display a variety of phenotypes as the capability of expanding their plant host range (Tsuchida, Koga & Fukatsu, 2004), protection to natural enemies (Oliver et al., 2003, 2010; Vorburger et al., 2010; Łukasik et al., 2013a,c; Cayetano & Vorburger, 2015), and tolerance to abiotic factors like extreme temperatures (Montllor, Maxmen & Purcell, 2002; Guay et al., 2009).

The advantageous traits related to SE infections have gained massive relevance in the last years because they could be essential for insect pests' adaptative potential (Janson et al., 2008; Feldhaar, 2011). As invasive species often arrive in low numbers, the genetic bottlenecks and founder effects can significantly reduce their genetic diversity (Schmid-Hempel et al., 2007; Puillandre et al., 2008). In aphids, extremely low genetic diversities have been reported in most of the invaded areas (Figueroa et al., 2005; Harrison & Mondor, 2011), but even though their low genetic diversity, aphids have shown to be highly invasive.

Sitobion avenae is a common cereal pest worldwide (Alkhedir, Karlovsky & Vidal, 2013). It can inflict important damages to cereal crops due to their sap-feeding diet, which causes physical damage to plants and the transmission of plant viruses (i.e., BYDV) (Dedryver et al., 2005). *Sitobion avenae* was presumably introduced to Chile from Europe and the Mediterranean zone (Blackman & Eastop, 2000). In Chile, *S. avenae* is the main aphid species infecting cereals (Starý, 1995) and is widely distributed in all cereal production areas. However, the genetic diversity of *S. avenae* have been shown to be exceptionally low (< 5%), with a few highly prevalent multilocus genotypes (superclones) representing almost 90% of the total genetic diversity (Figueroa et al., 2005).

Sitobion avenae have been shown to be infected by several SE including *Regiella insecticola*, *Hamiltonella defensa*, *Serratia symbiotica,* and *Rickettsia sp.* (Łukasik et al., 2013b; Luo et al., 2016; Sepúlveda et al., 2017; Hu et al., 2020). In Chile, *S. avenae* is mainly infected by *R. insecticola* and *H. defensa*, both showing a distinct and characteristic geographic distribution pattern (Sepúlveda et al., 2017).

The adaptative potential of SE facilitating insect pest invasiveness in a new area has been poorly studied. However, SE should be considered in biological invasions studies as they represent an important source of additional phenotypic variation that could compensate the low genetic diversity of introduced insect pests that reproduce asexually (Oliver et al., 2010). Comparative studies of the SE composition among the invaded and native areas could bring valuable knowledge on the SE's putative role during the invasion. Furthermore, any geographic pattern could be an important insight into the effects produced by SE in aphids, as the geography has shown to impact on SE distribution in several aphid species (Tsuchida et al., 2002; Luo et al., 2016; Sepúlveda et al., 2017; Hu et al., 2020).

In this work, we studied the spatial and temporal distribution of the most common SE found in *S. avenae*, considering an extensive area of the invaded (Chile) and native (France) areas during the spring of 2017 and 2018. Further, we studied the putative association among the main MLGs from both countries and SE species, giving some insights into SE's role during the aphid invasion.

3.3 Materials and Methods

3.3.1 Aphid Sampling

Aphid populations were sampled in wheat and corn crops during the Spring of 2017 and 2018 in Chile (September to December) and France (March to May) (chapter 2: Table 2) (Figure 1). A total of 69 populations were collected (25 from Chile and 44 from France). Chilean *S. avenae* were sampled along approximately 600 kilometers latitudinally. Two different agroclimatic zones were comprised; Central Chile, which presents a predominately warm Mediterranean climate (zone A), and Central-south Chile, characterized by a predominant temperate rainy climate (Zone B).

French populations of *S. avenae* sampled across a wide area of France, comprising nearly 800 kilometers latitudinally. The sampled fields contemplated the Northwest (NO), Northeast (NE), Southwest (SO), and Southeast (SE) zones in France. (Figure 1).

In each field, 5-25 adult individuals of *S. avenae* were sampled, separated by at least 10 meters, to limit the chances of sampling individuals belonging to the same colony. Aphids were kept in Eppendorf tubes with 95% ethanol for further molecular biology analyses.

3.3.2 MLG determination and facultative endosymbiont screening.

DNA was extracted from each aphid individual and then genotyped. DNA extractions were carried out by the prepGEM DNA extraction Kit (ZYGEM^{TM}). For aphid genotyping, we used a Multiplex PCR kit (Correa et al. in prep, appendix 7.1) to amplify the microsatellites loci Sm12, S3.43, S19, S5.L, Sm17, Sm10, and S3.R (Sunnucks et al., 1996; Wilson et al., 2004). Amplified PCR fragments were scored using

GeneMarker v1.75 (SoftGenetics LLC). We used the ten most frequent genotypes (MFMLGs) to study possible associations among SE species and *S. avenae* MLGs (chapter 1) (see Table 1).

We screened the seven most frequent facultative endosymbionts found in aphids: *Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, *Fukatsuia symbiotica*, *Rickettsia sp*., *Ricketsiella sp*., and *Spiroplasma sp*. following the protocol of (Peccoud et al., 2013; Sepúlveda et al., 2017). The obligate endosymbiont *B. aphidicola* was used as the positive control.

Table 1. The main MLGs (MFMLG) found in Chile and France.

Country	Main MLG	N° of copies	Number of populations present	Observed in
Chile	233	90	13	2018
	252	66	12	2017 and 2018
	234	47	15	2017 and 2018
	251	47	13	2018
	166	38	10	2017
	260	36	14	2017 and 2018
	181	23	8	2018
	249	16	8	2018
	165	14	10	2018
	182	8	5	2017
France	155	96	31	2017 and 2018
	111	81	29	2017 and 2018
	36	77	22	2017 and 2018
	122	51	24	2017 and 2018
	10	50	23	2017 and 2018
	219	31	7	2017 and 2018
	22	25	31	2017 and 2018
	205	16	11	2017 and 2018
	104	13	6	2018
	192	13	6	2017 and 2018

3.3.3 Data analysis.

The proportion of each secondary endosymbiont found was studied for every country separately. Hence, we evaluated the effect of aphids' geographic distribution through all populations in Chile and France and grouped by zones.

Temporal variation for SEs was also studied. Hence, we determined the effect of the sampling year (2017 and 2018) on the proportion of SEs.

All data were analyzed in the R software version 3.6.1 (Team, 2019). To test the effect of every zone, year, and MFMLG on the frequency of SEs, we used generalized linear models (GLMs).

The proportion of each SE was the dependent variable assuming a binomial distribution error. Due to some SEs were found in very low frequencies, we only considered single and double infections present in > 5 individuals.

Multiple comparisons were performed by the packages *multcomp* (Hothorn et al., 2020)

3.4 Results

3.4.1 Secondary endosymbiont frequencies

We detected six of the seven facultative endosymbionts previously reported for S*. avenae* in the 1,294 individuals analyzed (Chile=456, France= 838). *Rickettsia sp*. was the only facultative endosymbiont which was not detected in both countries, while *Fukatsuia symbiotica* and *S. symbiotica* were not detected in Chilean populations of *S. avenae*. *Regiella insecticola* (Chile= 339, France= 493) and *Hamiltonella defensa* (Chile= 45, France= 132) were the main facultative endosymbionts infecting *S. avenae* (Figure 2). Although facultative endosymbionts were mainly present as single infections, some low-frequency co-infections were also found; *R. insecticola-H. defensa* (Chile= 2)*, R. insecticola-Ricketsiella* (Chile= 2)*, R. insecticola-Spiroplasma* (France= 1), *R. insecticola-S. symbiotica* (France= 1)*, R. insecticola-Fukatsuia* (France= 8)*, H. defensa-Fukatsuia* (France= 7) *and H. defensa-Ricketsiella* (Chile= 1)*.*

3.4.2 Spatial and temporal distribution of facultative endosymbionts.

To better understand SE's distribution, we assessed the proportion of the more frequent single and double SE infections found in all populations (Chile=25: France= 44) (Figure 1).

Our results showed no evidence of significant variation in the proportion of SE between zones in Chile (χ^2= 6.490, df= 3, P= 0.090) (Figure 2). On the other hand, France showed an apparent effect of the zone

(χ^2=58.04, df= 21, P= < 0.001), but multiple comparisons showed that it was only due to significant variations in the proportion of facultative endosymbionts within each zone, but not among them (Figure 2). Higher frequencies of *R. insecticola* than other SE were found in all zones (Figure 2).

On the other hand, temporal variation in the presence and proportion of SE was detected in Chile (Figure 2C) (χ^2=24.804, df= 3, P= <0.0001). The differences were due to the absence of *H. defensa* during 2017 and a higher abundance of *R. insecticola* in 2017. Differences between years were also observed in France, with a significantly higher abundance of *H. defensa* in 2018, while the oppositive trend was observed for *R. insecticola* (Figure 2D). The proportion of uninfected individuals was also significantly higher in 2018 (Figure 2D).

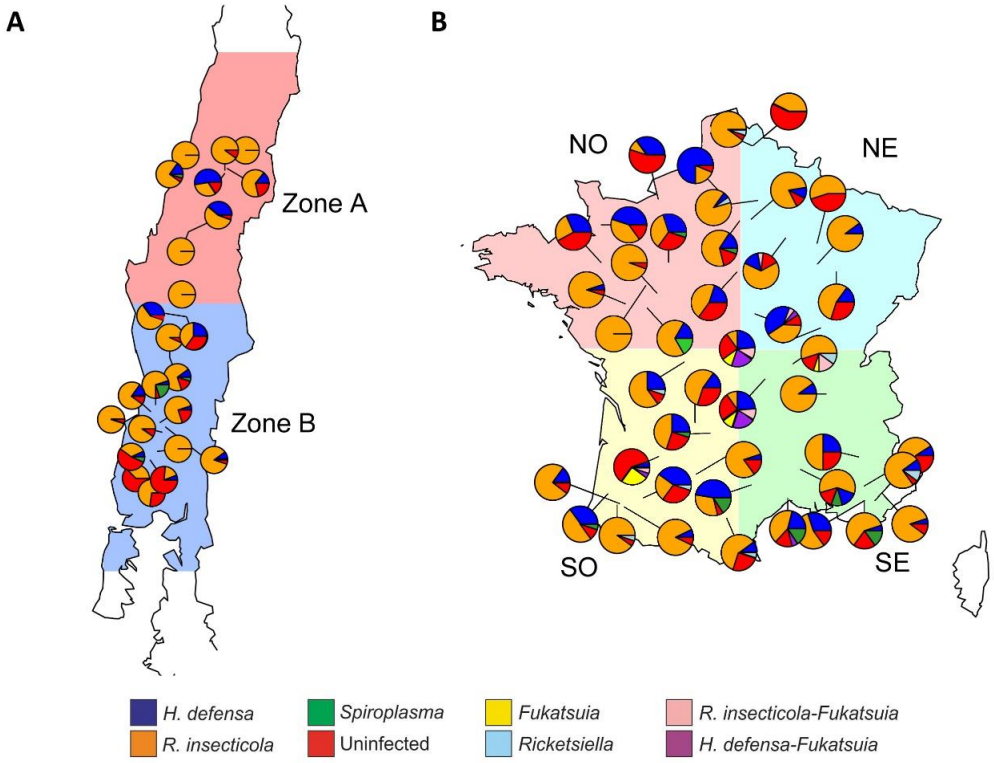

Figure 1. Frequency distribution of the SE in the populations of *S. avenae* in (A) Chile and (B) France.

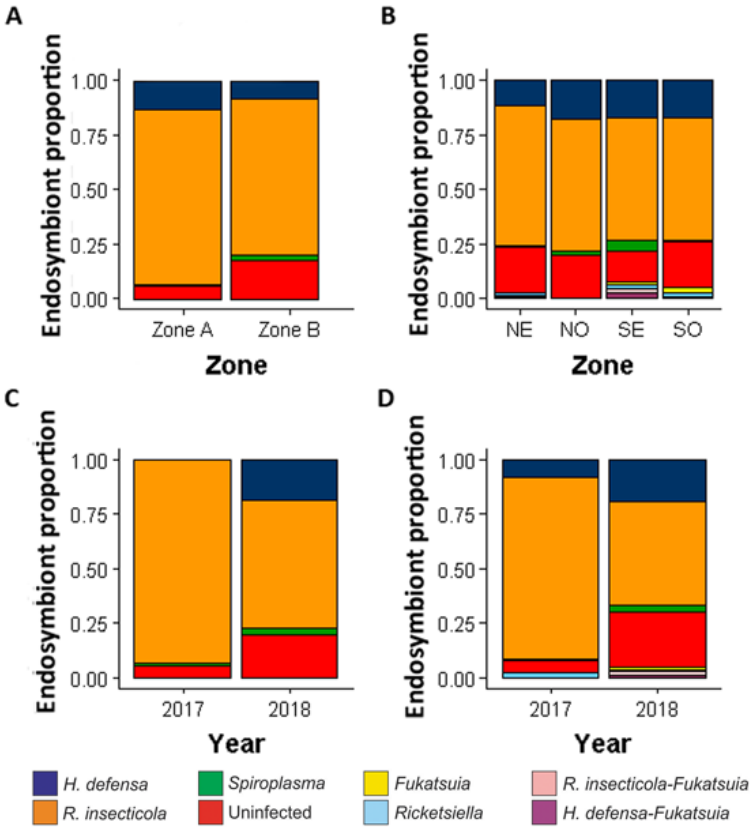

Figure 2. Frequency of SE found in Chile (A-C) and France (B-D) in zones (A-B) and years (C-D).

3.4.3 Frequent MLGs and secondary endosymbionts.

R. insecticola was the main facultative endosymbiont in all the MLGs studied in both countries (Figure 3). However, we found different patterns of *H. defensa* in MLGs from Chile. We detected *H. defensa* in six of the 10 Chilean MFMLGs studied and in 16 of the total 56 MLGs found in Chile (28%), although most of them were found in 2018 (except for MLG 260 and 299, which were also present in 2017, but in a lower frequency (see Chapter 2). In France, *H. defensa* was detected in all ten MFMLGs, and 74 MLGs in total from the 235 MLGs were detected (25%). *H. defensa* in the MFMLGs comprised 80% of the total *H. defensa* detected in Chile, while in France was only 14%. In Chile, the higher proportion of *H. defensa* was found in MLG 249 (40%), while the lowest was in MLG 260 (0.3%). We only detected one individual harboring *H. defensa* in that MLG. In France, the highest proportion of *H. defensa* was found in MLG 192 (23%) and the lowest in MLG 122 (10%).

When the effect of MFMLG on the proportion of SE was evaluated, we found a significant effect in Chile (χ^2=164.41, df= 27, P= <0.001). The differences were given by a higher proportion of *R. insecticola* in

MLG 252 (n=62), compared to MLGs 181 (n=11), 233 (n= 57) and 251 (n= 27). MLG 181, 233, and 251, unlike MLG 252, showed to be also infected by *H. defensa*, which probably reduced the proportion of *R. insecticola* found in these MLGs. A significant proportion of *R. insecticola* compared to other endosymbionts and uninfected individuals in MLGs 233, 252, 234, 166, and 260 was also found after multiple comparisons (Table 2). Contrastingly, MLGs did not affect the proportions of SE found in France (χ^2= 65.52, df= 63, P= 0.3895), as the three more frequent endosymbiont showed to be distributed similarly among the MLGs (Figure 3B).

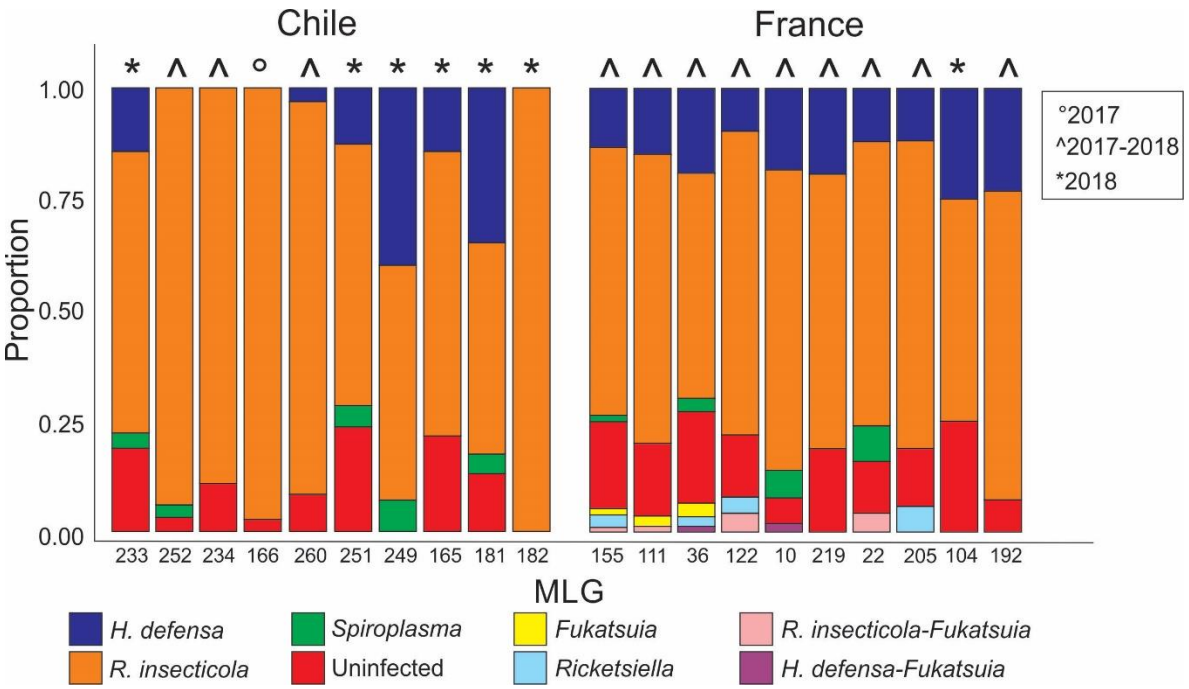

Figure 3. Main MLGs (MFMLG) from Chile and France and their SE frequencies.

Table 2. Multiple comparisons output of the proportions of SE in the Chilean MFMLGs. Different letters show significant differences at $p < 0.05$.

MLG	*H. defensa*	*R. insecticola*	*Spiroplasma*	Uninfected
233	de	bh	e	efg
252	abcdef	a	ef	ef
234	abcdef	ah	abcdef	efg
166	abcdef	ah	abcdef	efg
260	cde	ah	abcdef	efg
251	efg	ch	de	cde

249	cdfh	abcdg	bcde	abcdef
165	bcde	abcg	abcdef	bcde
181	bcdf	dgh	bcde	cde
182	abcdef	abcdef	abcdef	abcdef

3.5 Discussion

3.5.1 Secondary endosymbiont diversity and frequencies.

Selection and genetic bottlenecks during the colonization of a new area can affect aphids and their SE composition. Therefore, lower diversity of SE is expected in the invaded area compared to the native range. Our results show that the invaded area have a lower SE species diversity in *S. avenae* than their native area, with *S. symbiotica* and *Fukatsuia* completely absent from Chilean populations. Previously, a low SE diversity was detected in Chile 16S rRNA survey (Zepeda-Paulo et al., 2018), which support those observed by Sepúlveda et al. (2017) as *R. insecticola* and *H. defensa* are the most frequently SE found in *S. avenae.* Comparisons of the SE diversity between the invaded and native areas are scarce in the literature; however, some efforts were made by Desneux et al. (2018), who reported only partial evidence of the lower SE diversity in aphids in invaded areas. We explore two possible explanations for the lower diversity of SE species in Chile. First, a trade-off associated with harboring specific SE it is expected if natural selection act on those combinations of aphid genotypes and SE species (or SE genotypes) that could confer an advantage to face the new environmental conditions during the colonization process. Alternatively, the observed trend may result from an overrepresentation of some SE species and/or strains, as only the most frequent SE were established in the invaded area, while the less frequent SE were lost due to drift. As no apparent beneficial effect has been detected in the SE in *S. avenae* in Chile, both selection and drift could explain our results.

On the other hand*, Rickettsia sp.* was completely absent from both countries, which has also been reported in UK populations of *S. avenae* (Łukasik et al., 2013b). Contrarily to our findings, *Rickettsia sp.* was the most common SE found in Chinese populations of *S. avenae* (Hu et al., 2020). *Rickettsia* infection has been reported to confer aphids with protection against fungal pathogens but with strong fitness trade-offs, producing reduced fecundity, shorter lifespan, and reduced body weight (Sakurai et al., 2005; Simon et al., 2007; Łukasik et al., 2013a,c). Due to the trade-offs produced by harboring SE, low frequencies of this SE are expected and could explain its absence from our results, although the higher frequencies of *Rickettsia sp.* in China could be an insight of strong selection favoring the protective effect against fungal pathogens over the fitness costs.

To our knowledge, this is the first report on the presence of *Fukatsuia symbiotica* in *S. avenae,* even when it was detected in low frequency and only in the native area. *F. symbiotica* have shown to improve the recovery of the pea aphid after an event of heat stress (Heyworth, Smee & Ferrari, 2020), and have shown to give protection against fungal pathogens and parasitoids (Heyworth & Ferrari, 2015). A more robust protective effect to heat stress and parasitism has been reported when *F. symbiotica* and *H. defensa* co-infect the grain aphid (Guay et al., 2009), a combination that was also found in our results. Hence, the presence of *F.* symbiotica in French populations of *S. avenae,* could be explained by a putative strong protective effect. However, this protection may involve a trade-off due to the low frequency in which it was found in our results. More studies are needed to corroborate this idea.

A few infections with *S. symbiotica* were also detected in French populations of the grain aphid. Previously, *S. symbiotica* has been reported in low frequencies in *S. avenae* populations from Western Europe (Łukasik et al., 2013b) and even not detected at all in other study conducted in the UK (Henry et al., 2015). Contrarily, Chinese populations of *S. avenae* have shown to be frequently infected by *S. symbiotica* (Luo et al., 2016). *S. symbiotica* has been involved in conferring important tolerance to heat stress (Chen & Purcell, 1997; Montllor, Maxmen & Purcell, 2002), parasitoid resistance (Oliver et al., 2003), and provide with nutritional supplies in the absence of *B. aphidicola* in the pea aphid under laboratory conditions (Koga et al., 2003); however, their effects on *S. avenae* are unknown.

3.5.2 Spatial and temporal distribution of secondary endosymbionts.

Differences in the geographic distribution of SE have been detected in aphids, most of them related to climatic conditions (Tsuchida et al., 2002; Sepúlveda et al., 2017; Hu et al., 2020). In China, *R. insecticola* infecting *S. avenae* is negatively associated with mean annual temperature and mean annual precipitation (Hu et al., 2020). Similar results were found for the pea aphid infected with *R. insecticola*, which was also negatively correlated with the temperature and precipitation in different areas of Japan (Tsuchida et al., 2002). Despite the climatic differences among each zone in Chile, we did not detect any geographic difference in the SE distribution. Curiously, a previous study performed in Chile, including zones closer to those we studied, found that *H. defensa* was differentially distributed, with a higher frequency in South Chile (zone 2) than in Central Chile (zone 1); indeed, *H. defensa* was completely absent from central Chile (Sepúlveda et al., 2017). But we found no differences in the proportion of *H. defensa* between Chilean zones, although we found a higher proportion of *H. defensa* in Central Chile (zone 1) than in the South of Chile (zone 2). No differences in SE distribution were neither found in France, showing that both main SE, *R. insecticola* and *H. defensa,* were widely distributed acroos the territory. We are not in knowledge of extensive sampling efforts of *S. avenae* SE in France or any European country, whereby it is hard to compare the geographic distribution of SE of *S. avenae* found in France.

On the other hand, we found clear evidence of temporal changes in the frequency of SE of *S. avenae* in both countries*. H. defensa* was the SE that showed a more evident variation between sampling years in Chile, as it showed to be completely absent during season 2017. On the other hand, *H. defensa* also showed an increase from 2017 to 2018 in French populations. Temporal changes in SE frequencies in nature have been poorly studied, with no studies to compare our results. For instance, we sampled *S. avenae* through all the studied area in Chile in the springs of 2015-2017 (data not available), with no success in finding infected individuals harboring *H. defensa* to form aphid colonies, indicating that the presence of *H. defensa* seemed not to be time persistent in Chile.

3.5.3 MLGs and secondary endosymbionts

Our study shows that the season and the MLG of *S. avenae* are the main factors driving the proportion of *H. defensa* found in aphids feeding on cereal crops. Curiously, those MLGs that are infected with *H. defensa* were completely absent in 2017, which raises interesting questions. For instance, is the MLG which drives the presence of *H. defensa*, or is *H. defensa* which drives the presence of the MLGs associated with it? We detected an exciting dynamic among the MFMLG in Chile. Indeed, closely related MLGs seem to alternate their presence between seasons (see Chapter 2) as observed for MLG 233, which was absent in 2017, while MLG 234 (its closer MLG) was the second more frequent. Conversely, MLG 233 was the most frequent in 2018, while MLG 234 decreased drastically its frequency. A similar pattern was observed for MLG 251, 252 and 249; 166 and 165; and 181 and 182 (see Chapter 2).

The effects produced by SE are usually highly specific, involving for example genotype by genotype interaction that include not only the aphid and their SE, but also the parasitoid genotype (Cayetano & Vorburger, 2013). In the native region, SE can protect aphids agains specific natural enemies (Asplen et al., 2014). However, these natural enemies could not be present in the introduced range or the specific natural enemy's genotype for which the SE provides protection, thus becoming the SE neutral in the new environment and likely to be removed by drift. Moreover, several SE has shown to produce fitness trade-offs in the insect host (Dykstra et al., 2014), which could cause selection against SE in the invaded area or selection favoring those SE without fitness costs. Laboratory studies have shown that those SE conferring some advantage to aphids usually produce the strongest trade-offs and in the absence of a selective agent (e.g., parasitoids), they tend to be lost in populations under laboratory conditions (Luo et al., 2016; Desneux et al., 2018).

Whether *R. insecticola* or *H. defensa* confer any adaptative advantage to *S. avenae* remains unclear. However, some studies have reported different effects of *R. insecticola* in *S. avenae.* For instance, *R. insecticola* has shown having a negative effect on life-history traits in *S. avenae* when aphids are reared on rye, but with no effects when aphids are reared on wheat or oat (Da Wang et al., 2016). Similarly, adverse effects on life-history traits in *S. avenae* produced by *R. insecticola* were also detected by Luo et

al. (2017), in which *R. insecticola* showed to decrease the intrinsic rate of increase (r_m). Furthermore, *R. insecticola* has also reported producing higher susceptibility to parasitoid attacks (Luo et al., 2020) and predators (Ramírez-Cáceres et al., 2019). On the other hand, no protective effects of *H. defensa* against parasitoids have been detected (Łukasik et al., 2013b; Zepeda-Paulo, Villegas & Lavandero, 2017). The association found in Chilean MLGs with *H. defensa* could be the product of selection on genotype by SE species interaction. Hence, MLGs harboring *H. defensa* may increase their frequency due to particular selective agents, but because of its strong fitness trade-off, when the intensity of the selective agent is weaker or even disappear, those MLGs harboring *H. defensa* decrease their frequency drastically in the field. In fact, balancing selection is one of the main evolutionary forces that drive temporal dynamics of secondary endosymbionts, due to they can confer with protective effects on their hosts in the presence of natural enemies but produce fitness costs when the enemy is not present (Oliver et al., 2014). An alternative hypothesis is that the association found between MLGs and *H. defensa* is product of random processes after *S. avenae* introduction to Chile. Because of the parthenogenetic reproduction, distinct "lineages" of the same MLG were formed, some of them harboring *H. defensa.* Due to the predominance of *R. insecticola* in the 56 MLGs found in Chile (see Chapter 2), those MLGs harboring *H. defensa* could be at a lower frequency in the field and hence more challenging to be found. This latter hypothesis is supported by the strong drop of *R. insecticola* from 2017 to 2018, partially explaining that de appearance of *H. defensa* during 2018 was due to a decrease in those MLGs harboring *R. insecticola*.

On the other hand, Sepulveda et al. (2017) found *H. defensa* only in the south of Chile. The MLG 233, the most frequent MLG found in Chilean populations, is more frequently found in zone B, which correspond to the south of Chile; hence, probably those *S. avenae* individuals harboring *H. defensa* could correspond to MLG 233; therefore, their results could be related to the spatial variation in the frequency of MLGs harboring *H. defensa*. Previous variations in time of highly frequent Chilean MLGs (superclones) have already been reported (Figueroa et al., 2005). However, whether the SE drives the abundances of MLGs harboring specific SE species or is the MLG that modulates the frequency of SE found in our results remains unknown. More studies are required, putting efforts into exploring whether the SE found in Chile affects the fitness of *S. avenae*.

3.6 Conclusions

We report valuable information about the diversity of SE associated with *S. avenae* in both the invaded and native ranges. We found a higher diversity of species in the native range, which suggest a bottleneck that affected the SE diversity in *S. avenae* during the colonization of Chile. We did not find evidence of any geographical pattern of SE distribution at any country, which mean that the climate and geography should not be the main factors driving the SE frequencies in the studied zones. On the other hand, we provide valuable data on temporal variation in SE in both countries, which was more evident in Chilean *S.*

avenae, as *H. defensa* was completely absent from season 2017. Significant associations of certain MLGs and *H. defensa* in Chile give rise to several questions that could help to elucidate the factors that explain the success of *S. avenae* in Chile. Whether SE had a role in the success of *S. avenae* during the invasion remains unknown, and studies have failed to demonstrate any advantage of them over *S. avenae* phenotype in Chile.

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4. Chapter 4

Tittle:

Intraspecific diversity and antibiotic susceptibility of secondary endosymbionts in *Sitobion avenae*

Objetive:

Study the genetic diversity of different MLGs from *S. avenae* and implement an elimination of secondary endosymbionts protocol to assess the effect of secondary endosymbiont elimination in the intrinsic rate of increase, the body mass, and the developmental time.

4.1 Abstract

Endosymbionts are key players in insect pests' adaptative potential since they can confer diverse traits that help their hosts mediate with adverse conditions. Secondary endosymbionts (SE) in aphids have shown a wide variety of effects on aphid phenotype, such as protection against natural enemies and thermal tolerance; these effects can be determined by both the SE species and the SE strain. To test SE's effect in aphids, it is necessary to disinfect aphids before conduct any bioassay so that all experimental aphids have received the same treatment; however, disinfection protocols can variate depending on the host and the SE species and strain. In this research, we assessed the genetic diversity of SEs in different genotypes from an invaded and native area; moreover, we implemented an antibiotic protocol of SEs disinfection that ensure the survival of aphids for further bioassays. We studied the effect of eliminating SEs on life-history traits, including the intrinsic rate of increase, the body mass, and the developmental time. Our results showed the presence of a single strain of *Regiella insecticola* infecting Chilean genotypes of *S. avenae*; indeed, a unique strain of this SE was found, while a higher diversity was found in France. Interestingly, we detected different sensibilities to the antibiotic treatments depending on the SEs strains. Indeed, ST1-Reg, the unique strain found in Chile, required higher antibiotics doses for its elimination than other symbionts. The elimination of SEs from *S. avenae* genotypes did not affect life-history traits, suggesting that the effects of SEs in *S. avenae* can depend on different selective pressures.

Keywords: Endosymbiont, invasive pest, *Sitobion avenae*, endosymbiont strains, endosymbiont elimination, antibiotic treatment, life-history traits.

4.2 Introduction

Symbiosis is the most cohesive form of relationship in nature (Margulis, 1976). This is especially true for aphids, which maintain a close relationship with their bacterial symbionts (Oliver et al., 2010). Aphids harbor a primary endosymbiont, *Buchnera aphidicola*, which is crucial for their survival as it provides aphids with essential amino acids absent from the plants' phloem (Baumann, 2005). The relationship with this bacterium is so essential that aphids evolved specialized organs to protect it (Baumann, 2005). Aphids can also maintain close relationships with secondary endosymbionts (SE), which are not essential for their survival, but they can significantly impact their phenotype (Oliver et al., 2010). SE can provide aphids with protection against natural enemies (Oliver et al., 2003; Vorburger et al., 2010; Łukasik et al., 2013; Vorburger, 2018); tolerance to thermal stress (Montllor, Maxmen & Purcell, 2002), broaden their host plant range (Tsuchida, Koga & Fukatsu, 2004), and many other effects that continue appearing as the study of SE goes on. Effects of SE on the phenotype of aphids have shown to variate according to SE's strain (Oliver et al., 2009); for instance, the same SE but with a distinct strain could affect aphids' phenotype differently (Heyworth & Ferrari, 2015). Both primary and secondary endosymbionts are maternally inherited (Peccoud et al., 2009), but paternal transmission to the offspring during sexual reproduction has also been detected (Peccoud et al., 2013). When aphids invade a new area, they can be affected by genetic bottlenecks, genetic drift, and selective pressures from their new environment (Figueroa et al., 2005, 2018). For example, introduced populations of aphids can lose their ability to reproduce sexually, becoming mostly clonal (Figueroa et al., 2005). The effect of selective pressures during the colonization process upon the SE of aphids has been poorly studied. Furthermore, due to the advantageous effect on some SE on the aphid phenotype, they could be critical in the invasiveness potential of pest as aphids (Feldhaar, 2011; Lu, Hulcr & Sun, 2016).

The grain aphid of cereals, *Sitobion avenae,* invaded Chile in the 1960s, causing significant damages to cereal production. *S. avenae* was presumably introduced from Europe and the Mediterranean zone (Blackman & Eastop, 2000). In Chile, *S. avenae* have shown an extremely low genetic diversity and the loss of the ability to reproduce sexually (Figueroa et al., 2005). Moreover, previous studies in Chile have shown that *S. avenae* have a low SE diversity (Sepúlveda et al., 2017; Zepeda-Paulo et al., 2018), with only two SE species found: *Regiella insecticola* and *Hamiltonella defensa*.

Interestingly, SE strains can be as important as species diversity (Oliver et al., 2009; Vorburger et al., 2010; Heyworth & Ferrari, 2015). To identify SE's intraspecific diversity, it is crucial to evaluate their effects on aphids and give insights into their potential role during the colonization success of pest to new environments. Furthermore, comparing SE's intraspecific diversity of invasive and native populations can bring important information about the origin of the introduced aphid population and the selective forces

acting on SE, which could also bring valuable information about SE's role in the success of aphids as successful invaders.

The effects of SE on the aphid phenotype should be evaluated by comparing infected and uninfected individuals of the same genotype. Antibiotic treatments have been the most used to generate cured aphid lineages (Koga et al., 2007; McLean et al., 2011; Sochard et al., 2020); however, the effectivity of antibiotic treatments have shown to variate depending on the SE species, the strain, and the aphid genotype (Koga et al., 2007).

This study was aimed to identify the SE's intraspecific variation using different genotypes of *S. avenae* sampled in wheat fields in an invaded and a native area (Chile and France, respectively). We further evaluated different SE elimination methods in different genotypes of *S. avenae* and strains of SE, and we analyzed the effect of curing aphids with antibiotics on the reliability of measuring aphid life-history traits.

4.3 Materials and methods

4.3.1 Aphids lineages

Aphid individuals were collected from wheat fields during the spring of 2016 and 2017 in Chile and France. A total of 62 Chilean and 44 French lineages were established in the laboratory. All lineages were DNA extracted with the Salting-out protocol (Sunnucks et al., 1996), and the screening of the six more common SE in aphids was tested (Peccoud et al., 2013). We also genotyped aphid lineages by amplifying seven microsatellites (see Chapter 2) (Correa et al. in prep., appendix 7.1). We selected 22 aphid lineages to screen intraspecific SE variation and nine lineages for antibiotic treatments. Lineages were chosen based on their genotype and SE composition (Table 1). Hence, we selected multilocus genotypes (MLGs) with a high frequency in the field and different SE combinations. As *H. defensa* was not found in Chilean lineages (see Chapter 3), this SE was not included in the experiments.

Table 1. Aphids used in the different experiments.

Lineage ID	MLG	Country	Endosymbiont	Used in		
				SE sequencing	Antibiotic treatment	Fitness study
Clon 1	155	France	*R. insecticola*	x		
Clon 14	155	France	*H. defensa-R. insecticola*	x	x	x
Clon 15	155	France	*R. insecticola*	x	x	x
Clon 18	155	France	*H. defensa-R. insecticola*	x		
Clon 19	286	France	*R. insecticola*	x		
Clon 8	293	France	*R. insecticola*	x		
Clon 9	152	France	*R. insecticola*	x		

Clon 6	286	France	*R. insecticola*	x		
Clon 11	223	France	*R. insecticola*	x		
Clon 23	28	France	*R. insecticola*	x		
Clon 3	10	France	*R. insecticola*	x	x	x
Clon 7	239	France	*R. insecticola*	x		
Clon 4	122	France	*H. defensa*	x	x	x
Clon 17	122	France	*H. defensa*	x		
clon 12	223	France	*H. defensa*	x		
Clon 16	122	France	*H. defensa*	x		
Clon 21	120	France	*H. defensa*	x	x	x
Clon 26	260	Chile	*R. insecticola*	x	x	x
Clon 27	252	Chile	*R. insecticola*	x	x	x
Clon 28	166	Chile	*R. insecticola*	x	x	x
Clon 29	234	Chile	*R. insecticola*	x	x	x
Clon 31	253	Chile	*R. insecticola*	x		

4.3.2 Characterization of *R. insecticola* and *H. defensa* strains.

To assess the intraspecific genetic diversity of SE, we sequenced fragments of two bacterial housekeeping genes, acetyl-CoA carboxylase (*accD*) and Murein (*murE*) (Henry et al., 2013), following the same PCR protocol for SE screening (Peccoud et al., 2013). Amplicons were sequenced in both senses by the Sanger method. Sequences were manually inspected in Geneious Pro V.5.4.6 software (Biomatters Ltd.) looking for mismatched base pairs. Poor-quality ends were trimmed, the sequences of both housekeeping genes concatenated, and a neighbor-joining (NJ) tree with 1,000,000 bootstraps built. We used the lineage identity to show the obtained results (Table 1)

4.3.3 Elimination of facultative endosymbionts

Once the SE strain screening results were obtained, we selected nine aphid lineages (referred to as MLG), which were subjected to different antibiotic treatments (Table 2). We used three antibiotics that have shown previously used for SE elimination in aphids (Sochard et al., 2020), and we tested the better administration method and treatment to successfully eliminate different strains of *R. insecticola* and *H. defensa* in different MLGs of *S. avenae.* We first conducted T1-T3 as experimental treatments to establish a baseline of the adequate antibiotic administration method (artificial diet or leaf-dip method) and to standardize the range of antibiotic concentrations that do not kill *S. avenae* individuals. First, we tested the curing of *R. insecticola* and *H. defensa* with gentamicin and cefotaxime, respectively, and with a mixture of them with ampicillin (Table 2). Two different strains of each SE, MLG 155 and 260 for *R. insecticola* and MLG 120 and 122 for *H. defensa*, were tested as the target of the respective antibiotics

(C. Vorburger personal communication). Second, we performed T4 and T5, with all MLGs (Table 1) selected to improve the reliability of the experimental results.

For all antibiotic treatments, we used ten three-day old aphid nymphs, as the youngest aphids have shown to respond better (Sochard et al., 2020). We first tested the antibiotic administration method, evaluating the use of artificial diets and wheat leaves dipped into the antibiotic (T1-T3). The artificial diet was prepared with sucrose (30%) diluted in ultrapure water. One ml of the diet containing different concentrations of the antibiotics was set between two layers of parafilm membrane. This sandwiched diet was stretched to a Petri dish of 5 cm diameter and aphids set on the surface and left to feed. The administration through leaf-dip treatment was performed by inserting the stem of a wheat leaf (10 days old plant) into a 1.7 ml Eppendorf tube containing 1.5 ml of an antibiotic solution prepared in ultrapure water and protected by a Falcon 50 ml tube. All treatments were maintained at controlled conditions (20 ± 1°C, 65 ± 10 % RH, and D16/N8 photoperiod).

After each antibiotic treatment, the surviving aphids were counted and individually transferred to a four-seedling wheat plant (10 days-old) and left to grow and reproduce for two weeks. The last three newborn aphids from each treatment (G_0) were transferred to a new wheat plant, triplicating the number of the original survivors (G_1). This was done to avoid that SE could have inherited to some aphid offspring due to the telescopic reproduction of aphids (i.e., the development begins within their grandmothers). We tested the SE elimination success at G_4 as described in 4.3.1.

Table 2. Antibiotics treatments used in this study.

Administration mehod	Treatment	Antibiotic	Treatment duration (days)
Artificial diet	T1	Gentamicin 200 µg/mL	3
	T2	Cefotaxime 1 mg/ml	3
	T3	A mixture of gentamicin, cefotaxime, and ampicillin to 100 µg/ml each.	3 3
	T4	A mixture of gentamicin 200 µg/ml, cefotaxime, and ampicillin to 100 µg/ml.	3 3
	T5	A mixture of gentamicin 400 µg/ml, cefotaxime, and ampicillin to 100 µg/ml,	3 3
Dip-leaf	T1	Gentamicin 200 µg/ml	7
	T2	Cefotaxime 1 mg/ml	7
	T3	A mixture of gentamicin, cefotaxime, and ampicillin to 100 µg/ml each.	7 7

4.3.4 Effects of the elimination of facultative endosymbionts

The effect of MLGs and the SE presence or absence over the fitness of *S. avenae* was evaluated by the measure of the intrinsic rate of population increase (r_m), the pre-reproductive period, and the body mass to adulthood. We left to reproduce 20-30 wingless adult aphids of each MLG by 24 hours in a wheat plant; then, adults were removed, and the newborn aphids left to grow for five days before being transferred to a fresh 10 days-old wheat plant (this was done to avoid hurting the small aphid nymphs). Newly established aphids were observed daily until the first reproduction day and then left to reproduce for 8-15 days (depending on each individual's pre-reproductive period). Newborn aphids were counted and removed every two days until the end of the experiment. The pre-reproductive period was calculated as the number of days from the born until the first reproduction day and r_m as $0.738(\ln M_d)/T_d$ (Wyatt & White, 1977), where T_d is the pre-reproductive period (days), and M_d is the number of offspring produced in the time equivalent to the pre-reproductive period. Finally, the body mass was measured as the fresh weight at adulthood (i.e., the weight at their first reproduction day).

4.3.5 Data analysis

All statistical data analyses were performed with R version 6.3.1 (Team, 2019). To assess the effect of antibiotics treatments and MLGs in eliminating SE and their survival after treatments, we performed generalized mixed models (GLMM). The success of SE elimination was analyzed with a binomial distribution error; the administration method, the antibiotic treatment and MLG were the fixed effects, and a temporal block (four blocks) the random effect. Because not all MLGs were used in all the antibiotic treatments, we analyzed T4 and T5 separately from the other treatments, as well as the MLGs infected with *R. insecticola* and *H. defensa*.

To test the effect of symbiont elimination on the life-history traits studied, we first tested the assumptions of normality and heteroscedasticity of data. The r_m was normally distributed, and variances were homogeneously distributed, but not the body mass, for which a log-transformation was needed. The r_m and the log of body weight were analyzed with linear mixed models (LMMs). The pre-reproductive period was measure as the number of days from the born to the first reproduction day and analyzed with a generalized linear mixed model (GLMM) and a Poisson distribution error. The fixed factor for all the studied life-history traits studied was the aphid MLG lineage, i.e., each studied MLG infected or cured of from their SE (Table 1), and the random factor was the temporal block (three temporal blocks). An ANOVA type III was used to assess the effect of the combination of MLG and their SE status over the r_m and log of body weight, while a deviance Wald test was performed for the pre-reproductive period. Tukey Multiple comparisons were performed with the *Multcomp* package (Hothorn, Bretz & Westfall, 2008).

4.4 Results

4.4.1 Intraspecific diversity of facultative endosymbionts

We found four genotypes of *R. insecticola* and three of *H. defensa*, hereafter referred to as "strains." Our results show that all the Chilean lineages studied harbored the same *R. insecticola* strain ST1-Reg, while French lineages harbored all the four strains (Figure 1). Hence, ST1-Reg was frequently found in Chilean lineages, while ST2-Reg was predominant in the French lineages. Three *H. defensa* strains were found for French lineages, while no strain was detected for Chilean lineages as this SE was not detected in any lineage.

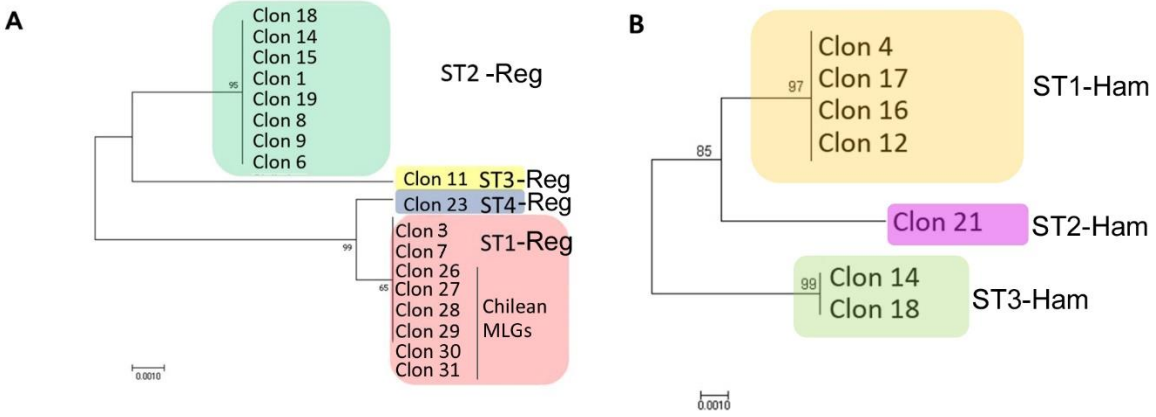

Figure 1. Neighbor-Joining tree of the SE strains. (A) *Regiella insecticola* and (B) *Hamiltonella defensa.*

4.4.2 Antibiotic treatments

4.4.2.1 *Regiella insecticola* elimination

Our results show that the success of facultative endosymbionts by the antibiotic treatments performed depended on the SE species and strains. Hence, the antibiotic treatments performed on MLG 155 and 260 which harbor *R. insecticola,* showed that the administration method (χ2= 0.1743, df= 1, P= 0.676) and the treatment (χ2= 3.3858, df= 1, P= 0.065), had no effect over the elimination success of *R. insecticola*, but there was a significant effect of the MLG (χ2= 31.0055, df= 1, 0.00000), but not of the interaction of the MLG with the administration method (χ2= 0.0000, df= 1, P= 0.999) and the treatment (χ2= 0.00, df= 1, P= 0.999). Differences between MLGs were due to a relatively high proportion of elimination in MLG 155, unlike MLG 260, which showed not a single cured individual at any treatment (Figure 2A). On the other hand, T4 and T5 treatments showed no significant effect on the treatment (χ2=

1.069, df= 1, P= 0.30117), but there was a significant effect of the MLG (χ2= 39.608, df= 5, P= <0.001) and the interactions between both factors (χ2= 10.577, df= 4, P= 0.03). Differences in *R. insecticola* elimination among MLGs could be explained by the higher proportion of cured individuals in MLG 155 than the others, which showed a poor SE elimination success (Figure 2B). Besides, differences in MLGs and the treatments' interaction can be related to the higher proportion of elimination success on T5 compared to T4.

4.4.2.2 *Hamiltonella defensa* elimination

Elimination of *H. defensa* in MLG 120 and 122 showed no significant effect of the administration method (χ2= 0.0037, df= 1, P= 0.951279) or the treatment (χ2= 0.0806, df= 1, P= 0.776), but there was a significant interaction between the two factors (χ2= 8.8771, df= 1, P= 0.002). Hence, the diet method showed to be more effective than the dip-leaf method in T3 eliminating *H. defensa* (Figure 2C). Besides, there was also a significant effect of the MLG (χ2= 17.6493, df= 1, P= < 0.001), but not in the interaction with the administration method (χ2= 0.0000, df= 1, P= 0.999), or the treatment (χ2= 0.0000, df= 1, P= 0.999). Hence, MLG 122 achieved up to 50% of elimination success in almost all treatments, with the exception of T2, while no successful elimination was possible for MLG 120.

T4 and T5 treatments shown not to be differentially affect the elimination of *H. defensa* (χ2= 0.2343, df= 1, P= 0.6283), neither the MLG (χ2= 0.0019, df= 2, P= 0.9990) or the interaction between both factors (χ2= 3.5195, df= 2, P= 0.1721); however, T4 and T5 seemed more effective eliminating *H. defensa* from MLG 120, that T2, and T3 (Figure 2D).

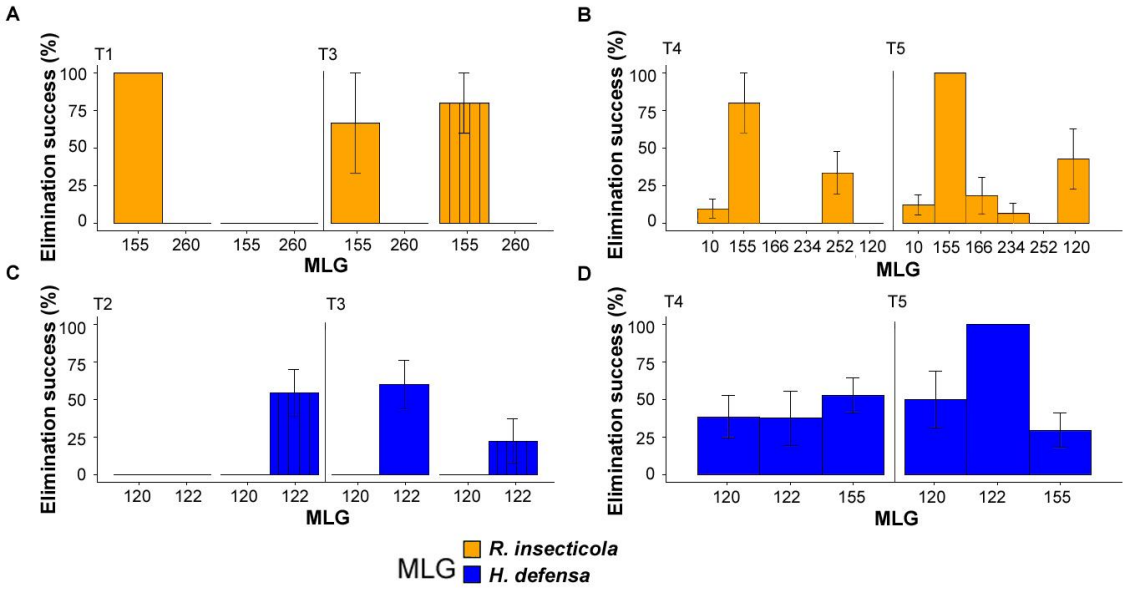

Figure 2. Elimination success rate (%) (mean ± SE) according to the administration method, diet and dip-leaf (hatched bars) (A and C), and the antibiotic treatments in different *S. avenae* MLGs infected with *Regiella insecticola* (A and B) and *Hamiltonella defensa* (C and D).

4.4.3 Survival to the antibiotic treatments

The survival of MLG 155 and 260 in T2 and T3 showed a significant difference among the administration method ($\chi 2$= 7.026, df= 1, P= 0.008), while the treatment ($\chi 2$= 0.302, df= 1, P= 0.582), and the MLG ($\chi 2$= 1.045, df= 1, P= 0.306) had no effect in the survival. The interaction between the administration method and the treatment ($\chi 2$= 2.1710, df= 1, P= 0.140), and administration method with the MLG ($\chi 2$= 3.517, df= 1, P= 0.060) or the treatment with the MLG ($\chi 2$= 2.860, df= 1, P= 0.090) had neither a significant effect on the survival. Hence the diet method seemed to have a smaller but significant better survival than the dip-leaf method (Figure 3A). Similar survivals were observed in T4 and T5 treatments ($\chi 2$= 2.248, df= 1, P= 0.133), with no effect of MLG ($\chi 2$= 6.9660, df= 5, P= 0.223) or the interaction between both factors ($\chi 2$= 3.611, df= 5, P= 0.6066) (Figure 3B).

On the other hand, no significant effect of the administration method ($\chi 2$= 0.414, df= 1, P= 0.5196), the treatments T2 and T3 ($\chi 2$= 1.172, df= 1, P= 0.278), or the MLG ($\chi 2$= 0.04628, df= 1, P= 0.8297) were detected for the survival after the antibiotic treatments for *H. defensa* elimination (Figure 3C). The interaction between the administration method and the treatment ($\chi 2$= 0.4393, df= 1, P= 0.507), the administration method and the MLG ($\chi 2$= 2.26691, df= 1, P= 0.132) and the treatment and the MLG ($\chi 2$= 2.26691, df= 1, P= 0.1322) had neither significant effect over the survival.

Similarly, no significant differences in survival were detected for treatments T4 and T5 ($\chi 2$= 0.091, df= 1, P= 0.762) or the interaction among factors ($\chi 2$= 4.072, df= 2, P= 0.130); contrarily, a significant effect of MLG was detected ($\chi 2$= 9.970, df= 2, P= 0.006). The differences among MLGs were to be due to a higher survival of MLG 155 compared to the others (Figure 3D).

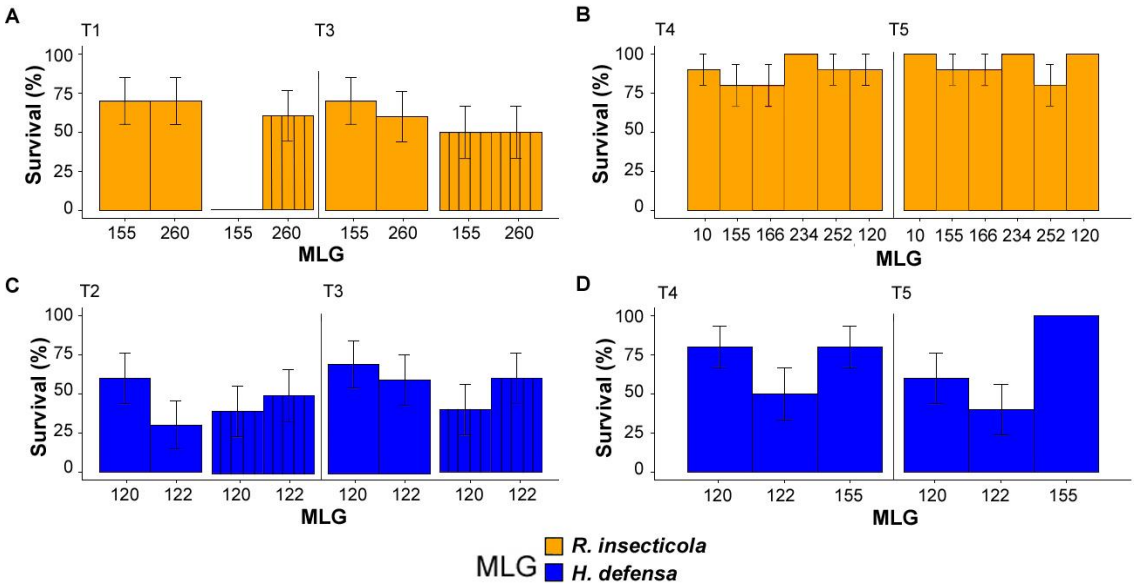

Figure 3 Survival rate (%) (mean ± SE) according to the administration method, diet and dip-leaf (hatched bars) (A and C), and treatments of *S. avenae* MLGs infected with *Regiella insecticola* (A and B) and *Hamiltonella defensa* (C and D).

4.4.4 SE elimination effect on life-history traits.

The aphid lineage (MLGs SE infected and SE cured) showed a significant effect on r_m values (F= 3.5365, df=16, P= < 0.001), but their differences were no between the same MLG (SE infected and SE cured); instead, they were due to a higher r_m of MLG 122 (SE infected and SE cured) compared to MLG 155 infected with *R. insecticola*, MLG 166 (SE infected and SE cured) and MLG 252 SE cured lineages (Figure 4A). On the other hand, the aphid lineage also significantly affected the weight of aphids (*F*= 2.514, df=16, P= 0.0019), but again, no differences were found between infected and cured aphids from the same MLG. Differences were mainly due to the loss of weight in MLG 252 infected with *R. insecticola* compared to MLG 234 cured and MLG 120 infected with *H. defensa* (Figure 4B). Finally, no effect of aphid lineage was detected in the pre-reproductive time (i.e., the developmental time) (χ2= 6.9539, df= 16, P= 0.9741).

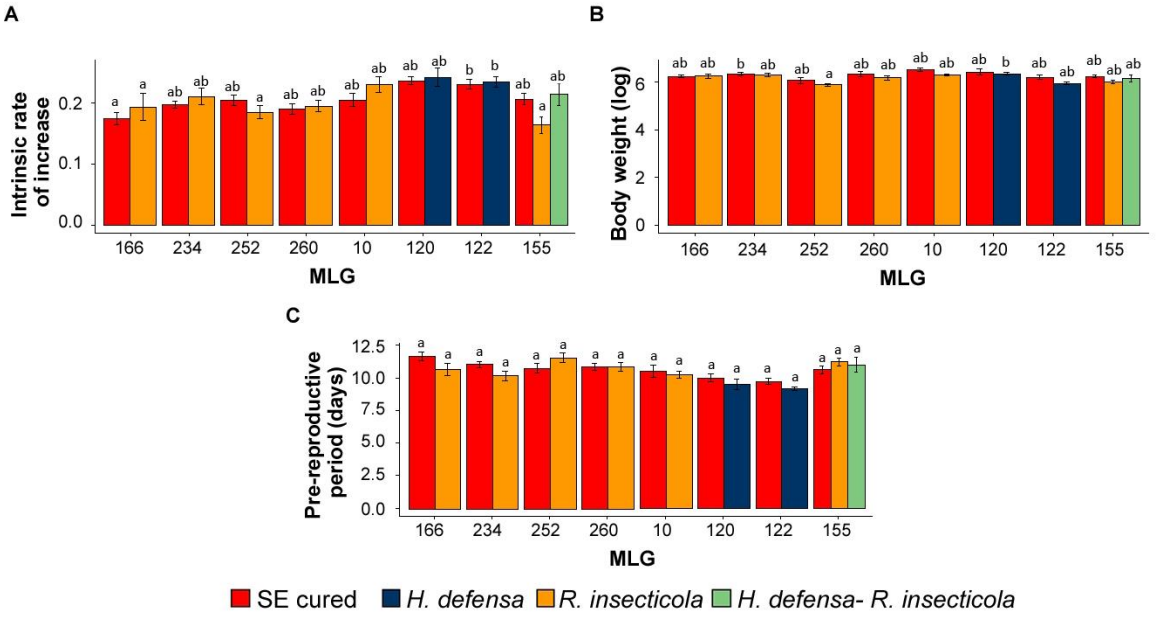

Figure 4. Life-history traits (mean ± SE) of infected and cured lineages of *S. avenae* MLGs. (A) the intrinsic rate of population increase (r_m), (B) log of body weight, and (C) Pre-reproductive period.

4.5 Discussion

4.5.1 Intraspecific diversity of SEs.

Our results revealed a low intraspecific genetic diversity of SE among the Chilean MLGs of *S. avenae* since only one single strain of *R. insecticola* was found infecting all the MLGs studied. Contrarily, French MLGs have higher intraspecific diversity in their SE for both *R. insecticola* and *H. defensa.* We propose three mechanisms that could account for the low genetic diversity found in Chilean SE of *S. avenae*. First, a lower diversity of SE species should be expected in introduced populations as SE could often represent high maintenance cost in terms of fitness (Vorburger & Gouskov, 2011; Vorburger, 2014), and natural selection could favor those SE with fewer fitness costs that allow a rapid establishment and spread of aphids in their new environment. Alternatively, strong selective forces during the introduction process may be filtering those combinations of aphid MLGs and SE strains that may confer a better adaptative response to the new environment in aphids, thus reducing both aphid and SE genetic diversity. And third, random processes affected by the low number of *S. avenae* propagules; new aphid invaders rapidly spread in the new environment and consequently spread the SE strain that they harbor, which could further be transmitted to other aphids through horizontal transmission. Unfortunately, studies comparing

SE between a natural and an introduced population of aphids are scarce, and more studies are required to test this latter hypothesis.

4.5.2 Antibiotic treatments success

Our results showed that *R. insecticola* ST2-Reg, harbored by the MLG 155, was the easiest SE to cure since it showed a high elimination success rate in almost all the administration methods and antibiotic treatments. In contrast, ST1-Reg showed a much lower success rate of elimination in all treatments and MLGs, including Chilean and French MLGs. Hence, the elimination of ST1-Reg was only achieved at higher gentamicin concentrations (T4 and T5). Therefore, our results seem to show more susceptibility to antibiotics treatments of ST2-Reg compared to ST1-Reg. Unfortunately, the MLG effect was not possible to isolate from the SE, as we did not perform combinations of the same MLG bearing the different SE strains. However, the French MLG 10, also bearing ST1-Reg, showed similar results to Chilean MLGs infected with that strain, which in part evidence the higher resistance to the treatments of ST1-Reg. Indeed, we selected the doses because the usual concentrations of antibiotic treatments reported (McLean et al., 2011; Liu, Lei & Chen, 2019) did not eliminate *R. insecticola* from Chilean MLGs (data not shown). On the other hand, *H. defensa* strains also differed in the antibiotic treatments, as it was more easily eliminated in MLG 122, which harbors ST1-Ham. *R. insecticola* and *S. symbiotica* are mainly located in secondary bacteriocytes, sheath cells and hemocoel (Moran et al., 2005). Secondary bacteriocytes are a small number of large cells that are intercalated between primary bacteriocytes (which harbor *B. aphidicola*), and sheath cells are small, flat cells located at the peripheries of the primary bacteriocytes. The target endosymbiont location can determine the effectivity of antibiotics; hence, some SE as *S. symbiotica* can be more complicated to eliminate as it has a thicker outer membrane (Moran et al., 2005; Koga et al., 2007). Whether the different strains of SE in our study are located differently in *S. avenae* body could then explain the differences found in the antibiotic treatments.

4.5.3 Effect of SE elimination

Our results did not show an effect of the SE removal in the life-history traits measured. However, SEs have shown only a differentially impact on aphid phenotype under certain conditions. For instance, different results have been found for *S. avenae* infected by *R. insecticola* in China. Hence, *R. inseticola* and *H. defensa* have been shown to improve *S. avenae* developmental time and fecundity on rye, but no effect was observed in wheat and oat (Da Wang et al., 2016; Li et al., 2018). On the other hand, the negative impacts of *R. insecticola* have also been detected in wheat, decreasing aphid increase rate and developmental time (Luo et al., 2017).

Moreover, the effects of *R. insecticola* can also depend on environmental conditions. *R. insecticola* showed the production of winged aphids inhibited at 25°C but not affected at 28°C and 31°C. Besides, *R.*

insecticola also decreased the intrinsic rate of increase at 25°C and 28°C, but not at 31°C, showing that the effects of SEs in *S. avenae* can be environmentally dependent, as previously reported (Liu, Lei & Chen, 2019). We only tested life-history traits when aphids are reared on wheat at a constant temperature of 20°C, and no significant differences between infected and cured MLGs were detected; this was probably due to determined conditions as a host plant and temperature are needed to detect differences between infected and cured MLGs.

4.6 Conclusions

As expected, lower genetic diversity in SE from Chilean populations was detected compared to the native area; unfortunately, we only counted with Chilean aphid lineages harboring *R. insecticola*. We could not assess the genetic diversity of *H. defensa*, as this endosymbiont has shown to be present in unstable frequencies in Chilean populations of *S. avenae* at the field level. Hence, our results show that the SEs genetic diversity reflects the low genetic diversity found in *S. avenae*. Whether this unique strain in Chile is the product of strong selection or drift remains unknown. Moreover, we detected variation in the disinfection rate among treatments that seemed to be related to SE strain. It is not clear why some endosymbionts are more sensitive to antibiotics, but studies suggest that it depends on their location in the aphid body, which could mean that the different SE strains in this study area are in different structures of *S. avenae* body. No apparent effect from the absence of SEs was detected in the studied genotypes; however, the effects produced by SEs depend on diverse factors, and a putative effect in *S. avenae* phenotype should not be excluded. As SEs have shown to affect the adaptative potential in invasive species, we emphasize the need for more studies that could reveal whether this unique strain of *R. insecticola* in Chile results from strong selection or just the effect of drift.

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5. Chapter 5

Title:

Spatial and Temporal Variation in the Aphid–Parasitoid Interaction under Different Climates

Objective:

Evaluate whether the aphid density, parasitism rate, and secondary endosymbiont frequency and composition are affected by the maximum temperature at field level in zones with different climates.

5.1 Abstract

Global warming will increase pest insect population sizes and diminish the effectiveness of biological control. This biological control failure scenario appears of particular of concern for areas with a significant increase in maximum temperatures, such as the increase experienced in the Central Valley of Chile over the last 40 years. We assessed the impact of different climatic zones and maximum temperatures in the coast and the Chilean Central Valley on *Sitobion avenae* density, parasitism rate, and facultative endosymbionts in wheat fields during the growing season in the springs of 2017 and 2018. A significant effect on aphid density due to zones and maximum temperatures was detected; however, this depended on the zone and year analyzed. Changes between zones and seasons were observed for parasitism rates, while maximum temperatures only significantly affected the parasitism rate in 2017. The main parasitoid wasp found was *Aphidius ervi* in both zones and seasons. *Regiella insecticola* infected 95% of the samples in both zones, although it does not seem to have a protective role at the field level. Our findings suggest that, at present, global warming does not significantly affect the grain aphid outbreaks and their biological control in Chile. However, this study points out the importance of pre-emptive monitoring to detect aphids and the synchrony loss of their parasitoid wasps.

Keywords: global warming; grain aphid; *Sitobion avenae*; *Aphidius ervi*; parasitism rate; facultative endosymbionts; biological control; introduced species

5.2 Introduction

Climate change (e.g., increased environmental temperature, increased atmospheric CO2, unstable climates, and altered frequency/intensity of extreme weather events) is the most serious concern for agriculture (Nelson et al., 2014; Campbell et al., 2016). Global warming, characterized by increased and extreme temperatures that will become more frequent, affects crop yields, mainly through crop pest biology and distribution changes, especially in invasive pest species (Ziska et al., 2011; Bebber, 2015; Furlong & Zalucki, 2017).

Aphids (Hemiptera: Aphididae) are a highly invasive pest species due to their broad phenotypic plasticity, which includes (a) their reproduction mode (see (Simon, Rispe & Sunnucks, 2002)), (b) the systematic development of insecticide resistance, (c) the development of defenses against plant chemistry and natural enemies, and (d) their symbiosis with obligate and facultative bacteria, which confer aphids with several relative advantages, including protection against natural enemies and heat tolerance (Figueroa et al., 2018; Simon & Peccoud, 2018). A worldwide pest of cereals, the English grain aphid, *Sitobion avenae*, (Fabricius) (Blackman & Eastop, 2000; Dedryver, Le Ralec & Fabre, 2010) originated from Eurasia and was introduced to Chile in the late 1960s, causing significant losses to cereal production (Starý, 1995). Mild winters in Chile favored asexual reproduction all year round, allowing aphid populations to distribute rapidly and widely across most of the cereal production area (Figueroa et al., 2005). Several Aphidiinae endoparasitoid species were introduced to Chile from different geographic origins during the late 1970s to cope with this pest as part of a governmental biocontrol program (Starý et al., 1993). Hence, a parasitoid assemblage (i.e., the set of parasitoid species attacking aphids) has provided an essential contribution to the suppression of high population densities of cereal aphids in Chile to date, with minimal input from insecticides (Zepeda-Paulo, Villegas & Lavandero, 2016). Indeed, Chile is considered one of the best examples of successful aphid biological control programs to combat the English grain aphid (Zuñiga et al., 1986).

Global warming, however, threatens the dynamics, synchronization, and temporal and spatial structures of the interactions among pests and their natural enemies (Welch & Harwood, 2014). Global warming can potentially increase the frequency of bacterial endosymbionts that provide aphids with abiotic protective effects, such as heat tolerance, even further (Montllor, Maxmen & Purcell, 2002; Heyworth & Ferrari, 2016; Doremus et al., 2017; Liu, Lei & Chen, 2019; Heyworth, Smee & Ferrari, 2020). If their transmission and geographic distribution increase at higher temperatures, the success of the biological control program may be threatened (Bensadia et al., 2006; McLean et al., 2016; Thierry, Hrček & Lewis, 2019; Liu, Lei & Chen, 2019).

Of particular concern are the maximum daily temperatures and frequency of warm events because these variables have the most critical effect on the life history traits of insect pests and biological control agents, making pest-derived problems likely to become more unpredictable (Bannerman & Roitberg, 2014; Ma, Hoffmann & Ma, 2015). The troubling fact is that Chile's central valleys have shown one of the most striking increases in maximum temperatures in recent decades, which threatens to continue (Falvey & Garreaud, 2009; Piticar, 2019).

The monitoring of the temporal dynamics of interacting species in the agroecosystems is crucial to anticipate climate change effects on aphid outbreaks and biocontrol populations, especially on those prone to global warming, highlighting the urgent need for these types of studies (Welch & Harwood, 2014; Macfadyen, McDonald & Hill, 2018). Hence, this study aimed to determine whether spatial and temporal variations in maximum temperatures modify the density of both *S. avenae* aphids and their parasitism rate in the field. We assessed the aphid density, parasitism rates, and frequency of facultative endosymbionts over two consecutive years throughout the wheat growing season, comparing the situation among field crops located in two areas—one with temperatures regulated by the Pacific Rim and the other with significant temperature increases in recent decades. We discuss the results in terms of management practices that ensure the continuity of the biological control of *S. avenae*.

5.3 Materials and Methods

5.3.1 Sampling Sites and Climates

Surveys were conducted in commercial insecticide-free spring wheat fields in two zones with contrasting climatic conditions (Figure 1) (Table S1, Supplementary Materials). Zone 1 is located close to the Pacific Rim and displays a coastal temperate Mediterranean climate, with rainfed wheat crops. Zone 2 is in the middle of the Chilean Central Valley, where a warmer Mediterranean temperate climate dominates, and wheat crops need periodic irrigation (four irrigation periods per season, occurring from September to December). The distance between fields in each zone ranged from 1.5 km to 19 km. Because of crop rotation, some wheat fields were different across the two years.

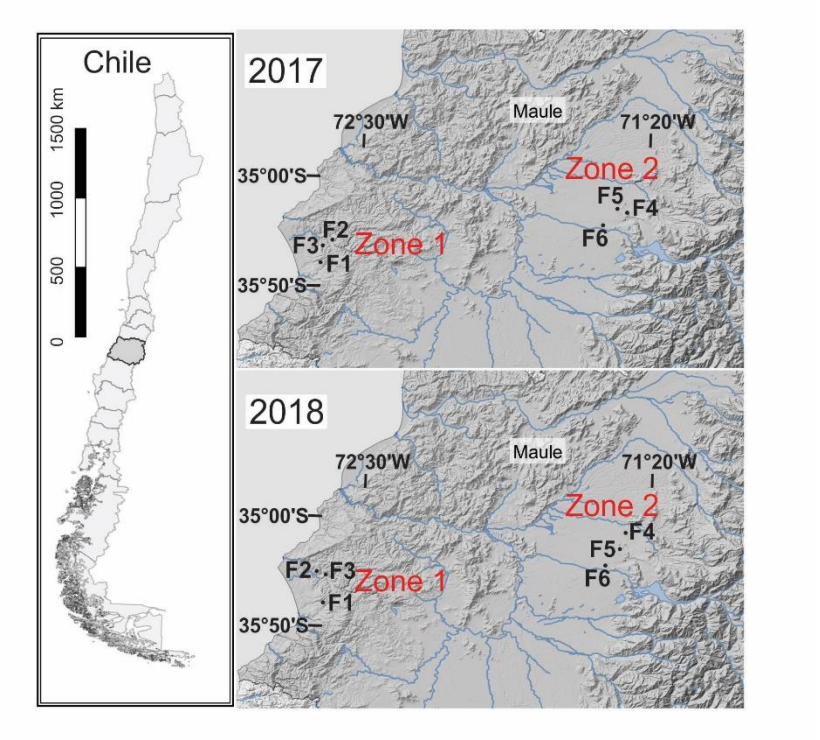

Figure 1. Location of the sampled zones for 2017 and 2018.

Meteorological data were collected from the national agrometeorological network (https://agrometeorologia.cl/). We selected wheat fields located close to a meteorological station to achieve a more accurate environmental temperature measurements. Hence, one meteorological station was used per zone (two in total). Figure S1 shows the temperatures recorded on each sampling date. We computed the average annual temperatures from the last three years of data (2015–2018) to develop a clearer picture of the climatic differences between the studied zones. Thus, Zone 1's temperatures ranged between 17.5 °C (max) and 7.8 °C (min) with 742 mm of rainfall, while Zone 2's temperatures ranged between 21°C (max) and 7.3 °C (min) with 668 mm of rainfall. Moreover, Zone 2 showed an alarming increase in its maximum temperature in recent decades (Piticar, 2019; Araya-Osses et al., 2020).

Because both zones had similar average temperatures, we determined the number of >30 °C events and the average maximum temperatures (2015–2018) during the austral spring (September–December), which is the growing period for most wheat cultivars in Chile, as indicators. Zone 1 showed no days with temperatures >30 °C and an average maximum temperature of 17.5 °C, while Zone 2 showed 37 days with temperatures >30 °C and an average maximum temperature of 22.6 °C.

5.3.2 Aphids and Parasitoids Samplings

Three wheat fields were sampled in each zone. To standardize the sampling effort, we collected aphids and parasitized aphids (referred to as "mummies") from a predefined area of 1 ha per field, avoiding borders (Table S2). Live aphids and mummies were collected every 14 days from the wheat tillering stage to the dough-ripening stage, which took place from September to December (austral spring), for two consecutive years (2017 and 2018). We sampled the total population of aphids and mummies observed on 20 wheat tillers in five randomly selected sampling points in each field, separated by at least 3 m from the field border and 10 m from one another (Gagic et al., 2012; Yang et al., 2017). The leaves and ears of the tillers that were infected with aphids were clipped off and carefully transferred to modified Petri dishes with ventilation and sealed with Parafilm®.

All fields were sampled on the same day and repeated every two weeks to complete six sampling dates (T1–T6). Once in the laboratory, aphids and mummies were counted and identified following taxonomic keys (Blackman & Eastop, 2000). Mummies were kept in Petri dishes until the emergence of wasps, and the parasitoids were identified using taxonomic keys (Starý, 1995). Live aphids were kept in cages containing wheat seedlings (*Triticum aestivum* cultivar Pantera) for 15 days and checked for mummification every two days. New *S. avenae* mummies were removed from the seedlings and kept in Petri dishes for counting and identification.

We collected an additional sample of 10–30 adult *S. avenae* aphids during each sampling date (field, zone, and year). We collected aphids through a linear transect to limit the chances of sampling individuals that belonged to the same parthenogenetic colony (i.e., clonal lineages) by taking one single wingless individual every 10 m. All aphids from those samples were stored in Eppendorf tubes with 95% ethanol until bacterial endosymbiont screening (700 aphids in total) (Table S2).

5.3.3 Aphid Bacterial Endosymbionts

The total DNA from each stored aphid was extracted by the salting-out method (Sunnucks et al., 1996). We screened for the presence of the most frequent endosymbionts harbored by aphids (*Hamiltonella defensa, Regiella insecticola, Serratia symbiotica, Fukatsuia symbiotica, Rickettsia sp., Ricketsiella sp*., and *Spiroplasma sp*.) using a PCR-based protocol described previously (Peccoud et al., 2013; Sepúlveda et al., 2017). The obligate endosymbiont, *Buchnera aphidicola,* was used as a positive control.

5.3.4 Data Analysis

All data were analyzed in the R software version 3.6.1. (Team, 2019). Differences in maximum temperatures were log-transformed and analyzed with a linear model.

To test the effect of every zone, year, and sampling date on *S. avenae*, parasitism rate, and facultative endosymbiont proportion, we used generalized linear mixed models (GLMMs).

The density of the English grain aphid *S. avenae* was analyzed as the number of individuals per 100 tillers in each field, with a Poisson distribution error. Due to the low number of emerged parasitoids in some fields and sampling dates, we did not perform statistical analysis for the composition of parasitoid species; therefore, data are only descriptive and were calculated as the proportion of each identified parasitoid species to the total of emerged parasitoids for every zone, year, and sampling date. The parasitism rate was calculated only for those fields with a frequency of *S.avenae* >6. Hence, the parasitism rate was calculated as the proportion of parasitized *S. avenae* individuals, i.e., the number of mummies found in 100 tillers/number of aphids + number of mummies found in 100 tillers (Gagic et al., 2012) for every zone, year, and sampling date, and analyzed with a binomial distribution error. Changes in the density of *S. avenae* during the season were evaluated for every zone and year.

Because *R. insecticola* was the predominant facultative endosymbiont (except for a single aphid individual bearing a co-infection with *R. insecticola* and *H. defensa*), we calculated the proportion of infected aphids as the number of aphids carrying *R. insecticola* divided by the total number of individuals collected per year, zone, and sampling date (Table S2), analyzed with a binomial distribution error.

We used the year, zone, and sampling date (T1–T6) as fixed parameters and the field in each zone as a random factor. The effect of each factor was evaluated by type II Wald chi-squared tests (Fox & Weisberg, 2019). Comparisons of each fixed factor for *S. avenae*, parasitism rate, and facultative endosymbiont proportion were performed with the R package multcomp (Hothorn et al., 2020). Because maximum temperatures differed between zones and years, the effect of maximum temperature on *S. avenae*, parasitism rate, and facultative endosymbiont proportion was analyzed separately for every zone and year using a GLMM with the maximum temperature as a fixed factor and the field as a random factor.

5.4 Results

5.4.1 Registered Temperatures in the Field

Our results show that Zone 2 registered higher maximum temperatures than Zone 1 in both seasons across the sampling dates (2017: F = 93.2, df = 1, P = < 0.001; 2018: F = 68.5, df = 1, P = <0.001) (Figure S1). In 2017, Zone 1 displayed a maximum temperature range of 11.8–21.2 °C, with an average maximum temperature of 16.1°C, while, in 2018, it displayed a range of 11.7–21.5 °C, with an average of

17.2°C. No extreme maximum temperature events were recorded for either of the studied seasons (>30 °C). Zone 2 displayed a maximum temperature range of 11.6–31.1°C in 2017 (with an average maximum temperature of 21.3 °C) and one single extreme high-temperature event (between T5 and T6), and 13.2–32.7°C in 2018, with an average maximum temperature of 22°C and two extreme high-temperature events (between T3–T4 and T5–T6). Data for the daily accumulated rainfall are provided in Figure S2.

5.4.2 *Sitobion avenae* Density

For the 2017 campaign, we collected 1305 aphids and mummies, from which 68.3% were identified as *S. avenae*. The *S. avenae* sample contained 192 mummies. For the 2018 campaign, we sampled 1128 aphids, 84.4% of which belonged to *S. avenae,* with 123 mummies (Table S2). We found a maximum of 109 *S. avenae* aphids per 100 wheat tillers (1.09 aphids per tiller on average).

When the effects of the zone, year, and sampling date on *S. avenae* density were tested, the results showed that *S. avenae* did not change significantly between the two years. However, the zone, the sampling date, and the interaction between the three factors had a significant impact (Table 1). The differences between zones were mainly due to the fact that there were significantly lower aphid densities in Zone 1 than Zone 2 (Table S2). In contrast, differences in zones by year were due to a decrease in the aphid population in Zone 1 from 2017 (n = 495) to 2018 (n = 343) (Table S2).

Table 1. Type II Wald chi-squared tests for *Sitobion avenae* density, parasitism rate, and *R. insecticola* proportion.

	Aphid			**Parasitism rate**			***R. insecticola* proportion**		
Effect	χ^2	**df**	**P**	χ^2	**df**	**P**	χ^2	**df**	**P**
Year	0.316	1	0.574	7.115	1	0.007	0.000	1	0.999
Zone	6.190	1	0.013	0.237	1	0.663	4.388	1	0.111
Sampling date	124.663	5	< 0.001	15.810	5	0.007	11.858	5	0.065
Year × zone	9.039	1	0.003	6.4722	1	0.010	0.000	1	0.999
Year × sampling date	13.126	5	0.022	5.309	5	0.379	0.000	5	1.000
Zone × sampling date	73.508	5	< 0.001	17.741	5	0.003	2.892	5	0.716
Year × zone × sampling date	72.122	5	< 0.001	22.391	5	< 0.001	0.000	5	1.000

Changes in the density of *S. avenae* during the season were analyzed separately for every zone and year. In 2017, a higher number of aphids was observed in Zone 1 at the beginning of the season (χ^2 = 20.94, df = 5, P = 0.0008) (T1; Figure 2A). However, this effect was not observed in Zone 2 (χ^2 = 6.08, df = 5, P = 0.30) (Figure 2B). In 2018, the density of *S. avenae* also changed throughout the season in Zone 1 (χ^2 = 17.03, df =5, P = 0.004), where a lower number of aphids was observed at T3 compared to T6 (Figure 2C). Similarly, the density of the *S. avenae* also changed throughout the season in Zone 2 (χ^2 = 22.8, df = 5, P = <0.01), with a marked decrease at T6 (Figure 2D).

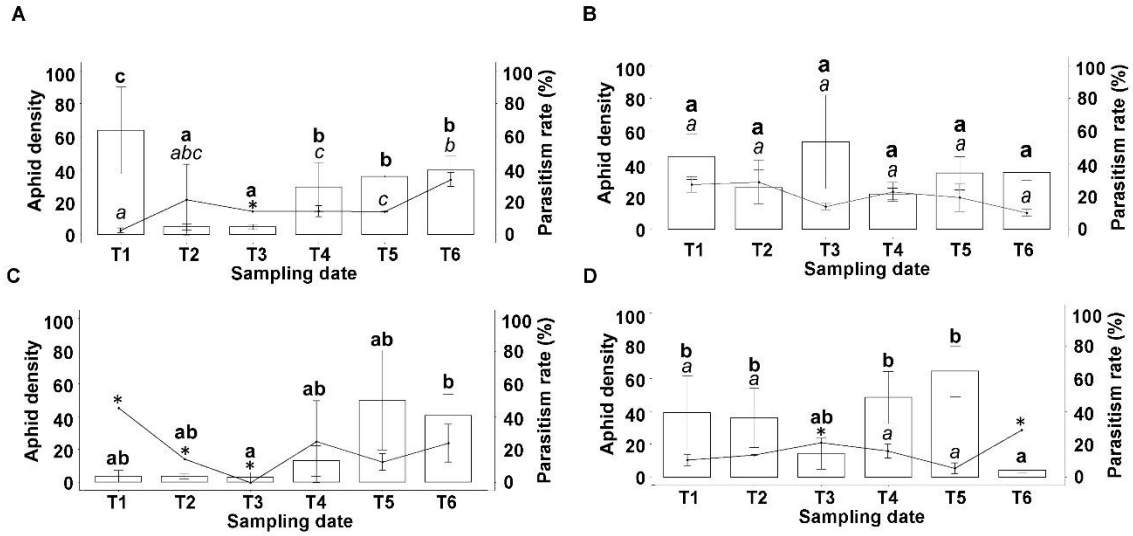

Figure 2. Aphid density (Mean ± SE, bars) and parasitism rate (Mean ± SE, lines) per 100 wheat tillers in three fields sampled during the sampling dates (T1–T6) in **(A)** Zone 1 and **(B)** Zone 2 throughout 2017; and **(C)** Zone 1 and **(D)** Zone 2 throughout 2018. Different letters show significant differences at $p < 0.05$ (bold letters for aphid density and italic letters for parasitism rate). *Data were obtained from one field, and hence multiple comparisons were impossible to calculate.

Overall, data analyses showed no significant effect of maximum temperatures in 2017 for any zone (Zone 1: $\chi^2 = 2.5244$, df= 1, P= 0.112; Zone 2: $\chi^2 = 0.5892$, df= 1, P= 0.443). In 2018, maximum temperatures showed a significant effect on *S. avenae* density, but only in Zone 1 (Zone 1: $\chi^2 = 8.2314$, df= 1, P= 0.004; Zone 2: $\chi^2 = 0.5532$, df= 1, P= 0.457) (Figure 3C), which indicates that the *S. avenae* density increased with the increase in maximum temperature (Figure 3).

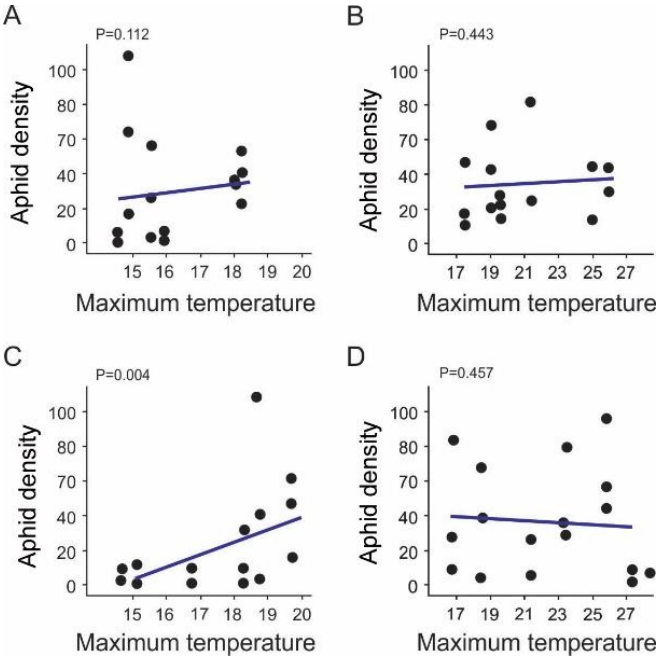

Figure 3. Effect of maximum temperature on aphid density (number of *S. avenae* per 100 wheat tillers). (A) Zone 1 and (B) Zone 2 throughout 2017; and (C) Zone 1 and (D) Zone 2 throughout 2018. P indicates the *p*-value obtained from a generalized linear mixed model (GLMM).

5.4.3 Parasitoid Composition

Seven parasitoid species belonging to the Braconidae family were found. Six species belonged to the *Aphidius* genus (*Aphidius ervi*, *A. uzbekistanicus*, *A. rhopalosiphi*, *A. avenae, A. matricariae*, and *A. colemani*), and one unidentified species belonged to the *Praon* genus (*Praon* sp.). From a total of 203 emerged parasitoids, the *A. ervi* wasp was the most frequent (58.6%), followed by *A. uzbekistanicus* (12.3%), *A. rhopalosiphi* (11.8%), *A. matricariae* (6.9%), *A. avenae* (4.4%), *A. colemani* (3%), and *Praon* sp. (3%) (Table S2). Figure 4 shows the variation in the parasitoid composition throughout the season.

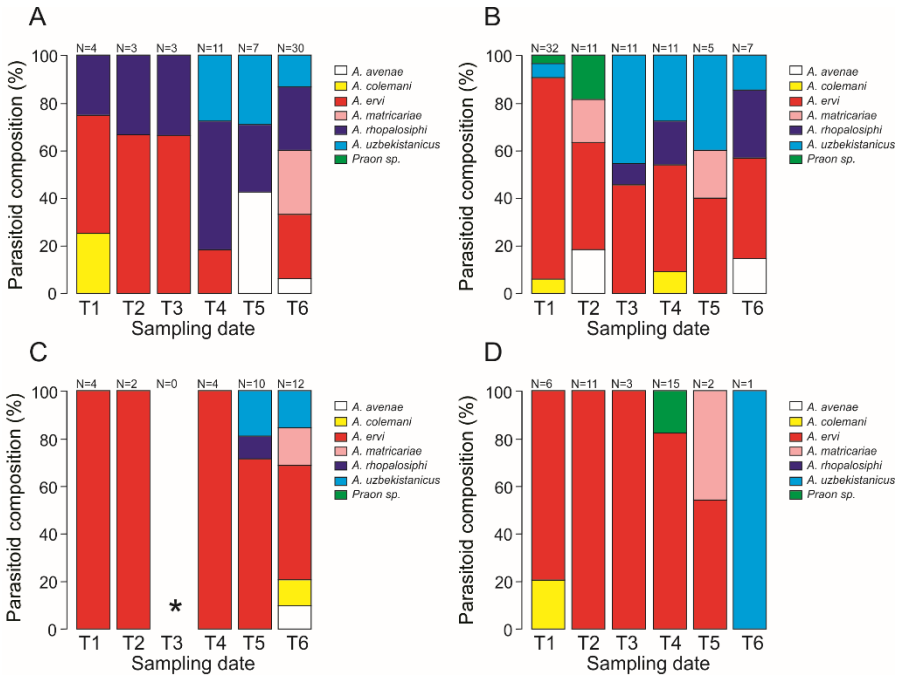

Figure 4. Parasitoid species composition according to the sampling date (T1–T6). (A) Zone 1 and (B) Zone 2 throughout 2017; and (C) Zone 1 and (D) Zone 2 throughout 2018. N = total number of individuals emerged. * No mummies were found.

5.4.4 Parasitism Rate

The zone had no effect on the parasitism rate; however, the year parameter had a small but significant effect. The sampling date and the interactions among the three factors were also significant (Table 1). A significant increase in the parasitism rate was observed in Zone 1 from 2017 to 2018 ($\chi^2 = 1948.2$, df = 1, P = <0.001; 17.19% and 20.65%, respectively), while the opposite occurred in Zone 2 ($\chi^2 = 6.2623$, df = 1, P = 0.012) (20.78% and 13.05%, respectively, for 2017 and 2018) (Table S2).

Changes in the parasitism rates at different sampling dates were analyzed separately for every zone and season. The sampling date showed a significant effect on the parasitism rate in 2017 for Zone 1 ($\chi^2 = 43.57$, df = 5, P = < 0.001), with most differences observed when T1 (which displays a lower parasitism rate) was compared with T4 and T6 (Figure 2A). In contrast, the parasitism rate did not change throughout the season in Zone 2 ($\chi^2 = 7.91$, df = 5, P = 0.16) (Figure 2B). In 2018, very low *S. avenae* densities were found in most of the fields belonging to Zone 1 (Table S2), making it impossible to collect enough data to calculate the effect of the sampling date on the parasitism rate in this season; therefore, we did not include comparisons for this season, but the data are still shown in Figure 2C.

No variation in the parasitism rate of *S. avenae* was detected throughout 2018 in Zone 2 ($\chi^2 = 6.74$, df = 5, P = 0.240). Because low aphid densities were detected at T3 and T6 in most of the fields in this zone,

we did not calculate parasitism rates for these sampling dates (Figure 2D), and they were not included in the multiple comparisons.

The maximum temperature influenced the parasitism rate in both studied zones, but only in 2017 (2018: Zone 1: $\chi^2 = 0.0054$, df= 1, P = 0.94; Zone 2: $\chi^2 = 0.238$, df= 1, P = 0.418). In Zone 1, there was an increase in the parasitism rate as the maximum temperature increased ($\chi^2 = 5445.2$, df = 1, P = < 0.001), while the opposite trend was observed in Zone 2 ($\chi^2 = 12.135$, df = 1, P = 0.005) (Figure 5).

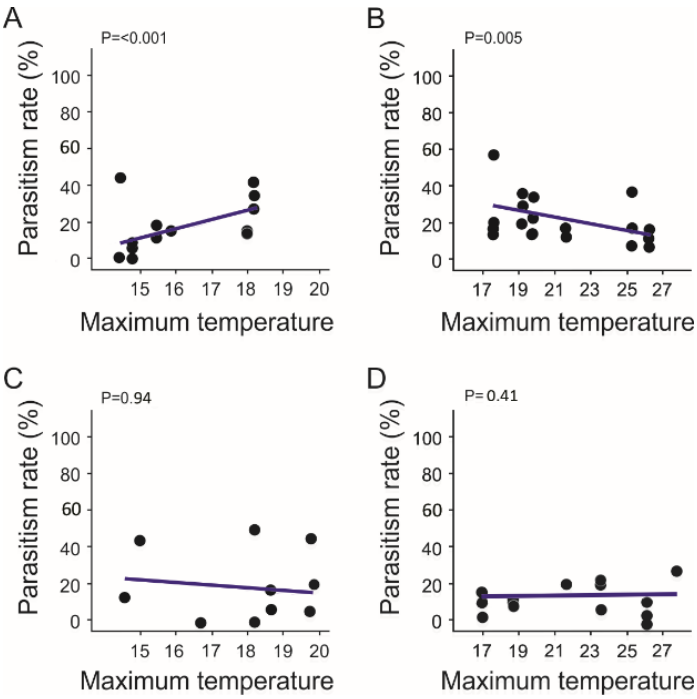

Figure 5. Effect of maximum temperature on parasitism rate in (A) Zone 1 and (B) Zone 2 throughout 2017; and (C) Zone 1 and (D) Zone 2 throughout 2018. P indicates the *p*-value obtained from GLMM.

5.4.5 Composition of Facultative Endosymbionts

Out of the seven facultative bacterial endosymbiont species screened, only *R. insecticola* and *H. defensa* were detected in both years and zones (Table S2). The bacteria *R. insecticola* was carried by 95% of the sampled aphids, mostly as single infections, while 4.9% of the aphids showed no infection. Co-infections with *R. insecticola* and *H. defensa* bacteria were observed in one individual, which was excluded from the analysis to avoid distortions. When the frequency of infections with *R. insecticola* was analyzed, no effects of the year, zone or sampling date were detected (Table 1). The maximum temperatures had no effect on *R. insecticola* proportions in any zone and year (Zone 1, 2017: $\chi^2 = 2.1$, df = 1, P = 0.145; Zone 2, 2017: $\chi^2 = 0.4$, df = 1, P = 0.517; Zone 1, 2018: $\chi^2 = 0.02$, df = 1, P = 0.884 and Zone 2, 2018: $\chi^2 = 0.003$, df = 1, P = 0.952).

5.5 Discussion

Climate can shape species' distribution and their interactions, and temperature is one of the most critical climatic factors. Changes in environmental temperatures may therefore impact the geographic distribution of pests and their biological control agents (Jeffs & Lewis, 2013).

The effect of high temperatures has been broadly studied, mainly under laboratory-controlled conditions. The potential impact of daily maximum temperatures, however, has been poorly studied at the field level. Because the daily maximum temperature has increased rapidly in recent years and has been shown to have a more substantial effect on pest insects and their interacting species (Ma, Ma & Pincebourde, 2021), it is a more realistic approach for evaluating global warming's effects on biological control. Our study considered two zones with differences in their maximum temperatures, and we assessed the impact of these factors on the temporal densities of *S. avenae* and their parasitoids. Furthermore, we considered the frequency of infections with facultative endosymbionts in *S. avenae*, which are expected to have a role in the adaptive potential of aphids to face global warming, and their interactions with parasitoids (Ma, Ma & Pincebourde, 2021).

5.5.1 Factors Shaping Spatial and Temporal *Sitobion avenae* Populations

Our results show a clear difference in *S. avenae* densities between zones, with a higher aphid density in the warmer zone (Zone 2). Even if temperatures above 30 °C reduce the fecundity and extend the physiological development period of pests, reducing their population growth (Han et al., 2019), an increase in temperature, as long as it does not exceed the thermal threshold of a given species, has been shown to have positive effects on aphid populations (Asin & Pons, 2001). In *S. avenae*, significantly higher population growth is reported at 25 °C than at 15 °C (Asin & Pons, 2001). Therefore, the temperatures recorded in Zone 2 seem to have a positive impact on *S. avenae* population numbers, which explains the higher density of S. avenae in that zone. It is important to note that Zone 2 also shows some extremely high temperature events (>30 °C). However, they do not seem to have negatively affected *S. avenae* (Figure S1) because no decrease in *S. avenae* density was observed after these events. The frequency of extremely high temperature events can negatively impact aphid biology because aphids may not have enough time and resources to recover from the potential heat damage produced by these events (Sentis, Hemptinne & Brodeur, 2013). For example, field experiments in China showed a decrease in *S. avenae* s when the frequency of extreme temperatures (>30 °C) was artificially increased by 60% compared with natural conditions (Ma, Rudolf & Ma, 2015). Intriguingly, in our study, maximum temperatures during the season only positively affect aphid density in Zone 1 for 2018 (from T4 to T6). As we previously described, Zone 1 is located close to the Pacific Ocean, within a coastal,

temperate Mediterranean climate with strong oceanic regulation. The increases in maximum temperatures observed in 2018 apparently did not exceed the thermal threshold of *S. avenae* [44], instead having a positive effect on *S. avenae* density. No effect of maximum temperatures was detected in Zone 2, probably because the maximum temperatures recorded throughout the seasons under study were not extremely high, but close to the optimal temperature that would allow for an *S. avenae* population increase.

Although our study was focused on the climate particularities of each zone, mainly temperatures, it is important to keep in mind other factors, which were not studied, that could also influence temporal variations in *S. avenae* density (i.e., host plant phenology, the effects of biological control agents, landscape, rainfall, etc.). Temporal changes in *S. avenae* density among sampling dates were detected for both zones and seasons. Our study considered zones with different climates and temperatures that could affect aphid performance and their host plants' phenology (Ciss et al., 2014). In cereals, the highest peak of aphid density is usually observed at the milk-ripening stage (Gagic et al., 2012), but the timing of this crop growth stage may vary according to the climate. Moreover, the synergy, additive, or antagonistic interaction of natural enemies, including parasitoids, predators, and pathogens (i.e., viruses, bacteria, and fungi) can decrease aphid density in fields and account for temporal variations in aphids throughout the season (Roy & Pell, 2000; Ramsden et al., 2017; Ortiz-Martínez & Lavandero, 2018). The complexity of the landscape could also impact aphid density because complex landscapes contain various grasses that could act as migration sources, thereby transferring aphids to wheat fields (Yang et al., 2019). Zone 2 is characterized by a higher area of arable land than Zone 1; however, we did not find a lower aphid density in this zone, as expected. Hence, landscape did not appear to be an important factor in explaining the *S. avenae* densities found in our results.

On the other hand, climate change and global warming favor drought in many areas worldwide (Spinoni et al., 2014), which may indirectly impact aphid density (Aslam, Johnson & Karley, 2013; Romo & Tylianakis, 2013; Beetge & Krüger, 2019). For instance, water deficits can increase the concentration of nitrogen and amino acid contents in the host plants of aphids, thus benefiting their growth and reproduction (Mattson & Haack, 1987; Cui et al., 2020). Moreover, water deficits can also decrease the parasitism rate by altering the parasitoid preference, thus impacting aphids' biological control (Tariq et al., 2013). Since 2010, central Chile has faced a dramatic sequence of dry years, with deficits in rainfall between 25 and 45%, referred to as the "Mega Drought," which is characterized by a few events of extreme precipitation, followed by long periods with no rain (Garreaud et al., 2020). Because Zone 2 has higher temperatures than Zone 1, this must mean that it bears the consequences of the drought of the

last decade more significantly, thereby increasing the water deficit on plants, which could also account for the higher density of *S. avenae* in this zone.

5.5.2 Parasitoid Assemblage

Most parasitoid wasps collected in wheat fields belonged to the sub-family *Aphidiinae,* in which *Aphidius ervi* represented 82% of the whole sample. This observation agrees with previous reports showing that *A. ervi* and *A. uzbekistanikus* are the most common parasitoids attacking cereal aphids in Chile (Ortiz-Martínez & Lavandero, 2018). Interestingly, the parasitoid assemblage changed as the season progressed. While the most frequent parasitoid in both zones and seasons was *A. ervi* early in the season, other parasitoid species gained relevance later; nevertheless, these results must be interpreted with caution because very low numbers of parasitoids emerged at some sampling dates. Changes in population dynamics throughout the season seem to be a feature of the parasitoid assemblages that parasitize cereal aphids, as previously observed in various European countries (Sigsgaard, 2002; Andrade et al., 2015; Eoche-Bosy et al., 2016). The seasonal activities of some parasitoid species seem to match their thermal tolerances. Some studies (Sigsgaard, 2000; Le Lann et al., 2011) have reported that the thresholds for *A. ervi* from egg to mummy were 2.2 °C and 6.6 °C for mummy to adult development, respectively, while *A. rhopalosiphi* were 4.5 °C and 7.2 °C, and those for *P. volucre* were 3.8 °C and 5.5 °C, respectively. Early parasitization is critical in preventing aphid outbreaks (Sigsgaard, 2002; Tomanović et al., 2008). A parasitoid able to develop at low temperatures could mean an earlier appearance in the field, which is essential for parasitizing aphids, as they start to multiply early. This feature does not appear to be affected by the climate conditions at present, and may explain the early presence of *A. ervi* in wheat fields found in our study and the biocontrol of *S. avenae* in Chile. Nevertheless, permanent temporal and spatial studies similar to ours are needed to monitor the biological control status for this pest and others in the coming years, as we discuss below.

5.5.3 Spatial and Temporal Changes in Parasitism Rate

The demographic balance between aphids and parasitoid wasps is critical for the efficacy of biological control. Nevertheless, this balance can suffer from increased environmental temperatures due to phenological desynchronization between aphids and parasitoids, which disrupts their trophic relationships and ultimately contributes to aphid outbreaks (Furlong & Zalucki, 2017; Harvey et al., 2020).

Our results show that the parasitism rate varied between seasons and zones. Zone 1 showed a higher parasitism rate in 2018 than in 2017, while the opposite trend was observed in Zone 2, although these results should be interpreted with caution since we had to remove data with a low number of aphids

to avoid overestimating parasitism rates (Table S2). It has been reported that *A. ervi* shows a better parasitism rate at 25 °C than at 15 °C or 20 °C, and the worst performance at 30 °C (Malina & Praslička, 2008). The rise in the maximum temperature from 2017 to 2018 in Zone 1 seemed to positively impact the parasitism rate because the colder zone (Zone 1) became a bit warmer (Figure S1). However, this produced a contrasting effect in the warmer zone (Zone 2), where temperatures are continuing to rise to levels at which the performance of parasitoid wasps significantly decays (Malina & Praslička, 2008; Furlong & Zalucki, 2017; Harvey et al., 2020). It is well known that species at higher trophic levels, such as parasitoids, are highly susceptible to temperatures close to 30 °C (Flores-Mejia et al., 2016; Ma, Ma & Pincebourde, 2021). Similar to those recorded for Zone 2 in 2018, high temperatures can decrease the parasitoid attack rate (Le Lann, Lodi & Ellers, 2014) and alter life history traits, such as the developmental time and lifespan of parasitoids (Jeffs & Lewis, 2013). Hence, during the last decade in central Chile, global warming-related phenomena could be following a sustained and troubling trend (Piticar, 2019), which explains the differences in the parasitism rates observed between zones and seasons in this study and alerts us to the outcomes of biological control programs in upcoming years. More monitoring is needed to test this hypothesis.

Intriguingly, the maximum temperatures recorded throughout the season only influenced the parasitism rate in 2017. Assessing the effect of temperature on the parasitism rate at the field level is challenging because a single species does not attack aphids, but an assemblage of parasitoid wasps. Several parasitoid species with different thermal tolerances can attack S. avenae aphids; therefore, the outcome of parasitism depends on each parasitoid species' thermal tolerances. A more random parasitism rate was found in 2018 according to maximum temperatures and sampling dates (which were higher than those in 2017); this could mean that temperatures may affect each parasitoid species differently. For instance, the parasitoid wasp A. avenae is more tolerant of high temperatures, whereas *A. rhopalosiphi* is more susceptible (Le Lann et al., 2011), while *A. ervi* seems to tolerate low temperatures, but not temperatures over 30 °C (Malina & Praslička, 2008; Ismail et al., 2013). Because *A. ervi* is the primary parasitoid attacking S. avenae in Chile, the predicted global warming could result in pernicious consequences for the biological control of *S. avenae* and wheat yields.

It is important to consider other factors that could impact parasitism rates, such as secondary parasitoids and predator attacks. Secondary parasitoids can cause a mortality rate of over 50% in parasitoid larvae (Mackauer, Völkl & Url, 1993), while predators can alter the parasitism rate by consuming parasitized aphids and adult wasps (Traugott et al., 2012). We only observed the appearance of secondary parasitoids at the last sampling date; hence, they probably did not impact our results significantly. On the

other hand, predators were rarely observed during the samplings; however, their effect should not be entirely excluded.

5.5.4 Facultative Endosymbionts

Global warming can also affect the insect microbiome because increased temperatures have been shown to alter the composition of bacterial endosymbionts, which mediate aphid–parasitoid interactions (Feldhaar, 2011; Flores-Mejia et al., 2016).

All aphids carry an obligated bacterial endosymbiont, *B. aphidicola*, which provides aphids with essential amino acids and vitamins that they cannot obtain from plant sap (Baumann, 2005). Moreover, aphids can harbor various facultative endosymbionts that are non-essential, but which can confer relative advantages to aphids (Oliver et al., 2010). Facultative endosymbionts can increase thermal tolerance in aphids (Montllor, Maxmen & Purcell, 2002), broaden the host plant range (Tsuchida, Koga & Fukatsu, 2004), and offer protection against natural enemies (e.g., parasitoids and predators) (Oliver et al., 2003; Vorburger et al., 2010; Łukasik et al., 2013). Facultative endosymbionts are considered a significant phenotypic variation source, mainly in introduced aphid pest species, which are frequently characterized by asexual populations and reduced genetic diversities (Figueroa et al., 2018). Hence, aphids can rapidly evolve in new environments in order to face global warming (Ma, Ma & Pincebourde, 2021), which also has important implications for their interactions with their natural enemies (Oliver et al., 2003, 2009; Degnan & Moran, 2008; Vorburger et al., 2010; Hansen, Vorburger & Moran, 2012). In warmer and dryer areas, Chilean populations of *S. avenae* are typically infected with *R. insecticola*, as the most frequent endosymbiont in central Chile (Sepúlveda et al., 2017; Zepeda-Paulo, Villegas & Lavandero, 2017). Our results show a high frequency (95%) of *R. insecticola* in all fields, zones, and on all dates sampled, supporting the previously reported high frequencies of this bacteria, regardless of the range of maximum temperatures recorded in the two studied zones. It is likely that, because of the differences in both zones' temperatures, these temperatures are not sufficient enough to affect the facultative endosymbionts of *S. avenae*.

Increased temperatures can affect the frequency of infections with facultative endosymbionts. For instance, the rate of vertical transmission of *R. insecticola* indicates failure at >28 °C. However, when high temperatures are maintained for several generations, the transmission rate of *R. insecticola* is increased by up to 100%, showing that *R. insecticola* can improve aphid responses to heat (Doremus et al., 2017). Facultative endosymbionts providing environmental adaptations, such as tolerance to increased temperatures, could improve their prevalence quickly, which may increase and/or modify the distribution of aphids (e.g., in warmer climates) (Chen & Purcell, 1997; Montllor, Maxmen & Purcell, 2002). Whether the *R. insecticola* strain(s) found in our study provide aphids with heat tolerance should be tested under laboratory conditions. However, the high prevalence of strain(s) of *R. insecticola* in the field during the

wheat growing season is an exciting observation, suggesting that there is no cost in relation to the fitness of *S. avenae* and no evidence of a strong selection against non-infected aphids.

5.6 Conclusions

Increases in the environmental temperatures can significantly improve aphid performance, positively impacting aphid population numbers (Liu, Lei & Chen, 2019). Adverse effects on parasitoid wasps' performance can disable biological control (Flores-Mejia et al., 2016). In classical biological control scenarios, when both the pest and its natural enemy are introduced species, they usually have reduced genetic diversity and limited evolvability due to the actions of evolutionary factors during their introduction in the form of founding effects, selection (e.g., insecticide applications, natural enemies, climate), and genetic drift (e.g., reduced population sizes) (Sakai et al., 2001; Figueroa et al., 2005; Zepeda-Paulo et al., 2016). Therefore, environmental stability appears to be an essential factor to maintain the balance of population sizes, which is threatened by global warming due to its potential to alter the herbivores in natural and managed systems and change the interactions between aphids and their natural enemies (Harmon, Moran & Ives, 2009).

Nevertheless, at present, the biological control of *S. avenae* by parasitoid wasps does not appear to be significantly affected in Chile since the densities of *S. avenae* are still below the threshold of economic damage (five aphids per tiller (Giller et al., 1995)). Furthermore, facultative endosymbionts such as *R. insecticola* do not appear to have any protective effect against parasitism at the field level (Zepeda-Paulo, Villegas & Lavandero, 2016). However, noteworthily, the warmer year in our study produced lower parasitism rates in the warmer zone, which could already be an indicator of the effects of global warming. Our research presents the current situation regarding the biological control of the English grain aphid in Chile at the field level, which may be useful for future comparisons. Finally, we suggest that periodic monitoring should be carried out to assess the densities of aphids and their facultative endosymbionts, as well as parasitoids and the inclusion of other biotic (e.g., hyperparasitoid and predator population dynamics) and abiotic (e.g., landscape composition, rainfall, etc.) factors that shape aphid–parasitoid interactions.

5.7 References

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6. General conclusions

This Ph.D. thesis work shows that *S. avenae* in Chile have a low genetic diversity compared to their native area. However, it was higher than those reported 20 years ago. Ten genotypes comprised a high proportion of the total genetic diversity. These ten genotypes showed to be closely related, as they were mainly grouped into four “genetic groups,” which means that each group proceeds from the same founder MLG. This finding supports the idea of evolution in *S. avenae* frequent genotypes (i.e., superclones) even under parthenogenetic reproduction. Curiously, closely related MLGs showed to variate their presence and frequency between 2017 and 2018; however, whether this shift is a random process or is explained by any selective force is not clear, and more studies comparing these MLGs are needed.

R. insecticola was confirmed as the most abundant secondary endosymbiont in Chile and France; however, this bacterium is not the most frequent in all *S. avenae* worldwide. Their presence in *S. avenae* is still not clear, as it has been shown to produce contrasting effects on the phenotype of *S. avenae*. Probably, the most intriguing finding was the temporal variation in the presence of *H. defensa*, which seemed to be closely related to the presence of certain MLGs. However, it is not clear whether selection drives its presence in *S. avenae* populations, as no advantageous effect has been detected. The temporal variation observed of *S. avenae* MLGs and *H. defensa* is an exciting finding, which could be a starting point to study its beneficial effect on *S. avenae*. Hence, Chile, as an invaded area of *S. avenae*, offers promising opportunities to study the role of secondary endosymbionts on the invasiveness potential of aphids, as strong selective forces can select the most suitable combinations of aphid genotype and SE

species (and strain) to deal with the environmental conditions in the new area. Moreover, because of the lack of sexual reproduction, aphids and SE can establish strong relationships in time, in which the concept of “holobiont” (i.e., the host plus all its symbiotic microbes) gains great relevance. Indeed, aphids would not be autonomous entities but rather a holobiont resulting from the interaction with their endosymbionts; this combination is where the selection would act. The holobiont formed by the aphid and their SE may develop a more close relationship in the absence of sexual reproduction, as sexual reproduction is the main mechanism of horizontal transference and loss of secondary endosymbionts.

On the other hand, a unique *R. insecticola* strain was detected in Chile's main MLGs, which can evidence intense selection. However, as any apparent effect in their removal was detected, the effect of genetic drift as a modulator of this unique strain cannot be ruled out.

Overall, this thesis evidenced that *R. insecticola* has no apparent effect protecting *S. avenae* from parasitism and temperature at the field level. However, this research reports important temporal variation patterns and associations with particular *S. avenae* genotypes that may help to elucidate secondary endosymbionts' role in invasive *S. avenae* populations.

Finally, endosymbionts not only represent an exciting study model by their advantages conferred to their hosts but also provide interesting opportunities for improving biological control. For instance, the manipulation of endosymbionts can alter the host range or tolerance of abiotic conditions, reduce insect competence to vector disease agents. Furthermore, disrupting primary endosymbionts' transmission can produce insect mortality, suppress insect growth, or reduce their fecundity. All these effects produced by endosymbionts can reduce the pest status of insect pests. Hence, endosymbionts can be used in agricultural biotechnology, as for example as biopesticides, contributing to the biological control of insect pests.

7. Appendices

7.1 Correa et al. Manuscript in preparation

Technical note - *Entomologia Experimentalis et Applicata*

Development of a novel multiplex microsatellite markers PCR method to characterize the grain aphid, *Sitobion avenae* (Hemiptera: Aphididae)

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Short Title: Novel Multiplex microsatellite markers kit for *Sitobion avenae*

Key words: Microsatellites, SSR, Aphids, *Sitobion avenae*, wheat, multiplex.

Introduction

Aphids (Hemiptera: Aphididae) are among the most important agricultural pests. The grain aphid, *Sitobion avenae* (Fabricius, 1775), is considered among the fifteen aphid species of most agricultural importance worldwide (Blackman & Eastop, 2017) and one of the most damaging pests of cereals globally (Alkhedir et al., 2013).

Microsatellite markers are the most suitable molecular markers for ecological and population genetic studies (Selkoe & Toonen, 2006) as they constitute versatile, informative, and cost-efficient molecular tools (Sunnucks, 2000). Even if next-generation sequencing has gained terrain in genetic studies, microsatellites remain helpful due to their polymorphism and easy data acquisition (Flanagan & Jones, 2019).

The use of individual microsatellite markers in PCR's (Simplex) is significantly more expensive and time-consuming than the use of several markers in the same polymerase chain reaction (Multiplex PCR). For example, the cost of multiplexing a set of microsatellite markers can become up to eight times cheaper than simplex reactions (Guichoux et al., 2011).

The first microsatellite markers for *S. avenae* started to be used around the 1990s and were first cited in the literature in 1996 (Sunnucks et al., 1996). In (Simon et al., 1999) provided a comprehensive list of primers to be used in single reactions and tested them on French populations. Even though, several genetic studies have been performed on *S. avenae* populations worldwide (Sunnucks et al., 1997; Wilson et al., 2004), their use has remained not optimized:

primers changes throughout the years, use of single-marker PCR reactions, lack of multiplex development, etc.

To optimize the existing *S. avenae* microsatellite loci already used in single PCR reactions and to allow their simultaneous amplification we developed and standardized a unique Multiplex PCR kit.

In this study, we described for the first time a Multiplex microsatellites PCR reaction to genotype *S. avenae* populations with seven microsatellite markers in one novel multiplex microsatellite PCR. This improvement enables high performance of population genetic studies on this aphid species by reducing time consumption and costs for data acquisition. The resulting multiplex was tested successfully on populations from its native range (France) and invaded range (Chile).

Materials and methods

Sitobion avenae sampling and DNA extraction

Populations of *S. avenae* from Chile and France were sampled from cereal fields during 2017 (Table 1). In each selected field, 5-25 wingless adult aphids were sampled, taken individual which were separated by at least 10 meters to limit the chances of sampling aphids belonging to the same parthenogenetic colony. Aphids were stored in Eppendorf tubes with 95% ethanol for further molecular biology analyses. Genomic DNA was extracted with the prepGEMInsect DNA extraction kit (ZyGEM, Lane Hamilton, New Zealand) without crushing the aphid body and following manufacturer recommendations.

Microsatellite markers polymerase chain reaction standardizations

The candidate microsatellites molecular markers for the multiplex kit were: S19, S3R, S3.43, Sm10, Sm11, Sm12, Sm17 and S5L (Sunnucks et al., 1996; Wilson et al., 2004). Due the importance of finding a common annealing temperature in the development of Multiplex PCR's, we first tested three annealing temperatures (58ºC, 60ºC and 62ºC) in standard PCR conditions with the candidate markers without fluorescent label. Individual polymerase chain reactions (PCR) for each primer pair were performed in a total volume of 10 µL containing 5µL of QIAGEN Multiplex PCR kit buffer, 1µL of each primer, and 1µL of extracted genomic DNA (approximately 45 ng) and 2 µL of water. The PCR cycles consisted of an initial denaturation step at 94 °C of 15 min, followed by 35 cycles with 30 s at 94 ºC for denaturation, 90 s at 58º, 60 º and 62ºC for hybridization and 1 min at 72 ºC for elongation, and a final extension step at 60 ºC for 30 min. Electrophoresis of each PCR product was launched in a Qiaxcel machine (Qiagen, Germany). The forward primer of markers producing a positive signal after electrophoresis were fluorescent-labeled (Applied Biosystems, Woolston, UK).

Fluorescent-labeled primers and Multiplex PCR reaction standardization

A new set of individual PCR's reactions were launched with the fluorescent 5' labeled forward primer and its associated reverse primer. PCR conditions were the same as mentioned above, but the standard annealing temperature was 58ºC. Two µL of the PCR products plus 9 µL of a mix of formamide and 500 LIZ GeneScanTM (Applied Biosystems, Woolston, UK) size standard (25 µL of LIZ 500 size standard for 1 mL of formamide) were separated by electrophoresis using an ABI 3700 sequencer (Applied Biosystems). Sizes of the amplified fragments were scored with GenemarkerTM version 1.75 software (SoftGenetics LLC). After verifying that primer pairs

provided a specific genotype, they were used in a multiplex PCR reaction (Table 2). Primer concentrations were then adjusted to reach homogeneous fluorescence intensities for all markers. The resulting multiplex reaction was used for genotyping the populations of *S. avenae* sampled.

Genetic diversity characterization of S. avenae populations

The number of alleles (Na) and the estimation of observed and expected heterozygosities were obtained with GeneClass (Piry et al., 2004). To test deviations from the Hardy-Weinberg equilibrium (HWE), compute F_{ST} estimates between populations, and test for linkage disequilibrium, the software Genepop v4.2 was used (Rousset, 2008). The HWE test P-values were corrected with standard Bonferroni correction.

Results

A successful multiplex PCR reaction of seven microsatellites markers for S. avenae

From the eight molecular markers tested with the standard PCR conditions for a further multiplex development at the three annealing temperatures (58ºC, 60ºC, and 62ºC), only Sm11 did not work. A second test for this marker was performed using 54ºC and 56ºC as annealing temperatures; in both cases, results were not satisfactory. Anyway, S19, S3R, S3.43, Sm10, Sm12, Sm17, and S5L had a clear performance at 58ºC.

The seven remaining fluorescent-labeled markers lead to unambiguous electropherograms after capillary electrophoresis in an automated sequencer. When used together in multiplex, the seven microsatellite markers provided electropherograms with the same allele sizes used in simplex

(Figure 1). Therefore, we validated a heptaplex microsatellite marker reaction for *S. avenae* (Table 2).

Genetic diversity of S. avenae characterized by the Multiplex PCR Kit

The multiplex PCR reaction was able to amplify the populations from Chile and France.

Alleles number varied between 2 to 8 depending on the locus and population. The Chilean population had an average of 3.14 allele number per loci with a minimum of 2 (for loci S3.43, S19 and Sm10) and a maximum of 5 (Sm17). On the other side, the French population had an average allele number of 5.86 with a minimum of 3 (S5L and Sm10) and a maximum of 8 (Sm17 and S3R). The lower diversity presented by Chile than France has been previously documented (Figueroa et al., 2005) (Correa *et al.* submitted). In Chile, populations of *S. avenae* have shown to be made up of a few genotypes (> 80%) highly predominant in time and space, usually referred to as "superclones" (Vorburger et al., 2003; Figueroa et al., 2005). Despite the low genotypic diversity (< 5%) found in populations of *S. avenae* in Chile, these aphids are widely distributed throughout different agro-climatic zones and are colonizing various cereal crops and wild grasses (Figueroa et al., 2005).

Observed heterozygosity ranged from 0.05 to 1.00, while expected heterozygosity (Nei's gene diversity) ranged from 0.322 to 0.841. Deviations from the Hardy-Weinberg Equilibrium (HWE), after False Discovery Rate (FDR) corrections on P-values (Benjamini & Hochberg, 1995) were detected for certain loci and populations, but only the locus Sm17 presented HWE deviation in both populations (Table 2). The two populations (T3 from Chile and 9SE from France) had an F_{ST} estimate of 0.1656.

Further studies to explain the success of *S. avenae* as an invader in different countries even though their low genetic diversity should be carried to improve the Integrated Pest Management of this species.

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Figure legends

Figure 1. electropherograms obtained in the single and the heptaplex reactions.

Figures

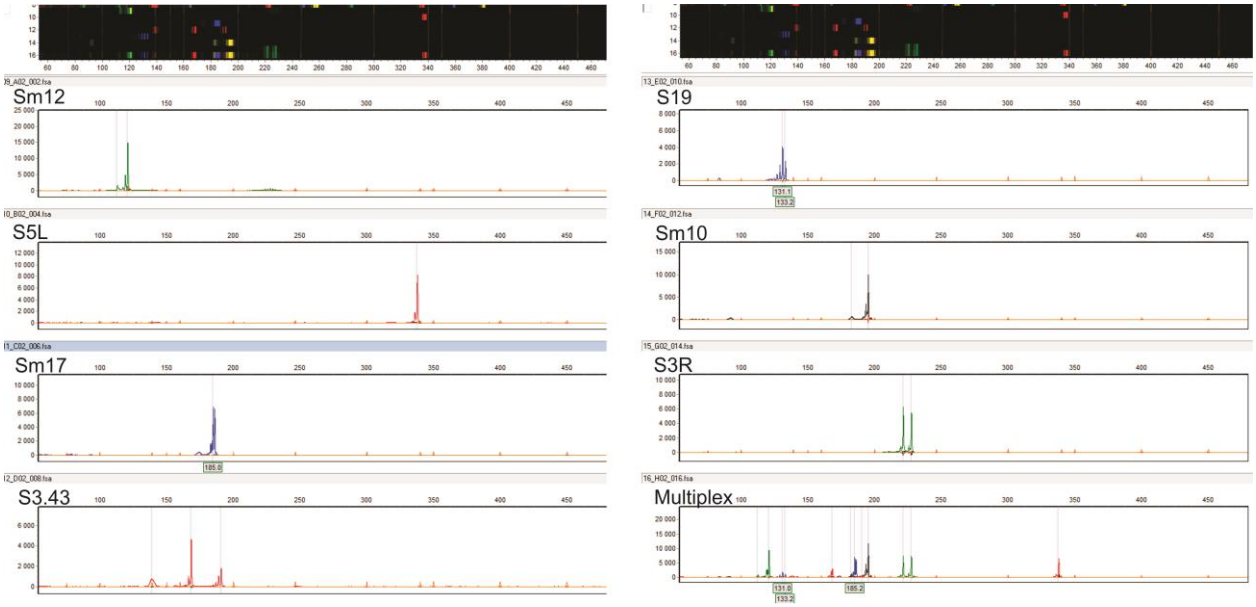

Table 1. Detail of populations sampled and analyzed in this study (n= number of individuals sampled).

Population code	N	Host	City	Locality	Country	GPS	GPS	Year
T3	20	Wheat (*T. aestivum*)	Ercilla	Araucanía	Chile	38°08'13.23 " S	72°19'32.39 "W	2017
9SE	20	Wheat (*T. aestivum*)	Fox Amphoux	Provence Alpes Côte d'Azur	France	43°35'27.6" N	6°05'44.0"E	2017

Table 2. Description of microsatellite markers composing the multiplex PCR reaction. N_A= numbers of alleles, H_o= observed heterozygosity, H_e= expected heterozygosity. , * indicates a significant deviation from HWE after Bonferroni correction.

Locus		Primer sequence (5´-3')	Fluorescent dye	Size range	Country	Na	H_o/ H_e	
Sm12	F:	GCGCATTGTGTAGCGAGC	VIC	119-153	T3	4	0.600/0.605	
	R:	CAAACATGTTATGTCACAATAC			9SE	6	0.368/0.669	*
S3.43	F:	CATCCGAGCGGTGGAATG	PET	165-207	T3	2	0.650/0.450	
	R:	CATTTCGTCATCATTTGCTACATG			9SE	7	0.650/0.768	*
S19	F:	GGCGAGACCCCTTAAAATCC	6FAM	106-160	T3	2	0.389/0.322	
	R:	GAGATACTCTTTTCGTCGTTAAACC			9SE	6	0.050/0.717	*
S5L	F:	TCT GCT GCA TTA CTG TTG GC	PET	221-225	T3	3	1.00/0.656	*
	R:	TCG TCT ACT TCG CCG TCA			9SE	3	0.600/0.581	
Sm17	F:	CAC CAT CGC GTT TCA TCT TA	6FAM	195-229	T3	5	1.00/0.677	*
	R:	ACT CCC AAC CTC TGA TGA GC			9SE	8	0.850/0.841	*
Sm10	F:	TTCTGGCTTCATTCCGGTCG	NED	154-170	T3	2	0.450/0.409	
	R:	CGTCGCGTTAGTGAACCGTG			9SE	3	0.632/0.482	
S3R	F:	GGACGACTCGTTAGTATAGGTGG	VIC	328-362	T3	4	1.00/0.641	*
	R:	CTATCTCTACCGTTTCGAATCG			9SE	8	0.350/0.667	
All loci					T3	3.143	0.727/0.537	
					9SE	5.857	0.500/0.675	

7.2 Supplementary material (Chapter 2)

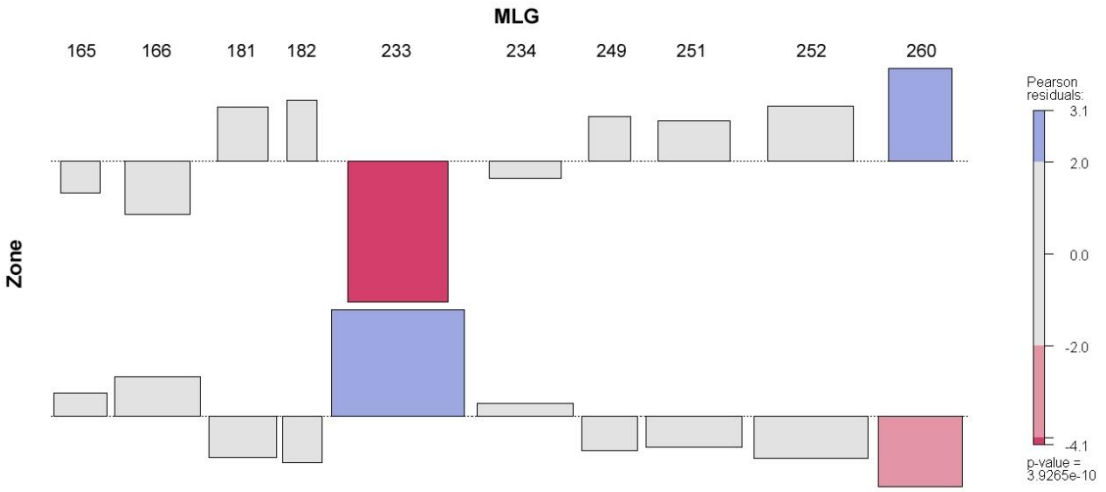

Figure S1. Association plots produced by R program showing the results from Chi-square tests of the Chilean MLGs between zones. These plots show the Chi-square analyses according to Pearson residuals (on the right side). This way, different colors in the plots indicate whether the data are over or under the expected values according to Chi- square expectations. Blue color indicates values which exceed the expected values in Chi-square test; dark and light pink indicate lower values than expected; gray color shows values which do not show any deviation from the expected. Height of the bars is proportional to the residual value (on the right side), and width is proportional to the square root of the expected counts, so that the area of the box is proportional to the difference in observed and expected frequencies (Meyer et al., 2006).

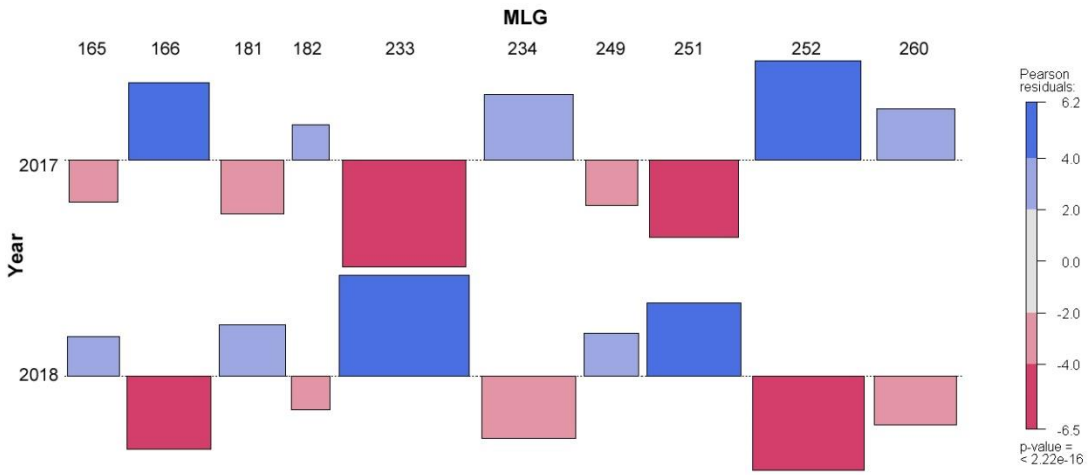

Figure **S2**. Association plots produced by R program showing Chi-square tests of the Chilean MLGs between years. Blue color indicates those values that exceed the expected values in Chi-square tests and dark and light pink color indicate lower values than expected and gray color those values which show any deviations from the expected.

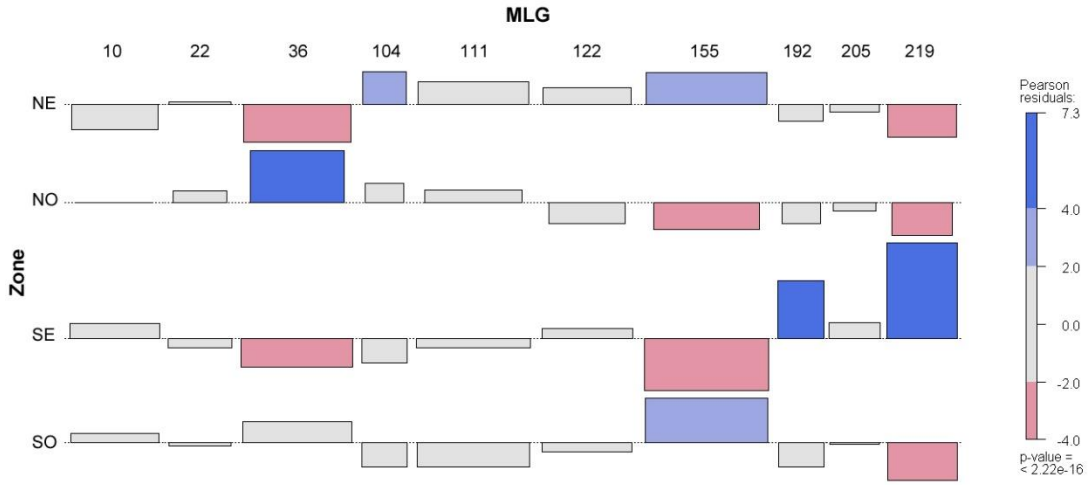

Figure **S3.** Association plots produced by R program showing Chi-squared tests in of the French MLGs between zones. Dark and light blue colors indicate those values which exceed the expected values in Chi-squared test, light pink indicates lower values than expected in Chi-squared tests and gray color those values which show any deviations from expected values.

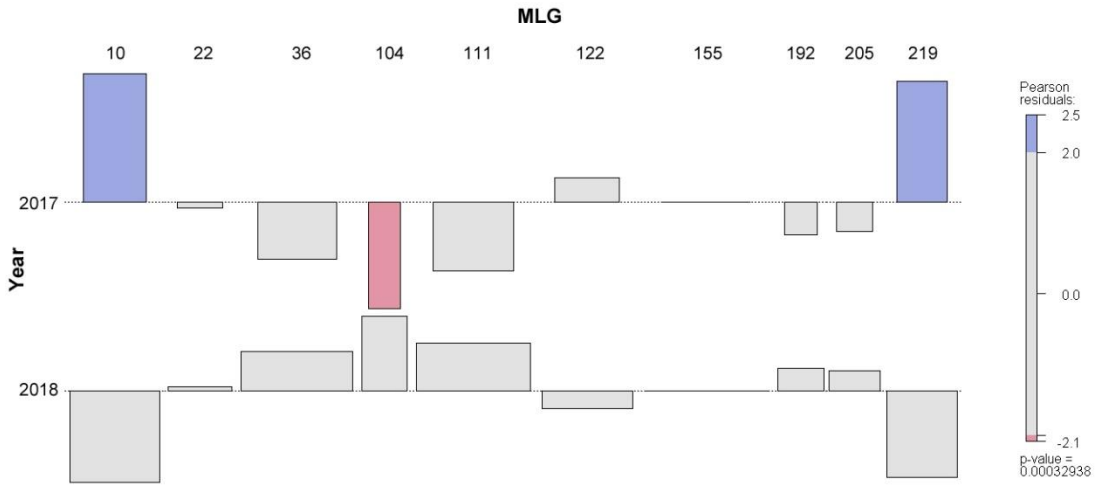

Figure **S4**. Association plots produced by R program showing Chi-squared tests in French MLGs between years. Dark and light blue colors indicate those values which exceed the expected values in Chi-squared test, light pink indicates lower values than expected in Chi-squared tests and gray color those values which show any deviations from expected values.

7.3 Supplementary material (Chapter 4)

Table S1 Geographical coordinates and area of the wheat fields sampled in the studied zones and years.

Year	Zone	Field	Latitude	Longitude	Area ($m^{2)}$
2017	Zone 1	Field 1	35°45'27.81"S	72°30'56.43"W	18418
		Field 2	35°40'15.74"S	72°30'51.43"W	13044
		Field 3	35°40'35.15"S	72°31'05.66"W	6020
	Zone 2	Field 4	35°34'2.55"S	71°23'55.57"W	42296
		Field 5	35°33'49.2"S	71°25'16.5"W	44911
		Field 6	35°35'34.36"S	71°28'27.35"W	37619
2018	Zone 1	Field 1	35°45'27.81"S	72°30'56.43"W	18418
		Field 2	35°39'43.88"S	72°31'38.71"W	20555
		Field 3	35°39'46.99"S	72°30'59.44"W	11589
	Zone 2	Field 4	35°30'14.07"S	71°24'26.01"W	178990
		Field 5	35°33'49.2"S	71°25'16.5"W	44911
		Field 6	35°35'34.36"S	71°28'27.35"W	37619

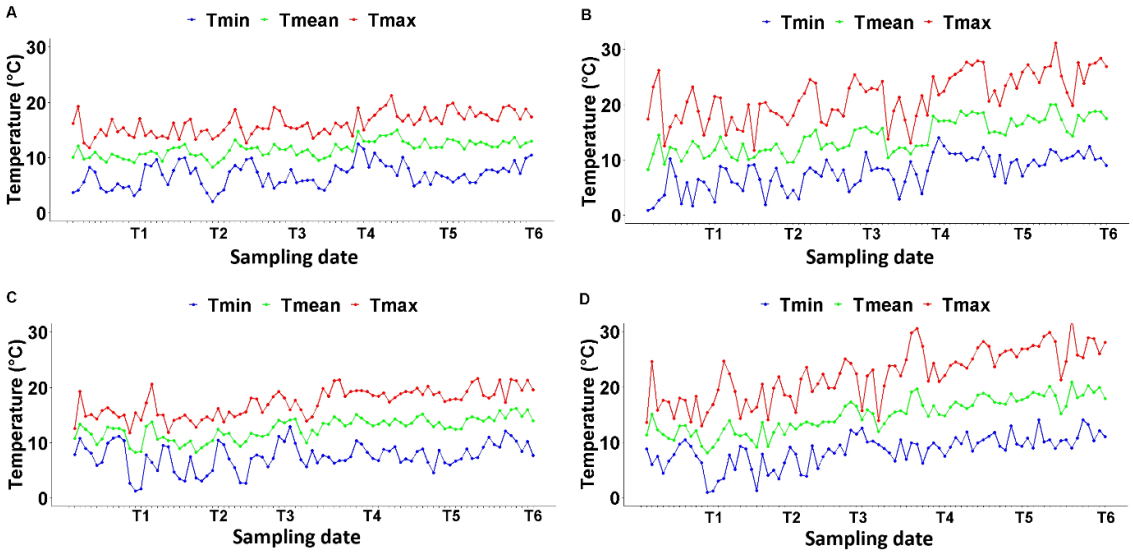

Figure S1. Daily temperatures registered for every zone and year throughout the season. **(A)** Zone 1 and **(B)** Zone 2 in 2017; and **(C)** Zone 1 and **(D)** Zone 2 in 2018. Tmin= minimum temperature, Tmean= mean temperature, and Tmax= maximum temperature.

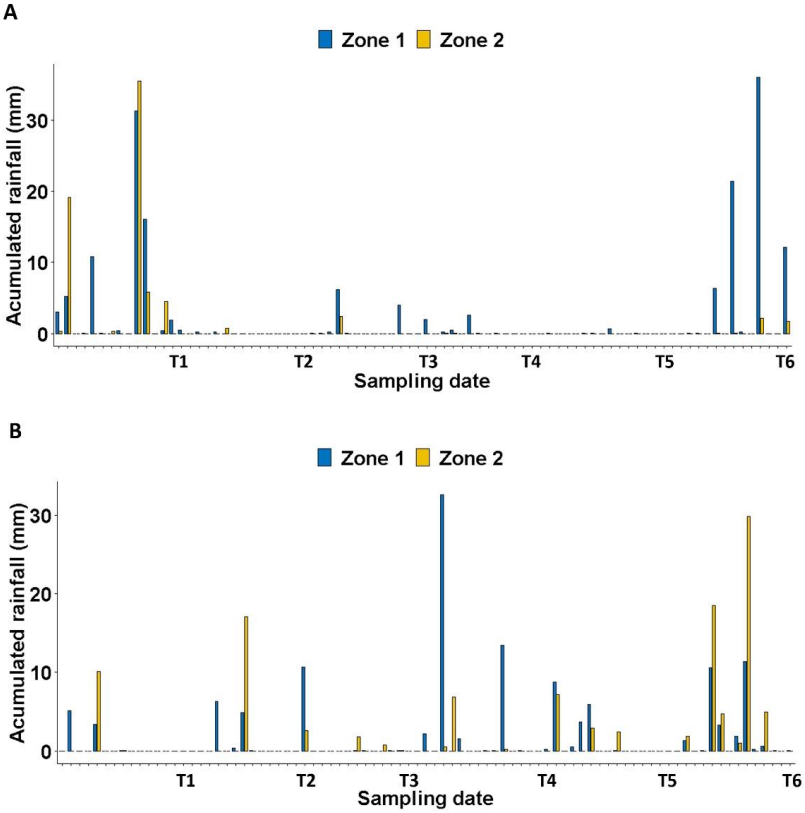

Figure S2. Daily accumulated rainfall for every zone and year throughout the season in (**A)** 2017; and **(B)** 2018.

Table S2. Data obtained in 2017 and 2018 for every zone and field.

Year	Zone	Field	Sampling date	*Regiella* proportion	Number of *S. avenae* per 100 tillers	Number of mummies	Parasitoid species							
							A. ervi	*A. uzbekistanicus*	*A. colemani*	*Praon sp.*	*A. rhopalosiphi*	*A. avenae*	*A. matricariae*	*no emerged*
2017	Zone 1	Field 1	T1	10 (10)	65	3	2							1
		Field 2	T1	10 (10)	17	0	NA	NA	NA	NA	NA	NA	NA	NA
		Field 3	T1	10 (10)	105	4			1		1			2
		Field 1	T2	9 (10)	1	0	NA	NA	NA	NA	NA	NA	NA	NA
		Field 2	T2	8 (10)	6	0	NA	NA	NA	NA	NA	NA	NA	NA
		Field 3	T2	10 (10)	4	3	2				1			0
		Field 1	T3	8 (10)	6	1	1							0
		Field 2	T3	10 (10)	3	2	1				1			0
		Field 3	T3	10 (10)	2	0	NA	NA	NA	NA	NA	NA	NA	NA
		Field 1	T4	7 (10)	4	0	NA	NA	NA	NA	NA	NA	NA	NA
		Field 2	T4	10 (10)	24	3	2							1
		Field 3	T4	10 (10)	46	10		3			6			1
		Field 1	T5	9 (9)	0	0	NA	NA	NA	NA	NA	NA	NA	NA
		Field 2	T5	8 (10)	30	5	0	2			1			2
		Field 3	T5	3 (10)	31	5					1	3		1
		Field 1	T6	28 (30)	17	6	1				3		2	0
		Field 2	T6	NA	32	22	5	2			4	1	5	5
		Field 3	T6	NA	27	14	2	2			1	1	1	7
	Zone 2	Field 4	T1	10 (10)	35	8	5	1						2
		Field 5	T1	9 (9)	15	6	5							1
		Field 6	T1	10 (10)	45	24	17	1	2	1				3
		Field 4	T2	10 (10)	9	2	1			1				0

		Field 5	T2	10 (10)	41	6				1		2	2	1
		Field 6	T2	10 (10)	8	10	4							6
		Field 4	T3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Field 5	T3	6 (10)	22	3	2							1
		Field 6	T3	17 (20)	69	13	3	5			1			4
		Field 4	T4	9 (10)	13	2	1							1
		Field 5	T4	9 (10)	18	9	2	3	1					3
		Field 6	T4	9 (10)	18	5	2				2			1
		Field 4	T5	10 (10)	37	7	1							6
		Field 5	T5	10 (10)	42	3							1	2
		Field 6	T5	9 (10)	9	5	1	2						2
		Field 4	T6	8 (9)	27	3					1	1		1
		Field 5	T6	9 (10)	28	2		1			1			0
		Field 6	T6	10 (10)	38	6	3							3
		Field 1	T1	5 (5)	11	5	4	0	0	0	0	0	0	1
		Field 2	T1	NA	0	0	NA	NA	NA	NA	NA	NA	NA	NA
		Field 3	T1	13 (13)	0	0	NA	NA	NA	NA	NA	NA	NA	NA
		Field 1	T2	10 (10)	2	0	NA	NA	NA	NA	NA	NA	NA	NA
		Field 2	T2	3 (3)	2	1	1							0
		Field 3	T2	17 (17)	7	1	1							0
2018	Zone 1	Field 1	T3	12 (12)	9	0	NA	NA	NA	NA	NA	NA	NA	NA
		Field 2	T3	5 (6)	0	0	NA	NA	NA	NA	NA	NA	NA	NA
		Field 3	T3	11 (11)	0	0	NA	NA	NA	NA	NA	NA	NA	NA
		Field 1	T4	10 (10)	31	0	NA	NA	NA	NA	NA	NA	NA	NA
		Field 2	T4	10 (10)	0	0	NA	NA	NA	NA	NA	NA	NA	NA
		Field 3	T4	10 (10)	8	4	4							0
		Field 1	T5	10 (10)	40	7	2				1			4

	Field 2	T5	10 (10)	3	0	NA	NA	NA	NA	NA	NA	NA	NA
	Field 3	T5	10 (10)	108	8	5	2						1
	Field 1	T6	10 (10)	16	1	NA	NA	NA	NA	NA	NA	NA	NA
	Field 2	T6	9 (9)	60	12		1						11
	Field 3	T6	10 (10)	46	21	6	1	1			1	2	10
	Field 4	T1	10 (10)	83	4	4							0
	Field 5	T1	10 (10)	27	4			1					3
	Field 6	T1	10 (10)	8	1	1							0
	Field 4	T2	7 (7)	67	9	5							4
	Field 5	T2	9 (10)	38	5	5							0
	Field 6	T2	10 (10)	4	1	1							0
	Field 4	T3	15 (15)	5	3	1							2
	Field 5	T3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Zone 2	Field 6	T3	15 (15)	24	5	2							3
	Field 4	T4	10 (10)	37	7	5							2
	Field 5	T4	10 (10)	29	6	3			2				1
	Field 6	T4	10 (10)	80	6	4			1				1
	Field 4	T5	10 (10)	44	5	1						1	3
	Field 5	T5	9 (10)	55	0	NA	NA	NA	NA	NA	NA	NA	NA
	Field 6	T5	10 (10)	95	4								4
	Field 4	T6	10 (10)	5	2		1						1
	Field 5	T6	10 (10)	1	0	NA	NA	NA	NA	NA	NA	NA	NA
	Field 6	T6	10 (10)	7	2								2

*NA= no data were available or it was not possible to calculate a result.

7.3 Scientific Publications

Sepúlveda DA, Barrueto G, Correa MCG, Castañeda LE, Figueroa CC. 2021. Spatial and Temporal Variation in the Aphid–Parasitoid Interaction under Different Climates. **(sended to *Agriculture*) Chapter 5**

Correa MCG, Sepúlveda DA, Briones LM, Castañeda LE, Simon J-C, Figueroa CC. Genetic diversity of invasive and native populations of the grain aphid, *Sitobion avenae,* related to the frequency and distribution of their secondary bacterial endosymbionts (In prep. To be sended to *Evolutionary applications*). **Chapter 2 and 3**

Correa MCG, Blin A, Sepúlveda DA, Briones LM, Castañeda LE, Figueroa CC.Development of a novel multiplex microsatellite markers PCR method to characterize the grain aphid, *Sitobion avenae* (Hemiptera: Aphididae) (In prep. To be sended to *Entomologia et applicata*).

Ballesteros GI, Sepúlveda DA, Figueroa CC. 2019. Identification and expression profiling of peripheral olfactory genes in the parasitoid wasp *Aphidius ervi* (Hymenoptera: Braconidae) reared on different aphid hosts. Insects 10. DOI: 10.3390/insects10110397.

Hlaoui A, Boukhris-Bouhachem S, Sepúlveda DA, Correa MCG, Briones LM, Souissi R, Figueroa CCCC. 2019. Spatial and temporal genetic diversity of the peach potato aphid *Myzus persicae* (Sulzer) in Tunisia. Insects 10. DOI: 10.3390/insects10100330.

Rubio-Meléndez ME, Sepúlveda DA, Ramírez CC. 2018. Temporal and spatial distribution of insecticide-resistance mutations in the green peach aphid *Myzus persicae* (Hemiptera: Aphididae) on primary and secondary host plants in central Chile. Pest Management Science 74:340–347. DOI: 10.1002/ps.4708.

Sepúlveda DA, Zepeda-Paulo F, Ramírez CC, Lavandero B, Figueroa CC. 2017. Loss of host fidelity in highly inbred populations of the parasitoid wasp *Aphidius ervi* (Hymenoptera: Braconidae). Journal of Pest Science 90:649–658. DOI: 10.1007/s10340-016-0798-8.

Sepúlveda DA, Zepeda-Paulo F, Ramírez CC, Lavandero B, Figueroa CC. 2017. Diversity, frequency, and geographic distribution of facultative bacterial endosymbionts in introduced aphid pests. Insect Science 24:511–521. DOI: 10.1111/1744-7917.12313.

7.3 Congresses attendance

XLVIII Reunión anual Sociedad de genética de Chile, November 2015. Efecto de la endogamia sobre el desempeño y la fidelidad al hospedero en el parasitoide *Aphidius ervi*.

IX reunión anual sociedad de evolución. Talca-Chile, October 2015. Diversidad, frecuencia y distribución geográfica de bacterias endosimbióticas facultativas en áfidos plagas introducidos.

International workshop "role of microbial symbionts on the biological interactions in agroecosystems." Talca-Chile November 2016. Insect associated microorganisms may facilitate pest invasiveness: the case of the grain aphid in Chile.

16th International Symposium on Insect-Plant Relationships. Tours-France, July 2017. The ecology and evolution of host preference in an aphid parasitoid

II Joint Congress on Evolutionary Biology. Montpellier-France, August 2018. The adaptability and resilience of a successful introduced pest aphid can be explained by functional symbioses.

International workshop "The role of microbial symbionts on the biological interactions in agroecosystems." Talca-Chile, October 2018. The adaptability and resilience of a successful introduced pest aphid can be explained by functional symbioses.

1er Simposio Latinoamericano, 4to Simposio Chileno De Control Biológico. Chillán-Chile, October 2019. Efecto de la temperatura ambiental y de la presencia de endosimbiontes facultativos sobre el éxito del parasitismo de *Aphidius ervi* sobre el áfido *Sitobion avenae*.

7.4 Teaching.

Co-rresponsable profesor, course “Genes y genomas”. Universidad de Talca August-November 2019.

Undergraduate thesis co-tutoring, Gonzalo Barrueto, Facultad de Ciencias Agrarias, Universidad de Talca, 2017-2018.“El incremento en la temperatura ambiental reduce el éxito del control biológico por parasitoides en el pulgón de los cereales *Sitobion avenae* Fabricius (Hemiptera: Aphididae)”.

7.5 Internships

INRAE, Institut de Génétique Environnement et Protection des Plantes (IGEPP). Rennes-France, March-July 2017.