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Exploring the potential for the selective breeding of *Nesidiocoris tenuis*: a study of its genetics and feeding behavior

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Exploring the potential for the selective breeding of *Nesidiocoris* tenuis: a study of its genetics and feeding behavior

PhD thesis submitted by **MILENA CHINCHILLA RAMÍREZ** in fulfilment of the requirements for the degree of doctor awarded by

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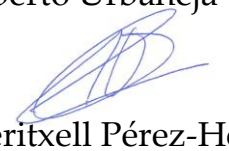

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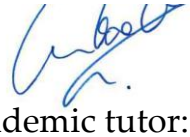

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To my parents, Teresa y Rolando.

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Summary

The application of augmentative biocontrol strategies in cultivated systems has increased over the last decades worldwide, and its tendency to grow is predicted to continue. Consumers and lawmakers are demanding healthier and more sustainable production systems, exerting important pressure for the biocontrol sector to develop more biological control agents. However, in the last decades the restrictions on the use of exotic fauna have also increased, affecting the biocontrol sector as well. Therefore, biocontrol practitioners have shifted their attention to the use and exploitation of locally available species. Yet, some of those species might not be the optimal at controlling certain pests, and/or could have traits that hinder their biocontrol services. In this context, there has been a revival on the idea of improving biocontrol agents by means of selective breeding, this time integrating genetic and genomic resources, with the aim of increasing its success rate and secure its uptake as an established discipline.

Nesidiocoris tenuis (Reuter) (Hemiptera: Miridae) is a cosmopolitan zoophytophagous predator, which zoophagy and phytophagy have given it both the status as biocontrol agent and pest, respectively. Thus, it fits into the category of biocontrol agents with traits that hinder its performance. In regions where it is valued as a biocontrol agent, it is mainly released in tomato crops to suppress key pests such as *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) and *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae). Its role in the activation of plant defenses has also been confirmed more recently. However, its phytophagous behavior can inflict damage on tomato crops under circumstances of prey scarcity. This can produce esthetical or yield losses, or lead to the application of pesticides to reduce its population, thus disrupting the biocontrol programs. Selective breeding in *N. tenuis* have been suggested as an alternative to minimize the detrimental effects of its phytophagy, however, no genetic or genomic information on the species was reported to date. The **first objective** of this theses was to investigate the population genetics of *N. tenuis* at a global scale by using 12 novel microsatellite loci, which were developed based on the genome of *N. tenuis*, and mitochondrial DNA (mtDNA) markers. Specifically, the genetic diversity and the population structure of 16 populations collected in four continents and three commercial rearings were determined. We found moderate-to-low diversity according to both markers. The population from

Vietnam showed the highest diversity (H_s) (0.760), whereas the lowest diversity was observed in Puerto Rico (0.401). Genetic differentiation was detected by both markers for several populations (F_{ST} with $p < 0.05$), with the population from China showing the highest F_{ST} value (0.429). A weak correlation was found between geographical distance and genetic differentiation, as indicated by Mantel test. The mtDNA data revealed a total of 23 haplotypes, with several unique haplotypes in Asia and a predominant and ubiquitous haplotype (haplotype 2) shared between Europe, America, Africa and commercial populations. Bayesian analyses and principal component analysis (PCoA) performed on the microsatellite data suggested two groups: one group with the American populations and a second group containing Asian, African, European and commercial populations. We concluded that geographical barriers and anthropogenic activities are the likely factors shaping the population structure of *N. tenuis*. The implications of these findings for agriculture and biocontrol are discussed within chapter 2.

Among the reasons preventing the adoption of selective breeding in biocontrol agents, it was recently noticed an important shortage of information regarding genetic variation of traits relevant for biocontrol. Additionally, the selection of the (biocontrol) traits to target in an eventual breeding program is also crucial. In *N. tenuis,* both its phytophagy and zoophagy are interesting candidate traits for selective breeding. The second objective of the present thesis was the quantification of the genetic variation in the phytophagy and zoophagy of *N. tenuis*. We compared nine isofemale lines on their capacity to produce necrotic rings and wilting on tomato plants as a proxy for phytophagy, as well as their efficacy to prey on *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs, as a proxy for zoophagy. Differences between isofemale lines in phytophagy and zoophagy indicated a genetic basis. Variation found in the zoophagy levels was larger than that in phytophagy levels. Our results showed that there is a genetic basis for the variation observed in the feeding behavior of isofemale lines of *N. tenuis,* highlighting the potential importance of selective breeding for such traits of biocontrol interest.

Another important aspect in a selective breeding program is to link phenotypic and genotypic data. However, a standard phenotyping methodology should be developed first, so that results from different groups can be compared directly. Moreover, in the case of zoophytophagous predators, measurement of traits comprising its interaction with the plant are of crucial importance due to the influence that the plant could exert on selected

lineages of the biocontrol agent. Therefore, the **third objective** of the thesis was to investigate the behavioral and mechanical components of the plant feeding of *N. tenuis*. For this, we compared the feeding behaviors of males, females and fifth-instar nymphs. Additionally, we investigated the type of stylet activities performed by each stage while probing in plant tissue, using the electrical penetration graph technique (EPG). Furthermore, stylectomy was performed and plant histology studied with the aim to correlate the feeding activities observed in the EPG recordings with stylet tip positions in specific tissues of the leaf petioles. Behavioral observations during a 30-min period showed that nymphs probed more frequently (38,6 ± 1,5 probes) than males and females (25,3 ± 1,1 and 24,3 ± 1,1 probes, respectively). Similarly, nymphs spent a higher proportion of time (656,0 ± 67,6 s) feeding on tomato apical sections compared to males and females (403,0 ± 48,8 s and 356,0 ± 43,7 s, respectively). The EPG recordings during 5 h indicated that cell-rupturing was the main stylet activity for all insect stages, and that fifth-instar nymphs spent a higher proportion of time on cell-rupturing events compared to adults. The histological studies revealed a trend of *N. tenuis* for the tissues within the vascular semi-ring. The stylet tips were found both in the vascular bundles and in the parenchyma of the interfascicular region. The findings of this chapter confirm an important role of fifth-instar nymphs feeding behavior in the phytophagy of *N. tenuis*. Moreover, the increased time spent on cell rupturing behaviour suggests that stylet laceration and enzymatic maceration of the saliva occurring during this event might greatly contribute to the inflict plant damage. A comprehensive understanding of the interactions of *N. tenuis* with the plant, at both the behavioral and mechanical levels, might shed light on new approaches to minimize its damage potential to tomato while maintaining its benefits as biocontrol agent.

Overall, throughout this thesis it is illustrated how genetic and genomic resources can be effectively incorporated into biocontrol with the aim of improving pest control strategies. Although selective breeding of biocontrol agents could be seen as the ultimate goal of combining these three fields, other important research lines, such as plant breeding, could also benefit from this combination of fields. Moreover, with the research hereby presented, perhaps other biocontrol initiatives that include zoophytophagous species in other latitudes could use the presented results for their benefit as well.

Resumen

La aplicación de estrategias de control biológico aumentativo en cultivos ha aumentado durante las últimas décadas en todo el mundo, y se prevé que continúe su tendencia a crecer. Los consumidores y legisladores exigen sistemas de producción más saludables y sostenibles, lo que ejerce una presión importante para que el sector de controladores biológicos desarrolle más agentes de control de plagas. Sin embargo, en las últimas décadas las restricciones al uso de fauna exótica también se han incrementado, afectando colateralmente al sector del biocontrol. Por lo tanto, los profesionales de este sector han centrado su atención en el uso y la explotación de especies disponibles localmente. Sin embargo, algunas de esas especies podrían no ser óptimas para controlar ciertas plagas y / o podrían tener rasgos que obstaculicen sus servicios de biocontrol. En este contexto, ha resurgido la idea de mejorar los agentes de control biológico mediante la cría selectiva, esta vez integrando recursos genéticos y genómicos, con el objetivo de incrementar su tasa de éxito y asegurar su asimilación como disciplina consolidada.

Nesidiocoris tenuis (Reuter) (Hemiptera: Miridae) es un depredador zoofófago cosmopolita, al que la zoofagia y la fitofagia le han otorgado el estatus de agente de biocontrol y plaga, respectivamente. Por tanto, se encuadra en la categoría de agentes de biocontrol con rasgos que obstaculizan su óptimo desempeño. En regiones donde se valora como agente de biocontrol, se libera principalmente en cultivos de tomate para suprimir plagas clave como *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) y *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae). Su papel en la activación de las defensas de las plantas también se ha confirmado más recientemente. Sin embargo, su comportamiento fitófago puede causar daños a los cultivos de tomate en circunstancias de escasez de presas. Esto puede producir pérdidas estéticas o de rendimiento, o llevar a la aplicación de pesticidas para reducir su población, interrumpiendo así los programas de biocontrol. Se ha sugerido la cría selectiva en *N. tenuis* como una alternativa para minimizar los efectos perjudiciales de su fitofagia, sin embargo, hasta la fecha no se ha reportado información genética o genómica sobre la especie. El **primer objetivo** de esta tesis fue investigar la genética poblacional de *N. tenuis* a escala global mediante el uso de 12 nuevos microsatélites, que se desarrollaron a partir del genoma de *N. tenuis*, y marcadores de ADN mitocondrial (mtDNA). Específicamente, se determinó la diversidad

genética y la estructura poblacional de 16 poblaciones recolectadas en cuatro continentes y tres crías comerciales. Encontramos una diversidad de moderada a baja según ambos marcadores. La población de Vietnam mostró la mayor diversidad (H_s) (0.760), mientras que la menor diversidad se observó en Puerto Rico (0.401). La diferenciación genética fue detectada por ambos marcadores para varias poblaciones (F_{ST} con $p < 0.05$), con la población de China mostrando el valor más alto de F_{ST} (0.429). Se encontró una correlación débil entre la distancia geográfica y la diferenciación genética, como lo indica la prueba de Mantel. Los datos de ADN mitocondrial revelaron un total de 23 haplotipos, con varios haplotipos únicos en Asia y un haplotipo predominante y ubicuo (haplotipo 2) compartido entre Europa, América, África y poblaciones comerciales. Los análisis bayesianos y el análisis de componentes principales (PCoA) realizados en los datos de microsatélites sugirieron dos grupos: un grupo con las poblaciones de americanas y un segundo grupo que contiene las poblaciones asiáticas, africanas, europeas y comerciales. Se concluye que las barreras geográficas y las actividades antropogénicas son los posibles factores que dan forma a la estructura de la población de *N. tenuis*. Las implicaciones de estos hallazgos para la agricultura y el biocontrol se analizan en el capítulo 2.

Entre las razones que impiden la adopción de la cría selectiva en agentes de biocontrol, recientemente se observó una importante escasez de información sobre la variación genética de rasgos relevantes para el biocontrol. Además, la selección de los rasgos (de control biológico) a los que apuntar en un eventual programa de reproducción también es crucial. En *N. tenuis*, tanto su fitofagia como su zoofagia son rasgos candidatos interesantes para la cría selectiva. El **segundo objetivo** de la presente tesis fue la cuantificación de la variación genética en la fitofagia y zoofagia de *N. tenuis*. Comparamos nueve líneas isogénicas en su capacidad para producir anillos necróticos y marchitamiento en plantas de tomate como referencia de la fitofagia, así como su eficacia para alimentarse de huevos de *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), como referencia de la zoofagia. Las diferencias entre las líneas isogénicas en fitofagia y zoofagia indicaron una base genética. La variación encontrada en los niveles de zoofagia fue mayor que en los niveles de fitofagia. Nuestros resultados mostraron que existe una base genética para la variación observada en el comportamiento de alimentación de las líneas isogénicas de *N. tenuis*, destacando un importante potencial de la cría selectiva para estos rasgos de interés en el biocontrol.

Otro aspecto importante en un programa de cría selectiva es vincular los datos fenotípicos y genotípicos. Sin embargo, primero se debe desarrollar una metodología estándar de fenotipado, de modo que los resultados de diferentes grupos puedan compararse directamente. Además, en el caso de los depredadores zoofitófagos, la medición de los rasgos que comprenden su interacción con la planta es de crucial importancia debido a la influencia que la planta podría ejercer sobre linajes seleccionados del agente de biocontrol. Por tanto, el **tercer objetivo** de la tesis fue investigar los componentes conductuales y mecánicos de la fitofagia de *N. tenuis*. Para ello, comparamos los comportamientos alimentarios de machos, hembras y ninfas de quinto estadio. Además, investigamos los comportamientos realizados con el estilete después de penetrar en el tejido vegetal, utilizando la técnica de gráfico de penetración eléctrica (EPG, por sus siglas en inglés). También se realizó una estilectomía y se estudió la histología de las plantas con el fin de correlacionar las actividades de alimentación observadas en los registros de EPG con las posiciones del extremo distal del estilete en tejidos específicos de los pecíolos de las hojas. Las observaciones del comportamiento durante un período de 30 minutos mostraron que las ninfas picaron la planta con más frecuencia (38,6 ± 1,5 picaduras) que los machos y las hembras (25,3 ± 1,1 y 24,3 ± 1,1 picaduras, respectivamente). De manera similar, las ninfas pasaron una mayor proporción de tiempo (656,0 ± 67,6 s) alimentándose de las secciones apicales de tomate en comparación con los machos y las hembras (403,0 ± 48,8 s y 356,0 ± 43,7 s, respectivamente). Las grabaciones de EPG durante 5 h mostraron que la ruptura celular fue la principal actividad del estilete para todos los estadíos de los insectos analizados, y que las ninfas del quinto estadio dedicaron una mayor proporción de tiempo a los eventos de ruptura celular en comparación con los adultos. Los estudios histológicos revelaron una preferencia de *N. tenuis* hacia los tejidos dentro del semi-anillo vascular. Los extremos distales de los estiletes se encontraron tanto en los haces vasculares como en el parénquima de la región interfascicular. Los hallazgos de este capítulo confirman un papel importante del comportamiento de alimentación de las ninfas de quinto estadio en la fitofagia de *N. tenuis*. Además, el mayor tiempo dedicado al comportamiento de ruptura celular sugiere que la laceración del estilete y la maceración enzimática de la saliva que ocurren durante este evento podrían contribuir en gran medida al daño a la planta. Una comprensión integral de las interacciones de *N. tenuis* con la planta, tanto a nivel conductual como mecánico, podría ayudar a encontrar nuevos enfoques para minimizar

su potencial de daño al tomate mientras se mantienen sus beneficios como agente de control biológico.

En general, a lo largo de esta tesis se ilustra cómo los recursos genéticos y genómicos se pueden incorporar eficazmente al biocontrol, con el objetivo de mejorar las estrategias de control de plagas. Aunque el mejoramiento selectivo de agentes de biocontrol podría verse como el objetivo último de combinar estos tres campos, otras líneas de investigación importantes, como el fitomejoramiento, también podrían beneficiarse de esta combinación de campos. Además, con la investigación que aquí se presenta, quizás otras iniciativas de biocontrol que incluyan especies zoofitófagas en otras latitudes también podrían utilizar los resultados aquí presentados para su beneficio.

Resum

Al llarg de les últimes dècades l´aplicació d'estratègies de control biològic augmentatiu s´ha vist incrementada a tot el món, i es preveu que aquesta tendència a créixer continue. Tant consumidors com legisladors exigeixen sistemes de producció més saludables i sostenibles, la qual cosa exerceix una pressió important per a què el sector de controladors biològics desenvolupe més agents de control de plagues. No obstant això, en les últimes dècades també s'han incrementat les restriccions a l'ús de fauna exòtica, afectant col·lateralment el sector del biocontrol. Per aquest motiu, els professionals d'aquest sector han centrat la seua atenció en l'ús i l'explotació d'espècies disponibles localment, malgrat que algunes d'eixes espècies podrien no ser òptimes per a controlar certes plagues i / o podrien tindre trets que obstaculitzen els seus servicis de biocontrol. En aquest context, ha ressorgit la idea de millorar els agents de control biològic per mitjà de la cria selectiva, aquesta vegada integrant recursos genètics i genòmics, amb l'objectiu d'incrementar la seua taxa d'èxit i assegurar la seua assimilació com a disciplina consolidada.

Nesidiocoris tenuis (Reuter) (Hemiptera: Miridae) és un depredador zoofitòfag cosmopolita, al qual la zoofàgia i la fitofàgia li han atorgat l'estatus d'agent de biocontrol i plaga, respectivament. Per tant, s'enquadra en la categoria d'agents de biocontrol amb trets que obstaculitzen el seu òptim compliment. En regions on es valora com a agent de biocontrol, s'allibera principalment en cultius de tomaca per a suprimir plagues clau com *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) i *Tuta absoluta* Meyrick (Lepidòptera: Gelechiidae) . Així mateix, també s'ha confirmat recentment el seu rol en l'activació de les defenses de les plantes. No obstant això, el seu comportament fitòfag pot causar danys als cultius de tomaca en circumstàncies d'escassetat de preses. Concretament, pot originar pèrdues estètiques o de rendiment, o portar a l'aplicació de pesticides per tal de reduir la seua població, interrompent així els programes de biocontrol. Com alternativa per a minimitzar els efectes perjudicials de la seua fitofàgia s´ha suggerit la cria selectiva de *N. tenuis*, encara que fins ara no s'ha reportat informació genètica o genòmica sobre l'espècie. El **primer objectiu** d'aquesta tesi va ser investigar la genètica poblacional de *N. tenuis* a escala global mitjançant l'ús de 12 nous microsatèl·lits, que es van desenrotllar a partir del genoma de *N. tenuis*, i marcadors

d'ADN mitocondrial (mtDNA). Específicament, es va determinar la diversitat genètica i l'estructura poblacional de 16 poblacions recol·lectades en quatre continents i tres cries comercials. Trobem una diversitat de moderada a baixa segons ambdós marcadors. La població de Vietnam va mostrar la major diversitat (Hs) (0,760), mentre que la menor diversitat es va observar a Puerto Rico (0,401) . La diferenciació genètica va ser detectada per ambdós marcadors per a diverses poblacions (F_{ST} amb P <0.05), amb la població de la Xina mostrant el valor més alt de F_{ST} (0,429). Es va trobar una correlació dèbil entre la distància geogràfica i la diferenciació genètica, com ho indica la prova de Tovalles. Les dades d'ADN mitocondrial van revelar un total de 23 haplotips, amb diversos haplotips únics a Àsia i un haplotip predominant i ubic (haplotip 2) compartit entre Europa, Amèrica, África i poblacions comercials. Les anàlisis bayesianes i l'anàlisi de components principals (PCoA) realitzades amb les dades de microsatèl·lits van suggerir dos grups: un grup amb les poblacions d'americanes i un segon grup que conté les poblacions asiàtiques, africanes, europees i comercials. Es conclou que les barreres geogràfiques i les activitats antropogèniques són els possibles factors que donen forma a l'estructura de la població de *N. tenuis*. Les implicacions d'aquestes troballes per a l'agricultura i el biocontrol s'analitzen al capítol 2.

Entre les raons que impedeixen l'adopció de la cria selectiva en agents de biocontrol, recentment es va observar una important escassetat d'informació sobre la variació genètica de trets rellevants per al biocontrol. A més a més, la selecció dels trets (de control biològic) als que apuntar en un eventual programa de reproducció també és crucial. En *N. tenuis*, tant la seua fitofàgia com la seua zoofàgia són trets candidats interessants per a la cria selectiva. El **segon objectiu** de la present tesi va ser la quantificació de la variació genètica en la fitofàgia i zoofàgia de *N. tenuis*. Comparem nou isolínies genètiques en la seua capacitat per a produir anells necròtics i marciment en plantes de tomaca com a referència de la fitofàgia, així com la seua eficàcia per a alimentar-se d'ous *d'Ephestia kuehniella* Zeller (Lepidòptera: Pyralidae), com a referència de la zoofàgia. Les diferències entre les isolínies genètiques en fitofàgia i zoofàgia van indicar una base genètica. La variació trobada en els nivells de zoofàgia va ser major que en els nivells de fitofàgia. Els nostres resultats mostraren que hi ha una base genètica per a la variació observada en el comportament d'alimentació de les isolínies genètiques de *N. tenuis*, destacant un important potencial de la cria selectiva per a aquests trets d'interès en el biocontrol.

Un altre aspecte important en un programa de cria selectiva és vincular les dades fenotípiques i genotípiques. No obstant això, primer s'ha de desenrotllar una metodologia estàndard de fenotipat, de manera que els resultats de diferents grups puguen comparar-se directament. A més a més, en el cas dels depredadors zoofitòfags, mesurar els trets que comprenen la seua interacció amb la planta és de crucial importància degut a la influència que la planta podria exercir sobre llinatges seleccionats de l'agent de biocontrol. Per tant, el **tercer objectiu** de la tesi va ser investigar els components conductuals i mecànics de la fitofàgia de *N. tenuis*. Per fer-ho, compararem els comportaments alimentaris de mascles, femelles i nimfes de quint estadi. A més, investigarem els comportaments realitzats amb l'estilet després de penetrar al teixit vegetal, emprant la tècnica de gràfic de penetració elèctrica (EPG, per les seues sigles en anglés). També es va realitzar una estilectomia i es va estudiar la histologia de les plantes a fi de correlacionar les activitats d'alimentació observades als registres d'EPG amb les posicions de l'extrem distal de l'estilet en teixits específics dels pecíols dels fulls. Les observacions del comportament durant un període de 30 minuts van mostrar que les nimfes picaren la planta amb més freqüència (38,6 ± 1,5 picades) que els mascles i les femelles (25,3 ± 1,1 i 24,3 ± 1,1 picades, respectivament). De manera semblant, les nimfes van passar una major proporció de temps (656.0 ± 67.6 s) alimentant-se de seccions apicals de tomaca en comparació amb els mascles i les femelles (403.0 ± 48.8 s i 356.0 ± 43.7 s, respectivament). Les gravacions d'EPG durant 5 h mostraren que la ruptura cel·lular va ser la principal activitat de l'estilet per a tots els estadis dels insectes analitzats, i que les nimfes del quint estadi van dedicar una major proporció de temps als esdeveniments de ruptura cel·lular en comparació amb els adults. Els estudis histològics van revelar una preferència de *N. tenuis* cap als teixits dins del semi-anell vascular. Els extrems distals dels estilets es trobaren tant als feixos vasculars com al parènquima de la regió interfascicular. Les troballes d'aquest capítol confirmen un rol important del comportament d'alimentació de les nimfes de quint estadi en la fitofàgia de *N. tenuis*. A més a més, el major temps dedicat al comportament de ruptura cel·lular suggereix que la laceració de l'estilet i la maceració enzimàtica de la saliva que ocorren durant aquest esdeveniment podrien contribuir al dany a la planta. Una comprensió integral de les interaccions de *N. tenuis* amb la planta, tant a nivell conductual com a mecànic, podria ajudar a trobar nous enfocaments per a minimitzar el seu potencial de dany a la tomaca mentre es mantenen els seus beneficis com a agent de control biològic.

Amb caràcter general, al llarg d'aquesta tesi s'il·lustra com els recursos genètics i genòmics es poden incorporar eficaçment al biocontrol, amb l'objectiu de millorar les estratègies de control de plagues. Encara que el millorament selectiu d'agents de biocontrol es podria veure com l'objectiu últim de combinar estos tres camps, altres línies d'investigació importants, com el fitomillorament, també podrien beneficiar-se d'aquesta combinació de camps. A més a més, amb la investigació que ací es presenta, potser altres iniciatives de biocontrol que incloguen espècies zoofitòfagues en altres latituds podrien utilitzar els resultats ací presentats per al seu benefici.

Table of contents

List of Figures

List of Tables

CHAPTER 1

General Introduction

1.1 Biological control

The use of a living organism to suppress another organism, or the detrimental effects associated with it, is known as biological control or biocontrol (Eilenberg et al., 2001). Biological control strategies are further classified into conservation, classical, and augmentative. In conservation biocontrol, the reduction of pest populations and/or their negative effects is attained through the manipulation of the environment to favor the natural enemies (Eilenberg et al., 2001). This type of biocontrol has been given more attention recently in an effort to strengthen pest control practices that are not pesticide-dependent (Holland et al., 2016). Classical biocontrol consist on the introduction of an exotic, usually co-evolved, biological control agent (BCA) with the aim of permanent establishment and long-term control (Eilenberg et al., 2001). Perhaps, one of the most successful and known cases of this type of biocontrol is the introduction of the *Rodolia cardinalis* (Mulsant) (Coleoptera: Coccinellidae) in California from Australia in 1888, to control the citrus pest *Icerya purchasii* (Maskell) (Hemiptera: Monophlebidae) (van den Bosch et al., 1982). The augmentative biocontrol strategy is based in the mass-rearing of natural enemies that are released in the crops (van Lenteren and Bueno, 2003). It is called inundative when large numbers of biocontrol agents are released to obtain a fast pest control, whereas it is called inoculative when the releases are periodical and throughout the growing season of the crop (van Lenteren and Bueno, 2003). It is mainly because of the success of this latter type of biocontrol, and its application in greenhouses, that an industry sector started the mass-rearing and distribution of BCAs in the 1970s that continues to grow until the present (van Lenteren et al., 2020).

There is an estimate of more than 30 million ha worldwide applying augmentative biocontrol in cultivated areas, and ca. 350 species of natural enemies available (van Lenteren et al., 2018). Several aspects explain the growing interest in biocontrol as a permanent strategy for pest control. For instance, the increasing concerns about the

impact of pesticides in the environment and human health, the ban of certain active ingredients of broad spectrum, and the insecticide resistance in some pests play an important role in the adoption of biocontrol measures in new crops and areas. The main groups of arthropods used in biocontrol are Hymenoptera (> 50%), Acari (ca. 15%), Coleoptera (ca. 12%) and Hemiptera (ca. 8%), and a trend for the use of generalist predators is noticeable over the last two decades (van Lenteren et al., 2020). However, although the use of biocontrol is largely accepted and positively valued, some concerns have also arisen from the risks associated to the use of exotic species to control native and/or invasive pests. Furthermore, stricter legislation on the importation of exotic species was put in place through the Nagoya Protocol on Access and Benefit Sharing from the Convention on Biological Diversity (Cock et al., 2010).

1.2. Improvement of biological control agents

The improvement or selection of organisms based on their (advantageous) attributes, is a practice that humans have applied to plants and animals during centuries. In short, it relies on the classical selection and crossing of parental lines with the desired traits or attributes that are expected for the offspring. For instance, numerous crop varieties and several mammal breeds (e.g. dogs and cattle) as we know them now, are the product of such selective/breeding processes. To a lesser extent, selection has also been applied to invertebrates. The silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae), and the honey bee, *Apis* spp. L. (Hymenoptera: Apidae), are cited as the first selected insects, with reports about their breeding starting centuries ago (Hoy, 1976). However, it is only in the last century that selection has been applied to BCAs (Hoy, 1986; Lirakis and Magalhaes, 2019) (Figure 1.1, lower panel).

1.2.1. What has been done?

Several traits in BCAs can be improved by means of selection. Most of the studies carried out to date have targeted traits such as insecticide resistance, thermal adaptation, foraging behavior, tolerance to pests, plant adaptation, desiccation tolerance, sex ratio, diapause, dispersal, developmental time, and fecundity (Figure 1.1, upper panel), with a predominance of BCAs from the groups Hymenoptera, Acari and Nematoda (Lirakis and

Magalhaes, 2019). It seems logical that most of BCA breeding studies have targeted insecticide resistance, considering the extensive use of pesticides in most modern cropping systems. Thus, the compatibility between pesticides and the use of BCAs has been long considered important for the implementation of integrated pest management (IPM) programs. Perhaps, one of the most successful cases of breeding is that of *Metaseiulus occidentalis* (Nesbit) (Acari: Phytoseiidae) (Hoy, 2009). In the 1970s, resistance to organophosphorus (OP) pesticides was common in populations of this predatory mite in apple orchards in Washington state (USA). This prompted research initiatives to select for strains with additional resistance to other pesticides. A carbaryl-, OP- and sulfur-resistant strain was developed and incorporated in a large-scale pest management program in almonds in California, which saved ca. $US 20 million per year to growers (Hoy, 2009). Moreover, this strain was further selected for non-diapause, thus allowing for pest control all year round (Field and Hoy, 1986, 1985). More recently, selective breeding for insecticide resistance has been carried out in *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae), a zoophytophagous species widely used in cropping systems across Europe (Balanza et al., 2019). Balanza et al., (2019) observed variation in the resistance of *O. laevigatus* to imidacloprid and thiamethoxan, and obtained more resistant strains of this predator after 40 cycles of selective breeding.

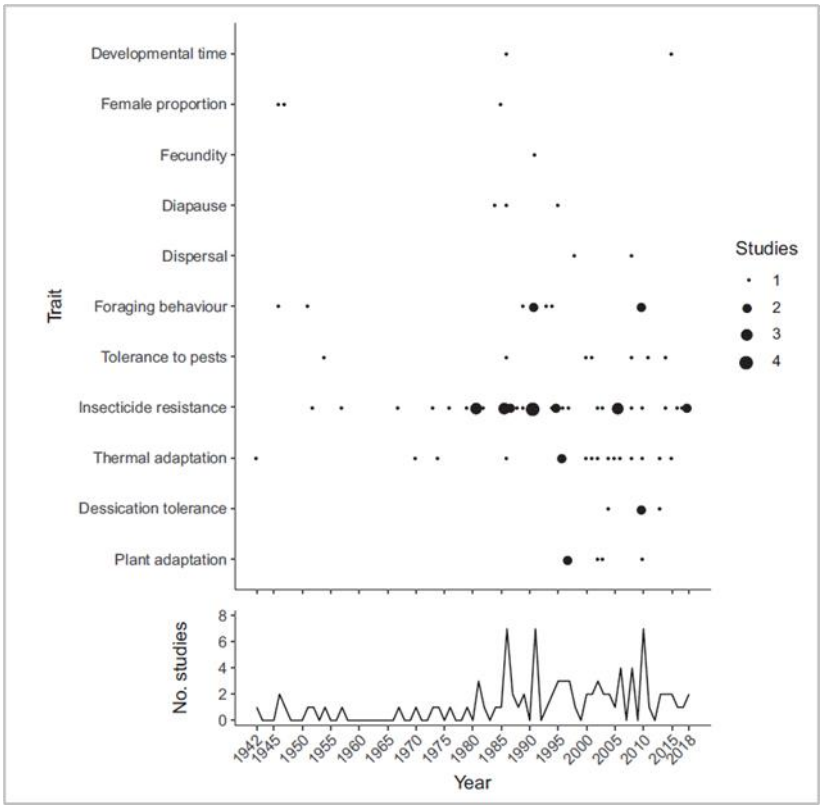

Figure 1.1. Number of studies dealing with a particular trait subject to experimental evolution or artificial selection in biological control agents across time, per trait (upper panel) and for all studies combined (lower panel). (From: Lirakis & Magalhães, 2019)

Adaptation of the BCAs to different temperatures has been of crucial importance in the context of classical biocontrol, when the BCA is imported from its center of origin to control invasive pests in the new invaded areas, where climatic conditions are likely different. Several attempts of selection for thermal adaptations have been performed with *Trichogramma* spp. (Hymenoptera: Trichogrammatidae) (Ashley et al., 1974; Carrière and Boivin, 2001; Jalali et al., 2006; Srivastava and Singh, 2015; Wang et al., 2004), as would be expected for one of the most studied groups of BCAs. However, not all the outcomes of these selection experiments yielded the expected results (Ashley et al., 1974; Jalali et al., 2006; Wang et al., 2004). Similarly, breeding efforts have been carried out with the nematodes *Heterorhabditis bacteriophora* (Nematoda: Heterorhabditidae) for heat tolerance (Shapiro et al., 1996), and *Steinernema feltiae* (Nematoda: Steinernematidae) for cold tolerance (Grewal et al., 1996). In the case of nematodes, adaptation to different temperatures have positive implications not only for their application in IPM, but also for their storage life.

Foraging behavior in BCAs is an essential trait for the success of biocontrol programs. Parasitoids and predators that are more efficient at finding and consuming prey have higher probabilities of suppressing the pest effectively. Some studies have tried to improve the host-finding abilities in *Steinernema carpocapsae* (Nematoda: Steinernematidae) (Gaugler and Campbell, 1991), and foraging-related traits in the predatory mite *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) (Nachappa et al., 2010). Nevertheless, and considering the importance of foraging behavior and/or feeding-related behaviors for biocontrol, it is surprising that it has been neglected in selection initiatives. For instance, in zoophytophagous species, which play an important role in biocontrol programs in Europe (van Lenteren et al., 2020), no selection initiatives related to those traits have been undertaken to date.

1.2.2. Challenges

Selective breeding in arthropods seems promising considering their smaller size and faster developmental life cycles relative to plants and livestock. However, and despite the research efforts carried out for selection or breeding of BCAs, this approach is not yet a common practice for biocontrol industry, and the reasons are diverse. One important first obstacle is the lack of sequenced genomes of BCAs. This could enable, for example, the identification of molecular markers for marker-assisted genetic selection (Dekkers and Hospital, 2002). Another aspect to be accounted for is (knowledge of) the target environment where the improved BCA is intended to be used. For instance, there could be other BCAs already present in the target location that are better adapted and which pest control is satisfactory. This was the challenge faced by an improved strain of the parasitoid *Aphytis lingnanensis* (Compere) (Hymenoptera: Aphelinidae), that was released in California but which performance was never properly evaluated due to the presence of *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae), which was already adapted to the local climate conditions (White et al., 1970). Additionally, other studies have also pointed at the importance of evaluating the genotype × environment interaction on the final performance of a selected BCA (Kruitwagen et al., 2018; Lommen et al., 2019). Another possible reason for the discontinuity of selection studies on BCAs is probably the loss of the selected trait once the selection pressure is relaxed (Gaugler et al., 1989), and might be due to phenotypic plasticity mistakenly interpreted as response

to selection, or perhaps because of a trade-off between the selected trait and other important trait(s). Overall, several obstacles for the selective breeding in BCAs have in common a lack of genetic and genomic knowledge and/or tools.

1.3. *Nesidiocoris tenuis*

Nesidiocoris tenuis (Reuter) is a zoophytophagous predator in the Miridae family (Hemiptera). It is a cosmopolitan species with a wide distribution in the Palearctic region and reports of its presence in different regions of America, Africa and south of Asia (Kerzhner and Josifov, 1999; Pérez-Hedo and Urbaneja, 2016). Its life cycle comprises egg stage, five nymphal stages and adult stage (Calvo and Urbaneja, 2004; J. G. Kim et al., 2016). The duration of its life cycle varies according to temperature and host plant (Calvo and Urbaneja, 2004; Urbaneja et al., 2005), with an average of 21.8 days at 25 °C when reared on tomato plants and with *Ephestia kuehniella* (Lepidoptera: Pyralidae) as prey (Sanchez et al., 2009). Each female is able to produce between 62-82 offspring in a temperature range of 20-35 °C (Sanchez et al., 2009).

1.3.1 Role as biological control agent

The release of *N. tenuis* in tomato crops to suppress some of its most important pests is an extended practice in (inoculative) augmentative biocontrol programs in the Mediterranean basin and some regions in Asia (Pérez-Hedo and Urbaneja, 2016; van Lenteren, 2012). Its ability to feed upon several pest species is one of the reasons for its success as BCA. It has been reported to feed on whiteflies, lepidopteran eggs and larvae, thrips, spider mites, leaf miners and aphids (Bhatt and Patel, 2018; Calvo et al., 2009; Calvo and Urbaneja, 2004; Desneux et al., 2010; Ebrahimi et al., 2019; Pérez-Hedo and Urbaneja, 2016; Zappalà et al., 2013). In particular, the increase in the use of *N. tenuis* in southern Spain was driven by the appearance of the invasive pest *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) in 2006, which prompted IPM programs with mirid predators an as essential strategy (Urbaneja et al., 2012).

Another benefit provided by *N. tenuis* is the activation of the plant defenses through its phytophagy and oviposition (Naselli et al., 2016; Pérez-Hedo et al., 2018, 2015b, 2015a). Pérez-Hedo et al., (2015b) demonstrated that the feeding punctures of *N. tenuis* on tomato induced the attraction of the parasitoid *Encarsia formosa* (Gahan) (Hymenoptera: Aphilinidae) through the activation of the jasmonate acid pathway, while at the same time induced the repellence of the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), as a consequence of the activation of the abscisic acid pathway. Moreover, in this study they also showed that plants punctured by *N. tenuis* produced volatiles capable of triggering defense responses in neighboring non-punctured plants. Another study showed that defensive responses in tomato are induced by all life stages of *N. tenuis* (Naselli et al., 2016). More recently, Pérez-Hedo et al., (2018) showed that the activation of plants with *N. tenuis* could also reduce the performance of *Tetranychus urticae* Koch (Acari: Tetranychidae) in tomato, with a 35% reduction of infestation levels on activated plants relative to control plants.

1.3.2. Controversy about its phytophagy

Despite its services in tomato as BCA, the phytophagy of *N. tenuis* is capable of inflicting important damages in plant tissues, thus giving this species the status of a pest in several regions (Bhatt and Patel, 2018; Castañé et al., 2011; Moerkens et al., 2020; Pérez-Hedo and Urbaneja, 2016). Damages have been reported in crops such as tomato, tobacco, cucurbits, sesame and ornamentals (El-Dessouki et al., 1976; Pérez-Hedo and Urbaneja, 2016). In tomato, the most visible damages inflicted by *N. tenuis* are the necrotic rings around stems and petioles, that are produced by the frequent insertion of the stylet and the subsequent suction of plant contents, affecting the transport of nutrients that leads to wilting (El-Dessouki et al., 1976; Raman et al., 1984). It can also feed on the fruits causing yellowish halos, and in more severe cases the phytophagy might lead to flower abortion and yield losses (Arnó et al., 2006, 2010; Sánchez, 2008) (Figure 1.2).

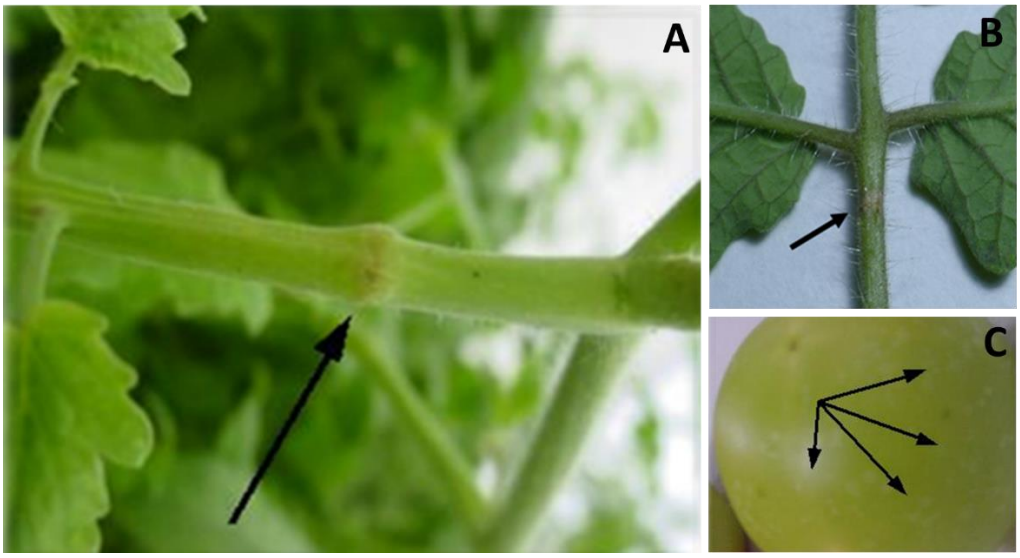

Figure 1.2. Damages inflicted by *Nesidiocoris tenuis*: A) necrotic rings in tomato stem, B) necrotic rings in tomato petiole, C) yellowish halos in fruit

Studies about the damage have been undertaken from different perspectives, most of them analyzing the severity relative to the interaction between *N. tenuis* and the pest. These studies have shown that the damage is prey-availability dependent, thus being more severe in the absence or scarcity of prey (Arnó et al., 2010; Calvo et al., 2009; Sanchez et al., 2009). It has also been shown that damage severity also depends on factors such as the temperature and tomato cultivar (Siscaro et al., 2019). Moreover, the role of the stylet morphology and saliva composition of *N. tenuis* have also been studied to try to explain the damage (Castañé et al., 2011).

More attention has been brought to its phytophagy detrimental effects due to increasing reports of its damage in greenhouse crops in northern Europe (Moerkens et al., 2020). This, together with its importance as BCA in other regions, confirms the need for more in-depth studies of the factors underlying the damage, from different perspectives, fields and techniques, with the aim to develop strategies to circumvent the detrimental effects of *N. tenuis* phytophagy.

1.4. New era of BCA improvement?

As pointed in sections 1.1 and 1.2 of this introduction, there is increasing interest in more BCAs made available to growers, but also increasing restrictions and challenges to

obtain new BCAs in the traditional way, that is, importing them from their native regions where they have likely co-evolved with the target pests. The exploitation of intraspecific variation for traits of interest in biocontrol has been proposed several times (Hopper et al., 1993; Hoy, 1986, 1976; Kruitwagen et al., 2018; Lommen et al., 2017; Narang et al., 1993), and has been put into practice in a number of studies, mainly over the last 50 years (see Lirakis & Magalhaes, 2019 for a review). New circumstances in the last 20 years are favoring again the interest in this approach. The stricter regulations on the trade/importation of exotic species deriving from the Nagoya Protocol, environmental and health concerns in consumers, increasing restriction on currently available pesticides, and development of resistance to pesticides by the pests, are just some of those circumstances prompting the renewed interest in the selective breeding of BCAs. Recent works have pointed the weaknesses of previous selection attempts and/or proposed a series of steps with the intention of increasing the acceptance and success of selective breeding approaches in biocontrol, with the incorporation of genetic and genomic tools as a common suggestion (Ferguson et al., 2020a; Kruitwagen et al., 2018; Leung et al., 2020; Lirakis and Magalhaes, 2019; Lommen et al., 2017).

One recent initiative for the incorporation of genetics and genomics into biocontrol was the BINGO (Breeding Invertebrates for the Next Generation Biocontrol) project (https://www.bingo-itn.eu/en/bingo.htm). The consortium BINGO was created as an International Training Network for early-stage researchers and is funded by the Marie Skłodowska Curie scheme of the EU Horizon 2020 programme. BINGO brought together participants from prominent universities, institutes and industry in nine European countries, and advisory board members from outside the EU. The network consisted of 24 researchers and 13 PhD students, who carried out their projects at the BINGO partners' facilities. Among the objectives of this initiative were 1) to improve current biocontrol practices through the exploration and exploitation of natural genetic variation present in native natural enemies, and 2) to extend the application of quantitative and population genetics to the invertebrate biocontrol field. One of the projects focused on the zoophytophagous predator *Nesidiocoris tenuis* and the arising concerns about the plant damage it can inflict in tomato. It is important to mention that zoophytophagous predators have not been common subjects of selective breeding and/or studies aimed at exploring/exploiting the genetic components of their (biocontrol) traits. It is only over the past five years that some investigation has been done around these possibilities in this

group of BCAs (Balanza et al., 2019; Dumont et al., 2017a, 2017b, 2016; Mendoza et al., 2020). One of the species studied is *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae), which variability in body size and pesticide resistance has been investigated and exploited via artificial selection (Balanza et al., 2019; Mendoza et al., 2020). The other species studied is *Campylomma verbasci* (Meyer) (Hemiptera: Miridae), which variability in zoophagy and diet specialization were investigated (Dumont et al., 2019, 2017a, 2017b, 2016).

To date, most studies on *N. tenuis* have mainly focused on its 1) biology (Sánchez, 2008; Sanchez et al., 2014, 2009; Urbaneja et al., 2005), 2) the phytophagy severity in tomato relative to the level of prey, temperature, tomato cultivar, and alternative food sources (Arnó et al., 2010; Calvo et al., 2012b, 2009; Perdikis et al., 2009; Siscaro et al., 2019; Urbaneja-Bernat et al., 2019), 3) characterization of its damage based on stylet morphology, saliva composition and histological studies (Castañé et al., 2011; El-Dessouki et al., 1976; Raman et al., 1984) and 4) plant defense induction (Naselli et al., 2016; Pérez-Hedo et al., 2018, 2015b, 2015a). However, there was a lack of information from the genetic and genomic perspectives, which limits the application of selection approaches to this species. Considering that *N. tenuis* is one of the polyphagous predator species which use, --and controversy over its phytophagy, has increased in the last two decades in Europe (van Lenteren et al., 2020), it remains important to continue investigating on ways to reduce the plant damage it can cause. Some studies point at the potential of selective breeding as an approach to improve zoophytophagous predators such as *N. tenuis* (Dumont et al., 2018; Pérez-Hedo et al., 2020). However, and with the aim of increasing the success of such improving attempts, it is necessary to study first the genetic and genomic aspects of this species that have not been studied yet. One key step towards the improvement of *N. tenuis* has taken place in the frame of the BINGO project. The sequencing of the whole genome of the species (Ferguson et al., 2020b) opens the possibility to study *N. tenuis* from its genomic and genetic perspective.

1.5. Research objectives

Recent studies highlight the importance of following certain steps before embarking on a selective breeding program in BCAs, with the objective of avoiding factors that might have contributed to the failure of past selection efforts (Bielza et al., 2020; Kruitwagen et al., 2018; Lirakis and Magalhaes, 2019; Lommen et al., 2017). Since there is a lack of information on *N. tenuis* regarding basic genetic aspects that are crucial for future selection programs, the goal of this thesis was to stimulate the development of knowledge concerning the selective breeding of the species, through the investigation of genetics aspects of *N. tenuis*, and the factors underpinning traits that could be improved by selection.

It is important to know the genetic diversity at the species level, to determine what is the genetic status of *N. tenuis* in different regions, and whether the genetic resources available could be further used in selective breeding programs. For this, in **Chapter 2** the objective was to determine the genetic diversity, genetic differentiation and population structure of *N. tenuis* across four continents and three commercial rearings using microsatellite markers, developed using the genome of *N. tenuis*, and mitochondrial DNA (mtDNA) COI markers.

Additionally, the most interesting traits to study should be chosen to quantify its genetic variation, and a suitable methodology to study them should be defined. In **Chapter 3**, the objective was to investigate whether there is genetic variation in two important traits of the feeding behavior of a wild population of *N. tenuis*, specifically phytophagy and zoophagy, by using an isofemale line approach.

Finally, gaining insight on the diverse factors underlying a (biocontrol) trait would facilitate the identification of approaches necessary to either promote or weaken that trait in a selective breeding program. Therefore, in **Chapter 4** the aim was to investigate the behavioral and mechanical components of the phytophagy of *N. tenuis* by studying the behavior and stylet activities of adult and nymphal stages on tomato.

CHAPTER 2

Population genetic structure of the cosmopolitan and controversial *Nesidiocoris tenuis* based on novel microsatellites and mitochondrial DNA

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Abstract

Nesidiocoris tenuis is a predatory mirid widely spread through the world, which zoophagous and phytophagous behavior have given it the status as both biocontrol agent and pest, respectively. Most of the research about this species have been centered in its biological and ecological aspects, and to a lesser extent on its genetics and genomics. In the present study we undertake the first population genetics study of *N. tenuis* at a global scale. To that end, we used 12 microsatellite loci, which were developed based on the genome of *N. tenuis*, and mitochondrial DNA (mtDNA) markers. The aim was to determine the genetic diversity and the population structure of 16 populations collected in four continents and tree commercial rearings. Our results show a moderate-to-low diversity according to both markers. Individuals from Vietnam showed the highest diversity (H_s) (0.760), whereas the lowest diversity was observed in those collected in Puerto Rico (0.401). Genetic differentiation was detected by both markers for several populations (F_{ST} with $p < 0.05$), with individuals from China showing the highest F_{ST} value (0.429). A weak correlation was found between geographical distance and genetic differentiation, as indicated by Mantel test. The mtDNA data revealed a total of 23 haplotypes, with several unique haplotypes in Asia and a predominant and ubiquitous haplotype (haplotype 2) shared between Europe, America, Africa and commercial populations. Bayesian analyses and principal component analysis (PCoA) performed on the microsatellite data suggested two groups: one group with the American populations and a second group containing Asian, African, European and commercial populations. Geographical barriers and anthropogenic activities are the likely factors shaping the population structure of *N. tenuis*. The implications of our findings for agriculture and biocontrol are discussed.

2.1. Introduction

Nesidiocoris tenuis (Reuter) (Hemiptera: Miridae) is a zoophytophagous predator widely used to control important pests in some Mediterranean and Asiatic regions (Pérez-Hedo and Urbaneja, 2016; Pérez-Hedo et al., 2020; van Lenteren, 2012). Its success as a biocontrol agent in tomato crops arises mainly from its ability to prey upon several arthropods (Bhatt and Patel, 2018; Calvo et al., 2009; Calvo and Urbaneja, 2004; Desneux et al., 2010; Ebrahimi et al., 2019; Urbaneja et al., 2005; Zappalà et al., 2013) and its capacity to trigger plant defenses through its interaction with the host plant via feeding and oviposition activities (Naselli et al., 2016; Pérez-Hedo et al., 2018, 2015b, 2015a). However, it is considered a serious pest in other regions, where the damages inflicted in crops such as tomato, tobacco and sesame outweigh its benefits as biocontrol agent (Bhatt and Patel, 2018; Castañé et al., 2011; Moerkens et al., 2020; Pérez-Hedo and Urbaneja, 2016; Roda et al., 2020). To date, most of the studies about *N. tenuis* have investigated its biology and ecology either in laboratory conditions or agroecosystems (Arnó et al., 2010; Calvo et al., 2012a, 2012b; Chinchilla-Ramírez et al., 2021; Mollá et al., 2014; Pazyuk et al., 2014; Perdikis et al., 2009; Sánchez, 2008; Sanchez et al., 2009; Urbaneja-Bernat et al., 2019; Urbaneja et al., 2005), whereas some other works have focused on the use of molecular tools to study its interactions in agroecosystems (Itou et al., 2013; J. Kim et al., 2016; Moreno-Ripoll et al., 2009). However, it is only in the last decade that research efforts have reached out to genetic and genomic approaches to enhance our knowledge about *N. tenuis* (Chinchilla-Ramírez et al., 2020; Ferguson et al., 2020b; Xun et al., 2016).

The increasing demand for biocontrol agents to suppress endemic and invasive pests, the stricter regulations on pesticides, and the growing concerns about health and environment, have prompted a renewed interest on the use of genetic and genomic tools for the improvement of biocontrol agents (Ferguson et al., 2020a; Kruitwagen et al., 2018; Le Hesran et al., 2019; Leung et al., 2020; Lirakis and Magalhaes, 2019; Lommen et al., 2017). The sequencing of the genome of *N. tenuis* (Ferguson et al., 2020b) is one of those tools that represents a big step towards the integration of state-of-the-art approaches of genetic and genomics into the applied field of biocontrol. Likewise, a recent study investigated the genetic variation in the feeding behavior (i.e. phytophagy and zoophagy)

of *N. tenuis* (Chinchilla-Ramírez et al., 2020), demonstrating that the knowledge about aspects of the genetic makeup of the species, and the underlying factors of its variation, could serve biological control purposes. In terms of population genetics of *N. tenuis*, to our knowledge, there is only one study performed at a local scale in China (Xun et al., 2016). In their study, Xun et al. (2016) observed high genetic diversity in 37 populations collected across China, and a population structure likely determined by topography, human-mediated activities and ecological factors such as temperature and air currents. Nevertheless, *N. tenuis* is a cosmopolitan species, found across five continents and commercially distributed in different countries (Pérez-Hedo and Urbaneja, 2016; Roda et al., 2020; van Lenteren, 2012), therefore, a global-scale study about its population genetics remained still pending.

A wider population genetics study of *N. tenuis* in the context of improvement/breeding of biocontrol agents would bring benefits to the practice of biocontrol in various aspects. The knowledge about the processes underpinning aspects such as genetic diversity, genetic differentiation, and population structuring, among others, are just some of those benefits. For instance, it would provide powerful tools for the assessment of the genetic diversity of mass-reared populations (Paspati et al., 2019). Also, it could improve our understanding of the factors that determine the genetic differentiation and structure in commercial and wild populations (Sanchez et al., 2012; Streito et al., 2017). Moreover, it will provide a broader overview of the genetic status of *N. tenuis* that is still lacking for potential breeding programs aimed at improving the biocontrol services of this species (Pérez-Hedo et al., 2020).

In the present study, we investigated the genetic structure of 16 populations of *N. tenuis* across four continents and three commercial rearings. To that end, we used 12 microsatellite markers, developed using the genome of *N. tenuis* (Ferguson et al., 2020b), and mitochondrial DNA (mtDNA) *COI* markers. We aimed to investigate the genetic diversity, genetic differentiation and population structure of this species.

2.2. Materials and Methods

The experiments of this work were carried out in two laboratories. Sample collection, genomic DNA extractions and mitochondrial analyses were performed in the entomology laboratories at Instituto Valenciano de Investigaciones Agrarias (IVIA) in Valencia, Spain. The microsatellite detection, primer design and microsatellite analyses were performed in the laboratory of genetics at Wageningen University (WUR) in Wageningen, The Netherlands.

2.2.1. Sample collection and genomic DNA extraction

A total of 61 individuals of *N. tenuis* were obtained from 16 different locations in Asia, Africa, Europe and America and from three commercial rearings (Table 2.1). The individuals were collected and sent in ethanol (70-100%) to IVIA facilities, where they were stored at -20 C° until DNA extraction. Genomic DNA was extracted from individual insects using a salting out method (Sunnucks and Hales, 1996) adapted from Monzó et al. (Monzó et al., 2011). Genomic DNA was stored at -20 C° until molecular analyses.

2.2.2. Mitochondrial DNA amplification and sequencing

Polymerase chain reaction (PCR) was performed to amplify a fragment of the mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene by using the standard primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCA GGGTGACCAAAAAATCA-3′) (Folmer et al., 1994). PCR amplifications were carried out in a total reaction volume of 20 µl containing 1 µl of DNA, 2 µl of 10X Reaction buffer, 0.65 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTP, 0.5 µl of 10 µM of each primer and 1 U of DNA polymerase (1 U/µl, Biotools) in a thermal cycler (Applied Biosystems, California, USA). Reactions were cycled as follow: initial denaturation at 94 °C for 120 seconds (s); 35 cycles of 95 °C for 60 s, 48 °C for 60 s, and 72 °C for 90 s; and a final extension at 72 °C for 600 s. PCR products were visualized on 2.0% agarose gels under UV light to confirm the presence of the *COI* band in all samples. PCR products were purified by using the Ultra Clean PCR Clean-up DNA Purification Kit (MO BIO Laboratories Inc., California, USA) and sequenced in both directions with

a 3130XL Genetic Analyzer (Applied Biosystems, California, USA). Chromatograms, including sense and antisense, were analyzed, edited and assembled using BioEdit v7.2.5 (Hall, 1999) to obtain a single consensus sequence for each sample.

Table 2.1. Sampling details of the *Nesidiocoris tenuis* used in this study.

Location	**Population**	**N_{COI}**	**N_{MST}**	**Origin / Host plant / Year**	**Coordinates**	**Source**
China, Hebei	CHIN	4	9	W / Tobacco / 2015	39°30' N, 116°35' E	This study
Vietnam, Me Linh	VIET	4	10	W / Tomato / 2016	21°10' N, 105°42' E	This study
India, Himachal Pradesh	IND	4	10	W / Sesame / 2015	30° 54' N, 77° 5' E	This study
USA, Texas	TX	4	10	W / -- / 2017		This study
Tunisia, Teboulba	TUN	3	8	W / Tomato / 2016	10°58' N, 35°39' E	This study
Spain, Peñíscola	SP-PN	4	-	L / Green bean / 2016	40°21' N, 0°24' E	This study
Italy, Catania	ITA	4	10	W / Tomato / 2015	37°29' N, 15°04' E	This study
Panama, Chiriquí	PAN	4	10	W / Tomato / 2015	8°48' N, -82°28" E	This study
Puerto Rico, Santa Isabel	PR	4	10	W / *Arivela viscosa* (L.) Raf. / 2017	17°57' N, -66°24' W	This study
Mexico, Sonora	MX	3	10	W / Tomato / 2017	30 47' N, -110 52' W	This study
Jordan	JOR	3	10	W / Tomato / 2017	32°04'N, 35°49'E	This study
Japan	JPN	4	6	L / Eggplant / 2015	-	This study
USA, Florida	FLO	4	6	L / Tobacco / 2015	-	This study
Commercial	BIOB	4	10	CR / 2016	-	This study
Commercial	KOP	4	10	CR / 2016	-	This study
Commercial	AGRB	4	10	CR / 2016	-	This study
Total from this study	***16***	***61***	***139***			
Japan	JPN-2	1	-	AB587603	-	Itou et al, Unpubl.
India	IND-2	1	-	MF140518	-	Sankarganesh et al, Unpubl.
Spain, Catalunya	SP-CT	1	-	HQ291844	-	Moreno-Ripol, Unpubl.
Italy, Serdegna	ITA-2	1	-	KY274649	-	Sánchez & Cassis, 2018
Spain, Tenerife	SP-TN	1	-	KY274652	-	Sánchez & Cassis, 2018
Reunion Island	REU	2	-	MG007860, MG007861	-	Streito et al, 2018.
Niger	NIG	8	-	MK607043 - MK607050	-	Garba et al, 2020.
China	CHIN-2	27	-	KF017246 - KF017265, KT598365 - KT598371	-	Xun et al, 2016.
Total from GeneBank	***8***	***42***	***-***			
Total analyzed	***24***	***103***	***139***			

N_{COI} = number of individuals/sequences for *COI* analysis; N_{MST} = number of individuals for microsatellite analysis; W = wild; L = laboratory rearing sourced from wild population; CR = commercial rearing.

2.2.3. Microsatellite detection, testing and experimental procedures

The microsatellites used in this study were mined from the genome of *N. tenuis* (Ferguson et al., 2020b) by using MsatCommander v.0.8.2 (Faircloth, 2008) with the default settings, except for the number of repeats, which was set to select for di-, tri-, tetra-, penta-, and hexa- repeats. The MsatCommander program uses Primer3 v1.1.1 to design locus-specific, flanking primers (Rozen and Skaletsky, 1999). The settings for Primer3 were as follows: no perfect repeats; product size 75-500; primer size: min 16, opt 20, max 24 bases; primer melting temperature: 56 °C - 64 °C. This yielded 1,835 primer sets. Poor primers were removed following these criteria: duplicate, positive for hairpins, high complementarity, and high likelihood of selfing (Rozen and Skaletsky, 1999), after which ca. 700 primer sets remained. The markers with the corresponding set of primers were sorted in descendent order according to number of repeats, as markers with fewer repeats can be less polymorphic. A total of 20 primer sets were initially tested in single-reaction PCRs to verify efficacy and optimal annealing temperatures. For this, single-reaction PCRs were done in a final volume of 20 µl containing 0, 75 µl of 1U/µl DNA polymerase (1 U/µl, Biotools), 1 µl DNA, 0,60 µl of 50 mM $MgCl_2$, 2 µl 10X reaction buffer, 0,4 µl of 10 mM dNTP and 0,4 µl of 10 mM of each primer. The cycling conditions were as follows: 2 min denaturation at 94 °C, 36 cycles of 94 °C for 15 s, 56–64 °C for 30 s, 72 °C for 30 s and followed by 10 min at 72 °C. A final set of 12 primer pairs successfully amplified their corresponding microsatellite loci and were chosen for the analysis (Table S 2.1). Fluorescent labels 6FAM (emission range: 519 λmax/nm), HEX (559 λmax/nm), and ATTO-550 (576 λmax/nm) (Biolegio, Nijmegen, The Netherlands) were assigned to these primer pairs according to product size to reduce overlap of emission in the fluorescent fragment analysis (Table S 2.1).

The 12 microsatellite markers were amplified for the 15 populations (139 individuals) of *N. tenuis* in two rounds of multiplex PCRs using the Qiagen Multiplex PCR kit (Qiagen). Multiplex PCRs were done in a final volume of 50 µl containing 25 µl of 1X master mix, 1 µl of DNA, 5 µl of primer mix (each primer at 0.2 µM), and 19 µl of MQ H_2O. The cycling conditions were as follows: 15 min denaturation at 94°C, 35 cycles of 35 s at 94°C, 90 s at 60 °C and 60 s at 72°C, followed by 30 min at 72°C. PCR products were diluted 250 times and then analyzed in an ABI 3730 Bioanalyzer (Agilent, Santa Clara, USA) with a GeneScan 500 LIZ ladder (GeneScan, Freiburg, Germany).

2.2.4. Data analyses

Mitochondrial data analysis

Additional mtDNA *COI* sequences of *N. tenuis* were retrieved from GeneBank and included in the analysis (Table 2.1) to determine the genetic diversity parameters between populations (i.e. consensus sequences of this study and GeneBank sequences) and groups (i.e. continents and commercial). Consensus and GeneBank sequences were aligned with MUSCLE algorithm under default settings, and trimmed to 658 bp using MEGA X v10.1.8 (Kumar et al., 2018) For each population and continent, the number of haplotypes (H), haplotype diversity (*Hd*) and nucleotide diversity (π), were calculated using Arlequin v3.5.2.2 (Excoffier and Lischer, 2010) . For inferring the evolutionary relationships among haplotypes, a median-joining network of the mtDNA haplotypes was constructed with PopArt (Bandelt et al., 1999) with default parameters. The genetic differentiation was assessed by calculating the pairwise genetic distances (F_{ST}) with 10,000 permutations between populations and groups using Arlequin v3.5.2.2. the same software was used to investigate the demographic history changes for *N. tenuis* by estimating Tajima's *D* (Tajima, 1989) and Fu's F_S (Fu, 1997) for the populations and the defined groups. Under the assumption of neutrality, the population expansion produces significantly negative values, whereas processes such as a population subdivision or recent population bottlenecks would be suggested by significantly positive values.

Microsatellite data analysis

Genotyping was done using Geneious v10.3 and Geneious Primev2019.2.1 (Peakall and Smouse, 2012, 2006), where peaks were initially called using the automatic peak detection within Geneious v10.3 and then confirmed manually. Hardy-Weinberg equilibrium (HWE) and genotypic linkage disequilibrium (LD) were computed with GenePop 4.2 on the Web (https://genepop.curtin.edu.au). GenAlEx v6.51b2 was used to calculate genetic diversity parameters such as the mean number of alleles (*Na*), the effective number of alleles (*Ne*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), and unbiased expected heterozygosity (*uHe*). Gene diversity (*Hs*) was calculated with GenePop 4.2 on the Web. To measure the degree of genetic differentiation, pairwise F_{ST} was computed using GenAlEx v6.51b2. Isolation by distance (IBD) was determined

using Mantel test in GenAlEx v6.51b2 to test for the correlation between geographical and genetic distance (Nei D_A). The software STRUCTURE v2.3.4 (Evanno et al., 2005a) was used to identify clusters of genetically similar populations based on the Bayesian approach. A first run for *K*=2 to *K*=15 set to 30,000 Markov Chain Monte Carlo (MCMC) and 30,000 burn-in, admixture assumed, correlated allele frequencies within populations and 15 iterations. Results of this run were then analyzed with STRUCTURE HARVESTER web version v0.6.94 (Earl and vonHoldt, 2012), which relies on the Evanno method (Evanno et al., 2005b), to identify the optimal number of populations (K). A second run in STRUCTURE v2.3.4 with the optimal K size was set to an MCMC and burn-in of both 200,000 and 100 iterations and the output was visualized and interpreted using CLUMPAK (Kopelman et al., 2015). Additionally, principal component analysis (PCoA) based on the covariance of the F_{ST} genetic distance matrix was implemented in GenAlEx v6.51b2.

2.3. Results

2.3.1. Mitochondrial data

Genetic diversity

A 658 bp fragment of the *COI* gene was sequenced for 61 *N. tenuis* individuals collected for this study. The *COI* sequences contained 10 variable sites of which 7 were parsimony informative. A total of 103 *COI* sequences (61 sequences from this study and 42 sequences retrieved from the GeneBank) generated 23 haplotypes (Table 2.2). The higher number of haplotypes was found in Asia (18) whereas the lowest number was found in America (2). Haplotype 2 was the most frequent (37 individuals) and the most widespread, as it was present in all groups except in Asia. Haplotype 4 is the second most common (18 individuals) and is found in Asia and Africa. Haplotype 5 is the third most common (16 individuals), and was exclusive to Asia. Several haplotypes (haplotypes 1, 3, 5-19) were exclusively found in Asia. Haplotype 23 was exclusive to PR in the American continent. Haplotypes 2 and 22 were found in the commercial populations, with haplotype 2 found in AGRB and BIOB, and haplotype 22 found in KOP (Table 2.2, Figure 2.1). For populations, the haplotype diversity (*Hd*) and nucleotide diversity (π)

ranged between 0.989 – 0.500 and 0.00478 – 0.00076, respectively, while they were most frequently nil (i.e. a single haplotype was detected) (12/16 populations of this study, 6/8 populations retrieved from the GeneBank). For groups, haplotype diversity (*Hd*) and nucleotide diversity (π) ranged between 0.842 – 0.105 and 0.00394 – 0.00039, respectively (Table 2.2). The overall haplotype diversity (*Hd*) and nucleotide diversity (π) was 0.821 and 0.00346, respectively.

The median-joining haplotype network showed a star-like pattern for most Asian populations, with haplotype 5 at the center and most derivatives connected to it corresponding to haplotypes exclusive to China and retrieved from GeneBank. A second and smaller cluster of Asian populations were connected to haplotype 3, represented mostly by JPN individuals. Most populations from NIG and REU, from the sub Saharan region, grouped together as haplotype 4, and were distant from the north African population TUN, represented by haplotypes 20 and 21. Most populations from Europe, America and commercial rearings clustered together and distant from Asia (Figure 2.1).

Table 2.2. Distribution of haplotypes, genetic diversity indices and neutral tests among different populations of *Nesidiocoris tenuis* from four continents and three commercial rearings based on mtDNA COI sequences

Population	N / H	Haplotype	*Hd* ± SD	π ± SD	Tajima's *D*	Fu's Fs
By location						
CHIN	4 / 1	5	0	0	0	-
VIET	4 / 2	4, 5	0.500 ± 0.265	0.00076 ± 0.00040	-0.61237	0.172
JPN	4 / 1	3	0	0	0	-
IND	4 / 1	5	0	0	0	-
JOR	3 / 1	4	0	0	0	-
TUN	3 / 2	20, 21	0.667 ± 0.314	0.00347 ± 0.00151	0	1.609
REU	2 / 2	2, 4	1.000 ± 0.500	0.00304 ± 0.00152	-	0
NIG	8 / 1	4	0	0	-	-
ITA	4 / 1	2	0	0	0	-
SP-PN	4 / 3	2, 20, 22	0.833 ± 0.222	0.00173 ± 0.00051	-0.70990	-0.887
FLO	4 / 1	2	0	0	0	-
TX	4 / 1	2	0	0	0	-
MX	3 / 1	2	0	0	0	-
PAN	4 / 1	2	0	0	0	-
PR	4 / 2	2, 23	0.500 ± 0.265	0.00172 ± 0.00081	-0.70990	1.099
KOP	4 / 1	22	0	0	0	-
BIOB	4 / 1	2	0	0	0	-
AGRB	4 / 1	2	0	0	0	-
This study	*61 / 9*		*0.702 ± 0.054*	*0.00280 ± 0.00030*	*-0.4028*	*-1.194*
SP-CT	1 / 1	2	0	0	-	-
SP-TN	1 / 1	2	0	0	-	0
ITA-2	1 / 1	2	0	0	-	-
IND-2	1 / 1	1	0	0	-	-
JPN-2	1 / 1	3	0	0	-	-
CHIN-2	27 / 18	1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	0.989 ± 0.015	0.00478 ± 0.00058	-1.70196*	-13.899***
By group						
Asia-global	48 / 18	1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	0.842 ± 0.036	0.00394 ± 0.00048	-1.710**	-11.200***
Asia-this study	19 / 3	3,4,5	0.608 ± 0.089	0.00185 ± 0.00038	1.134	1.015
Europe global	11 / 3	2, 20, 22	0.345 ± 0.172	0.00068 ± 0.00033	-1.430	-1.246*
Europe-this study	8 / 3	2,20,22	0.464 ± 0.200	0.00076 ± 0.00117	-1.310	-1.410
Africa	13 / 4	2, 4, 20, 21	0.526 ± 0.025	0.00197 ± 0.00294	-0.626	-0.406
America	19 / 2	2, 23	0.105 ± 0.119	0.00039 ± 0.00131	-1.511*	0.021
Commercial	12 / 2	2, 22	0.485 ± 0.106	0.00091 ± 0.00050	1.066	1.003
Total	***103 / 23***		***0.821 ± 0.026***	***0.00346 ± 0.00031***	***-1.772****	***-15.644******

N = sample size; H = number of haplotypes; *Hd* = haplotype diversity; π = nucleotide diversity; SD = standard deviation; * = P < 0.05, ** = P < 0.02, *** = P < 0.001

Genetic differentiation

The pairwise F_{ST} values for populations based on mitochondrial data ranged between -1 – 1. A significant genetic differentiation was observed in 100 of the 276 comparisons between populations (Table S 2.2). For groups, the F_{ST} values ranged between 0.020 – 0.693, and the genetic differentiation was significant for all comparisons except Europe vs. commercial and Europe vs. America (Table 2.3). This result is consistent with the clustering observed for haplotype 2 in the median-joining network.

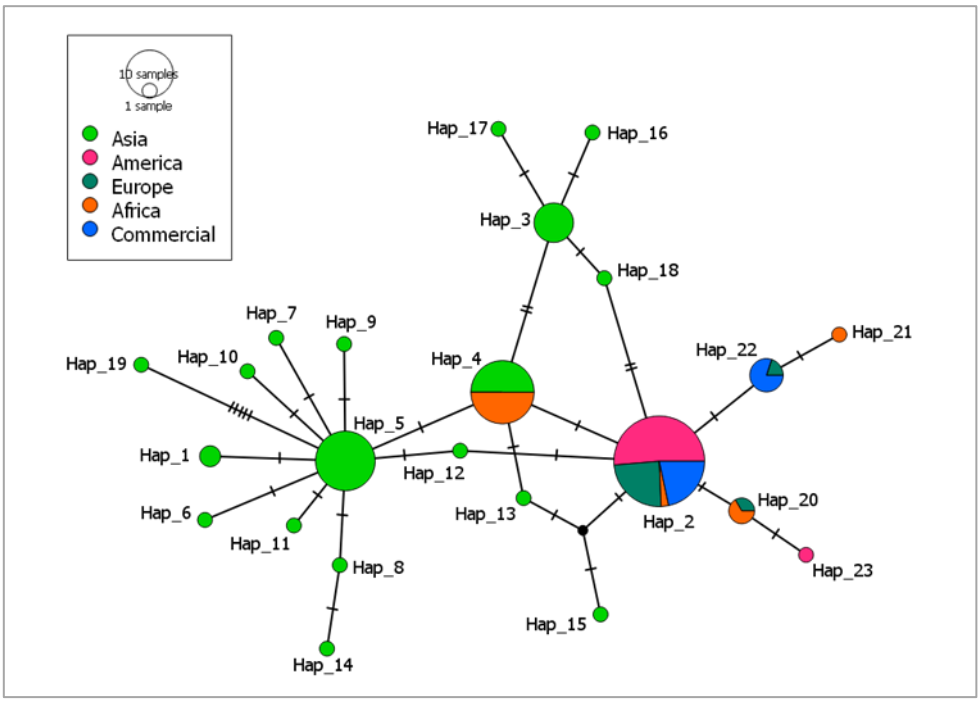

Figure 2.1. Median-joining haplotype network of the 23 haplotypes based on the *Cytochrome oxidase subunit I* (*COI*) sequences from *Nesidiocoris tenuis*. Tick marks over lines indicate the number of mutations between haplotypes. Black circles indicate intermediate, missing haplotypes.

Population structure and demographic history

Tajima's *D* and Fu's *Fs* varied across groups and for populations (Table 2.1). For all samples pooled together, both Tajima's *D* and Fu's *Fs* were negative and significant. The Asian group including GeneBank sequences also exhibited significantly negative Tajima's *D* and Fu's *Fs*, whereas the Asian group that only included our samples yielded

non-significant positive values. For the European group, a significantly negative value was only observed for Fu's *Fs* when the GeneBank sequences were included. The group composed by American samples, all being samples from this study, exhibited a significantly negative Tajima's *D* value. The African, the commercial group, and a group composed by all the samples from this study showed non-significant results.

Table 2.3. Pairwise F_{ST} among groups for *Nesidiocoris tenuis* based on mtDNA *COI* gene sequences (below diagonal). Bold indicates significant values (P < 0.05).

	Asia	**Europe**	**Africa**	**Commercial**	**America**
Asia	***				
Europe	**0.340**	***			
Africa	**0.186**	**0.523**	***		
Commercial	**0.296**	0.020	**0.467**	***	
America	**0.446**	0.022	**0.693**	**0.242**	***

2.3.2. Microsatellite data

Genetic diversity and genetic differentiation

A total of 139 individuals from 15 populations were genotyped at 12 microsatellite loci. The Hardy-Weinberg expectations (HWE) test showed that 65 from the 180 locus-population combinations deviated significantly, and no linkage disequilibrium (LD) was found for any pair of loci.

There was variation in the parameters of genetic diversity across populations (Table 2.4). For instance, the highest mean number of alleles (*Na*) was observed in IND (5.667) and the lowest in PR (2.500). Similarly, for the effective number of alleles (*Ne*) IND also displayed the highest value (3.841) and PR the lowest (1.757). The observed heterozygosity (*Ho*) was moderate and similar across populations with a range between 0.200 – 0.338. The expected heterozygosity (*He*) ranged from 0.676 – 0.372. The population with the highest genetic diversity (*Hs*) was VIET (0.760) whereas PR (0.401) had the lowest genetic diversity. Significant genetic differentiation based on among-

population F_{ST} values was detected for all pairwise-comparisons ($p < 0.05$) except for TUN-ITA. The F_{ST} values between populations based on the microsatellite analysis ranged from 0.036 to 0.429 (Table S 2.2). The Mantel test revealed a significant but weak evidence of isolation by distance (IBD) ($R^2 = 0.0185$, $P = 0.010$) (Figure S 2.1).

Table 2.4. Genetic diversity of 15 populations of *Nesidiocoris tenuis* at 12 microsatellite loci. Codes of populations are given in Table 2.1.

Population	***Na***	***Ne***	***Ho***	***He***	***uHe***	***Hs***
CHIN	3.333	2.297	0.203	0.480	0.515	0.541
FLO	2.667	2.196	0.200	0.438	0.481	0.512
IND	5.667	3.841	0.316	0.663	0.703	0.728
ITA	4.583	3.168	0.338	0.611	0.653	0.676
JOR	4.250	2.915	0.222	0.592	0.627	0.654
JPN	3.417	2.949	0.203	0.557	0.610	0.654
KOP	4.667	3.119	0.300	0.600	0.637	0.659
BIOB	4.167	3.016	0.276	0.562	0.594	0.613
MX	2.667	1.966	0.225	0.387	0.408	0.419
PAN	3.500	2.503	0.311	0.467	0.494	0.505
PR	2.500	1.757	0.234	0.372	0.392	0.401
AGRB	4.083	3.150	0.292	0.600	0.637	0.663
TUN	3.833	3.023	0.282	0.573	0.617	0.645
TEXAS	4.083	2.871	0.248	0.566	0.597	0.618
VIET	4.750	3.369	0.237	0.676	0.721	0.760
Average	3.878	2.809	0.259	0.543	0.579	0.603

Na = mean number of alleles; *Ne* = the effective number of alleles; *Ho* = observed heterozygosity; *He* = expected heterozygosity; *uHe* = unbiased expected heterozygosity, *Hs* = gene diversity.

Population structure

The optimal K was determined to be K = 2 (ΔK = 474.82), which inferred one cluster composed by the populations from America and a second cluster composed by the populations from Asia, Africa, Europe and commercial rearings. Visualization of clusters at K = 3 (ΔK = 64.06) separated again America in one group, a second group composed by a mix of Asian, African, European and commercial populations, and a third group containing CHIN, JPN and VIET in one group (Figure 2.2).

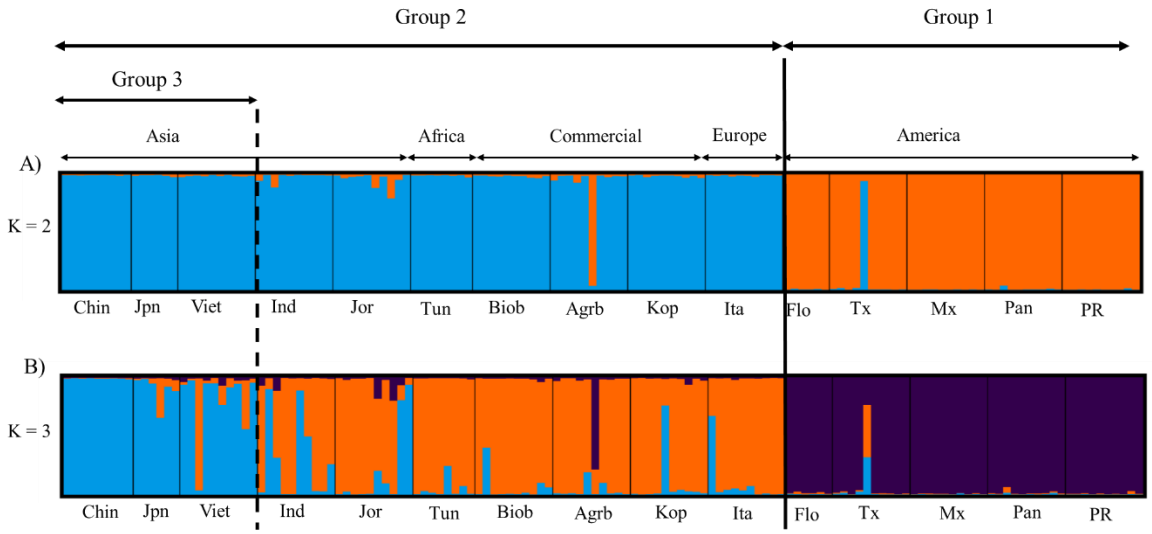

Figure 2.2. Population structure inferences of 139 individuals of *Nesidiocoris tenuis* based on Bayesian clustering. Bayesian assignment probabilities for K = 2 (A) and K = 3 (B). Codes of populations and number of individuals per sample are given in Table 2.1.

This structuring was further supported by the PCoA, that indicated two distinct groups. One of the groups clustered all the populations from the American continent, whereas the second group clustered the populations from Asia, Africa, Europe and the commercial rearings. Within this second group, a smaller third group composed by CHIN, JPN and VIET was distinguished and separated from the rest of the populations, which is consistent with the Bayesian clustering at K = 3. The first and second axes accounted for 31.19% and 16.85 of the variance, respectively (Figure 2.3).

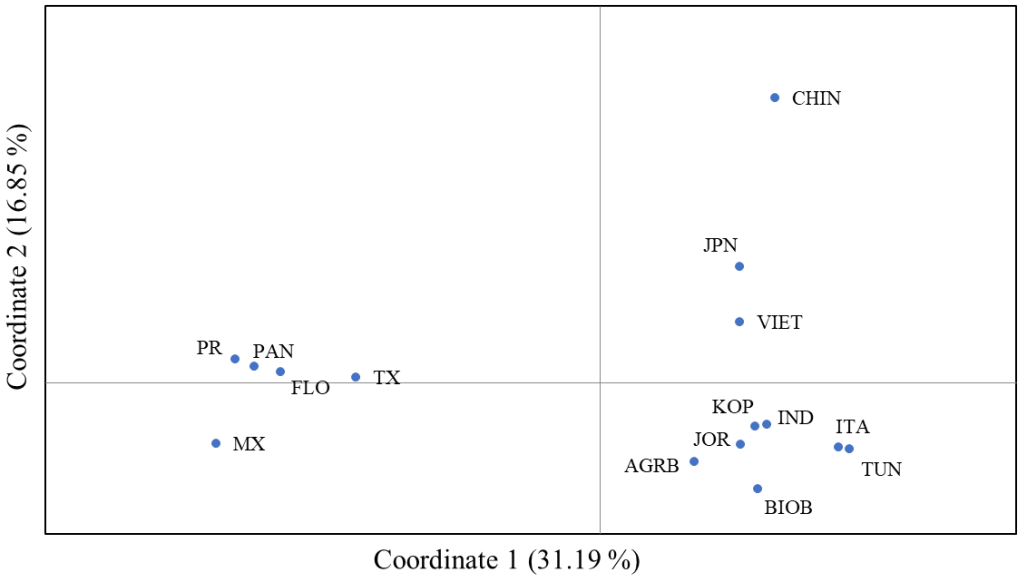

Figure 2.3. Principal component analysis (PCoA) based on the covariance of the F_{ST} genetic distance matrix for microsatellite data representing the relationships among 15 populations of *Nesidiocoris tenuis*. Codes of populations are given in Table 2.1.

2.4. Discussion

2.4.1. Genetic diversity and genetic differentiation

The objective of this study was to investigate the population genetics of the zoophytophagous predator *N. tenuis* across four continents and three commercial rearings. The mtDNA analysis indicated a moderate-to-low genetic diversity in the individuals collected and analyzed for this study. The low number of individuals analyzed per population (3-4 individuals) could partly explain this low diversity. This is consistent with previous studies that found a correlation of the mitochondrial diversity with the sampling effort (Valade et al., 2009). Nevertheless, it is remarkable that the highest diversity in our samples was that observed in SP-PN, considering that the individuals analyzed were sourced from a laboratory rearing that was started with few wild individuals (ca. 25 individuals) (Chinchilla-Ramírez et al., 2020). The presence of three different haplotypes out of four samples analyzed confirms high diversity, which was likely higher in the original wild population collected. For instance, one of the haplotypes

reported for SP-PN is only found in one other location, TUN (haplotype 20). The ubiquitous haplotype 2 was also found in SP-PN, and the third haplotype observed corresponds to one that is only found in KOP (haplotype 22). Hence, dispersal of commercial individuals to this area (where no commercial introductions were done before our collecting date) may also be possible. However, this can also be an indication that the source for this commercial rearing could have been in neighboring areas of SP-PN.

The presence of one exclusive haplotype in KOP (haplotype 22) is possibly explained by the genetic isolation to which mass-reared species are exposed (Paspati et al., 2019; Rasmussen et al., 2018), which could have favored specific mutations that have not occurred in the other commercial populations (BIOB and AGRB, both displaying the haplotype 2 only). Likewise, the geographical isolation in PR (i.e. an island) could have also favored a similar process on individuals of this region that did not happen in other locations, thus explaining the exclusive haplotype 23 found in PR only. Similar observations were done about populations of *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae), another biocontrol agent, collected from and island (Sanchez et al., 2012). However, it is important to mention that the samples from PR were collected in *Arivela viscosa* (L.) Raf. (Brassicales: Cleomaceae) hence it cannot be discarded that host associated differentiation (HAD) processes might be underlying mutations in this region to some extend (Medina, 2012). Indeed, the successful spreading and colonization of haplotype 2, which is found in all continents except Asia, could be mediated by the host plants in which *N. tenuis* can survive and reproduce. Previous studies have demonstrated that haplotype ubiquity and success in *Dalbulus maidis* (Delong & Wolcott) (Hemiptera: Cicadellidae) was facilitated by its association with its host plant, maize, that is extensively cultivated (Bernal et al., 2019). In the case of *N. tenuis*, it has been reported in a wide range of plants from the Solanaceae family such as tomato, sweet pepper, potato, eggplant and tobacco (Pérez-Hedo and Urbaneja, 2016), which are also extensively cultivated in many regions worldwide. Therefore, our results suggest that haplotype 2 could represent a well-adapted lineage to different solanaceous crops, using them to spread and colonize, while displacing other local haplotypes at the same time.

Due to its high polymorphism, faster mutation rates and increased sensitivity to detect recent evolutionary events (Putman and Carbone, 2014; Selkoe and Toonen, 2006), microsatellites are considered more informative than other molecular markers for

intrapopulation studies. Our results of genetic diversity based on 12 microsatellites loci indicated an overall moderate genetic diversity in the populations of *N. tenuis* analyzed. The diversity observed in *N. tenuis* is similar to that reported for *M. pygmaeus*, which is also widely used in cultivated systems (Sanchez et al., 2012; Streito et al., 2017). The consistency of PR as the population displaying the lowest diversity values is probably explained by its geographical conditions as an island, and the subsequent reduction of genetic diversity suffered by species when colonizing such environments. This process was also deemed as the likely cause of the reduced genetic diversity observed in *M. pygmaeus* collected from the Tenerife island in Spain (Sanchez et al., 2012). The higher diversity observed in VIET and IND populations is consistent with the expectation of increased diversity in or in the proximity of the putative center of origin of a species, which has been suggested to be in Asia (Wheeler and Henry, 1992).

Genetic differentiation between populations was confirmed by both mitochondrial and microsatellite F_{ST} values. However, the geographical distance between the populations was not the main reason explaining the differentiation, as supported by the weak positive correlation shown by the Mantel's test. This is in agreement with a population structure study of *N. tenuis* carried out in China (Xun et al., 2016). Xun et al., (2016) found that the distance was not the main driver of differentiation between populations, and they proposed topography, and anthropogenic activities among the possible reasons for the differentiation they observed. It is likely that the use of *N. tenuis* in biocontrol has facilitated its spreading and therefore influenced the population structure, as a consequence of plant/produce trading and agricultural practices. This is further supported by the ubiquity and abundance of haplotype 2 shown by the mitochondrial data. The topography and geographical barriers might also explain part of the differentiation observed in our data. For instance, the fact that the most significant differentiation was observed in CHIN and PR suggests that the Tibetan/Himalayan Plateau, in the case of CHIN, and hydrographic barriers (i.e. seas), in both locations, could be acting as strong barriers for those populations, thus promoting the isolation of lineages in both regions and further increasing their differentiation. However, as mentioned before, HAD might be occurring in PR, and additional experiments with individuals sourced from different host plants are necessary to test this hypothesis. At the commercial rearings level, the three populations analyzed were not among the most differentiated, although BIOB had higher F_{ST} values than AGRB and KOP. This might be an indication that the

genetic drift/bottlenecks have not been as severe in *N. tenuis* as in other biocontrol commercial populations (Paspati et al., 2019), or that longer time of rearing would be necessary to see a more pronounced differentiation. Paspati et al., (2019) studied the predatory mite *Amblyseius swirskii* (Athias-Henriot) (Acari: Phytoseiidae), which has been used in biocontrol about the same as *N. tenuis* (van Lenteren, 2012), but which life cycle is faster (ca. 5 days) than that of *N. tenuis* (ca. 21 days) under standard rearing conditions. The overall negative values from the neutrality tests Tajima's *D* and Fu's *Fs*, although not all values were significant, suggests that *N. tenuis* departed from neutrality and experienced population expansion. This is further reinforced by the star-shape of the Asian haplotypes in the Median-joining network, which is indicative of population expansion. It remains pending to investigate the roles that intensive agriculture and augmentative biocontrol practices, based on just one/few lineages, might be playing in its expansion and population structuring.

2.4.2. Population structure

Our results based on both mtDNA and microsatellites revealed genetic structuring of the *N. tenuis* populations analyzed. The haplotype network showed that Asian and African populations clustered together and shared more haplotypes between them than those shared with the group composed by the European, American and commercial populations. Moreover, the location of the African haplotypes in the network suggest that populations of this region are more closely related to the Asian populations, thus suggesting more gene flow between Asia-Africa than between Asia-Europe. A possible explanation for this could be an increased trade of plants/agricultural products between Africa and Asia, which could facilitate the dispersion and constant exchange of the lineages of *N. tenuis*. Similarly, the clustering of haplotypes from Europe, America and commercial rearings suggest a more constant gene flow of *N. tenuis* lineages, namely haplotype 2, between those groups. Interestingly, JPN segregates apart from the rest of the Asian populations in the haplotype network, which is in agreement with our observations about differentiation (i.e. F_{ST}). On the other hand, the genetic structuring inferred with both STRUCTURE and PCoA based on the microsatellite data divided all individuals into two clusters. The first cluster encompassing the populations from America and the second cluster encompassing the remaining populations. The geographic barrier imposed by the

Atlantic Ocean between the two clusters is likely the strongest driver of *N. tenuis* genetic structuring in this scenario. Moreover, the third cluster observed at K = 3, and also distinguishable in the PCoA, further supports the geographical barriers (i.e. the Tibetan/Himalayan Plateau) as an important structuring factor between *N. tenuis* populations in the second group, composed by the Asian, European, African and commercial populations. Other studies have underlined the role of topography in the creation of genetic structure in populations (Knoll et al., 1996; Yuan et al., 2012)

2.4.3. Implications for agriculture and biological control

Overall, our findings suggest a moderate genetic diversity and a population genetic structure of *N. tenuis* likely influenced by geographical barriers and anthropogenic activities. To the best of our knowledge, this is the first study to undertake a global-scale analysis on the population genetics of this zoophytophagous predator. Considering the controversy around *N. tenuis* and its status as biocontrol agent and as a pest, we believe our study shed light on important aspects of the evolutionary ecology of this species that are useful for both perspectives. On the one hand, in regions where *N. tenuis* is considered a pest, knowledge about its genetic diversity and the factors affecting its differentiation, dispersal and structuring, could be a starting point to design control programs aimed at reducing its expansion, as well as pointing/preventing those human-mediated activities that favor it. Moreover, the usefulness of microsatellite markers of *N. tenuis* hereby demonstrated, could enable local-scale analyses of populations from natural and cultivated stands, to determine whether plant domestication is mediating the creation of (more phytophagous) lineages. This is of particular importance considering previous evidence on how some agricultural-related practices are favoring the creation of pests (Alvarez et al., 2007; Bernal and Medina, 2018). On the other hand, in regions where *N. tenuis* is valued as a biocontrol agent, our findings might serve different purposes as well. For instance, it could be used for the monitoring of its spatial distribution in areas where it is released. Although it is a species mainly used in greenhouses, movements of individuals between the inside of the greenhouses and the surroundings have been confirmed at a molecular level for *M. pygmaeus*, which is used in the same way as *N. tenuis* for pest control (Streito et al., 2017). Furthermore, our findings on the genetic diversity of three commercial populations might serve as a starting reference to monitor

and prevent the negative effects that the mass-rearing usually exerts on the genetic makeup of species (Paspati et al., 2019; Rasmussen et al., 2018; Sørensen et al., 2012). Finally, our study adds important tools to the broader field of genetic improvement of biocontrol agents. An overview of the genetic diversity and population structure of *N. tenuis*, as the one hereby presented, prompts the studying of this species from the genetic and genomic perspective, with the aim of disentangling whether the detrimental effects of its phytophagy could be further reduced at gene levels. Our findings add to previous studies on the population genetics of biocontrol species (Paspati et al., 2019; Sanchez et al., 2012; Streito et al., 2017), and offer an analysis with populations from representative regions where *N. tenuis* is currently present. However, to better understand its patterns of gene flow, dispersal ability and adaptation to different climates and natural- or agro-ecosystems, it would be interesting to include more populations from additional regions and wild and domesticated host plants/stands.

Author contributions: Conceptualization MC-R, AU, MP-H and BP; methodology MC-R and KF; validation AU, MP-H and BP; formal analysis MC-R, KF and BP; investigation MC-R and KF; resources AU, MP-H and BP; data curation MC-R and KF; visualization MC-R, AU and MP-H.; supervision AU, MP-H and BP; project administration AU, MP-H and BP; funding acquisition AU, MP-H and BP; writing—original draft preparation MC-R; writing—review and editing AU, MP-H, KF and BP.

CHAPTER 3

Genetic variation in the feeding behavior of isofemale lines of *Nesidiocoris tenuis*

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Abstract

Zoophytophagous predators provide biocontrol services in various major crops of modern horticulture due to the combination of its predatory capacity and the induction of plant defenses derived from its phytophagy. However, under certain conditions of prey scarcity these natural enemies can inflict plant damage. Exploitation of genetic variation and subsequent selective breeding on foraging traits is a potential alternative to overcome this inconvenience. In this study, we quantified the genetic variation of phytophagy and zoophagy of *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae), a zoophytophagous predator widely used in tomato crops to suppress key pests. We compared nine isofemale lines on their capacity to produce necrotic rings and wilting on tomato plants as a proxy for phytophagy, as well as their efficacy to prey on *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs as a proxy for zoophagy. Differences between isofemale lines in phytophagy and zoophagy showed a genetic basis. Variation found in zoophagy levels was larger than that in phytophagy levels. Our results showed that there is a genetic basis for the variation observed in the feeding behavior of isofemale lines of *N. tenuis*, highlighting the potential importance of selective breeding for such traits of biocontrol interest.

3.1. Introduction

The importance of zoophytophagous species to suppress pests in agroecosystems has increased over the last decades (Alomar et al., 2006; Calvo et al., 2012a; Mcgregor et al., 1999; Pérez-Hedo et al., 2017; Pérez-Hedo and Urbaneja, 2016; Urbaneja et al., 2012; van Lenteren et al., 2020; Zappalà et al., 2013). However, the assessment of genetic variation on traits of biocontrol interest in these species is rather recent (Dumont et al., 2018, 2016). Dumont et al. (Dumont et al., 2016) tested the hypothesis that zoophytophagous populations consist of a mix of specialized genotypes (i.e. zoophagous, phytophagous and generalists) instead of only one highly plastic genotype. In this study, Dumont et al. (Dumont et al., 2016) demonstrated genetic differences in the feeding behavior of *Campylomma verbasci* (Meyer) (Hemiptera: Miridae), specifically in their zoophagy on two different prey species this zoophytophagous feeds upon in apple orchards. These results shed light on the possibility to explore and exploit intraspecific genetic variation of interesting biocontrol traits in commercially available zoophytophagous species.

Nesidiocoris tenuis (Reuter) (Hemiptera: Miridae) is a cosmopolitan zoophytophagous predator that has been extensively used in tomato crops in the Mediterranean basin to control different key pests (Pérez-Hedo and Urbaneja, 2016). Its major contribution in the biocontrol programs in tomato is attributed to its efficacy against the ubiquitous whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) and the invasive South American pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelichiidae) (Cabello et al., 2013; Calvo et al., 2012a; Calvo and Urbaneja, 2004; Desneux et al., 2010; Mollá et al., 2014; Pérez-Hedo and Urbaneja, 2016; Sanchez et al., 2014; Urbaneja et al., 2012; Zappalà et al., 2013). However, its status as a biocontrol agent is sometimes controversial because of the damage it can inflict in plant tissues when prey levels decrease (Arnó et al., 2010; Calvo et al., 2009; Cano et al., 2009; Pérez-Hedo and Urbaneja, 2016; Sánchez and Lacasa, 2008). The exploitation of the genetic variation in the feeding behavior of *N. tenuis* has been suggested as a mean to improve its predatory efficiency while mitigating the detrimental effects derived from its phytophagy (Dumont et al., 2018).

Most natural enemies currently used in augmentative biological control programs have been selected based on differences between species (interspecific variation) to effectively control pests. Nevertheless, there is a growing interest in the exploitation of genetic differences in traits of interest within species (intraspecific variation) of natural enemies (Bielza et al., 2020; Kruitwagen et al., 2018; Le Hesran et al., 2019; Leung et al., 2020; Lommen et al., 2017). This approach has been applied before in predatory insects, predatory mites and parasitoids, with successful selection on traits such as pesticide resistance, increased fecundity, host preference, sex ratio and improved climatic tolerance (Balanza et al., 2019; Ferguson et al., 2020a; Hoy, 1986; Lirakis and Magalhães, 2019). However, only few of these examples went beyond laboratory or pilot tests, and most of them were not continued after a few years despite their positive outcomes. Financial, technical and legal limitations have been deemed as likely causes preventing the development of genetic improvement in biocontrol agents (Lirakis and Magalhães, 2019; Lommen et al., 2017). Therefore, the current efforts are focused on the generation of knowledge and optimization of procedures that has been missing for arthropods, to promote artificial selection on biocontrol agents (Kruitwagen et al., 2018; Le Hesran et al., 2019; Lommen et al., 2017).

A first step for selection to be feasible is the presence of variability in the target trait, but more relevant is the existence of a genetic basis for at least part of the variation observed in that trait (Beukeboom and Zwaan, 2005; Lirakis and Magalhães, 2019). Once the genetic variation has been explored and confirmed, multiple genetic tools can be further applied to identify the gene(s) and factors involved in their expression on a certain population, and to choose the optimal approach for artificial selection (Leung et al., 2020). For instance, the recent sequencing of the genome of *N. tenuis* (Ferguson et al., 2020b) is one of those tools that enables the genetic exploration of this species for improvement purposes of its biocontrol traits.

In this study, we aimed to investigate whether there is genetic variation in the feeding behavior of wild individuals of *N. tenuis*, for both phytophagy and zoophagy, by using an isofemale line approach. Necrotic rings and wilting inflicted in plant tissue, as well as consumption of *E. kuehniella* eggs, were used as a proxy for phytophagy and zoophagy respectively. Our study is the first to investigate the genetic basis of the feeding behavior

of *N. tenuis*, and the results shed light on the potential to use the genetic variation in these two traits to enhance the biocontrol services of this zoophytophagous predator.

3.2. Materials and Methods

3.2.1. Isofemale lines and plants

Wild adults and nymphs of *N. tenuis* were collected with an aspirator from two outdoor tomato farms located in Peñíscola, Castellón (Spain) (Field-1: 40° 23′ 39.33″ N/0° 24′ 24.79″ E; Field-2: 40° 22′ 44.5″ N/0° 23′ 49.71″ E) (ca. 2 km distance between fields) in September of 2016. The tomato plants in these farms were grown organic and no previous releases of *N. tenuis* had been done before the time of collecting, but wild *N. tenuis* had been reported to appear in these crops every season. The province of Castellón is considered a transition zone between the two regions were mirid predators are used in tomato farms: the warmer southern Spanish territories, where mostly *N. tenuis* is released in tomato crops for pest control, and the temperate eastern Spanish territories where *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae) is the predominant species used in tomato crops for pest suppression (A. Urbaneja, personal communication). Wild populations of *N. tenuis* were scarce during the season of 2016 and only a few insects (adults and nymphs) were captured (Field 1: $n = 9$, Field 2: $n = 16$), hence it was necessary to mix them to secure the establishment of the laboratory colony with the maximum diversity available at the moment of collecting. Individuals collected in both farms were brought to the laboratory, mixed and placed in a plastic cage (30 x 30 x 30 cm) with green bean pods (*Phaseolus vulgaris* L.) and eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) provided twice a week as oviposition substrate and food source, respectively. This colony was kept in the laboratory during five generations at 25 ± 2 °C, 50% ± 10% RH and 14L:10D h photoperiod (1 generation ~ 22 days at these experimental conditions). Individuals from the colony were allowed to randomly mate and increase population numbers necessary to found the isofemale lines. Isofemale lines were initiated as follows: one male and one virgin female, both less than three-days-old, were allowed to mate in a Petri dish with mesh on the lid for ventilation (90 mm on diameter), with an entire green bean pod (ca. 15 cm long) as oviposition substrate and *E. kuehniella* eggs *ad*

libitum supplied twice a week until the female died. Fifteen isofemale lines were founded but only nine of them survived (extinct isofemale lines did not survive beyond generation three). Each bean pod bearing eggs laid by the founder females was then placed in an individual muslin cage (25 x 25 x 25 cm) with new bean pods and *E. kuehniella* eggs. Insects of the same isofemale line were allowed to mate randomly but parents and progeny were kept separated at all times by removing the bean pods bearing laid eggs twice a week and placing them in a new cage. *E. kuehniella* eggs were supplied twice a week as food source. Frozen *E. kuehniella* eggs for all rearings and experiments were provided by Koppert Biological Systems (Almería, Spain).

Tomato plants cv Raf Supermarmande (Mascarell Seeds, Spain) used in this experiment were in vegetative stages V5 to V7 (ca. 30-40 cm height). Plants were grown in pots (8 x 8 x 8 cm) filled with soil and vermiculite (3:1) and watered every two days. Plants were kept in pest-free climatic chambers until the start of the experiment, at the same experimental conditions previously described for the isofemale lines. This cultivar is known to be susceptible to the feeding damage of *N. tenuis*, hence it was selected for this study to allow for visualization and measurement of the phytophagy parameters explained bellow.

3.2.2. Phytophagy experiment

The isofemale line approach is convenient to study the genetic variation in traits of interest. It consists of the establishment of several strains or lines, each from a single mated female, and the quantification of the trait in several offspring produced by each female (Parsons, 1980; Wajnberg, 2004). Considering that rearing and experimental conditions for all isofemale lines are identical, the variation observed between them for the mean values of the trait can be considered of genetic origin (Beukeboom and Zwaan, 2005; David et al., 2005). This experiment was carried out in generation 11 due to the limited availability of individuals in earlier generations that prevented the attainment of the minimum of replicates necessary, and to secure homozygosity in the isofemale lines, which increases with the generations in the isofemale lines (Hoffmann and Parsons, 1988).

As a proxy for phytophagy, we quantified the number of necrotic rings and wilting percentage produced by ten adults of each isofemale line per replicate on two types of leaves of a tomato plant. Adult insects used in this experiment were three-five days-old. The age of the experimental individuals was chosen to allow sufficient time for the females to mature, mate and oviposit, in order to secure enough progeny and survival of the isofemale lines for the following zoophagy experiment (see below). The phytophagy parameters were evaluated in leaves attached to entire plants. During the experiment, the plants were placed in trays inside a pest-free climatic chamber and watered every two days. The space between plants in the trays was enough to avoid differences in development or leaf quality due to light competition, as well as to reduce the possibility of plant injuries during manipulation of the plants and trays. Provided that the apical part of the tomato plant is preferred by *N. tenuis* (Arnó et al., 2010; Perdikis et al., 2014), the two youngest fully developed leaves in the upper section of the plants were selected for this experiment, and classified according to its position from the apical bud: the *young* leaf was the closest to the apical bud and the *old* leaf was the second-to-last from the apical bud. The apical bud was not used to test for phytophagy because of 1) the heterogeneity in their size and structure and 2) limitations on the maximum number of individuals available in each isofemale line at a given generation. Each leaf was enclosed in a muslin bag (15 x 21 cm) and ten adults of the corresponding isofemale line were placed inside the muslin bag without supplementary food source or prey. The insects were allowed to feed for five days. Dead individuals were removed daily from the muslin bags to prevent necrophagy during this experiment and mortality per isofemale line was recorded. Dead individuals were not replaced with new individuals. After the feeding period, the remaining insects were removed, and the level of phytophagy was evaluated. Necrotic rings visible in the petiole and rachis were recorded. For wilting evaluation, each leaflet (five leaflets per leaf, all of similar size) was assigned the percentage of wilting in a 20% of the total leaf surface.

This experiment was carried out in ten consecutive blocks (all with individuals from generation 11). In each block, all isofemale lines were included with a minimum of one replicate per isofemale line (except isofemale lines 5 and 10 in blocks 6 and 7, respectively) (Table S 3.1). A replicate consisted of one leaf enclosed in a muslin bag with ten adults (five males and five females). A total of 240 individuals were used in this experiment, with the number of individuals tested per isofemale line ranging between 21-

33. One tomato plant always contained two replicates, one on the *young* leaf and one on the *old* leaf, and replicates on the same plant never belonged to the same isofemale line to exclude any possible plant effect. Each block consisted of similar-aged adults (three-five days old) and plants of similar developmental stage as described above. Similar-aged adults were obtained from the bean pods which were replaced in the cages twice a week as previously described.

3.2.3. Zoophagy experiment

In a separate experiment, we quantified the number of *E. kuehniella* eggs preyed by adults of each isofemale line during 24 hours as a proxy for zoophagy. This experiment was carried out in generation 15 because of the decline in the number of individuals in all isofemale lines after the phytophagy experiment, in which most adults of each isofemale line were used to achieve enough replicates. Isofemale line 5 was excluded from the zoophagy experiment because the number of individuals were not enough for the minimum of replicates needed for this experiment. A total of 193 individuals were used in this experiment, with 22-26 individuals tested per isofemale line. For this, females and males of *N. tenuis* less than five-days-old were starved for 24h with a moist cotton plug for water supply. After the starvation period, the insects were placed individually in a Petri dish (55 mm on diameter), and *E. kuehniella* eggs (n = 120) were offered to each individual in a piece of sticky cardboard. Insects were allowed to feed for 24 hours. After the feeding period, the *N. tenuis* adults were removed and the number of preyed eggs was counted with the help of a dissecting microscope. Experimental conditions were 25 ± 2 °C, 50% ± 10% RH and 14L:10D h photoperiod.

3.2.4. Statistical analysis

The variation for necrotic rings, wilting and zoophagy was analyzed in two steps. For necrotic rings, we first fitted a linear mixed-effect model (LMM) on the square-root-transformed data to estimate the genetic variation between isofemale lines. In this model, *leaf* type (*young* or *old*) and *mortality* were entered as fixed effects, and *block* and *line* (9 isofemale lines) were entered as random effects. This model estimates the variance components by REML, which allow for the estimation of the broad-sense heritability (H^2)

in isofemale lines (Falconer and Mackay, 1996; Wajnberg, 2004). To analyze possible differences in the number of necrotic rings between the isofemale lines, a second LMM was fitted with *block* in the random structure and *leaf*, *mortality* and *line* in the fixed structure. *Line* fitted as a fixed effect allows for the comparison of the number or necrotic rings between the isofemale lines.

Similarly, wilting percentage was also analyzed following these two steps. First, genetic variation was estimated with an LMM fitted on arcsine-transformed data, with *leaf* and *mortality* as fixed effects, and *block* and *line* as random effects. The comparison of wilting percentages between isofemale lines was done by fitting a second LMM with *block* as random effect and *leaf*, *mortality* and *line* as fixed effects. For zoophagy, genetic variation was estimated by fitting an LMM on square-root-transformed data, with *sex* as fixed effect and *line* as random effect. Comparison between isofemale lines was done after fitting a linear model on the square-root-transformed-data with *sex* and *line* as fixed effects. Model selection was done based on the Akaike Information Criterion (AIC); models with the lowest AIC were selected as best-fitted models (Bolker et al., 2009) (Table 3.1). Significant fixed effects were followed by multiple comparison between isofemale lines with Bonferroni correction ($\alpha = 0.05$). Finally, correlations between zoophagy, necrotic rings and wilting percentage were estimated within isofemale lines. All statistical analyses were performed in R (version 3.6.1) (R Core Team, 2009).

Table 3.1. Akaike Information Criterion (AIC) for different linear mixed-effect models (LMM) for number of necrotic rings and wilting percentage inflicted on tomato plants (n = 240 individuals from 9 isofemale lines) and zoophagy (i.e. consumption of *E. kuehniella* eggs in 24h) (n = 193 individuals from 8 isofemale lines) by *Nesidiocoris tenuis* adults. Values in bold indicate the model selected based on the lowest AIC.

Fixed effects	**Random effects**	**Akaike Information Criterion (AIC)**		
		Necrotic rings	**Wilting**	**Zoophagy**
Leaf + Mortality	Block + Line	457.21	**304.56**	
Leaf + Mortality	Line	493.52	314.91	
Leaf + Mortality	Block	463.03	310.01	
Leaf	Block + Line	**451.19**	308.96	
Mortality	Block + Line	468.64	321.92	
Sex	Line			**714.31**
Sex				769.89

3.2.5. Heritability

As an estimate of the genetic variance, the broad-sense heritability (H^2) was calculated, which is the ratio of the total genetic variance (V_G) (i.e. additive, dominance and epistatic) to the total phenotypic variance (V_P) (Falconer and Mackay, 1996) (Table 3.2). The ratio V_G / V_P expresses the extent to which the phenotype of an individual is determined by its genotype, hence it is also known as the degree of genetic determination (Falconer and Mackay, 1996). For necrotic rings and wilting, V_G was represented by *between-line* variance and V_P was represented by the sum of *between-line* variance and environmental variance (i.e. block variance and residual variance). For zoophagy, V_G was represented by *between-line* variance and V_P was represented by the sum of *between-line* variance and *within-line* variance. Additionally, we calculated the coefficient of genetic variation (V_G) for phytophagy and zoophagy traits from the estimated genetic components with the formula

$$CV_G = 100 * \frac{\sqrt{V_G}}{\bar{X}}$$

where V_G is the genetic variance and $\bar{X}$ is the trait mean. The coefficient of genetic variation (V_G) is another parameter used as an indication of the ability of a population to respond to natural or artificial selection, i.e. the evolvability of a trait (Houle, 1992).

Table 3.2. Estimates of the means and standard errors (SE), genetic variation (VG), environmental variation (VE), broad-sense heritability (H2) and coefficient of genetic variation (CVG) for infliction of necrotic rings and wilting on tomato leaves, and zoophagy (i.e. consumption of *Ephestia kuehniella* eggs in 24h) by *Nesidiocoris tenuis*. Estimates are based on squared-root-transformed data for necrotic rings and zoophagy, and arcsine-transformed data for wilting.

Parameter	***n***	**Mean ± SE***	**V_G**	**V_E**	***H^2***	**CV_G (%)**
Necrotic rings	240	1.177 ± 0.044	0.070	0.444	0.16	22.54
Wilting	240	1.254 ± 0.032	0.039	0.217	0.18	15.77
Zoophagy	193	7.961 ± 0.131	1.190	3.231	0.37	13.70

3.3. Results

3.3.1. Phytophagy

The number of necrotic rings was significantly different between *old* leaves and *young* leaves ($F_{1, 221} = 17.86$, $p < 0.0001$), with *young* leaves showing more necrotic rings (2.1 ± 0.2) than *old* leaves (1.4 ± 0.2). The number of necrotic rings inflicted on the tomato plants also differed across isofemale lines ($F_{8, 221} = 3.82$, $p = 0.0003$). Isofemale line 14 inflicted the highest number of necrotic rings for both types of leaves, with an average of 2.9 ± 0.5 for *young* leaves and 2.1 ± 0.4 for *old* leaves. The isofemale line producing the least necrotic rings on both types of leaves was isofemale line 10, with an average of 1.5 ± 0.3 for *young* leaves and 1.1 ± 0.2 for *old* leaves (Figure 3.1). The broad-sense heritability for necrotic rings infliction was $H^2 = 0.16$ (likelihood-ratio test: $\chi^2_1 = 7.74$, $p = 0.005$) (Table 3.2).

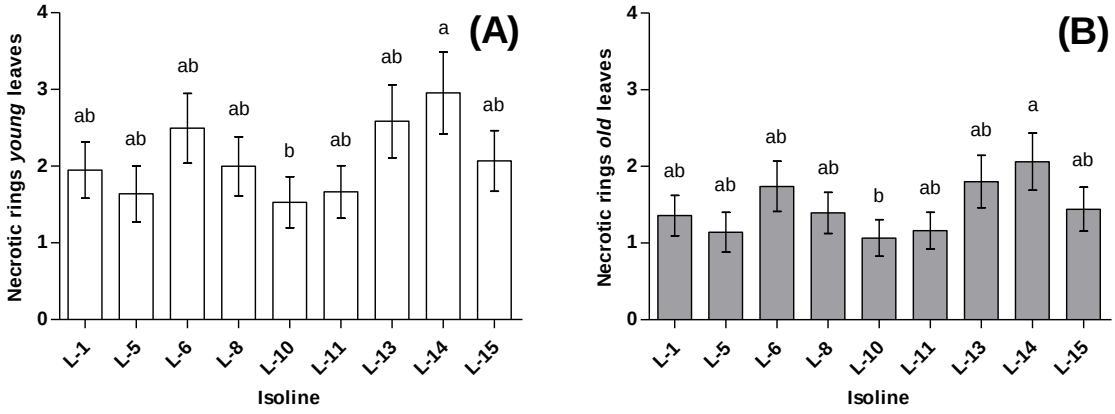

Figure 3.1. Average necrotic rings inflicted by *Nesidiocoris tenuis* on tomato plants. **(A)** Average necrotic rings in *young* leaves, **(B)** Average necrotic rings in *old* leaves. Bars sharing letters are not significantly different; error bars represent SE.

For wilting percentage, significant differences were observed between leaf types ($F_{1, 220} = 27.78$, $p < 0.0001$). In *young* leaves the percentage of wilting reached an average of 42.3 ± 5.9 % whereas in *old* leaves the wilting average was 25.5 ± 5.5 %. Differences were also observed between isofemale lines ($F_{8, 220} = 518$, $p < 0.0001$). Isofemale line 13 produced the highest wilting proportion on both *young* and *old* leaves (80.3 ± 9.8 and

63.1± 13.9, respectively) whereas the lowest proportion of wilted leaves was produced by isofemale line 15, with 28.6 ± 12.2 for *young* leaves and 14.4 ± 7.7 for *old* leaves (Figure 3.2). Mortality showed a significant effect on the wilting percentage ($F_{1, 220}$ = 15.96, p = 0.0001), with the highest observed mortality in isofemale line 8 (5.79 ± 0.35) and the lowest in isofemale line 15 (2.85 ± 0.36). The estimated broad-sense heritability for wilting was H^2 = 0.18 (likelihood-ratio test: χ^2_1 = 7.45, p = 0.006) (Table 3.2)

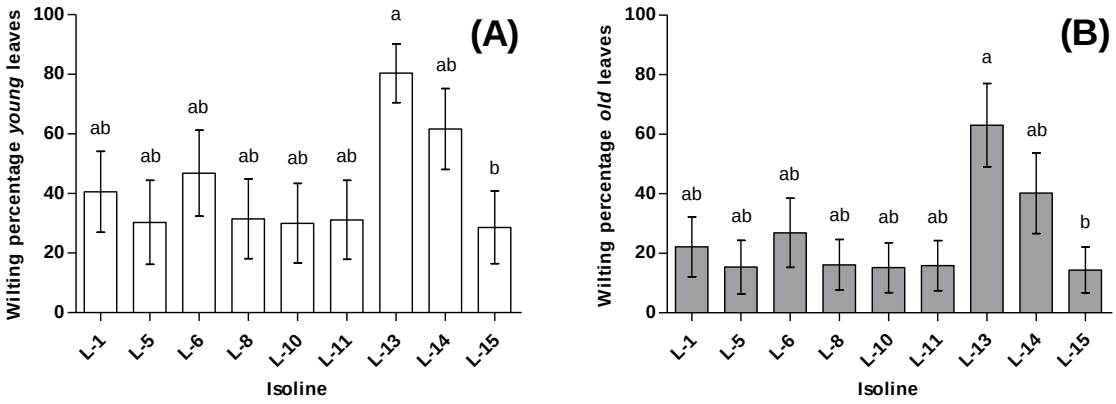

Figure 3.2. Average wilting percentages inflicted by *Nesidiocoris tenuis* on tomato plants. **(A)** Average wilting percentage in *young* leaves, **(B)** Average wilting percentage in *old* leaves. Bars sharing letters are not significantly different; error bars represent SE.

3.3.2. Zoophagy

The consumption of *E. kuehniella* eggs in 24h significantly differed between sexes ($F_{1,184}$ = 24.18; p < 0.0001). For females, isofemale line 15 showed the overall highest predation rate with 99.2 ± 5.0 eggs preyed in 24 hours, whereas isofemale line 13 preyed the lowest rate with 44.3 ± 5.2 eggs (Figure 3.3 A). In the case of males, the highest predation was also observed in isofemale line 15 (84.7 ± 5.4), whereas isofemale line 13 showed the lowest predation rate with 34.8 ± 5.1 eggs in 24h (Figure 3.3 B). The amount of *E. kuehniella* eggs preyed upon also differed across isofemale lines ($F_{7,184}$ = 14.45; p < 0.0001) (Figure 3.3 A-B). Broad-sense heritability estimate for zoophagy was H^2 = 0.37 (likelihood-ratio test: χ^2_1 = 57.57, p < 0.0001) (Table 3.2).

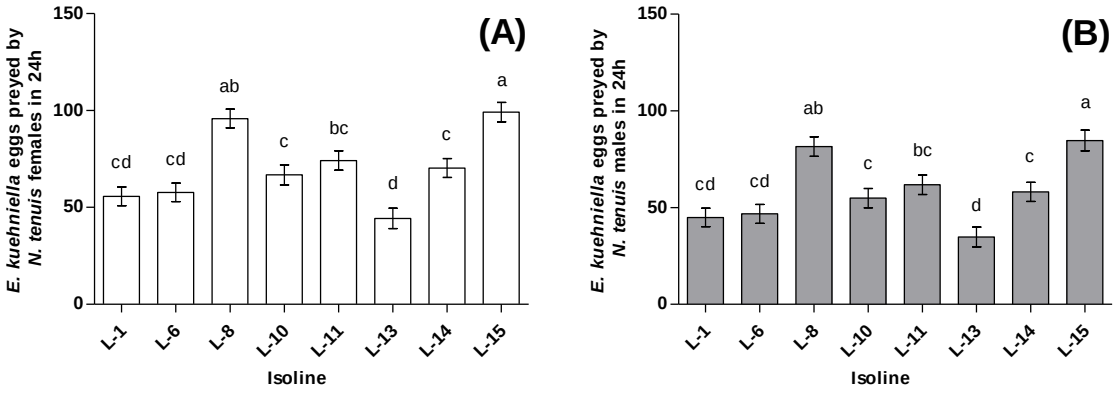

Figure 3.3. Average *Ephestia kuehniella* eggs preyed by *Nesidiocoris tenuis* in 24 hours. **(A)** Average *Ephestia kuehniella* eggs preyed by *Nesidiocoris tenuis* females **(B)** Average *Ephestia kuehniella* eggs preyed by *N. tenuis* males. Bars sharing letters are not significantly different; error bars represent SE.

The number of necrotic rings was positively correlated with the wilting percentage (Spearman's rho = 0.46, $p < 0.0001$) (Figure 3.4). Wilting percentage and zoophagy were negatively correlated (Spearman's rho = -0.19, $p = 0.0041$) and there was no significant correlation between necrotic rings and zoophagy (Spearman's rho = -0.06, $p = 0.3442$).

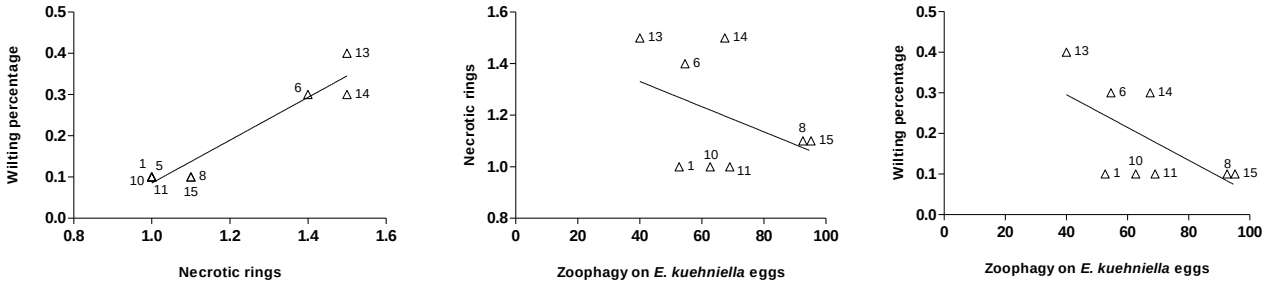

Figure 3.4. Correlation between wilting percentage and necrotic rings, necrotic rings and zoophagy on *Ephestia kuehniella* eggs, and wilting percentage and zoophagy on *Ephestia kuehniella* eggs by *Nesidiocoris tenuis* on tomato leaves. Numbers represent the isofemale lines ID.

3.4. Discussion

The presence of genetic variation in a trait is fundamental for selection processes to take place. In biocontrol agents, the existence of natural genetic variation in traits of interest provides the opportunity to select for lower or higher values in those traits that could be useful in the biocontrol practice (Ferguson et al., 2020a; Kruitwagen et al., 2018; Leung et al., 2020; Lirakis and Magalhães, 2019; Lommen et al., 2017). To the best of our knowledge, this is the first study to quantify the genetic variation in the feeding behavior of isofemale lines of *N. tenuis*. Our study adds to the growing body of literature reporting quantitative values of genetic variation, needed to enable successful genetic improvement of biological control agents (Ferguson et al., 2020a). The phytophagy experiment showed that *N. tenuis* ability to produce necrotic rings and wilting on tomato plants differed across isofemale lines and leaf types. Similarly, differences were also observed for zoophagy across isofemale lines in their consumption of *E. kuehniella* eggs.

Necrotic rings in the stems and petioles of tomato plants are the most visible injuries produced by *N. tenuis* (Pérez-Hedo and Urbaneja, 2016). Our results showed that these injuries were more abundant in *young* leaves than in *old* ones. Similarly, wilting percentage on *young* leaves was higher than that of *old* leaves. This is consistent with previous studies that reported higher number of necrotic rings in younger and softer plant tissues (Castañé et al., 2011; Perdikis et al., 2014). Thus, this suggest that the differences in the level of damage observed between leaf types are likely related to a gradient in tenderness and/or susceptibility of the leaf tissues. However, an experiment on the feeding behavior of the isofemale lines in different plant tissues is necessary to confirm this, since morphological differences (e.g. trichomes, exudates) could also influence the predator behavior, and thus the damage level. Interestingly, the phytophagous behavior of the isofemale lines was consistent across the two types of leaves for the two phytophagy parameters evaluated, i.e. isofemale lines inflicting more damage in *young* leaves were also the isofemale lines inflicting more damage in *old* leaves. Moreover, the positive correlation between necrotic rings and wilting also revealed consistency in the damage inflicted per isofemale line, with the isofemale lines inflicting more necrotic rings as the isofemale lines causing more wilting too, thus reinforcing the presence of an isofemale line effect.

Our results show variability in the levels of necrotic rings and wilting percentage inflicted across the isofemale lines. However, the genetic component in both of these phytophagy parameters is rather limited, as suggested by the low H^2 values. Besides the drawbacks commonly experienced by isofemale lines maintained for a number of generations in the laboratory, such as genetic drift, (Hoffmann and Parsons, 1988), other factors specific to zoophytophagous species can also explain low genetic variation in some of their traits. Castañé et al. (Castañé et al., 2011) pointed at the complex interaction of physiological, behavioral and morphological aspects of the insect, the host plant and the environment that leads to the damage occurrence when zoophytophagous mirid predators are present.

For instance, as many hemipterans, *N. tenuis* is a zoophytophagous insect that needs plant tissue for both feeding and oviposition (Wheeler, 2001). Phytophagy is regarded as essential for extraoral digestion and survival of zoophytophagous species rather than facultative (Castañé et al., 2011), and securing a favorable environment for their offspring is fundamental for several species (Thompson and Pellmyr, 1991), especially when the eggs are inserted in the plant tissue as is the case for *N. tenuis*. Thus, it is possible that the low genetic variation observed in the phytophagy-related traits is a by-product of selection acting on other related traits (Hedrick and Riechert, 1989), such as reproduction. In addition, low heritability is consistent with strong selection pressure (Merilä et al., 2001). It is suggested that behavioral traits might be under the same type of selection as life history traits (Mousseau and Roff, 1987), which heritability values are low often times. Thereby, the H^2 values observed for phytophagy might be an indication of strong selection controlling this specific feeding strategy. Furthermore, genetic variation of species associated to cropping systems tends to be limited as a consequence of a homogeneous habitat (i.e. monocultures) that favors some genotypes of the populations (Mitter and Futuyma, 1983). The individuals for our initial population were collected from tomato farms, where plant resources are homogeneous and readily available through the year. Hence, individuals collected could be those genotypes already adapted to better exploit plant resources, thus preventing the capture of a wider diversity for phytophagy traits. Nevertheless, the estimates of evolvability (CV_G) for the phytophagy traits reveal that in the case of necrotic rings, the low H^2 value could be likely the consequence of a larger environmental variance and not necessarily due to low genetic variance. Thus, some degree of response to selection might still be expected for necrotic rings.

Zoophagy in *N. tenuis* is essential for its development (De Puysseleyr et al., 2013; Urbaneja et al., 2005). In the present study, we found genetic variation for zoophagy among the *N. tenuis* isofemale lines. Larger differences between isofemale lines for this trait and higher H^2 values suggest a greater genetic basis for the differences found in zoophagy than for those observed in phytophagy traits. This larger variation is consistent with the conditions of constant fluctuations in prey densities and prey species faced by *N. tenuis* in cultivated systems. Other studies also argued the spatial and temporal changes in prey levels in the agricultural systems as an important cause for the genetic variation in other predatory invertebrates (Dumont et al., 2016; Nachappa et al., 2010). In addition, the larger variation and larger H^2 values observed in this trait suggests that zoophagy is probably under less strong selection pressure (Merilä et al., 2001), hence allowing for faster responses to changing environments (Nachappa et al., 2010), such as those experienced in agroecosystems in terms of prey. Interestingly, and opposite to the estimates for necrotic rings, the estimate of evolvability (CV_G) for zoophagy is low relative to a moderate value of H^2. This is likely explained by a lower environmental variance relative to the genetic variance observed for this trait.

Previous studies with natural populations of *N. tenuis* have shown that plant damage decreases when prey availability increases in tomato plants (Sánchez, 2008), and greenhouse experiments with commercial strains have also shown a negative correlation between phytophagy and zoophagy (Calvo et al., 2009; Castañé et al., 2011). In the present study, the correlation of phytophagous traits and zoophagy was negative, but it was significant only for wilting percentage, not for necrotic rings. However, there is a trend for the most zoophagous isofemale lines to be also those which inflict less damage. An additional experiment that evaluates zoophagy and phytophagy of the isofemale lines simultaneously would provide a more clear view of this correlation.

The results of our study suggest an interesting potential of the genetic variation in the feeding behavior of *N. tenuis,* and of the isofemale lines approach as a tool to be exploited in genetic improvement programs of biological control agents. Although the broad-sense heritability (H^2) calculated on isofemale lines can sometimes lead to overestimations of the actual heritability in natural populations (Hoffmann and Parsons, 1988), the existence of a genetic component in feeding-related traits of a biocontrol agent is promising. The lower values of H^2 and the smaller differences between isofemale lines observed in

phytophagy, in addition to the diverse nature of the factors influencing its variation, suggest this trait could be more challenging to target in breeding programs. Although the evolvability observed for necrotic rings is still a good sign for potential selection and should be further investigated. Conversely, the larger variation observed in zoophagy represents a positive output for biocontrol improvement. The existence of isofemale lines showing higher predation rates could favor a decrease in the predator: prey ratio currently used in crops (Dumont et al., 2018). Hence, the use of less individuals of these "zoophagous" isofemale lines would still allow a successful pest control while reducing the exposure of crops to phytophagy and potential damage. It would be important to replicate these experiments with individuals collected in different environments and host plants, especially from non-agricultural settings, to increase the possibilities of capturing higher diversity of wild populations.

3.5. Conclusions

The presence of genetic variation in traits of biocontrol interest is fundamental for selection of improved biocontrol agents. Our study is the first to quantify the genetic variation of the feeding behavior of *N. tenuis*. Phytophagy-related traits might be more challenging to select against due to the interaction of several factors, whereas the variation found for zoophagy-related traits is promising for the biocontrol practice. As such, our study adds to the growing body of literature reporting quantitative values of genetic variation, needed to enable the successful genetic improvement of biological control agents.

Author Contributions: Author contributions: Conceptualization MC-R, AU, MP-H and BP; methodology MC-R; validation AU, MP-H and BP; formal analysis MC-R and BP; investigation MC-R; resources AU and MP-H; data curation MC-R and BP; visualization MC-R, AU and MP-H.; supervision AU and MP-H; project administration AU and MP-H; funding acquisition AU, MP-H and BP; writing—original draft preparation MC-R; writing—review and editing AU, MP-H and BP. All authors have read and agreed to the published version of this chapter.

CHAPTER 4

Plant feeding by *Nesidiocoris tenuis*: Quantifying its behavioral and mechanical components

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Abstract

Zoophytophagous predators play an important, though sometimes controversial, role in pest management programs in different crops. In tomato crops, damage caused by phytophagy of the mirid *Nesidiocoris tenuis* has mainly been reported at high predator population levels or when prey is scarce. Previous research has focused on predator/prey ratios, stylet morphology and saliva composition to explain plant damage by *N. tenuis*. In this study, we investigated the behavioral and mechanical components of the phytophagy. For this, we compared the feeding behaviors of males, females and fifth-instar nymphs of *N. tenuis*. Additionally, we investigated the type of stylet activities performed by each stage while probing in plant tissue, using the electrical penetration graph technique (EPG). Furthermore, stylectomy was performed and plant histology studied with the aim to correlate the feeding activities observed in the EPG recordings with stylet tip positions in specific tissues of the leaf petioles. Behavioral observations during a 30-min period showed that nymphs probed more frequently (38.6 ± 1.5 probes) than males and females (25.3 ± 1.1 and 24.3 ± 1.1 probes, respectively). Similarly, nymphs spent a higher proportion of time (656.0 ± 67.6 s) feeding on tomato apical sections compared to males and females (403.0 ± 48.8 s and 356.0 ± 43.7 s, respectively). The EPG recordings during 5 h indicated that cell-rupturing was the main stylet activity for all insect stages, and that fifth-instar nymphs spent a higher proportion of time on cell-rupturing events compared to adults. The histological studies revealed a trend of *N. tenuis* for the tissues within the vascular semi-ring. The stylet tips were found both in the vascular bundles and in the parenchyma of the interfascicular region. The findings of this study confirm an important role of fifth-instar nymphs feeding behavior in the damage potential of *N. tenuis*. Moreover, the increased time spent on cell rupturing behaviour suggests that stylet laceration and enzymatic maceration of the saliva occurring during this event might greatly contribute to the inflicted damage. A comprehensive understanding of the interactions of *N. tenuis* with the plant, at both the behavioral and mechanical levels, might shed light on new approaches to minimize its damage potential to tomato while maintaining its benefits as biocontrol agent.

4.1. Introduction

The use of zoophytophagous predators for biological control of pests in agroecosystems has increased over the last decades (van Lenteren et al., 2018). *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) is one of these predators widely used in current biocontrol programs in Southern Europe, where it is occurring naturally and can spontaneously colonize vegetable crops (Arnó et al., 2010; Pérez-Hedo and Urbaneja, 2016). *Nesidiocoris tenuis* is commercially available and performs a crucial role in integrated pest management (IPM) programs in tomato (Albajes et al., 2006; Calvo and Urbaneja, 2004; Pérez-Hedo and Urbaneja, 2016; van Lenteren, 2012; van Lenteren et al., 2018). Advantages such as preying upon several key pest species, high predation efficiency and its capacity to stay in the crop under prey shortage conditions (Urbaneja et al., 2009, 2005) are some of the primary reasons this predator is considered a successful biocontrol agent in Southern Europe. Moreover, recent studies have demonstrated the benefits deriving from its phytophagy in terms of activation of plant defenses that enhance biological control (Bouagga et al., 2020, 2018a; Naselli et al., 2016; Pérez-Hedo et al., 2018, 2015b, 2015a). However, despite its services as biocontrol agents, under certain conditions damage caused by their phytophagy has also been reported. Plant damage ranges from necrotic rings in stems and petioles, to abortion of small fruits and flowers, reduced vegetative growth, and blemishes in fruits (Arnó et al., 2010; Calvo et al., 2009; Calvo and Urbaneja, 2004; Castañé et al., 2011; El-Dessouki et al., 1976; Pérez-Hedo and Urbaneja, 2016; Sánchez, 2008; Sánchez and Lacasa, 2008). The damage caused by *N. tenuis* can become very important in tomato crops cultivated in heated greenhouses and/or with low pest pressure. For instance, in northern Europe, where these conditions are common to tomato production, *N. tenuis* is considered a serious pest (Ferguson et al., 2020; Moerkens et al., 2020; Pérez-Hedo and Urbaneja, 2016).

Regardless of its damage potential, the success and widespread use of *N. tenuis* as a biological control agent in cultivated systems have prompted researchers to investigate the mechanisms underlying its phytophagy, and ways to reduce its negative impacts. For instance, research first focused on predator-prey interactions. Several studies have demonstrated that damage occurs mainly at high predator population levels and its severity is prey density-dependent, with an increase in number of necrotic rings as prey

populations decrease (Arnó et al., 2010; Calvo et al., 2009; Sánchez, 2008). The role of temperature has also been explored, and it was shown that the severity of the damage inflicted by *N. tenuis* increased at higher temperatures (Sánchez, 2008; Siscaro et al., 2019). Stylet morphology and saliva composition of important zoophytophagous species, including *N. tenuis*, have been studied aiming at finding the mechanisms underlying plant damage, but these factors alone did not explain *N. tenuis* damage potential (Castañé et al., 2011). Histological studies on stained tissues *N. tenuis* fed upon have also been carried out to characterize the damage (Raman and Sanjayan, 1984).

More recently, research trying to explain the mechanisms causing plant damage by zoophytophagous predators has changed the focus from general to more specific approaches. Hence, more attention has been given to biotic factors such as plant cultivar and plant interaction with microorganisms (Cabello et al., 2013; Garantonakis et al., 2018; Siscaro et al., 2019). For instance, mixed results have been reported regarding the influence of tomato cultivar on damage incidence by *N. tenuis*, with significant differences between cultivars reported by Cabello et al. (2013), whereas differences between cultivars found by Siscaro et al. (2019) were not significant. Moreover, the role of microorganisms associated to the plants in damage caused by zoophytophagous predators has been demonstrated for *N. tenuis* by Garantonakis et al. (2018), who reported that tomato plants inoculated with the endophytic strain *Fusarium solani* K had significantly less damage than non-inoculated plants. However, the behavioral aspects and the stylet activities of *N. tenuis* while piercing the plant remain unexplored.

Direct behavioral observations are a practical approach that has been applied to zoophytophagous species to study their phytophagous behavior (Bouagga et al., 2018a, 2018b). For *N. tenuis*, its behavior on sweet pepper plants was recently described by Bouagga et al. (2018a), however, its behavior on tomato has not been described yet. Additionally, the feeding behavior of piercing-sucking insects can be studied with the electrical penetration graph (EPG) technique. In brief, this technique consists of incorporating the plant and the insect as components of an electrical circuit: one of the electrodes holds a wired insect (EPG probe) and the other electrode is a copper post that is inserted in the soil of the potted plant. When the insect pierces the plant, the circuit is closed and the different activities of the stylets in different tissues are recorded as waveforms, hence allowing for an *a posteriori* biological interpretation (Tjallingii 1978).

Although EPG has been most often used to study feeding behavior of aphids (Fereres and Collar, 2001; Garzo et al., 2016; Jiménez et al., 2019; ten Broeke et al., 2013; Tjallingii, 1985, 1978) and other piercing-sucking insects (AB Ghaffar et al., 2011; Antolinez et al., 2017; Guedes et al., 2018; Jin et al., 2012; Lucini and Panizzi, 2016), its application to other Hemiptera, such as Miridae, is rather recent (Backus et al., 2007; Cervantes et al., 2016; Cline and Backus, 2002).

In the present work, the behavior and stylet activities (i.e. cell rupturing and ingestion) of *N. tenuis* on tomato were investigated in order to determine their role in phytophagy. First, the feeding behavior of males, females and fifth-instar nymphs of *N. tenuis* on excised tomato apical sections was quantified and compared. Second, the stylet activities of males, females and fifth-instar nymphs of *N. tenuis* during probing events (i.e. the time the stylets remain inserted in the plant tissue) were evaluated with EPG. Finally, stylectomy and histological preparation of tomato petiole sections containing the inserted portion of the cut stylets of *N. tenuis* were performed to identify the plant tissues reached.

4.2. Materials and Methods

The experiments were performed in three different laboratories. The behavior observation experiment was carried out at the entomology laboratories of Instituto Valenciano de Investigaciones Agrarias (IVIA) in Valencia, Spain. The EPG recordings were performed in the entomology laboratories of Wageningen University in Wageningen, The Netherlands. The stylectomy experiment, the histological work and waveform characterization and identification was conducted at the entomology laboratories of Instituto de Ciencias Agrarias - Consejo Superior de Investigaciones Científicas (ICA-CSIC) in Madrid, Spain.

4.2.1. Behavioral observation

Plants and insects

A rearing of *N. tenuis* was established in the laboratory in a plastic insect cage (60 x 60 x 60 cm) (BugDorm-2 insect tents; MegaView Science Co., Ltd, Taichung, Taiwan). Green bean pods (*Phaseolus vulgaris* L.) and eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) were provided twice a week as oviposition substrate and food source, respectively. Cohorts of similar age were obtained every week by placing three green bean pods in the rearing cage for females to oviposit during 3 days. After this time period, the green bean pods bearing mirid eggs were removed from the rearing cage and placed in plastic containers (14 x 14 x 8 cm), with an opening in the lid covered with fine mesh for ventilation. One fresh green bean pod and *E. kuehniella* eggs *ad libitum* were provided twice a week to the cohorts in each plastic container until they were at the developmental stage required for the experiments. Both the rearing and the cohorts were kept at 25 ± 2 °C, constant relative humidity of 50 ± 10 % RH and 14L:10D photo:scotoperiod. *N. tenuis* and *E. kuehniella* eggs were supplied by Koppert Biological Systems (Águilas, Murcia, Spain).

Tomato plants *cv.* Raf Supermarmande (Mascarell Seeds, Spain) used in this experiment were in vegetative stages V6 to V7 (ca. 30-40 cm height). Plants were grown in plastic pots (8 x 8 x 8 cm) and kept in pest-free climatic chambers until the start of the experiment, at the same experimental conditions previously described for the *N. tenuis* rearing.

Behavioral test

Insects used were isolated in test tubes and starved during 24 h, with water supplied through moistened cotton plugs. Less than 3-day-old females (presumably mated) and males, and fifth-instar-nymphs (N5) were used. One individual with its respective tomato plant apical section was considered a replicate. A total of 20-22 replicates per developmental stage were recorded. Previous studies have demonstrated the preference of *N. tenuis* for the apical part of the tomato plant (Castañé et al., 2011; Perdikis et al., 2014); hence only apical sections (i.e. the apical bud and the two youngest fully developed

leaves) were used for this experiment. The apical sections were excised and immediately placed inside a Petri dish (150 mm diameter) and covered with its lid. Then, one mirid was gently released inside the horizontally placed Petri dish at the base of the excised apical section. A piece of dry synthetic sponge was used to cover the excision point, to prevent the insects from feeding on the exudates produced by the cut or the water in the sponge. A new apical section was used for each replicate. Visual observation of feeding and trivial behaviors of the individuals started when the insect made the first contact with the plant tissue. Total observation time for each individual was 30 minutes. All behaviors exhibited by the insects and the time spent on each activity were documented. Observations were done under a Leica M165 C stereomicroscope with the Petri dishes in horizontal position. The time spent on each location inside the Petri dish was also documented. The locations were defined as follows:

- Apical bud (AB): apical bud
- Leaf 1 (L1): first fully developed leaf from the apical bud.
- Leaf 2 (L2): second fully developed leaf from the apical bud.
- Stem (ST): stem section to which the apical bud and the leaves were attached.
- Out of plant (OP): the insect was in contact with the Petri dish but not with the plant tissues.

Behavior descriptions were adapted from Bouagga et al. (2018a) and defined as follows:

- Feeding (F): the predator inserts its stylets into the plant tissue for more than two seconds. Stylets movements can be observed.
- Probes (P): the predator inserts the stylets for less than two seconds.
- Resting (R): the predator stands motionless.
- Searching (S): the predator is at rest but moves its antennae and/or taps on the plant with the stylets/proboscis tip.
- Walking-Searching (WS): the predator walks over the plant tissue, moves its antenna and taps on the plant with the stylets/proboscis.
- Cleaning (C): the predator uses forelegs or hindlegs to clean mouthparts and/or other parts of the body

- Out of plant (OP): the insect left the plant tissue and is in contact with the Petri dish only.
- Out of sight (X): when the insect reached parts of the plant tissue that were out of the sight of the observer from any possible angle, even after adjusting the Petri dish position (without disturbing the insect).
- Oviposition (O): The predator bends the abdomen and inserts the ovipositor into the plant tissue to lay an egg.

4.2.2. Electrical Penetration Graph (EPG) recordings

Plants and insects

A *N. tenuis* rearing was established with individuals provided by Wageningen UR Greenhouse Horticulture (Bleiswijk, The Netherlands), which were originally sourced from Koppert Biological Systems (Águilas, Murcia, Spain). Green bean pods (*Phaseolus vulgaris* L.) and *E. kuehniella* eggs (Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands) were provided twice a week as oviposition substrate and food source *ad libitum*, respectively. One tomato plant *cv.* Moneymaker was supplied weekly to the rearing for the mirids to get experience with the plant tissues used during the EPG recordings. The rearing was kept in a muslin cage (25 x 25 x 25 cm) at the same environmental conditions as described above for the behavior experiment.
Tomato plants *cv.* Moneymaker used in this experiment were in vegetative stages V5-V6 (ca. 30 cm height). Plants were grown in plastic pots (6 x 6 x 6 cm) and kept in a greenhouse compartment at 19–21 °C, 60-70% RH and 16L:8D h photo:scotoperiod.

EPG recordings

The EPG recordings were carried out inside Faraday cages to prevent electromagnetic interference. For wiring, the mirids were first anesthetized on ice for approximately one minute and then placed at the tip of a pipette tip (200 µl) connected to a vacuum under low suction. Then, a 2-3-cm-long gold wire (18 µm diameter) was attached to the dorsum of the insect using a small drop of water-based silver glue (EPG

Systems, Wageningen, The Netherlands). Insects used in this experiment were individually starved during hanging on their respective wires for 5 h. Water was not supplied during starvation since cotton plugs provided surface and traction for the insects to detach from their wires. To start the EPG recordings, the wired insect was carefully placed on the petiole of the second or third fully developed leaf from the apical bud of a potted tomato plant. We used less than 3-day-old females (presumably mated) and males, and N5 nymphs for the recordings. Fifteen replicates were recorded for males and females, and fourteen for N5-nymphs, for a total of 44 individuals. A new plant was used for each individual. EPG recordings were obtained with a Giga-8 DC-EPG device (EPG Systems) during an undisturbed 8-h period. EPG data acquisition and analysis were conducted using Stylet+ Software for Windows (EPG Systems).

The broad waveform classification into probing and non-probing behaviors for this experiment was done following Backus (2000), who defined as probing behaviors all behaviors from the start of the style insertion into the plant tissue until stylet withdrawal. The non-probing behaviors comprise all other behaviors that do not involve stylet penetration (Backus, 2000). Probing behaviors in cell rupture feeders are further classified into probing waveforms: cell rupturing (CR), transition (T) and ingestion (I) (Cervantes et al., 2016). The identification and classification of the probing waveforms for *N. tenuis* was based on the waveform library of the mirid species *Lygus lineolaris* (Palisot de Beauvois) and *Lygus hesperus* (Knight) (Hemiptera: Miridae) (Cervantes et al., 2016). Since waveforms T are suggested to be species dependent (Cervantes et al., 2016), and were scarce and not clearly distinguishable in our recordings, waveforms resembling T patterns were included as CR. For the purposes of this study, non-probing behaviors were not included in the analysis.

4.2.3. Stylectomy and plant tissue histology

Histological thin-section analysis was performed to correlate the position of the stylet tips with the cell rupturing and active ingestion waveforms observed during the EPG recordings. For this study, additional adults of *N. tenuis* were monitored on tomato petioles with a Giga-4 DC-EPG device (EPG Systems) under conditions similar to those of the previous EPG recordings. When the respective waveform of interest was observed,

the feeding activity was artificially terminated by stylet amputation with a tungsten needle of a Zapper RF micro-cautery unit (www.aphidzapper.com) following the methodology proposed by Downing and Unwin (1977). Petiole segments (ca. 0.5 cm) containing the severed stylets of *N. tenuis* were carefully removed from the plant with a scalpel. Then, the petiole segments (hereafter samples) were immersed in Karnovsky fixative at room temperature and placed under a low vacuum for 1 h to prevent air bubbles in the tissues. Afterwards, the samples were dehydrated in graded ethanol series (10-100%) and then infiltrated and embedded in paraffin. Serial transverse sections (15-20 µm thick) were cut on a Leica 1512 microtome and stained in 0.05% toluidine blue solution for 10 minutes. Permanent slides were prepared in mounting medium DePeX (SERVA Electrophoresis GmbH, Heidelberg, Germany) and examined using a Nikon Eclipse E800 microscope. Digital images were captured using the same microscope coupled with a Canon EOS 6D Mark II camera. Mounted transverse-sections were examined for any indication of salivary sheath and to determine the position of the stylet tips inside the petiole tissues. Petiole tissues examined were: epidermis, ground tissue (i.e. parenchyma between epidermis and vascular tissue), vascular tissue (i.e. vascular bundles and interfascicular region [i.e. parenchyma between vascular bundles]). In tomato petioles the vascular tissue is arranged in a semi-ring shape (Maiti et al, 2012).

Tomato plants *cv.* Moneymaker used in this experiment were in vegetative stages V3-V4 (ca. 15 cm height), smaller than those used for the EPG recordings of the previous experiment because of size restrictions of the stylectomy equipment. Plants were grown in pots (6 x 6 x 6 cm) in a climatic chamber at 14L:10D °C, 60-70% RH and 16L:8D h photo:scotoperiod.

4.2.4. Statistical analysis

Behaviors were analyzed with Generalized Lineal Models (GLM) with Poisson and quasipoisson error distributions, by using the function *glm* to assess differences in behaviors between developmental stages/sex (hereafter Stages/Sex). Stage/Sex was entered as independent variable for all behaviors except for oviposition. Significant differences between Stages/Sex were followed by multiple comparisons with Bonferroni correction ($\alpha = 0.05$), by applying the *emmeans* function. Differences in time spent on

each location were analyzed with GLM with quasipoisson error distribution. In this model, Location and Stage were entered as independent variables. Multiple comparisons were applied with the *emmeans* function (Bonferroni correction $\alpha = 0.05$) for the variables with significant differences.

The EPG data analysis was conducted based on 5 h out of the 8 h of recording time due to mortality of experimental insects observed after the 5th hour. EPG parameters were calculated for every mirid tested using the EPG analysis worksheet created by Sarria et al. (2009). Description of *N. tenuis* feeding behaviors was performed based on the variables defined by Backus et al. (2007). These variables were calculated for each waveform type (CR and I) and each cohort (in this study, cohort = N5-nymphs, males or females and N = number of individuals of the same cohort tested): total probing duration (TPD = sum of probing time per cohort/N), total waveform duration (TWD = sum of time spent by all individuals of the same cohort performing one waveform), number of waveform events per insect (NWEI = sum of events of one waveform type per cohort/N), waveform duration per insect (WDI = TWD/N), waveform duration per event per insect (WDEI = mean time spent in one waveform type per cohort/N), and time to first probe from the start of the EPG recording. Comparison of variable means across insect Stages/Sex were performed with nonparametric Kruskal-Wallis test followed by Dunn's test for multiple comparisons when variables did not meet normality assumptions. One-way ANOVA followed by Tukey's test for multiple comparisons, and Student's t-test were applied for variables following normality assumptions before or after transformation by ln(x), ln(x+1), sqrt(x), sin(x) or $1/x^2$. All statistical analyses were performed in R software (version 3.4.3).

4.3. Results

4.3.1. Behavioral observation

Significant differences were found between Stages/Sex for number of probes, feeding, resting and searching (Table 4.1). In contrast, no significant differences were found between Stages/Sex for time allocation to walking-searching, cleaning, out of plant and out of sight (Table 4.1). Multiple comparisons for significant Stages/Sex effect

revealed that N5-nymphs spent longer time feeding than both males and females (Z = -3.07, $P < 0.05$ and Z = -3.82, $P < 0.05$, respectively). Similarly, nymphs probed more frequently on the plant tissues than both males and females (Z = -7.16, $P < 0.05$ and Z = -7.98, $P < 0.05$, respectively). Resting time was higher in males than females (Z = -3.36, $P < 0.05$), but similar to that of nymphs (Z = 1.60, P = 0.329). Time spent searching was higher in females than males (Z = 2.79, $P < 0.05$), but did not differ from nymphs (Z = 0.61, P = 1.00).

Table 4.1. Number observed or duration in seconds (mean ± SE) spent by females, males and fifth-instar nymphs of *Nesidiocoris tenuis* performing eight different behaviors on tomato apical sections during 30 min observation periods. Significant differences between *Stages/Sex* are indicated by different letters (Bonferroni correction α = 0.05).

Behavior	***Nesidiocoris tenuis***			**Statistics**		
	Females (n = 22)	**Males (n = 20)**	**Nymphs (n = 20)**	**df**	***F***	***P***
Number of probes	24.3 ± 1.1 b	25.3 ± 1.1 b	38.6 ± 1.5 a	2	5.84	0.004
Feeding	356.0 ± 43.7 b	403.0 ± 48.8 b	656.0 ± 67.6 a	2	8.21	< 0.001
Resting	30.6 ± 17.2 b	232.2 ± 49.8 a	126.1 ± 39.8 ab	2	9.19	< 0.001
Searching	228.0 ± 30.5 a	117.0 ± 23.0 b	200.0 ± 32.5 ab	2	4.01	0.023
Walking-searching	698.0 ± 63.4 ab	813.0 ± 71.8 a	569.0 ± 65.2 b	2	3.11	0.052
Cleaning	209.0 ± 26.9 a	184.0 ± 26.4 a	122.0 ± 23.4 a	2	2.7	0.076
Out of plant	42.6 ± 21.3 a	78.2 ± 30.2 a	90.6 ± 35.3 a	2	1.12	0.333
Out of sight	14.7 ± 8.16 a	39.2 ± 13.97 a	22.0 ± 11.35 a	2	1.73	0.187
Oviposition	193.7 ± 28.5	-	-	-	-	-

Time spent on each apical section varied across locations (F_4 = 45.60, $P < 0.001$) but no differences were found among Stages/Sex (F_2 = 0.90, P = 0.390) and no Stages/Sex × location interaction was found (F_8 = 1.50, P = 0.163). All stages spent most of their time on Leaf 2 (L2) (56%) and the least Out of plant (OP) (4%) (Figure 4.1).

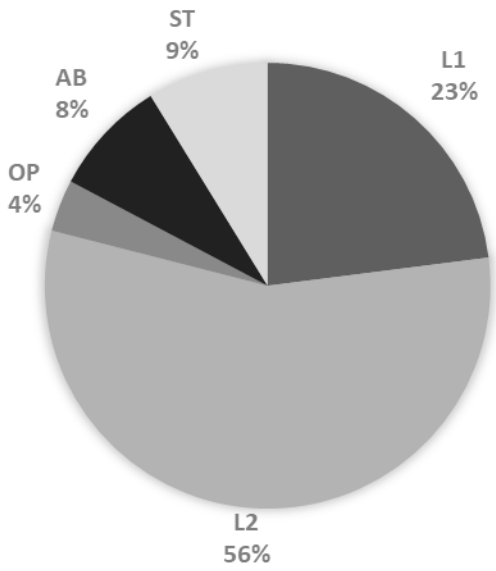

Figure 4.1. Proportion of time (percentage) spent by *Nesidiocoris tenuis* on different apical sections of tomato in the behavior experiment. AB: apical bud, ST: stem, L1: leaf 1 from the apical bud, L2: leaf 2 from the apical bud, OP: out of plant

4.3.2. Electrical Penetration Graph (EPG) recordings

Probing events recorded for *N. tenuis* (Figure 4.2a) showed irregular patterns for cell rupturing (CR) (Figure 4.2b), and regular, peak-and-wave patterns for ingestion (I) (Figure 4.2c). Variability in the fine structure of I was also observed (Figure 4.2d-i). Males, females and N5-nymphs spent proportionally more time on CR compared to I (Table 4.2). No significant differences were found across insect Stages/Sexes for total probing duration (TPD) and time-to-first probe since the start of the EPG recording (Table S 4.1).

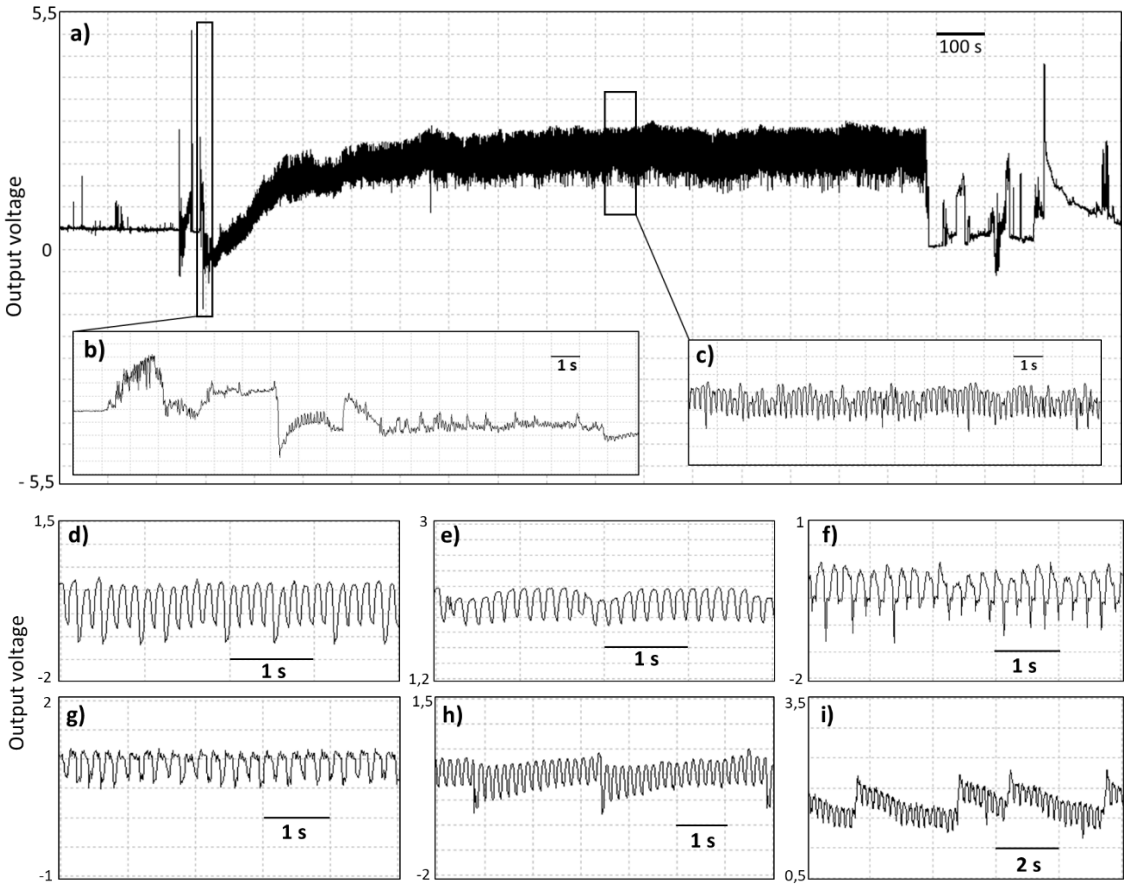

Figure 4.2. Overview of cell-rupturing (CR) and ingestion (I) waveforms produced by *N. tenuis* on tomato stems during EPG recordings; **(a)** probing event by *Nesidiocoris tenuis* with details of CR waveforms **(b)** and I waveforms **(c-i)**.

Probing behaviors: Cell Rupturing (CR)

Waveform duration per insect (WDI) was similar for N5-nymphs, females and males (H = 0.19; P = 0.910) (Figure 4.3A). The mean waveform duration per event per insect (WDEI) differed (F_2= 20; P < 0.0001), with N5-nymphs displaying the highest mean, followed by males and with females showing the lowest mean value (Figure 4.3C). Significant differences were found in the mean number of waveform events per insect (NWEI) (F_2= 12; P = 0.029), with females and males showing higher means compared to that of N5-nymphs (Figure 4.3E).

Table 4.2. Calculated total waveform duration (TWD) for CR and I waveforms in males, females and N5-nymphs of *Nesidiocoris tenuis*.

Insect stage	Waveform	TWD (s)	Percentage of time
Male (n = 15)	Cell rupturing	192,198.02	78
	Ingestion	54,654.2	22
Female (n = 15)	Cell rupturing	181,185.76	77
	Ingestion	52,782.32	23
N5-nymph (n = 14)	Cell rupturing	190,645.56	89
	Ingestion	24,455	11

Probing behaviors: active Ingestion (I)

No significant differences were found for WDI across insect Stages/Sexes for I (H = 2.0; P = 0.365) (Figure 4.3B). Similarly, the WDEI mean values were not significantly different across insect Stages/Sexes (H = 3.1; P = 0.217) (Figure 4.3D). Significant differences were observed for NWEI (F_2= 19; P < 0.0001), with females showing more ingestion periods, followed by males, and N5-nymphs showing the lowest number (Figure 4.3F).

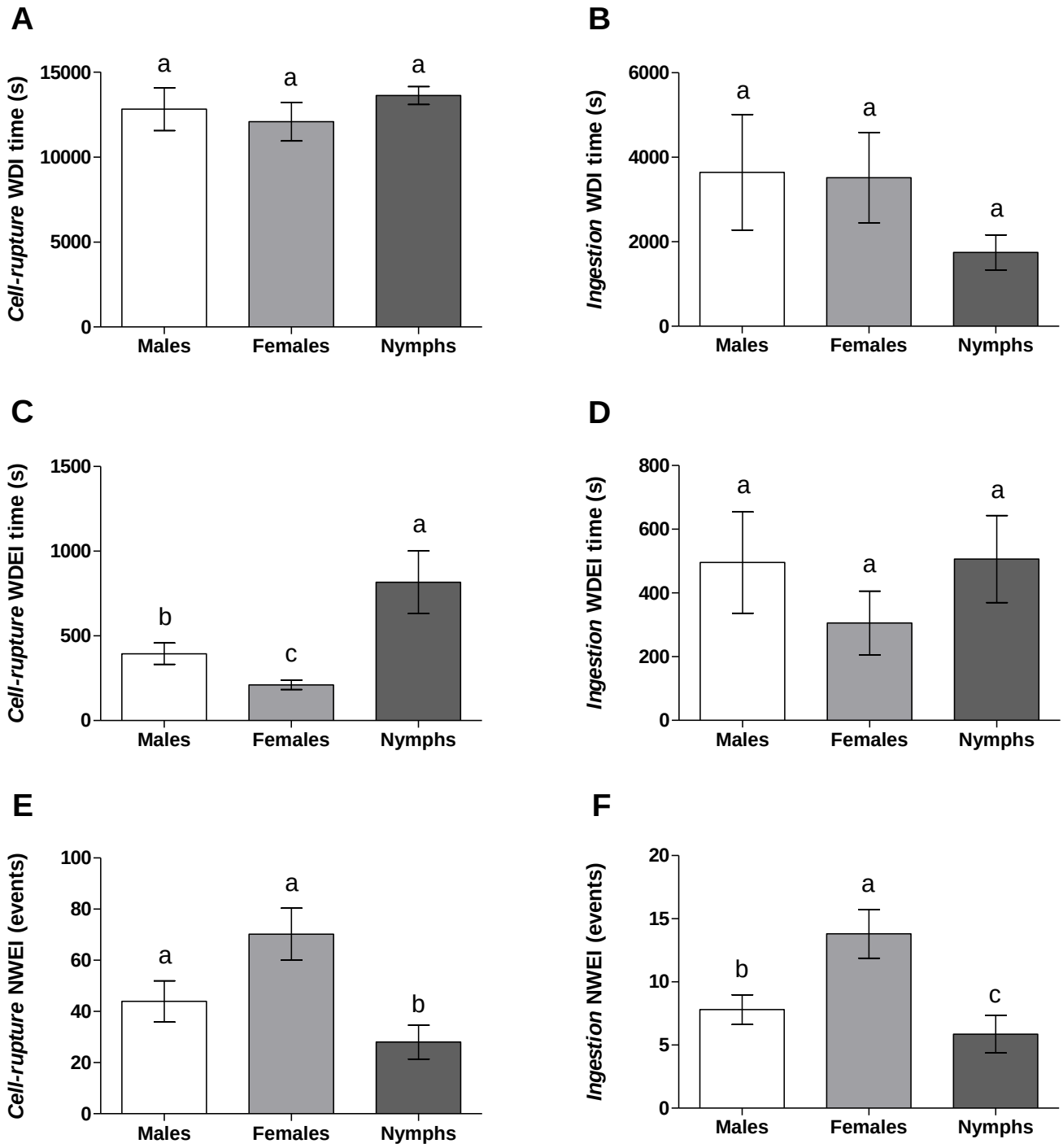

Figure 4.3. Calculated waveform duration per insect (WDI), waveform duration per event per insect (WDEI) and number of waveform events per insect (NWEI) for CR and I waveforms (means ± SE). Different letters indicate significant differences (Tukey test or Dunn's test, $\alpha = 0.05$).

4.3.3. Correlation between EPG waveforms and stylet tip positions in the plant tissue

Plant histological studies confirmed that *N. tenuis* does not generate a salivary sheath while performing CR waveform or I waveform in tomato petioles. The stylet tips during the CR waveform (n = 3) were located in the interfascicular region (i.e. parenchyma between vascular bundles, inside the vascular semi-ring) (n = 2) (Figure 4.4A), and in the vascular bundle (n = 1) (Figure 4.4B). For the I waveforms (n = 3) the stylet tips were located in the vascular bundle (n = 1) (Figure 4C) and in the interfascicular region (n = 2) (Figure 4.4D).

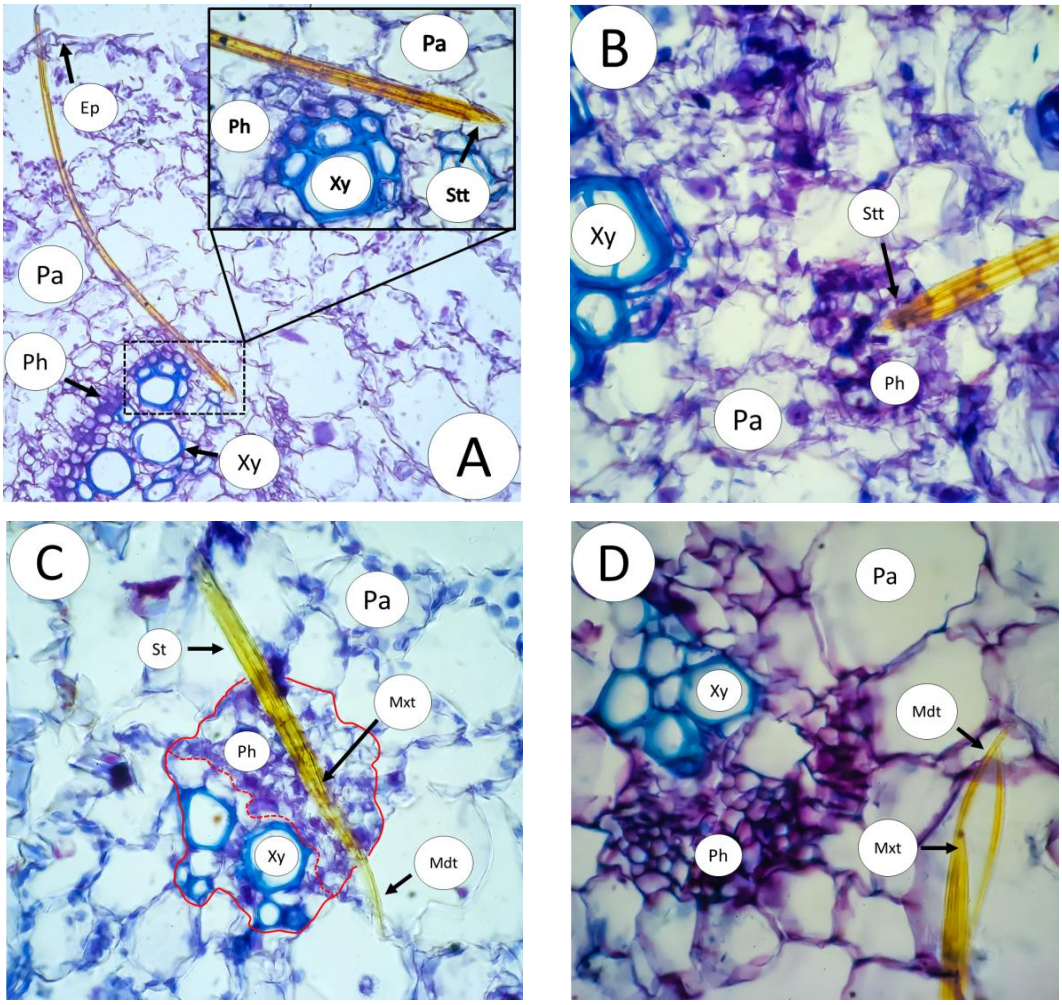

Figure 4.4. Light micrographs of cross-sections of tomato petioles containing severed stylets of *Nesidiocoris tenuis*. Stylet tip in: **(A)** parenchyma tissue (200x, and 1000x in expanded image), and **(B)** vascular bundle (1000x) during CR waveform. Stylet tip in: **(C)** vascular bundle (1000x) and **(D)** parenchyma (1000x) during I waveform. **Pa**: parenchyma, **Xy**: xylem, **Ph**: phloem, **Ep**: epidermis, **St**: stylet, **Stt**: stylet tip, **Mdt**: mandibular stylet tip, **Mxt**: maxillary stylet tip. In (C) the red solid line surrounds a vascular bundle, and the dashed line indicates the separation between phloem and xylem.

4.4. Discussion

In this study, the feeding behavior and stylet activities of immature and adult stages of *N. tenuis* in tomato were quantified, and their role in the plant feeding was investigated. Our findings show that N5-nymphs perform significantly more plant feeding activities than adult stages. Moreover, results of EPG studies suggest a primary role of cell rupturing behavior in the plant feeding compared to ingestion behavior, in all insect stages

analyzed in this study. Furthermore, histological studies revealed a trend of *N. tenuis* adults for probing and feeding from cells within the vascular ring region.

Previous studies reported a higher damage potential of *N. tenuis* nymphs compared to that of the adults (Arnó et al., 2006; Calvo et al., 2009; Perdikis et al., 2009). For instance, Arnó et al., (2006) demonstrated a two-fold difference in the number of necrotic rings caused by nymphs relative to adults in tomato side shoots. The present results revealed that N5-nymphs probe (i.e. insertion of the stylet) and feed for longer time in tomato than the adults, thus suggesting an important mechanical component in the damage potential of the different stages of *N. tenuis*. According to Hori (2000), the mechanical destruction is likely the primary cause of plant damage in heteropterans, with the rupture of cells by the stylets as the first step in the injury process. Contrary to salivary-sheath feeders (e.g. aphids, mealybugs), in which there is minimum disruption of plant cells (Miles, 1968), *N. tenuis* is a cell rupture feeder. In this common feeding strategy in mirids, the insect lacerates the plant tissue with the stylet movements, and injects watery saliva in the surrounding cells, forming pockets of diluted cell contents that will eventually be ingested (Backus et al., 2007, 2005; Cervantes et al., 2016; Hori, 2000). Therefore, the higher number of probes observed in N5-nymphs is likely among the main causes that could explain its higher damage ability, due to the continuous piercing of the plant tissues. However, although feeding time in N5-nymphs was found to be significantly higher than that of adults in the behavior experiment, conclusions about the damage potential of *N. tenuis* cannot be made on the basis of feeding time alone. This specific result is in conflict with the total probing duration (TPD) in the EPG experiment (i.e. feeding time = TPD: time the stylets remains inserted in the plant tissue), where no differences were found across insect stages. The 24 h starvation to which all insects were subjected before the behavior experiment could have affected N5-nymphs more severely than adults, thus likely explaining longer feeding time observed in this stage during the first minutes of plant contact (30 min of behavior experiment). In contrast, TPD results suggest that feeding time is similar across life stages when time of plant contact increases (5 h of EPG recording). Moreover, the starvation time before the EPG experiment was shorter (i.e. 5 h), which could also partially explain the similarities in TPD due to less severe conditions experienced by the insects. The lack of differences in the time to first probe suggest a similar acceptance of the host plant by all stages and sexes of *N. tenuis* evaluated.

The findings of the present study also revealed a preference of both N5-nymphs and adults of *N. tenuis* for the L2 leaf (second fully developed leaf from the apical bud). Although a conclusion about the influence of trichomes on *N. tenuis* location preference cannot be made on the basis of the data collected for this study, it is important to highlight the faster and smoother mobility along the petiole/leaflets of L2 for all insect stages (M. Chinchilla-Ramírez, personal observation). One study about the biomechanics of the interaction between *Dicyphus errans* Wolff (Hemiptera: Miridae) and several plant species revealed that performance of this omnivorous species on hairy plant surfaces was positively influenced by trichome length and diameter (Voigt et al., 2007). Hence, trichome characteristics of the different plant locations in tomato cannot be discarded as a factor influencing location preference. Further research addressing *N. tenuis* feeding behavior on tomato cultivars with different trichome density/types could provide valuable information for a more precise prediction of the damage location.

In the cell rupture feeders, the CR waveforms represent the probing behavior in which the plant cells are lacerated and macerated by the action of the stylet movements, and injection of watery saliva, respectively (Cervantes et al., 2016). The EPG results show that CR is performed about 77-89% of the total waveform duration (TWD) for the insect stages and sexes evaluated. This suggests a prominent role of CR behavior in the overall plant feeding of *N. tenuis*. These results are consistent with those from Tuelher et al. (2020), who noted that CR behavior in *L. lineolaris* were the primary reason for leaf damage in cotton. They argued a combination of probing-related wounding, and saliva-mediated solubilization over time, as the mechanisms underlying such damage. In our experiment, the remarkably longer CR events (WDEI) contrast with the low number of CR counts (NWEI) in N5-nymphs. This suggests that when plant tissues are exposed to N5-nymphs they endure fewer but longer periods of laceration and maceration than when exposed to adults, hence partially explaining the increased damage capacity of nymphs observed in previous studies (Arnó et al., 2006; Calvo et al., 2009; Perdikis et al., 2009). This also suggests that nymphs might be deploying a “quality over quantity” strategy, with longer CR events allowing for better enzymatic digestion of cell contents previous to I events, thus providing the nymphs with ingestion of more readily available nutrients. Cervantes et al. (2016) observed several periods of walking/waiting between single CR events and I events in *Lygus* spp, and argued the enabling of more salivary degradation on cell contents as a likely reason for this behavior. These longer CR events could also

explain the increased feeding time observed in N5-nymphs relative to adults in the beavioral observation experiment.

During the ingestion (I) waveforms, the cell-rupture feeder uses its cibarial pump to swallow the pre-digested cell contents mixed with watery saliva through the stylets (Cervantes et al., 2016). Overall, I activity was numerically lower than CR as demonstrated by WDI, WDEI and NWEI mean values. Moreover, ingestion was performed only about 11-23% of the TWD by all insect stages and sexes evaluated in this study. Hence, the role of ingestion activity is presumably minor in the plant feeding behavior of N5-nymphs and adults of *N. tenuis*, compared to CR behavior. The low proportion of time spent on I activity found in this study are consistent with those reported for different life stages of *Lygus* spp. (Cervantes et al., 2016; Cline and Backus, 2002). It is worth mentioning that although not all parameters for I activity showed significant differences, there was a trend for N5-nymphs that these were numerically lower than for adults. This could mean that N5-nymphs are less efficient at ingestion as a consequence of smaller size and/or characteristics of the saliva, as suggested by Tuelher et al. (2020). Deficient ingestion in nymphs could also mean more enzymatic saliva left in the plant tissue compared to more efficient ingestion in adults, thus causing more damage over time due to maceration. The decreased ingestion efficiency in N5-nymphs is further supported by its TWD, which is < 50% of that observed in adults. Shorter stylets in immature stages have been suggested as limiting factor for feeding (Cooper & Spurgeon, 2013), and it is likely an additional reason for this decreased efficiency.

The histological studies revealed a trend of *N. tenuis* to perform both CR and I in the tissues comprised in the vascular semi-ring when piercing on the petiole. Stylet tips corresponding to either CR or I waveforms were all found in vascular bundles or in parenchyma cells of the interfascicular region. Similar results were reported in previous studies based on histological sections of stained tissues with damage inflicted by *N. tenuis* (Raman and Sanjayan, 1984). Different position of mandibular stylet tips relative to maxillary stylet tips was observed in some samples from both CR and I events, hence laceration is likely occurring during both probing activities, and in the different tissues reached by the stylets. Our results suggest that *N. tenuis* does not feed on a specific cell type within the vascular semi-ring. Instead, *N. tenuis* creates pre-digested pockets of mixed contents from cells in this region, which could vary in nutrient contents depending

on its proximity to the phloem. This “unspecific” cell selection is further supported by the fine structure and polarity of the I waveforms observed during the EPG recordings. The peak-and-wave structure is common in active ingestion (contrary to passive ingestion typical of phloem feeders) where the regular pattern is attributed to the rhythmical pumping and swallowing produced by the cibarial muscle (Cervantes et al., 2016; Dugravot et al., 2008; Lucini and Panizzi, 2016). Additionally, the positive polarity of the probes observed in our recordings is contrary to the negative polarity expected from intracellular stylet penetrations (Walker, 2000). Further studies with more histological samples are necessary to confirm these results, and to determine whether other tissues are also targeted under other circumstances, such as prey availability.

The findings of this study provide insights about the role of feeding and probing behavior in the plant feeding by *N. tenuis*. CR probing events stand out as a primary mechanical component of the overall phytophagy of the insect stages evaluated. The increased number of probes and longer CR events observed in N5-nymphs could be the mechanisms underlying the higher damage potential of this life stage. Based on EPG results and the histological observations, most CR events are then expected to occur in the vascular region, thus probably comprising important damage to plant nutrient transport as well. Overall, this study broadens the understanding of the mechanical aspects underlying the phytophagy of *N. tenuis* on tomato. This could be useful in the development of new methods aimed at diminishing its negative impacts. For instance, plant breeders could benefit from this knowledge to target specific plant tissues and develop cultivars less susceptible to suffer from *N. tenuis* phytophagy.

Author contribution: MC-R: conceptualization, methodology, formal analysis, investigation, data curation, visualization, writing-original draft. EG: conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, visualization, supervision. AF: conceptualization, validation, formal analysis, visualization, supervision. JG-V: methodology, investigation. CtB: conceptualization, validation, supervision. JvL: validation, resources, supervision. AU: conceptualization, validation, resources, visualization, supervision, project administration, funding acquisition. MP-H: conceptualization, validation, resources, visualization, supervision, project administration, funding acquisition.

CHAPTER 5

Discussion and conclusions

5.1. General Discussion

The implementation of biological control in agricultural systems to suppress pests has experienced an important growth over the last century. This growth have led to the professionalization of the sector (van Lenteren, 2012) and its expansion is expected to continue considering the several challenges that agriculture is faced with. For instance, invasive pests, the transformation of secondary pest into key pests as a consequence of the limitation or ban of certain pesticides, or the development of resistance to pesticides still permitted, are just some of those challenges. Equally important is the pressure from consumers that demand products cultivated more sustainably and with less pesticide residues. However, the biocontrol sector is also experiencing challenges as the pressure for new BCAs increases. For example, recent studies have raised concerns about the introduction of exotic species for classical and augmentative biocontrol (De Clercq et al., 2011). Additionally, global regulations on the use and/or importation of exotic species have become stricter (Cock et al., 2010; van Lenteren, 2012). As a consequence, there has been an important shift from the use of exotic species to indigenous species as BCAs in the last 20 years (van Lenteren et al., 2020). The combination of the challenges affecting agriculture and the biocontrol sector has promoted a renewed interest in the selective breeding of BCAs as a tool to improve plant protection strategies.

Although the attributes of arthropods, such as small size and short life cycles, make these organisms the perfect candidates for selective breeding programs, this approach has not yet been widely adopted by the biocontrol sector. One of the obstacles faced by biocontrol researchers and industry when attempting to improve species by selection is the lack of genetic information/resources. For instance, the sequencing of arthropod genomes has been mainly centered in model organisms, vectors of diseases and agricultural pests, whereas BCAs genomes are still neglected (Li et al., 2019). Recently, within the frame of the BINGO project (https://www.bingo-itn.eu/en/bingo.htm), the genome of *N. tenuis* was sequenced and annotated for the first time (Ferguson et al.,

2020b), thus facilitating the study of this species at the genomic and genetic levels. One of the immediate applications of the sequenced genome is reflected in **chapter 2** of this thesis, in which **novel microsatellite markers were developed to study the population genetics of *N. tenuis***. The availability of these markers would facilitate, for example, the monitoring of the spatial distribution of the species, especially in the context of climate change, that is enabling the activity and invasion of species from warmer regions to temperate zones, where *N. tenuis* is usually considered a pest (Moerkens et al., 2020; Pérez-Hedo and Urbaneja, 2016). Therefore, plant breeders could tailor their new tomato varieties accounting for the population genetic aspects of *N. tenuis* present in those regions.

Traditionally, the design of pest control strategies has focused on the detailed knowledge of the pests and the (environmental and genetic) factors determining their biology. However, an effective control program should also encompass the same knowledge about all the species interacting in the agroecosystem (Narang et al., 1993), especially the BCAs. In chapter 2, we determined that **the genetic diversity, genetic differentiation and population structure of *N. tenuis* at a global scale is likely shaped by anthropogenic activities (i.e. plant/produce trade and augmentative biocontrol) as well as geographical barriers**. The existence of the ubiquitous highly common haplotype (haplotype 2), revealed by the mtDNA COI, suggests that the commercialization and release of *N. tenuis* as BCA might be contributing to a regular gene flow between several regions where this predator is present, thus favoring this haplotype 2, that could also be pre-adapted to the exploitation of different Solanaceous crops. Similar expansion and ubiquity of a haplotype linked to agricultural practices have been reported for *D. maidis* in maize (Bernal et al., 2019). Conversely, the microsatellite results showed that geographic barriers (i.e. oceans and Tibetan/Himalayan Plateau) are important factors shaping the genetic structure and diversity of *N. tenuis* across populations. Other studies also pointed at geographic barriers like drivers of genetic diversity (Sanchez et al., 2012; Wang et al., 2020; Xun et al., 2016). Although *N. tenuis* is already commercialized as a BCA in some regions, our results on the population genetics of this zoophytophagous species may shed light for new initiatives to use *N. tenuis* as BCA in other locations. For example, by the facilitation of information about the genetic makeup and interconnection of the populations locally available in those locations, thus helping increase the success of future pest control programs. On the other

hand, for regions where *N. tenuis* is already mass-reared and released, our study provides information on the current genetic status of commercial populations and tools to monitor their genetic diversity.

A key aspect for the success of selective breeding programs is to know the extent to which the observable differences in certain trait are, at least, partly determined at the genetic level. The renewed interest in the application of selective breeding in BCAs establishes the quantification of the genetic variation in traits of biocontrol interest as a crucial step (Ferguson et al., 2020a; Kruitwagen et al., 2018; Lirakis and Magalhães, 2019; Lommen et al., 2017). Moreover, it is also important that the methodology used to quantify the genetic variation could be easily replicated in more applied settings. In **chapter 3** of this thesis, **an isofemale line approach was used for the first time in a commercially available zoophytophagous predator, to quantify the genetic variation in its feeding behavior**. A similar approach was successfully applied in the quantification of genetic variation in the zoophagy of *C. verbasci*, another zoophytophagous mirid predator that is not commercially available (Dumont et al., 2016). However, it is important to mention that further attempts to quantify the genetic variation on feeding-related traits, or other biocontrol traits in *N. tenuis* or other BCAs, a special emphasis should be made on collecting large numbers of individuals, preferably from different plant hosts, to secure the capturing of a broader genetic variation and to minimize the effects of genetic drift. In chapter 3 **we demonstrated the existence of genetic basis for the differences observed in zoophagy and phytophagy of *N. tenuis***, though we would expect to see larger differences in these traits as the initial population size and diversity increase. Interestingly, and despite our initial population for the founding of the isofemale lines (chapter 3) was small, in our chapter 2, the mtDNA markers revealed the presence of 3 different haplotypes (2, 20 and 22) out of 4 samples analyzed from such founding population (SP-PN = Spain, Peñíscola). This particular result indicates a high genetic diversity in this population, which further supports our findings on the genetic variation of *N. tenuis* feeding behavior presented in chapter 3.

Moreover, quantifying the genetic variation also allows to get insight on the magnitude of the non-genetic components of the variation, as well as that of selective forces acting on a trait. This can provide researchers with a preliminary idea of the potential and/or limitations for selection on certain traits. For instance, in chapter 3, the

low broad-sense heritability (H^2) observed in phytophagy is likely due to the multiple factors influencing that trait (i.e. large environmental variance). Therefore, researchers looking to mitigate the detrimental consequences of its phytophagy via selective breeding will need to account for factors associated to the plant host besides those associated to the BCA per se. The low H^2 should also warn insect breeders about the probability of strong selection forces acting on the trait, thus suggesting increased challenges to select against it. Conversely, finding traits with higher H^2 values, such as that observed for zoophagy in chapter 3, might be indicative of less strong selective forces and/or lower environmental variances for the trait, thus redirecting the research investment on that direction. This could be of special interest in the context of the emerging interest on heteropteran predators observed in recent decades (van Lenteren et al., 2020). For example, in regions where zoophytophagous predators are being evaluated as potential BCA candidates (Silva et al., 2017), a timely quantification of the genetic variation on phytophagy (chapter 3) , coupled with good knowledge of the population genetics of the candidate species (chapter 2), would certainly lead to the selection and mass-rearing of the BCA that maximizes biocontrol services while minimizing its detrimental phytophagy.

Besides the existence of genetic variation, it is also essential to explore other factors that influence the expression of a (biocontrol) trait that would likely play a role in a potential breeding program of a BCA. In the case of zoophytophagous predators, the expression of the trait of interest, and the performance of the BCA, not only depends on the BCA itself, but also on other trophic levels, such as the prey and the host plant. Considering that previous studies demonstrated that saliva composition and stylet morphology of *N. tenuis* did not explain the damage infliction on tomato (Castañé et al., 2011), in **chapter 4** of this thesis we investigated the behavioral and mechanical components of its feeding behavior. Our findings showed that **the increased plant damage previously reported for nymphal stages compared to that of the adults of *N. tenuis* could be partly explain by i) increased mechanical damage as a consequence of higher number of probes in the plant, and ii) increased maceration of the plant tissue due to longer cell rupturing events**. This characterization of the feeding behavior is relevant because it enhances our understanding of the interaction between *N. tenuis* and the tomato plant, and also because it demonstrates the usefulness of these two methods (i.e. behavior observation and EPG) for future studies on *N. tenuis* phytophagy in the context of selection programs. For instance, our quantification methods could be applied

to individuals of the different populations identified in our population genetics study (chapter 2), for a standardized comparison of the feeding behavior of *N. tenuis* that could help in the identification of phenotypes with different levels of phytophagy across its range of expansion. More importantly, this phytophagy quantification coupled with our results from chapter 3 on the genetic variation for the phytophagy trait, get the breeding efforts one step closer to the delineation of the genetic architecture of the trait. For example, behavioral observation, EPG experiments and gene expression analyses (e.g. transcriptomics), can be performed for the two extreme phenotypes of our isofemale lines (chapter 3), to estimate whether differences in phytophagy between them persist and whether patterns of gene expression could explain such differences.

Another relevant finding from chapter 4 indicated that ***N. tenuis*** **tends to perform cell rupturing events in the vascular region of the petioles.** The low number of replicates achieved in our study does not allow to draw a definitive conclusion regarding the preferred plant tissue for *N. tenuis* to feed upon, yet it could provide an interesting cue for disciplines like plant breeding. A common application of EPG studies is the location of resistance factors in plant tissues (Alvarez et al., 2006; Garzo et al., 2002). In chapter 4 of this thesis, the combined use of EPG and histological techniques shed light on the interaction of *N. tenuis* with the tissues of tomato plants, giving valuable insight about the stylet activities (i.e. cell rupturing and ingestion) after penetrating the plant epidermis, and a likely tissue to be attacked in absence of prey (i.e. vascular tissue). Therefore, this can set a basis of knowledge for a more in-depth investigation from the plant breeding perspective, with the aim to develop tomato varieties with resistant factors that could counteract the detrimental effects of *N. tenuis* phytophagy.

Further steps of the studies hereby presented would include a molecular analysis of the isofemale lines in chapter 3 (i.e., microsatellite and/or mtDNA markers), to determine whether the phenotypic differences and genetic variance we found (chapter 3) hold at the molecular level too. Consistency in the differences at both levels would suggest a strong potential of *N. tenuis* feeding behavior as a very interesting trait for selection programs. Moreover, should the differences between isofemale lines stand at molecular level, the further step would be the application of behavioral observation and EPG methodologies (chapter 4) for the quantification of the phytophagy. These results would elucidate the

role of the mechanical and/or maceration components in the differences observed in damage level.

Overall, the findings presented in this thesis demonstrate how some genetic and genomic tools can be integrated into biological control, i.e., chapters 2 and 3, adding to the growing body of investigation made towards the genetic improvement of biocontrol agents. The sequencing of *N. tenuis* genome made possible the development of novel microsatellites that permitted the study of the population genetics of this predator (chapter 2). Additionally, a genetic basis was revealed and broad-sense heritability (H^2) calculated for the variation in the phytophagy and zoophagy of *N. tenuis* (chapter 3). Finally, the mechanical and behavioral characterization of the phytophagy (chapter 4) would facilitate the phenotyping of populations and/or isofemale lines for future experiments linking phenotypic and genotypic data.

5.2. Conclusions

Population genetic structure of the cosmopolitan and controversial *Nesidiocoris tenuis* based on novel microsatellites and mitochondrial DNA markers

i. A moderate-to-low genetic diversity was observed across populations of *N. tenuis* from its global range of expansion based on microsatellite and mtDNA COI markers.
ii. The trade of plants and produce, and the augmentative biocontrol practice based on one/few lineages, might be mediating the ubiquity and abundance of one of the haplotypes (haplotype 2) in Europe, Africa and America.
iii. Geographic and anthropogenic factors are the likely drivers of the genetic differentiation across populations of *N. tenuis.*
iv. The commercial populations analyzed do not show the lowest genetic diversity compared to that of wild populations.

Genetic Variation in the Feeding Behavior of Isofemale Lines of *Nesidiocoris tenuis*

i. The differences observed in the levels of phytophagy and zoophagy across isofemale lines of *N. tenuis* have a genetic basis.
ii. The genetic variation for zoophagy is larger than that of phytophagy likely due to more factors influencing the occurrence of the latter.
iii. Broad-sense heritability (H^2) is higher for zoophagy than phytophagy, indicating a promising implication for selective breeding efforts of the former trait.
iv. There is a moderate correlation between necrotic rings and wilting percentage across isofemale lines

i. Fifth-instar nymphs of *N. tenuis* probe more frequently on tomato apical sections than adults.
ii. Fifth-instar nymphs, males and females of *N. tenuis* spend a higher proportion of time on cell rupturing behaviors than on ingestion behaviors during a probing event
iii. Adults of *N. tenuis* tend to perform both cell rupturing and ingestion activities on the vascular region.
iv. *N. tenuis* does not target a specific cell type on the vascular region while performing cell rupturing or ingestion behaviors as revealed by the stylet position tips in both vascular bundles and parenchyma cells.

5.2. Conclusiones

Genética de poblaciones del controversial y cosmopolita *Nesidiocoris tenuis* basada en nuevos microsatélites y marcadores de AND mitocondrial

I. Se observó una diversidad genética de moderada a baja en las poblaciones de N. tenuis en su rango de expansión global basada en microsatélites y marcadores mitocondriales COI.
II. El comercio de plantas y productos, y la práctica de biocontrol aumentativo basada en uno o pocos linajes, podrían estar mediando la ubicuidad y abundancia de uno de los haplotipos (haplotipo 2) en Europa, África y América.
III. Los factores geográficos y antropogénicos son los posibles impulsores de la diferenciación genética entre las poblaciones de *N. tenuis*.
IV. Las poblaciones comerciales analizadas no poseen la menor diversidad genética en comparación con las poblaciones silvestres.

Variación genética en el comportamiento alimenticio de isolíneas de *Nesidiocoris tenuis*

i. Las diferencias observadas en los niveles de fitofagia y zoofagia en las isolíneas de *N. tenuis* tienen una base genética.
ii. La variación genética de la zoofagia es mayor que la de la fitofagia, probablemente debido a mayor cantidad de factores que influyen en la aparición de los sítomas de esta última.
iii. La heredabilidad en sentido amplio (H^2) es mayor para la zoofagia que para la fitofagia, lo que indica un potencial prometedor para eventuales esfuerzos de selección de la zoophagia.
iv. Existe una correlación moderada entre los anillos necróticos y el porcentaje de marchitamiento entre las isolíneas

i. Las ninfas de quinto estadio de *N. tenuis* pican con más frecuencia en las secciones apicales de tomate que los adultos.
ii. Las ninfas de quinto estadio, los machos y las hembras de *N. tenuis* dedican una mayor proporción de tiempo a comportamientos de ruptura celular que a comportamientos de ingestión durante un evento de picada.
iii. Los adultos de *N. tenuis* tienden a realizar actividades tanto de ruptura celular como de ingestión en la región vascular.
iv. *N. tenuis* no se dirige a un tipo de célula específico en la región vascular mientras realiza comportamientos de ruptura celular o ingestión, tal como lo revela la posición de las puntas del estilete tanto en los haces vasculares como en las células del parénquima.

Appendix

Table S 2.1. Microsatellite loci used in this study

Marker	Motif	No. Repeats	Predicted size (bp)	primer sequences (5'-3')	Dye
MNES02	AG	8	185	(F) CTTTAACGGGCTGAGTGGAC	6FAM
				(R) CCATGTTCTCTCGCGTGATG	
MNES03	AC	8	194	(F) AGCAGCCATCCTAGTTCTCG	6FAM
				(R) CGTCGGGTTTGATAGTGTGC	
MNES04	AC	8	203	(F) ATTCCATTGCGTGCCTCATC	ATTO-550
				(R) TGGTTGGCATTTACGGTGTG	
MNES06	AG	8	207	(F) CGGCAGGTCGAAGGAGAG	ATTO-550
				(R) CGCGATGAATAGCTACGGAAC	
MNES07	AG	8	220	(F) CATCTATTTGTGCTGCCCGG	HEX
				(R) CGAAGCAGCCGTTTATCCTC	
MNES09	AG	8	236	(F) TTGACTCCTGAGCCTCTGAC	6FAM
				(R) TGACTATGAGGTGCTGGTCG	
MNES10	AG	8	246	(F) ATTCCGTCTCCTTCCTTCCG	HEX
				(R) TTACAGGCCTCTTCCACCAC	
MNES12	AG	16	335	(F) GCACAGTCTTCAGCAACCAG	6FAM
				(R) ATTGGGACTGAACTCCTCCG	
MNES13	AT	16	346	(F) ACTGAGCGCAAACAATGTCC	ATTO-550
				(R) TATTGGGATTTCATGGCGGG	
MNES16	ACG	8	127	(F) GCCGTGACCTATCTTCAACG	HEX
				(R) AAAGTGCTGAGACCCGTCAG	
MNES17	AAT	8	130	(F) AATGAACGTTGGCTGCTCAG	HEX
				(R) CTCCAAGCAAGCCAGAACAG	
MNES20	AAC	16	419	(F) AACGTCGGCTGATCCTACTG	ATTO-550
				(R) TGCTGTTGAGGAGTTGGGAC	

(F) = forward; (R) = reverse

Table S 2.2. The pairwise F_{ST} among populations of *Nesidiocoris tenuis* based on 12 microsatellite loci (above diagonal, 15 populations) and mtDNA *COI* gene sequences (below diagonal, 24 populations). Non-reported values in the microsatellite matrix correspond to population sequences retrieved from GeneBank for mtDNA *COI* analysis. Bold indicates significant values ($p < 0.05$).

	CHIN2	**VIET**	**TX**	**TUN**	**SP-TN**	**SP-PN**	**KOP**	**AGRB**	**REU**	**PAN**	**PR**	**NIG**	**MX**	**BIOB**	**JPN2**	**JPN**	**JOR**	**ITA2**	**ITA**	**IND2**	**IND**	**FLO**	**CHIN**	**SP-CT**
CHIN2	***	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VIET	0.049	***	**0.172**	**0.124**	-	-	**0.098**	**0.138**	-	**0.196**	**0.290**	-	**0.264**	**0.121**	-	**0.098**	**0.121**	-	**0.117**	-	**0.101**	**0.236**	**0.204**	-
TX	**0.336**	**0.750**	***	**0.175**	-	-	**0.168**	**0.129**	-	**0.090**	**0.187**	-	**0.150**	**0.193**	-	**0.193**	**0.168**	-	**0.163**	-	**0.139**	**0.082**	**0.289**	-
TUN	**0.144**	0.429	**0.724**	***	-	-	**0.064**	**0.080**	-	**0.251**	**0.323**	-	**0.314**	**0.113**	-	**0.168**	**0.106**	-	0.019	-	**0.060**	**0.251**	**0.270**	-
SP_TN	0.060	0.500	0.000	0.333	***	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SP_PN	**0.098**	0.333	0.167	0.089	-0.667	***	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KOP	**0.336**	**0.750**	**1.000**	**0.724**	1.000	0.444	***	**0.049**	-	**0.187**	**0.263**	-	**0.255**	**0.082**	-	**0.146**	**0.067**	-	**0.074**	-	**0.062**	**0.197**	**0.236**	-
AGRB	**0.336**	**0.750**	0.000	**0.724**	0.000	0.167	**1.000**	***	-	**0.192**	**0.258**	-	**0.239**	**0.077**	-	**0.211**	**0.116**	-	**0.061**	-	**0.095**	**0.197**	**0.262**	-
REU	-0.054	0.245	0.385	0.208	-1.000	-0.191	0.724	0.385	***	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PAN	**0.336**	**0.750**	0.000	**0.724**	0.000	0.167	**1.000**	0.000	0.385	***	**0.193**	-	**0.162**	**0.250**	-	**0.233**	**0.213**	-	**0.247**	-	**0.218**	**0.127**	**0.353**	-
PR	**0.206**	0.500	0.000	0.429	-1.000	-0.067	**0.750**	0.000	-0.081	0.000	***	-	**0.259**	**0.299**	-	**0.331**	**0.257**	-	**0.310**	-	**0.249**	**0.224**	**0.394**	-
NIG	**0.290**	**0.781**	**1.000**	**0.834**	1.000	**0.719**	**1.000**	**1.000**	0.628	**1.000**	**0.839**	***	-	-	-	-	-	-	-	-	-	-	-	-
MX	**0.306**	0.707	0.000	0.667	0.000	0.077	**1.000**	0.000	0.250	0.000	-0.091	**1.000**	***	**0.258**	-	**0.334**	**0.260**	-	**0.289**	-	**0.243**	**0.206**	**0.429**	-
BIOB	**0.336**	**0.750**	0.000	**0.724**	0.000	0.167	**1.000**	0.000	0.385	0.000	0.000	**1.000**	0.000	***	-	**0.226**	**0.088**	-	**0.107**	-	**0.084**	**0.246**	**0.306**	-
JPN2	-0.015	0.500	1.000	0.333	1.000	0.167	1.000	1.000	0.000	1.000	0.500	1.000	1.000	1.000	***	-	-	-	-	-	-	-	-	-
JPN	**0.295**	**0.750**	**1.000**	**0.724**	1.000	**0.583**	**1.000**	**1.000**	0.724	**1.000**	**0.750**	**1.000**	**1.000**	**1.000**	0.000	***	**0.148**	-	**0.166**	-	**0.133**	**0.267**	**0.241**	-
JOR	**0.187**	0.613	**1.000**	0.667	1.000	**0.520**	**1.000**	**1.000**	0.250	**1.000**	0.707	0.000	1.000	**1.000**	1.000	**1.000**	***	-	**0.079**	-	**0.036**	**0.216**	**0.281**	-
ITA2	0.060	0.500	0.000	0.333	0.000	-0.667	1.000	0.000	-1.000	0.000	-1.000	1.000	0.000	0.000	1.000	1.000	1.000	***	-	-	-	-	-	-
ITA	**0.336**	**0.750**	0.000	**0.724**	0.000	0.167	**1.000**	0.000	0.385	0.000	0.000	**1.000**	0.000	0.000	1.000	**1.000**	**1.000**	0.000	***	-	**0.040**	**0.240**	**0.252**	-
IND2	0.024	0.500	1.000	0.333	1.000	0.167	1.000	1.000	0.000	1.000	0.500	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	***	-	-	-	-
IND	**0.222**	0.000	**1.000**	**0.724**	1.000	**0.583**	**1.000**	**1.000**	0.724	**1.000**	**0.750**	**1.000**	**1.000**	**1.000**	1.000	**1.000**	**1.000**	1.000	**1.000**	1.000	***	**0.212**	**0.234**	-
FLO	**0.336**	**0.750**	0.000	**0.724**	0.000	0.167	**1.000**	0.000	0.385	0.000	0.000	**1.000**	0.000	0.000	1.000	**1.000**	**1.000**	0.000	0.000	1.000	**1.000**	***	**0.343**	-
CHIN	**0.222**	0.000	**1.000**	**0.724**	1.000	**0.583**	**1.000**	**1.000**	0.724	**1.000**	**0.750**	**1.000**	**1.000**	**1.000**	1.000	**1.000**	**1.000**	1.000	**1.000**	1.000	0.000	**1.000**	***	-
SP_CT	0.060	0.500	0.000	0.333	0.000	-0.667	1.000	0.000	-1.000	0.000	-1.000	1.000	0.000	0.000	1.000	1.000	1.000	0.000	0.000	1.000	1.000	0.000	1.000	***

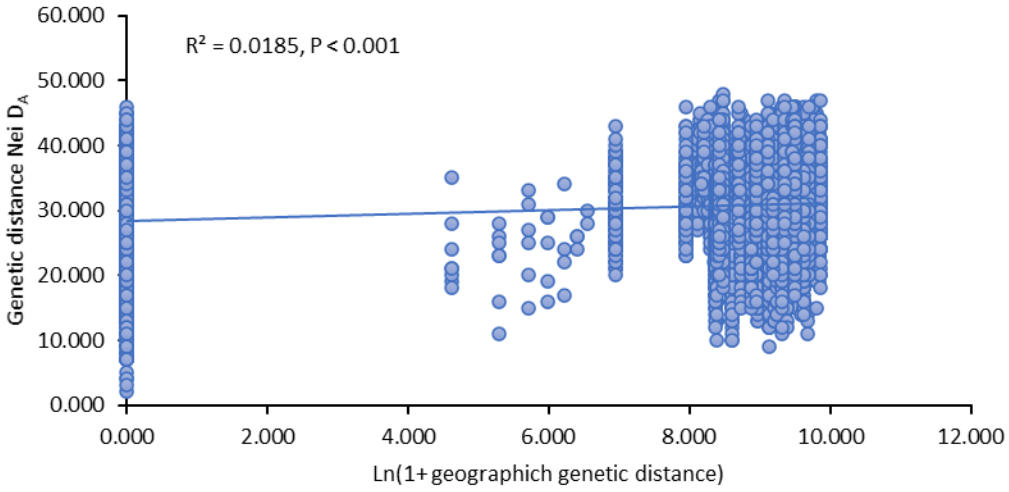

Figure S 2.1. Correlation analysis between the genetic (Nei D_A) and logarithmic geographic distance based on microsatellite data

Table S 3.1. Number of replicates per isofemale line in each block and per leaf type tested in the phytophagy experiment. One replicate consisted of one leaf enclosed in a muslin bag with ten *Nesidiocoris tenuis* adults (five males and five females).

Isofemale line	Leaf type	Block										Total replicates per leaf	Total replicates per isofemale line
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**		
1	*Young*	2	1	2	2	–	3	3	1	2	1	17	33
	Old	1	1	1	1	1	4	5	1	–	1	16	
5	*Young*	1	–	1	1	1	–	1	3	2	1	11	21
	Old	2	1	1	1	–	–	–	2	–	3	10	
6	*Young*	1	1	2	1	–	1	2	3	2	2	15	28
	Old	2	1	1	–	1	1	2	3	1	1	13	
8	*Young*	2	1	–	1	1	2	2	3	–	1	13	30
	Old	2	1	1	2	1	2	1	3	2	2	17	
10	*Young*	2	–	2	2	1	–	–	1	2	2	12	23
	Old	1	1	1	1	1	1	–	1	3	1	11	
11	*Young*	1	1	1	1	1	1	1	3	1	2	13	27
	Old	2	–	–	2	2	1	1	3	2	1	14	
13	*Young*	1	2	–	2	1	1	2	1	2	1	13	26
	Old	2	–	1	1	1	1	2	1	2	2	13	
14	*Young*	2	–	1	1	1	1	1	3	1	1	12	26
	Old	1	1	2	2	1	–	–	3	2	2	14	
15	*Young*	2	1	1	2	1	1	–	3	2	2	15	26
	Old	1	1	1	1	–	–	1	3	2	1	11	
		Total replicates of the experiment											**240**

Table S 4.1. Calculations of total probing duration (TPD) and time to first probe in males, females and N5-nymphs of *Nesidiocoris tenuis*. Values expressed in seconds (mean ± SE).

Insect stage	TPD	Time to first probe
Male	16,457.0 ± 743.0	72.0 ± 9.6
Female	15,598.0 ± 332.5	114.4 ± 22.2
N5-nymphs	15,364.0 ± 660.0	174.9 ± 45.1
	F_2= 0.91; P = 0.409	H = 4.5; P = 0.110

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