Universidad de Concepción

Dirección de Postgrado

Facultad de Ciencias Forestales – Programa de Doctorado en Ciencia Forestales.

ESTUDIO DEL PARASITISMO DE *DELADENUS SIRICIDICOLA* BEDDING (NEMATODA: NEOTYLENCHIDAE) Y SU EFECTO SOBRE POBLACIÓN E INTENSIDAD DE ATAQUE DE LA PLAGA *SIREX NOCTILIO* FABRICIUS (HYMENOPTERA: SIRICIDAE)”, EN LA REGIÓN DEL BIOBÍO, (CHILE).

Tesis para optar al grado de Doctor en Ciencias Forestales

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CONCEPCIÓN CHILE

2019

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DEDICATORIA

A mi amada esposa Yoyita.

A mis queridos hijos, Micky y Dany.

AGRADECIMIENTOS

A Forestal Mininco S.A., por su apoyo durante mis estudios y por dar las facilidades para el desarrollo de esta tésis y sus respectivas publicaciones.

A Jean Pierre Lasserre, Luis De Ferrari y Gastón González, por su permanente motivación.
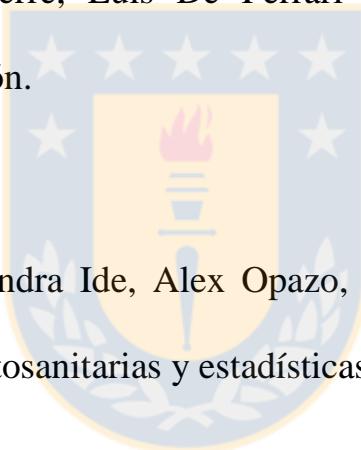
A Gloria Molina, Sandra Ide, Alex Opazo, Felipe Vargas, por su apoyo técnico en materias fitosanitarias y estadísticas.

A Margarita Figueroa por su gentil orientación y permanente apoyo en materias formales del post grado.

A mis profesores, por el soporte técnico, motivación y compromiso.

TABLA DE CONTENIDO

INDICE DE FIGURAS

INDICE DE TABLAS.

RESÚMEN

El 2010 se inició en la Región del Biobío Chile, un intenso programa de control de *S. noctilio* mediante el nematodo esterilizante de hembras, *Deladenus siricidicola*, Una cepa "no esterilizante (NE)" de *D. siricidicola*, fue reportada en Europa, Japón, Nueva Zelanda y América del Norte. Entre el 2012 a 2017 se realizó en la región del Biobío el presente estudio, que tuvo como objetivo evaluar el parasitismo de *D. siricidicola*, estimar su efecto sobre la población y nivel de ataque de la plaga, y explorar la presencia de las cepas Kamona y "NE" del nematodo. El parasitismo fue determinado por disección de hembras emergidas desde trozas de *P. radiata*; la población fue estimada a partir de la emergencia de individuos desde esas trozas, el nivel de daño mediante parcelas establecidas en rodales de *P. radiata* (1 / 5 ha^{-1}) y la determinación de cepas Kamona y "NE", se realizó mediante PCR-RFLP, utilizando, Acil, Rsal y BsrGl. En el período de estudio, el parasitismo de *D. siricidicola* aumentó desde 29,6% hasta 93,1%, la población de la plaga disminuyó en 3,4% y el nivel de ataque incrementó de 0,3 a 11,0% (2015) y el 2017 disminuyó a 5,9% de árboles afectados. Se determinó la cepa kamona en el 100% de hembras de *Sirex* parasitadas y se detectó por primera vez en Chile la cepa de *D. siricidicola* "NE" parasitando a niveles de 30%.

ABSTRACT

In 2010 year, winth the Biobío Region of Chile, an program of control of *S. noctilio* was initiated with the sterilizing nematode of females, *Deladenus siricidicola*, A "non-sterilizing (NS)" strain of *D. siricidicola* was reported in Europe, Japan, New Zealand and North America. Between 2012 and 2017, the present study was carried out in the Biobío, which was aimed to evaluate the parasitism of *D. siricidicola*, estimate its effect on both population and attack level of the pest, and explore the presence of Kamona and "NS" strains of the nematode. The parasitism was determined through the dissection of females emerged from *P. radiata* logs. The wasp population was estimated from the emergence of individuals from those same logs, the damage level through by plots established in stands of *P. radiata* (1/5 ha^{-1}) and the determination of Kamona and NS was performed through PCR-RFLP, using Acil, Rsal and BsrGl. In the study period, the parasitism of *D. siricidicola* increased from 29.6% to 93.1%, the population of the pest decreased in 3.4% and the attack level increased from 0.3% to 11.0% (2015) and then decreased to 5.9% of the affected trees in 2017. The Kamona strain was determined in 100% of the parasitized *Sire* females, and the "*NS*" strain of *D. siricidicola* were detected for the first time in Chile, parasitizing at levels of 30%

CAPITULO I. INTRODUCCIÓN GENERAL.

La avispa, *Sirex noctilio* Fabricius*,* (Hymenoptera Siricidae), originaria de Eurasia es una de las más invasivas taladra la madera de árboles en pie, pertenecientes principalmente al género *Pinus* y ocasionalmente a especies de *Pseudotsuga*, *Abies, Larix* y *Picea* (Bain, 2005); siendo *Pinus radiata* D. Don la especie más susceptible (Aguilar y Lanfranco, 1988).

El ataque de esta avispa*,* se inicia cuando la hembra adulta taladra con su aparato ovipositor aserrado, a través de la corteza, el fuste de árboles vivos susceptibles y deposita sus huevos en la madera, junto a ello inyecta un mucus fitotóxico y artrosporas de un hongo bacidiomicete simbionte llamado *Amylostereum areolatum* (Chaillet ex Fries) Boidin (Taylor, 1981; Newmann et al, 1987; Bain, 2005). El mucus mata los tejidos de la albura y la deja disponible para el crecimiento del basidiomicete (Bain, 2005), el que ocasiona pudrición blanca (Newman et al., 1987); en tanto que las larvas de *S. noctilio* emergidas a partir de los huevos, construyen galerías en la madera del tronco infestado y obtienen su alimento desde las hifas del hongo (Newmann et al, 1987; Bain, 2005). La acción combinada del mucus y hongo, dañan el sistema vascular, ocasionando la muerte de los árboles infestados

(Talbot, 1977; Kroll et al, 2013), en tanto que las galerías construidas por las larvas y la pudrición asociada, degradan la calidad de la madera (Taylor, 1981; Newmann et al., 1987; Bain, 2005).

La avispa de la madera *Sirex noctilio* Fabricius (Hymenoptera: Siricidae), originaria de Eurasia (Talbot, 1977) y su presencia fuera de su origen ha sido reportada en Nueva Zelanda (1900), Australia (1952), Uruguay (1980), Argentina (1985), Brasil (1988), Sud África (1994), Chile (2001), Estados Unidos de América (2004), Canadá (2005) y China (2013) (Bain, 2005; Bedding, 2009; Hoebeke et al., 2005; De Groot et al., 2006; Li et al., 2015).
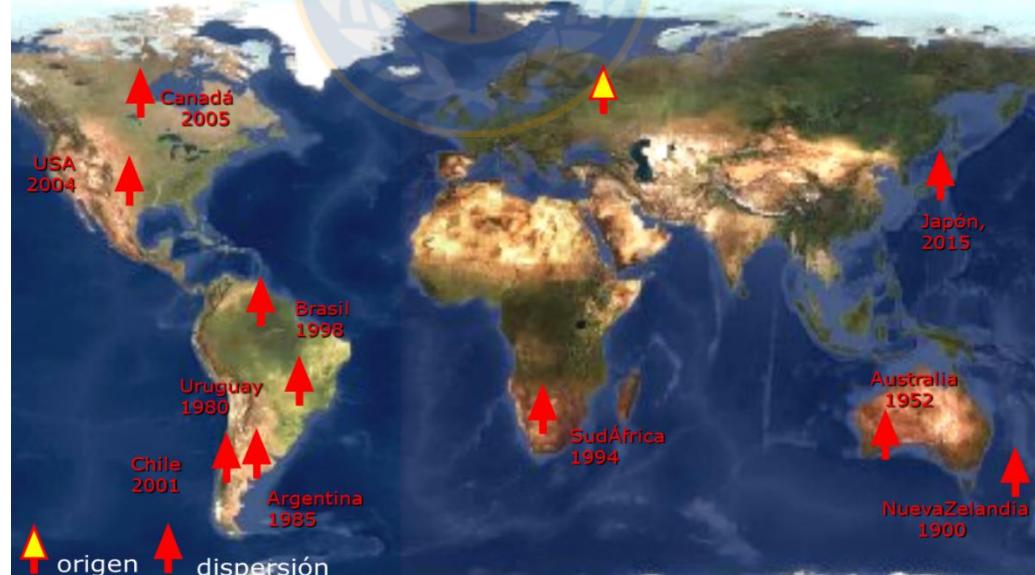
Figura 1.1. Distribubión de *Sirex noctilio* en el mundo (Bedding, 2009; Yu et al, 2009).

La avispa de la madera es considerada una de las plagas de mayor relevancia económica en los países del hemisferio sur, en los que se ha dispersado en ausencia de enemigos naturales nativos (Williams & Hajek, 2017, habiéndose reportado pérdidas económicas de relevancia, (Bain, 2005; Hurley et al.,, 2007; Bedding, 2009), llegando incluso a causar mortalidad de hasta un 80% de los árboles en las zonas más afectadas (Bedding, 2009). En América del norte se ha estimado que la plaga representa alto riesgo para las especies de *Pinus*, tanto introducidas como nativas (Yemshanov et al., 2009). En tanto que, en su distribución natural, la plaga no representa riesgo, ni económico, ni ecológico (Yu et. al, 2009), probablemente por la presencia de sus enemigos naturales.

1.1.- *Sirex noctilio*, morfología, ciclo de vida.

Las avispas son grandes de cuerpo cilíndrico y robusto (Ruíz, 2006). La hembra adulta de *S. noctilio, mide* el cuerpo es de 25 mm a 40 mm de largo, es de color azul oscuro metálico, excepto las alas y las patas que son ámbar, sus antenas antenas tienen 21 segmentos y posee una vaina que protege el aparato ovipositor que se proyecta de 2 a 3 mm más allá del abdomen (Newman & Minko, 1981). En la base del ovipositor se ubican micangios que contienen las esporas del hongo simbionte asociado, Cerca de los cuales

se encuentran un par de glándulas productoras del mucus fitotóxico (Aguilar y Lanfranco, 1988; Ruíz, 2006). El macho adulto es azul oscuro metálico, excepto en los segmentos abdominales de tres a siete, las patas delanteras y medias son de color marrón anaranjado, mientras que las patas traseras son gruesas y negras, las alas son de color ámbar y mide de entre 9,3 a 35 mm de largo. Las antenas tienen 20 segmentos y son 6.8 mm de largo. Una prominente espina está presente en el último segmento abdominal de ambos sexos (Newmann & Minko, 1981).

Los huevos son blancos, suaves, lisos, alargados y tienen una longitud de 1,55 mm y una anchura de 0,28 mm (Newmann y Minko, 1981). Las larvas son de color blanco cremoso, profundamente segmentadas, con una cabeza bien desarrollada dotada de fuertes mandíbulas oscuras y dentadas; y presentan, 3 pares de patas toráxicas cortas, rudimentarias y una prominente espina supraanal esclerosada en el extremo del abdomen (Newman y Minko, 1981; Ruíz, 2006). Las pupas son de color blanco cremoso y gradualmente adquieren el color de los adultos (Neumann & Minko, 1981; Ruíz, 2006).

Normalmente el ciclo biológico de *S. noctilio* se completa en el transcurso de un año, pero se ha reportado en Australia y en Nueva Zelanda, que el ciclo en algunos individuos puede durar sólo 2 o 3 meses, especialmente cuando

el ataque se concentra en árboles de diámetro pequeño y las condiciones climáticas le son favorables; este ciclo corto se presenta entre verano y mediados de otoño, cuando existe alto déficit hídrico (Aguilar & Lanfranco, 1988, Ruíz, 2006).

La emergencia de los adultos en el hemisferio sur va desde octubre hasta mayo, alcanzando generalmente las máximas poblacionales a mediados del verano (Ruíz, 2006). Poisson et al., (2016), dererminaron para la región del Biobío Chile un período de vuelo comprendido entre diciembre a abril, que con el porcentaje más alto de emergencia, correspondiente a un 75,7% durante el mes de enero. Las hembras depositan sus huevos en forma aislada o de a pares, los que son incubados en la madera por un período de 9 a 14 días, siendo factible encontrar esta fase, en el hemisferio sur, entre mediados de diciembre y hasta comienzos de abril; pudiendo en condiciones desfavorables para la eclosión de las larvas permanecer en dormancia varios meses (Aguilar y Lanfranco, 1988). Los adultos no se alimentan y sólo dependen de las reservas energéticas acumuladas en su fase larvaria, llegando a vivir un período de 5 días en el caso de hembras y 12 días en el caso de machos (Aguilar y Lanfranco, 1988). Según Ruíz (2006) en base a descripción de Morgan (1968), la larva emergida se alimenta de *A. aerolatum*

y su período de desarrollo dura alrededor de un año, durante el cual aumenta de tamaño sin cambiar de forma, pasando por 6 a 7 estadios larvales, el mayor de los cuales alcanza 3 cm de largo. La larva se desarrolla construyendo galerías hacia el duramen, para luego dirigirse nuevamente a la parte más cercana a la corteza y pupar, permaneciendo en ese estado entre 3 y 5 semanas (Iede *et al*., 1993 en Ruíz, 2006).

1.2.-*Sirex noctilio* Fabricius: síntomas, signos y susceptibilidad de hospederos.

Los síntomas del ataque de *S. noctilio*, corresponden a una clorosis progresiva del follaje, que finalmente toma un color café rojizo, para luego desprenderse, siendo más evidente en primavera-verano y adicionalmente se observa en la parte alta de la copa marchitamiento foliar repentino. En el fuste de árboles atacados, como indicador de ovoposición, se observa bolsas o escurrimientos de resina y bajo la resina en el punto de oviposición se aprecia una mancha oscura que corresponde al hongo. Adicionalmente, en la corteza, se observan orificios de emergencia de adultos de 3 a 7 mm de diámetro (Newmann et al., 1987; Aguilar y Lanfranco, 1988).

Figura 1.2. A: árbol *P. radiata* marchito, atacado por *S. noctilio*; B:necrosis subcortical causada por *Sirex*; C: mancha café en madera causada por *A. aerolatum;* D:Fuste con resinación producto ovipostura; E: fuste con orificios de emergencia de *S. noctilio*.

El daño de *S. noctilo*, se asocia preferentemente a árboles que presentan algún nivel de deterioro en su condición fisiológica debido a la acción de diversos factores limitantes de su desarrollo normal, sean de origen biótico o abiótico; como, daños por otras plagas, o ataques recurrente del mismo *S. noctilio*, sequía, excesiva competencia, etc. (Newmann et al., 1987; Aguilar y Lanfranco, 1988). Los árboles en este estado de estrés fisiológico, se encontrarían en una "fase de predisposición", atraerían a la avispa y su ovipostura (Newmann et al., 1987; Aguilar y Lanfranco 1988) y la duración de esta fase dependería de la severidad y persistencia del estrés fisiológico. Así por ejemplo, Madden (1971), demostró que árboles intensamente podados adquirirían susceptibilidad después de 9 a 12 días y que permanecerían en esa condición por muchos meses (Neumann et al, 1987). Los árboles estresados en estado de "predisposición" estarían caracterizados por; bajo rango de división celular en el fuste, pero altos niveles de transpiración y respiración; alto nivel de tensión de agua en el sistema vascular asociado a baja turgencia celular y baja presión de resina; floema del tallo con bajo nivel nutricional, deshidratado y con baja presión osmótica de resina y sobre-permeabilidad de la de la corteza con altos rangos de emisión de atrayentes volátiles de pino. (Newmann et al., 1981).

Adicionalmente, Taylor (1981), al respecto indica que en el tallo de árboles bajo estrés, el rango de actividad respiratoria del floema incrementa, hasta que ocurre una declinación de la presión osmótica en la zona de máxima actividad respiratoria, la que correspondería a la sección fustal que es la primera y más intensamente a atacada por *S. noctilio*, disminuyendo el contenido de humedad significativamente en la sección media fustal de árboles atacados por *S. noctilio*, donde además se concentra el mayor porcentaje de larvas del insecto en toda la sección fustal.

Aun cuando, los árboles más vigorosos tienen reservas físicas y químicas que le permiten resistir o tolerar mejor el ataque (Taylor, 1981); en condiciones de altos niveles poblacionales, también pueden ser dañados (Aguilar y Lanfranco, 1988). En general, los mecanismos de resistencia por el cual actúan estos árboles vigorosos, corresponden a, i) inundación con resina de los orificios de ovipostura lo que produce mortalidad de huevos y larvas y por aislamiento del hongo simbionte a través de una barrera química de polifenoles (Coutts y Dolezal, 1969; citado por Taylor 1981). La resina, según lo demostraran, Kile y Turnbull (1974), tendría un efecto inhibitorio o tóxico sobre *Amylostereum aerolatum* (Taylor, 1981). Adicionalmente, debe considerarse que la hembra de *S. noctilio*, en ocasiones inyecta en el fuste de

árboles sanos, sólo el mucus fitotóxico y el basidiomicete simbionte, con el propósito de preparar a su huésped para futuras colonizaciones (Hurley et al., 2007).

1.3.-Manejo de la plaga *S. noctilio*: Control silvicultural y Control biológico.

Para un control eficiente de *S. noctilio*, se debe integrar medidas con bases en la silvicultura a modo de mejorar el vigor de las plantaciones, complementando con el uso de controladores biológicos (Baldini et al., 2005).

El daño de *S. noctilio* no ocurre masivamente en un rodal, afecta y daña mayormente a aquellos que tienen menor vigor, debido a diversos factores limitantes de su desarrollo normal; sequía, daños de otras plagas, y ataque recurrente de la misma plaga en cuestión, excesiva competencia, etc. (Aguilar y Lanfranco, 1988); de ahí que la silvicultura, en tanto permita un desarrollo adecuado del cultivo, sea muy eficaz en mitigar los daños de la plaga. Algunos tratamientos silvícolas, pueden incrementar el vigor de los árboles, lo que aumentará su resistencia al ataque de *S. noctilio*, como el raleo por ejemplo, que disminuye competencia y elimina árboles dañados, reduciendo

con ello el ataque de la avispa sobre árboles selectos-remanentes (Coutts, 1965); el establecimiento de plantaciones en sitios adecuados, evitando aquellos de baja fertilidad o que dificulten el manejo (Newmann et al., 1987), evitar podas en períodos de vuelo del insecto, ya que árboles podados son susceptibles al ataque por muchos meses (Madden,1971).

Un novedoso método de control mecánico de la plaga, fue planteado recientemente por Poisson et al., (2016), que consiste en el establecimiento de mega trampas cebo de un cuarto de hectárea, en que se atrae la ovipostura de la plaga mediante el debilitando de sus árboles por medio del uso de herbicida. En su estudio Poisson et al., (2016), demostró que parcelas establecidas en Chile, resultaron efectivas, particularmente las que fueron instaladas en noviembre logrando capturar 5,4 veces más individuos que la parcela control. Los árboles deben ser eliminados antes del próximo período de vuelo de la avispa para evitar su dispersión en el bosque.

El control biológico en los países en que la plaga ha sido detectada, ha considerado el uso del complejo de especies, integrado por insectos del Orden Hymenoptera: *Rhyssa persuasoria, Ibalia leucospoides, Megarhyssa nortoni* (Cresson) y el nematodo de la familia Neotylenchidae *Deladenus siricidicola*; especies que según Lanfranco et al., (1990), actúan en diferentes

etapas de la historia de vida de *S. noctilio* y que tanto en Australia como en Nueva Zelandia, son responsables en conjunto de alcanzar un 90% de control de la plaga.

De acuerdo a la descripción de los modos de acción de las especies de Hymenopteros, realizada por Lanfranco et al., (1990): *Ibalia leucospoides* (Hockenwarch) (Hymenoptera-Ibaliidae**),** es un parasitoide de huevos a punto de eclosionar y de larvas de primer y segundo estadio. Las hembras ovipositan, sólo un huevo en el hospedero, por el mismo de orificio dejado por la hembra de *S. noctilio*. Es endoparásito inicialmente, pero en su tercer estadio se alimenta externamente de la larva de la avispa, emergen en verano y una fracción en otoño; *Rhyssa persuasoria* (Linanaeus.) (Hymenoptera-Ichneumonidae) y *Megarhyssa nortoni* (Cresson), son especies que atacan estadios más avanzados de *S. noctilio,* cuando las larvas en sus galerías se sitúan más hacia el interior del fuste, para ello están provistas de un largo ovipositor, siendo más largo en *Megarhyssa*, por lo que no compiten por su alimento. Ambas especies, primero paralizan a la larva de *S. noctilio* inyectando un veneno, luego oviponen sobre ellas y sus larvas comen externamente (ectoparasitoides) y luego pueden pupar en el mismo lugar; una parte de la población pupa rápidamente y emerge en el verano o bien las

larvas pueden entrar en diapausa, pupar a la primavera siguiente y emerger en el verano respectivo.

Bedding (2009), señala que mientras varios de los insectos parasitoides han sido introducidos en el hemisferio sur, con la excepción de *Ibalia leucospoides* han tenido un limitado efecto. El nematodo es intensamente dependiente de la densidad y puede alcanzar niveles de parasitismo de 100% y es reconocido como el principal agente controlador en el hemisferio sur (Bedding, 2009).

1.4.-*Deladenus siricidicola* Bedding.

El nematodo *Deladenus siricidicola* fue descubierto en Nueva Zelandia en 1962 por Zondang, infectando huevos, larvas, pupas y adultos, machos y hembras de *S. noctilio* (Zondang, 1962) y posteriormente fue descrito por Bedding en 1968 (Bedding, 2009). Ha sido ampliamente usado como agente de control biológico clásico, siendo introducido y establecido en, Australia, Nueva Zelandia, Brasil, Uruguay, Argentina, Chile y Sud África (Bain, 2005; (Bedding, 2009) y más recientemente en Estados Unidos de Norteamérica (Williams & Hajek, 2017).

Deladenus siricidicola, presenta dos estrategias de desarrollo, cada uno con hembras adultas dimórficas entre si; uno es de vida libre en el que el nematodo se alimenta del simbionte asociado con *Sirex noctilio, Amylostereum areolatum* (Fr.) Boidin, y el otro parasítico, en el que hembras infectivas ya fecundadas, penetran a la cavidad del cuerpo de la larva de *S. noctilio* y allí crecen, mudan y luego lanzan juveniles al líquido hemocélico, los que a su vez migran a los órganos reproductores de *S. noctilio* cuando éste ha alcanzado el estado de pupa (Lanfranco y Aguilar, 1990). En las hembras adultas de *S. noctilio* los nematodos invaden ovarios, oviductos y huevos, provocando su esterilidad y afectando la viabilidad de los huevos (Lanfranco y Aguilar, 1990). En los machos adultos el parasitismo de *D. siricidicola*, les produce una hipertrofia testicular, pero no los esteriliza; en estos, los espermatozoides pasan desde los testículos a las vesículas seminales antes que los nematodos juveniles invadan los testículos y los nematodos no juveniles no pueden pasar a través de los conductos deferentes en las vesículas seminales; en consecuencia, estos machos no transmiten los nematodos durante la cópula y sus espermatozoides son absolutamente normales y viables; (Lanfranco y Aguilar, 1990; Bedding, 2009) truncando con esto la vida de los nematodos. El nivel de esterilidad de la hembra de

Sirex parasitadas por el nematodo, podría depender de la sincronización entre la liberación de los nemátodos juveniles en la homocele y el desarrollo del huevo del insecto, si estos son liberados cuando el huevo está en avanzado estado de desarrollado los nemátodos no pueden penetrar (Bedding, 1972; Williams & Hajek, 2017).

Es entonces, sólo a través de las hembras de *Sirex* que *Deladenus* asegura su continuidad y dispersión (Lanfranco y Aguilar, 1990). Las hembras de *S. noctilio* parasitadas emergen desde árboles infestados y dispersan los nematodos (Bediding, 2009), ovipositan normalmente, introduciendo en los árboles *A. aerolatum* y sus huevos estériles, cada uno de los cuales contiene sobre 200 nematodos juveniles que migran a la madera, se alimentan del hongo, se transforman en adultos, manteniéndose en su estado de micetófagos (vida libre), y oviponen en las traqueidas cercanas a los orificios de ovipostura de *Sirex;* allí, en la vecindad de larvas saludables, los nuevos nematodos juveniles pueden desarrollarse en hembras adultas infectivas, las que se aparean y penetran larvas del insecto originados a partir de huevos fértiles iniciando un nuevo ciclo parasítico (Taylor, 1981). De esta manera la dispersión del nematodo depende de la ovipostura de individuos sanos sobre el árbol previamente atacado por una hembra de la avispa infectada,

(Williams & Hajek, 2017), por lo que la dispersión del nematodo sería denso dependiente de la población de la avispa (Bedding, 2009).

Otros efectos de la infección de *D. siricidicola* sobre *S. noctilio*, fueron descritos por, Bedding y Akhurts (1974), quienes reportaron que la competencia temprana por alimento fungoso entre la larva de *S. noctilio* y nematodos, probablemente resulte en disminución de tamaño de los adultos del sirícido. Adicionalmente, cuando la densidad de nematodos en la madera es alta, algunas larvas de sirícidos pueden morir de hambre, habiéndose reportado en Tasmania mortalidad de larvas antes de que alcanzaran su tercer instar, en árboles que contenían nematodos (Taylor, 1981), en esta línea, Zongad (1962), señala que, en algunos casos los nematodos pueden matar a su hospedero prematuramente, observándose en árboles infectados, mortalidad de larvas y pupas en la madera, que aparecían como sacos de líquido negro, en cuyo interior contenían varios estados intermedios de nematodos.

Deladenus siricidicola, es la especie clave para el control de *S. noctilio* y algunas evaluaciones indican que pueden controlar más del 90% de las poblaciones de la plaga (Bedding, 2009); ha sido ampliamente usado como agente de control biológico clásico, principalmente en el hemisferio sur;

aunque los nematodos pueden ser trasladados entre bosques o plantaciones, por las hembras de *S. noctilio* parasitadas, puede ser que esto ocurra demasiado tarde para prevenir serias irrupciones, por lo que deben ser introducidos en nuevas áreas artificialmente, tan pronto como *Sirex* sea detectado; para ello el nematodo puede ser cultivado y masificado en su estado de vida libre o micetófago sobre *A. aerolatum* en condiciones de laboratorio y luego inoculado artificialmente en árboles atacados por *S. noctilio*, hasta que se constate su establecimiento. (Lanfranco y Aguilar, 1990; Bedding, 2009).

La selección de *D. siricidicola* y su procedimiento de uso en programas de control biológico, fue desarrollado en Australia por Congress of Scientific and Industrial Research Organization (CSIRO) durante la década 1960-1970 (Bedding, 2009). En este programa se realizaron colectas de varias especies de *Sirex* que afectaban a coníferas en Europa, Estados Unidos de América, Canadá, India, Pakistan, Turkia, Marruecos y Japón, aislándose a partir de ellas, a lo menos siete especies de *Deladenus* (Bedding y Akhurst, 1974), de las que se seleccionó *D. siricidicola* - procedencia Sopron, por su capacidad de esterilización de hembras, altos niveles de parasitismo, y mínimo impacto sobre el tamaño de adultos de *S. noctilio* (Bedding 2009). En Australia, *D.*

siricidicola - Sopron, fue masificado en laboratorio a partir de su estado de vida libre y se utilizó exitosamente en el control de *S. noctilio.* Sin embargo, el prolongado tiempo de cultivo *in vitro* del nemátodo (20 años), usando sólo el ciclo micetófago, ocasionó la selección de una cepa mermada en su capacidad de control (Bedding, 2009). Para subsanar este problema, en 1991 se re-aisló la cepa original desde la localidad Kamona en Tasmania, donde originalmente fue liberada la cepa Sopron en la década del 70, siendo denominada actualmente como *D. siricidicola* – Kamona, usada ampliamente en el control de esta plaga (Bedding, 2009; Williams & Hajek, 2017). La procedencia Sopron de *D. siricidicola*, fue introducido a Brasil desde Australia en 1989 y luego en 1995, fue reemplazada y reintroducida la cepa Kamona, por lo que en Brasil y quizás otros países de la región, podría existir una población residual de la raza "defectuosa australiana" de *D. siricidicola* (Bedding 2009). Hurley et al., (2007), señalan que en 1995 nemátodos aislados de individuos de *S. noctilio* fueron enviados a Argentina y años más tarde, cultivos de este nemátodo de la cepa Kamona fueron transferidos desde Brasil a Uruguay y Chile.

Adicionalmente a la anomalía en pérdida de habilidad parasítica de *D. siricidicola*, en la década del 70 en Nueva Zelandia fue detectada la existencia

de una cepa de *D. siricidicola* "No Esterilizante (NE)" (Zondag, 1975), luego también fue descrita en Europa, Japón (Bedding & Akhurts, 1978), Canadá y Estados Unidos (Yu et al., 2009; Williams et al., 2009). La cepa no NE de *D. siricidicola* permanece asociada a los ovarios femeninos de *S. noctilio,* pero no penetra a los huevos (Yu et al., 2009; Williams et al., 2009; Kroll et al., 2013), por lo que la hembra permanece fértil.

La inoculación artificial de *D. siricidola,* se realiza, mediante la aplicación de nematodos concentrados, suspendidos, en un gel sobre árboles cortados o trozas, a razón de 4 a 5 inyecciones de 1 ml gel con 2.500 nematodos, por cada metro de longitud de la troza (Lanfranco y Aguilar, 1990). El medio para la suspensión de nematodos, puede ser gel de polyacrylamide (Australia, Sudáfrica) o gelatina (SudAmérica) (Bedding, 2009) ver si va aquí o en otro lugar.

Los resultados de establecimiento del nematodo han sido variables (Hurley et al., 2007). En Australia, en la zona de Victoria, se reportó parasitismo cercano a 100%; en Brasil (Encruzilhado do Sul) de 90% (Bedding, 2009) y en Sudáfrica (Cape town) de 96% (Tribe & Cillié, 2004), después de dos, cuatro y tres años de haber realizado la inoculación, respectivamente. Entre los casos de resultados insatisfactorios, se cuenta entre otros, el de Sudáfrica

(Eastern Cape y KwaZulu-Natal), donde después de haber inoculado *D. siricidicola* dos años consecutivos (2004-2005), se alcanzó parasitismo de entre 5 a 10% (Hurley et al., 2007).

El parasitismo de *D. siricidicola* y su capacidad de esterilización de huevos de *Sirex*, se ven afectados además de por las condiciones inherentes al nematodo, como pérdida de habilidad parasítica y esterilizante, anteriormente descritos; por otras condiciones del medio, como el contenido de humedad de la madera que aunque se desconoce el mecanismo a través del cual actúa, se considera que podría afectar directamente la supervivencia y movilidad del nematodo en la madera; alternativamente, podría afectar el establecimiento del hongo simbiótico *A. areolatum* en que le sirve de alimento a *D. siricidicola* en su forma de vida libre, lo que sería consistente con Taylor (1981), quien señaló que el crecimiento de *A. areolatum* se ve impedido por un exceso o escasez de agua (Hurley et al., 2008). Zondag (1969) añade que habría más nematodos presentes en la madera húmeda (Hurley et al., 2008).

El insecto *Ips grandicollis* (Eichhoff) (Coleoptera: Curculionidae), también ha sido reportado afectando el parasitismo de *D. siricidicola* al atacar a árboles trampa e introducir al hongo de la madera *Ophiostoma ips* (Rumbold)

Nannfelt (Ophiostomatales: Ophiostomataceae), el que interactúa compitiendo con *A. aerolatum* lo que puede inhibir la migración del nematodo dentro del árbol (Yousuf et al., 2018). *Ophiostoma ips*, según lo señalan Yousuf et al., (2014) crece más rápido que *A. areolatum* en medios artificiales y en madera; en cultivo cuando ambos hongos se inocularon simultáneamente, *O. ips* colonizó más sustrato que *A. areolatum* y, posteriormente, impidió que *A. areolatum* colonizara el espacio; en todo caso a pesar de las diferencias en el crecimiento radial, *A. areolatum* mostró fuertes capacidades de defensa y nunca fue reemplazado por *O. ips* En el estudio de Yousuf *et al.*, (2014), se determinó que no había galerías de larvas en la región que *O. ips* infectaba la madera; sin embargo, las galerías de larvas estaban presentes en la región colonizada por *A. areolatum*, lo que sugiere que *S. noctilio* parece estar confinado a la madera libre de *O. ips*, señalándose a partir de estos resultados que la presencia de *O. ips* se correlaciona con la ausencia de galerías larvales. La falta de galerías de larvas en la región de *O. ips* podría deberse a la posibilidad de que los huevos de *S. noctilio* no se incuben en presencia de *O. ips* o que las larvas mueran de hambre debido a la falta de disponibilidad de alimentos (Yousuf et al., 2014).

1.5. *Sirex noctilio*, detección en Chile.

En Chile, *S. noctilio* fue detectado en enero del año 2001 en la región de Valparaíso, en un área que no contenía plantaciones comerciales de *Pinus radiata*, siendo erradiacada (Ciesla, 2003). Posteriormente, el mismo año, la plaga fue detectada en la región de Los Lagos, desde donde se distribuyó a otras partes del país (Poisson et al., 2016), afectando en la actualidad a una superficie aproximada de 1.400.000 ha de *Pinus radiata*, ubicadas entre las Regiones de Valparaíso (33°24′44″ S, 70°37′12″ W) y Aysén (47°04′23″ S, 71°57′46″ W) (SAG, 2012, 2017).

En la Región del Biobío, *S. noctilio* fue detectada el año 2009, iniciándose el año 2010 un intenso programa de control biológico mediante *D. siricidicola* procedencia Encruziliada do Sul. (Beeche et al., 2012), inoculándose con el nematodo en el área de estudio a lo menos 76.000 árboles naturalmente atacados por la plaga, durante los años 2010 a 2016.

Conocida la relevancia económica de la plaga, el alto costo y la expectativa cifrada en el programa de control biológico con *D. siricidicola* se plantea el presente estudio, tendiente a evaluar el parasitismo del nematodo, posibles anomalías y su efecto en la población y nivel de ataque de plaga en la Región del Biobío, entre los años 2012 a 2017.

1.6.-HIPÓTESIS.

El nematodo *Deladenus siricidicola* usado en el control de *Sirex noctilio* conserva su capacidad esterilizante, alcanzando en la región del Biobío niveles de parasitismo sobre 90% después de tres años de su aplicación, afectando la población de la plaga y con ello su nivel de ataque.

1.7.-OBJETIVO.

Caracterizar la dinámica y posibles anomalías del parasitismo de *D. siricidicola* sobre *S. noctilio*, su efecto en la población y nivel de ataque de plaga en la Región del Biobío, entre los años 2012 a 2017.

1.7.1.-.-Objetivos específicos.

- Evaluar el parasitismo de *S. noctilio* por *D. siricidicola* en la Región del Biobío, las temporadas, 2012-2013, 2013-2014, 2014-2015, 2015-2016 y 2016-2017.

- Caracterizar-estimar el efecto del parasitismo de *D. siricidicola* sobre la población de *S. noctilio*, y el nivel de ataque de la plaga en las temporadas 2012-2013, a la 2015-2017, en cuatro zonas inoculadas el año 2011.

- Evaluar la participación de *D. siricidicola* cepas Kamona en el parasitismo de hembras de *S. noctilio* en el área de estudio –Región del Biobío, Chile.

- Evaluar la presencia de *D. siricidicola* cepa no esterilizante en el área de estudio –Región del Biobío, Chile.

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CAPÍTULO II. BIOCONTROL OF *SIREX NOCTILIO* BY THE PARASITIC NEMATODE *DELADENUS SIRICIDICOLA:* A FIVE SEASON FIELD STUDY IN SOUTHERN CHILE.

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2.1.-ABSTRACT

In 2009, the woodwasp *Sirex noctilio* was detected in *Pinus radiata* plantations in the Biobio region of southern Chile. Subsequently, an intense biological control program using the female sterilizing nematode *Deladenus siricidicola* was implemented in 2010. During five seasons between 2012 and 2017, we studied the parasitism of *D. siricidicola* nematode and its effect on woodwasp populations and infestation of *P. radiata* in different locations within the Biobio region. Parasitism was assessed by dissecting adult females of *S. noctilio* obtained from infested *P. radiata* logs. The total population of *S. noctilio* was determined by the emergence of individuals from the same logs. The level of damage caused by the *S. noctilio* pest was determined by establishing plots in stands of *P. radiata* at an intensity of 1 plot every 5 ha-1. During the study period, parasitism of *S. noctilio* by the nematode *D. siricidicola* increased from 29.6% in 2012 to 93.1% in 2016, while pest population decreased 3.4% in the same time period. Infestation increased from 0.3 to 11,6% of trees between 2012 and 2015, but subsequently decreased to 5.9% by 2017. We confirmed establishment of the nematode in the region under study and its natural dispersion to non-inoculated areas.

Finally, we determined that the effect of inoculation age (antiquity) on parasitism levels reached 90% after three years of inoculation.

2.2.-INTRODUCTION

The woodwasp *S. noctilio* Fabricius (Hymenoptera: Siricidae) drills the wood of standing trees belonging to the *Pinus* genus and occasionally *Pseudotsuga, Abies, Larix* and *Picea* species (Bain, 2005)[1], with the most susceptible species being *Pinus radiata* D. Don (Aguilar & Lanfranco, 1988) [2]. *Sirex noctilio* attack begins when females oviposit in the stems of live trees, injecting a phytotoxic mucus and spores of the symbiotic fungi *Amylostereum areolatum* [1] (Bain, 2005) along with the egg. The combined action of the mucus and basidiomycete fungus obstructs the vascular system, resulting in the death of infested trees [3,4] (Hurley et al., 2007; Madden, 1988). Moreover, decay associated with the establishment of larvae galleries deteriorates the quality of the wood (Newmann et al., 1987)[5]. The wood decaying symbiont dries the wood substrate providing a more suitable micro.environment for eggs and larvae development. The wood degradation by the fungus facilitates tunneling of the larvae.

Sirex noctilio is originally from Eurasia and Northern Africa (Hurley et al., 2007; Spradbery & Kirk, 1978) [3,6] where it presents no ecological or

economic damage (Yu et al., 2009) [7]. However, the pest has a major economic impact in southern hemisphere countries where *S. noctilio* has unintentionally been introduced (Hurley et al., 2007) [3], resulting in up to 80% mortality of infested trees in heavily infected areas (Bedding, 2009) [8]. *Sirex noctilio* has been reported outside its natural distribution in New Zealand (1900), Australia (1952), Uruguay (1980), Argentina (1985), Brazil (1988), South Africa (1994), Chile (2001) (Bain, 2005; Bedding, 2009) [1,8], the United States (2004), Canada (2005) (de Groot et al., 2006 [9], and China (2013) (Li et al., 2015) [10].

In the southern hemisphere, biological control is the most common strategy for management of *S. noctilio* (Hurley et al., 2008) [11], particularly with the parasitic nematode *D. siricidicola* (Bedding, 2009) [8,12]. This nematode, first described by Bedding in New Zealand in 1968 (Yu et al., 2009) [7], was found to infect *S. noctilio* eggs, larvae, pupae, and adults (male and female) (Zondag, 1962) [13]. The life cycle of the nematode includes a mycetophagous state in which it feeds on the fungi *A. areolatum* and an infective state involving parasitic larvae and the pre-pupae of *S. noctilio* Yu et al., 2009; Bedding, 2009; Bedding, 1972; Zondag, 1969) [7,8,14,15]. Once inside the host, the female nematode produces juveniles, which develop in

the hemocele and migrate to the reproductive organs of *S. noctilio* as it pupates. The juveniles subsequently invade the ovaries and eggs, resulting in sterilization of adult female woodwasps and compromising the viability of already laid eggs (Lanfranco & Aguilar, 1990; Zongan, 1979) [16,17]. In the male, the nematode produces a non-sterilizing hypertrophy of the testes (Lanfranco & Aguilar, 1990) [18]. Parasitized *S. noctilio* females emerge normally from infested trees and disperse the nematodes (Bedding, 2009; Williams & Hajek, 2017) [8,19,20].

The first formal biological control program using *D. siricidicola* began 1970¨s with work by the Australian Congress of Scientific and Industrial research Organization (CSIRO) using a nematode strain originally collected in Sopron, Hungary. Subsequently, the original strain was replaced by the Kamona strain (Williams & Hajek, 2017) [19]. To date, the Kamona nematode has been used for biocontrol purposes, in Australia, New Zealand, Brazil, Uruguay, Argentina, Chile, South Africa (Hurley et al., 2007; Bedding, 2009) [3,8] and the United States (Williams y Hajek, 2017) [19] with variable results (Hurley et al., 2007) (3). Parasitism levels close to 100% were reported in Victoria Australia, two years after initial nematode inoculation, comparated to 90% in Encruzilhado Do sul, Brazil, after four

years (Bedding, 2009) [19] and 96% in Cape locality, South Africa after three years (Tribe & Cillé, 2004) [21]. In contrast biocontrol programs in Eastern Cape & KwaZulu-Natal Reports, South Africa, reported only 5 to 10% parasitism after two consecutive years of *D. siricidicola* inoculation (Hurley et al., 2007) [3]. The varied establishment of D. siricidicola highligst the need for region –specific control and evaluation programs.

In 2001, *S. noctilio* was detected in Chile, and an Official Control Program was developed and implemented in the same year (Beeche et al., 2012) [22]. Currently *S. noctilio* is present in fragmented areas over an approximate surface of 1,400,000 hectares of pine plantations located between the Valparaíso Region (32°30'37.76"S, 71°26'59.42"O) to Aysen 46°49'52.89"S, 71°59'36.12"O (SAG, 2012, 2017) [23, 24]. In November 2009, we confirmed the presence of *S. noctilio* in *P. radiata* plantations belonging to Forestal Mininco S.A. in the Biobio Region. Pest evaluation during 2010 revealed an affected surface of 200 ha which expanded to 20,000 ha by 2017. Between the months of April and September for the years 2010-2016, *D. siricidicola* was successfully inoculated, as has been reported previously (Hurley et al., 2007; Bedding, 2009) [3,8]. Due to the significant economic burden wood wasp represents for the forest industry coupled with the high

cost of biological control of *S. noctilio* using *D. siricidicola*, we aimed to determine the level of *S. noctilio* parasitism by *D. siricidicola* over five seasons. In addition, we estimated the effect of parasitism on *S. noctilio* populations and resulting levels of infestation in *P. radiata* plantations in the Biobio region of Chile.

2.3.-MATERIALS AND METHODS.

2.3.1.-Inoculation of *Deladenus siricidicola* in *Pinus radiate*.

This study was performed between 2012 and 2017 in *S. noctilio* infested *P. radiata* plantations on the property of Forestal Mininco S.A., located in different sites within the Biobio Region. The extent of *S. noctilio* infestation varied annually, with 3,100 ha, 5,800 ha, 11,900 ha, 19,500 ha, 20,000 ha, and 20,000 ha recorded for the years 2012, 2013, 2014, 2015, 2016 and 2017, respectively (unpublished data). Between the months of April and September for the years 2010-2016, *D. siricidicola* was inoculated in 2,500, 4,000, 11,455, 12,500, 15,000, 15,000 and 10,000 trees naturally infested by *S. noctilio*, respectively. Each tree was inoculated with 250,000 to 300,000 nematodes, as reported previously (Bedding, 2009) [3,8]. The inoculation procedure was performed as described by Bedding (2009) [8]. Trees were selected for inoculation based on diameter (greater than 15 cm), and the

absence of emergency orifices The presence of *S. noctilio* larvae was confirmed as previously through dissection of a stem segment. Trees were debranched and drilled at intervals of 30 cm in two parallel lines along the axe. Cylindrical perforations of 1 cm in diameter were made at a depth of 2 cm. *D. siricidicola* was applied in each perforation. This process was carried out between the months of April to September of each year, without rain and at environmental temperatures between 7°C and 24°C. The temperature or moisture inside the inoculated trees was not measured.

2.3.2.-Selection of sampling sites to evaluate parasitism of *S. noctilio* by *D. siricidicola*

With the aim of evaluating the efficacy of the biological control program in the macroarea of the Biobio region in southern Chile, samples sites were selected across the zone of *S. noctilio* colonization, advancing from the south to the north. Figure 2.1 illustrates a map of the sampling area prepared by Forestal Mininco using the software ArcView 3.2. The first detection of *S. noctilio* occurred in 2009 in southern localities with detection continuing through 2015 in northern localities. The Sample Units (SUs) for the evaluation of parasitism of *D. siricidicola* were established annually as new localities were colonized by the pest (Table 2.1) and in stand older than ten

years regardless of *D. siridicola* inoculation status. Each SU consisted of 3 wooden logs 1 m in length extracted from 3 randomly selected trees with evidence of *S. noctilio* infestation, including the presence of eggs of *S. noctilio* on the tree stem, chlorosis, and fading or death of the foliage (Aguilar & Lanfranco, 1988; Neumann et al., 1987) [2,5]. Non-infected trees were not sampled since they were not counted in the infected population. Therefore, the infestation data represented the population of the pest in infected trees, expressed as individuals attacked/ m^3. We established 78, 85, 92, 104, and 93 SU for the five years between 2012 and 2016, respectively (Figure 2.1, Table 2.1). In some localities, the number of sampling points decreased between years due to reduction of the pest, forest fires, and tree harvests.
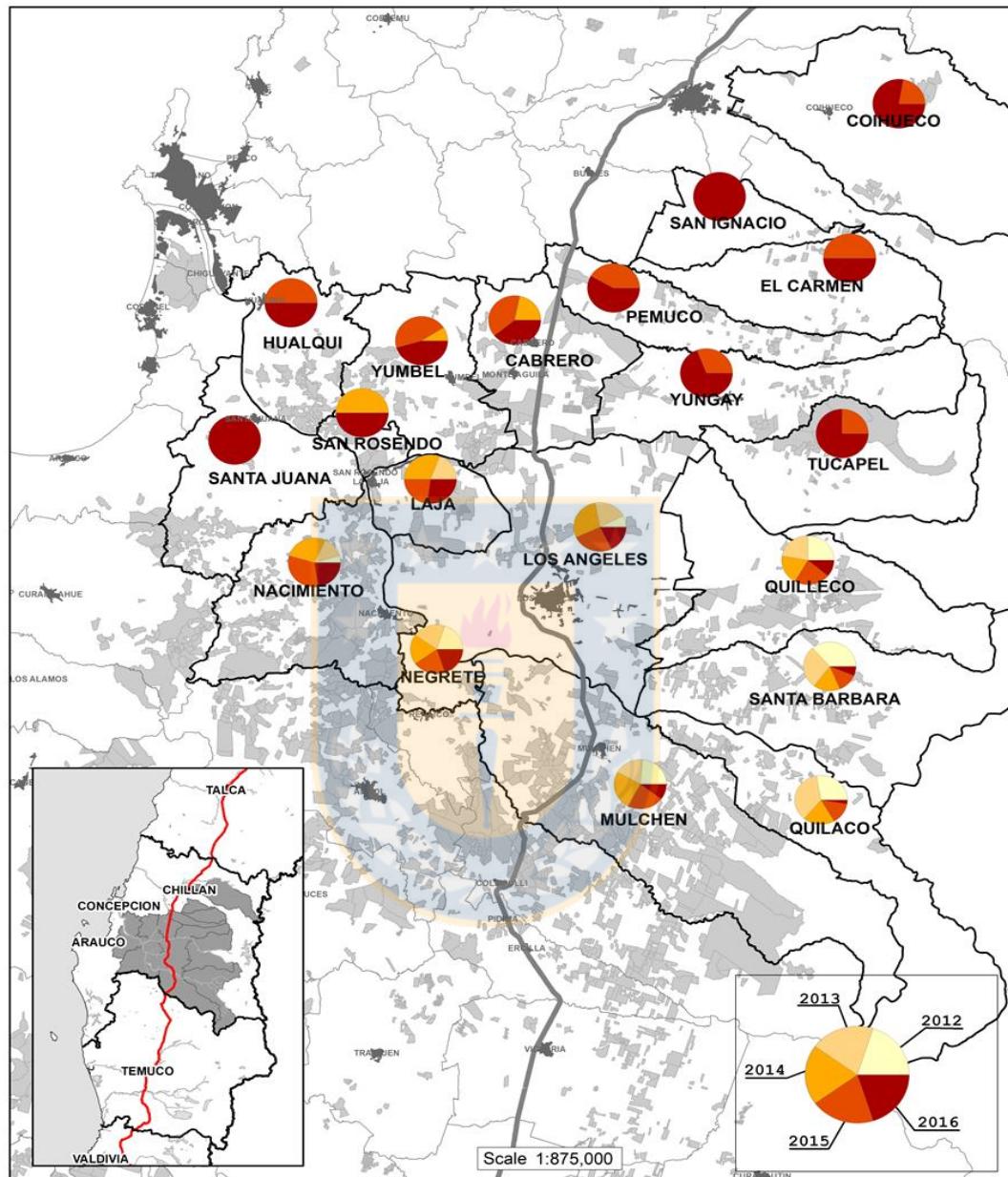
Figure 2.1. Area of sampling for evaluation of *D. siricidicola* parasitism in the Biobío region, Chile. Republished from Luis De Ferrari (personal communication) under a CC BY license, with permission from Luis De Ferrari original copyright 2018.

Table 2.1. Number of annual sampling units per locality established in *P. radiata* plantations to evaluate *D. siricidicola* parasitism of *S. noctilio.*

Locality	*D. siricidicola* Year of inoculation	Sampling units (N°)				
		2012	**2013**	**2014**	**2015**	**2016**
Quilaco	2010	10	13	7	5	1
Santa Barbara	2010	35	27	16	12	6
Los Ángeles	2011	6	16	23	20	14
Mulchen	2011	16	12	17	15	7
Nacimiento	2011	1	3	6	7	5
Negrete	2011	1	1	1	1	1
Quilleco	2011	9	8	7	8	4
Laja	2012	.	5	8	6	7
Cabrero	2013	.	.	5	9	9
Coihueco	2013	.	.	.	2	7
Pemuco	2013	.	.	.	5	7
San Rosendo	2013	.	.	1	.	1
Yumbel	2013	.	.	1	5	5
Tucapel	2014	.	.	.	1	3
Yungay	2014	.	.	.	4	9
El Carmen	2015	.	.	.	3	3
Hualqui	2015	.	.	.	1	1
San Ignacio	2016	.	.	.	.	1
Santa Juana	2016	.	.	.	.	3
Total		**78**	**85**	**92**	**104**	**93**

Selected trees from each SU were manually felled in October of each year. A meter-long log was extracted from the medial section of the stem. Prior to extraction, the presence of S. noctilio was determined by splintering the upper and lower ends of the shaft. When the insect was not detected in the stem shaft, the tree was exchanged for another infected individual.

2.3.3.-Evaluation of parasitism of *S. noctilio* by *D. siricidicola*

P. radiata logs were labeled and the ends were sealed with solid paraffin until adults hatched according to the methods described by Goycoolea et al. (2015) [25]. The tree logs from the same SU were arranged vertically in a breeding chamber comprised of a cardboard drum (1.1 m of height x 0.7 m width) with a metal mesh cover, avoiding contact between the logs and the walls of the drum. The breeding chambers were kept in a shed with semi-shade mesh (50% coverage) and a polyethylene roof for up to 30 days after the emergence of the last adult *S. noctilio* specimen. Breeding chambers were observed weekly and adult *S. noctilio* emergence was recorded. *S. noctilio* specimens were preserved in 70% alcohol along with to the other individuals that emerged from the same drum or camera. *Deladenus siricidicola* parasitism was determined in the lab by dissecting adult *S. noctilio* females using methods described by Zondang (1969) [15]. Briefly, the abdomen was cut and placed in a clock glass. Distilled water was added and the abdomen was dissected longitudinally under a dissecting scope (10x). The presence of nematodes was observed directly in the tissue, hemocele, and reproductive organs. Parasitism of *S. noctilio* by *D. siricidicola* per SU was calculated as a ratio between N° of parasitized in relation to N° total dissected.

2.3.4.-Effect of inoculation antiquity and geography on parasitism of *S. noctilio*.

To assess the effect of time passed since initial inoculation on parasitism levels, we surveyed parasitism in SU in sites with different inoculation dates. We included SUs from distinct regions (coastal mountain, central valley, and Andes mountain) in consideration of possible geographic differences including Nacimiento from the Nahuebulta mountain chains, Los Ángeles from the South Central Valley, Laja from the North Central Valley and Quilleco from the Pre-Andean site. With the exception of Laja, in which the SUs were inoculated in 2012, all localities were inoculated in 2011. For each SU, we calculated the time in years between inoculation and sampling. For each season, we aggregated parasitism levels for SUs inoculated in the same year. Data was analyzed using the Duncan multiple comparison test ($p \leq 0.05$) executed in the R version 3.4.2.

2.3.5.-Evaluation of the effect of *D. siricidicola* parasitism on *S. noctilio* population and *P. radiata* infestation.

We selected the localities Nacimiento, Los Ángeles, Quilleco, and Mulchén, which were inoculated with *D. siricidicola* in 2011, to allow for the longest possible temporal analysis of parasitism evaluation, *S. noctilio* population,

and *P. radiata* damage assessment. Sirex noctilio population was determined according to the emergence of adult specimens in the growth chambers and expressed as the number of *S. noctilio* specimens per cubic meter of infected trees. The volume was obtained by applying the formula for a cylinder

Sirex noctilio infestation of P. radiata was evaluated between 2012 and 2017. The infestation evaluation unit was comprised of lineal parcels of 30 trees. Evaluation units were established every five hectares. *Sirex noctilio* infestation was confirmed according to the signs and symptoms described by Newmann et al. (1987) and Aguilar & Lanfranco (1988) [2,5]. Results were expressed as the percentage of infested trees versus healthy trees (i.e. infestation intensity). The relationship between infestation intensity by *S. noctilio* and *D. siricidicola* parasitism was analyzed using the Duncan multiple comparison test as described above

2.4.-RESULTS

2.4.1.- *Sirex noctilio* emergence.

Total adult *S. noctilio* emergence (male and female adult specimens) between 2012 and 2016 was 3,374, 2,259, 4,033, 3,100 and 1,597 specimens, respectively. The specimens/m3 in the 2012 was of 478.3 (±22.4), in the 2013 was of 374.5 (±25.1), in the 2014 was 721.5 (±41.1), in the 2015 was 678.9 (±38.3) and in the 2016 was 461.7 (±22.9), respectively.

During the first four seasons, S. noctilio adult emergence occurred between December and April. During the fifth season, adults emerged between November and March. For the 2012– 2013 and 2015–2016 seasons, emergence peaked in January, with 41% and 60% of total emergence occurring during that month, respectively. For all other seasons, emergence was highest in December. Until the fourth season, male emergence was higher than female emergence with male to female ratios of 2.5:1, 1.6:1, 2.2:1, 2:1, and 1:2 for each season, respectively (Fig 2.2).
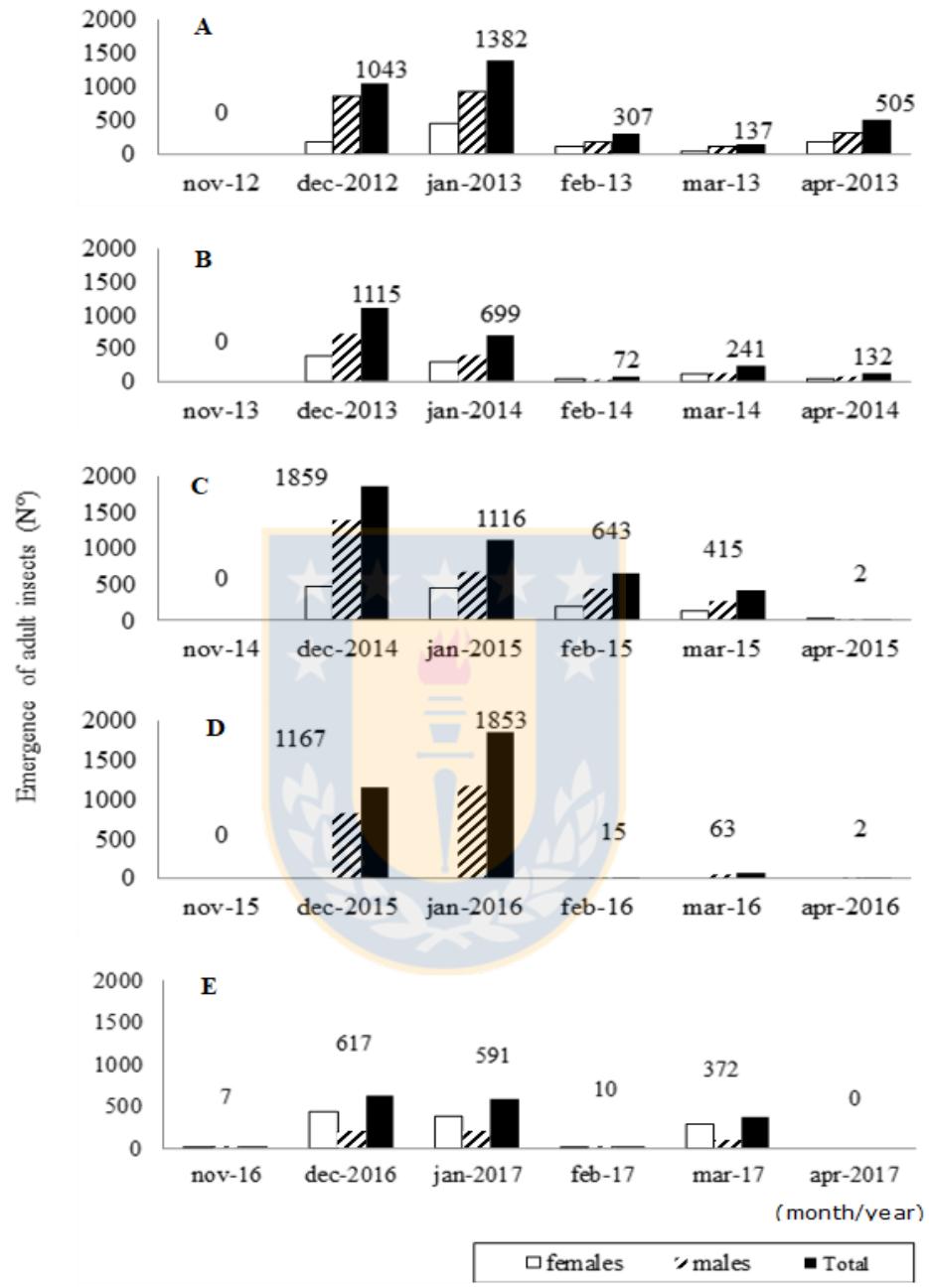
Figure 2.2. Seasonal emergence of adult *S. noctilio*. (A) Season 2012-2013; (B) Season 2013-2014; (C) Season 2014-2015; (D) Season 2015-2016, (E) Season 2016-2017

2.4.2.-*Deladenus siricidicola* parasitism of *S. noctilio* females.

Deladenus siricidicola parasitism of *S. noctilio* females reached averages of 29.6%, 61.9%, 93.6%, 96.5% and 93.1% for years 2012, 2013, 2014, 2015 and 2016, respectively (Table 2.2). The number of locations with *D. siricidicola* parasitism increased over time, varying from 57% during the first season to 100% in the third season. *D. siricidicola* parasitism increased with time in all study locations, exceeding 85% from 2014 onward (Table 2.2). During 2015 we observed *D. siricidicola* parasitism of *S. noctilio* in the non-inoculated locations of El Carmen and Hualqui (Table 2.2). In addition, we observed *D. siricidicola* parasitism in several SUs in seven non-inoculated sites in 2012 and nine non-inoculated sites in 2013.

Table 2.2. *Deladenus siricidicola* parasitism of female *S. noctilio* by locality during five seasons.

Locality – year of inoculation	Parasitism of *D. siricidicola* over female *S. noctilio* (%)				
	2012	**2013**	**2014**	**2015**	**2016**
Quilaco - 2010	**53.4** (13.9)[a]	**85.0** (9.1)[ab]	**100.0** (0.0)[a]	**96.7** (3.3)[a]	**100.0** (NE)
Santa Bárbara - 2010	**47.6** (7.2)[a]	**89.3** (6.1)[a]	**97.5** (2.5)[a]	**100.0** (0.0)[a]	**100.0** (0.0)[a]
Los Ángeles - 2011	**0.0** (0.0)[a]	**31.3** (11.3)[ab]	**90.8** (3.4)[a]	**96.7** (2.9)[a]	**94.4** (0.0)[a]
Mulchén - 2011	**17.3** (11.7)[a]	**43.3** (13.7)[ab]	**94.3** (2.7)[a]	**96.9** (2.1)[a]	**100.0** (0.0)[a]
Nacimiento - 2011	**0.0** (0.0)[a]	**0.0** (0.0)[b]	**96.9** (3.1)[a]	**90.0** (7.2)[a]	**86.3** (7,9)[b]
Negrete - 2011	**0.0** (0.0)[a]	**0.0** (0.0)[b]	**93.3** (0.0)[a]	**100.0** (0.0)[a]	**80.0** (0.0)[a]
Quilleco - 2011	**30.8** (12.4)[a]	**53.8** (18.0)[ab]	**95.8** (2.8)[a]	**100.0** (0.0)[a]	**94.7** (5.4)[a]
Laja - 2012	.	**58.3** (20.6)[ab]	**85.0** (10.6)[a]	**100.0** (0.0)[a]	**85.4** (5.5)[a]
Cabrero - 2013	.	.	**100.0** (0.0)[a]	**93.4** (4.2)[a]	**97.9** (1.1)[a]
Coihueco - 2013	.	.	.	**100.0** (0.0)[a]	**85.7** (14.3)[a]
Pemuco - 2013	.	.	.	**85.4** (13.8)[a]	**88.9** (4.3)[a]
San Rosendo - 2013	.	.	**91.3** (0.0)a	.	.
Yumbel - 2013	.	.	**100.0** (0.0)a	**100.0** (0.0)[a]	**100.0** (0.0)[a]
Tucapel - 2014	.	.	.	**100.0** (0.0)[a]	**97.4** (2.6)[a]
Yungay - 2014	.	.	.	**98.2** (1.8)[a]	**98.9** (7.7)[a]
El Carmen - 2015	.	.	.	**100.0** (0.0)[a]	**92.3** (NE)
Hualqui - 2015	.	.	.	**91.3** (0.0)[a]	**90.0** (NE)
San Ignacio - 2016	.	.	.	.	**100.0** (NE)
Santa Juana - 2016	.	.	.	.	**100,0** (NE)
Total	**29,6** (±2,6)	**61.9** (±3,0)	**93.6** (±0,5)	**96,5** (±0,4)	**93,1** (±0,9)

Values in parentheses indicate standard error. NE indicated Not evaluated standar error. The different letters indicate statistically significant differences between mean parasitism levels per locality within the same year of evaluation (p≤0.05).

2.4.3.-Effect of time since inoculation on *D. siricidicola* parasitism of *S. noctilio*

Parasitism levels in relation to time since inoculation were different for years 2012 and 2013 in comparison to years 2014, 2015 and 2016. During 2012 and 2013 parasitism levels increased as a function of time since inoculation. For the year 2013, parasitism levels were 52.3%, 62.5, and 91.5% for one, two, and three years after *D. siricidicola* inoculation, respectively. Table 2.3 presents data collected from SUs of the 2011 inoculation area. Between the years 2014 and 2016 parasitism levels where higher, more homogenous, and independent of time since inoculation (Table 2.3). Parasitism levels in relation to time since inoculation increased progressively from the years 2012 to 2014. For one, two, and three years after inoculation, parasitism levels increased from 21.5% to 90.9%, 60.7% to 96.9%, and 91.5% to 90.6%, respectively. From 2015 onward, parasitism levels were higher and more homogeneous than previous years with values around 90% (Table 2.3).

Table 2.3. Female *S. noctilio* parasitism according to the age of *D. siricidicola* inoculation.

Age of inoculation with *D.siricidicola* (Years)	*S. noctilio* female parasitism (%)									
	2012		2013		2014		2015		2016	
	%	n	%	n	%	n	%	n	%	n
1	**21.5**(6.1)[b]	30	**52.3** (15.2)[b]	11	**90.9** (8.5)[a]	8	**100.0** (0.0)[a]	7	**92.6** (5.5)[a]	18
2	**60.7** (15.2)[a]	5	**62.5** (7.6)[b]	35	**96.9** (3.1)[a]	8	**98.5** (1.5)[a]	7	**94.7** (3.4)[a]	5
3	.	.	**91.5** (7.2)[a]	8	**90.6** (3.7)[a]	28	**100.0** (0.0)[a]	6	**97.9** (1.5)[a]	7
4	.	.	.	.	**100.0** (0.0)[a]	3	**94.3** (3.4)[a]	21	**95.0** (5.0)[a]	5
5	.	.	.	.	.	.	**94.4** (5.6)[a]	3	**82.3** (9.7)[a]	10
6	.	.	.	.	.	.	.	.	**100.0** (NE)	1

Values in parentheses indicate standard error. NE indicated not evaluated standard error. The lowercase letter n indicates the number of SUs evaluated. The different letters indicate statistically significant differences between the mean corresponding to time since inoculation (rows) in each year of evaluation (columns) ($p \leq 0.05$).

2.4.4.-Geographical effect on *D. siricidicola* parasitism.

Deladenus siricidicola parasitism of S. noctilio was determined in four localities representing different geographical zones. No parasitism was observed in Nacimiento from the Nahuebulta mountain chain during the years 2012 and 2013. Los Angeles from the South Central Valley also showed no parasitism during 2012. From the year 2014 onward we observed parasitism in all localities with increased levels of parasitism in comparison

to earlier years reaching values ranging between 90.8 and 100% (Table 2.2).

Increases in parasitism levels were generally of greater magnitude between 1 to 2 and 2 to 3 years after inoculation compared to later time points (Table 2.4). The largest increases were reported in the South Central Valley (59%) during the third year and in the Nahuelbuta mountain chain (90.7%) during the second year year (p≤0.05) (Table 2.4).

Table 2. 4. Annual levels of *D. siricidicola* parasitism of female *S. noctili*

Location	Increase in *D. siricidicola* parasitism over female *S. noctilio* per location (%), between inoculation years.							
	Year (2-1)		Year (3-2)		Year 4-3		Year 5-4	
	(%)	(n)	(%)	(n)	(%)	(n)	(%)	(n)
North Central Valley (Laja)	22.1 (28.0)^a	5	4.4 (4.4)^b	5	-10.8 (±6.4) b	4	.	.
South Central Valley (Los Ángeles)	25.0 (19.4)^a	5	59,0 (16,0)^{ab}	13	13.5 (5.7)^a	12	-2.2 (±1.5) ab	10
Nahuelbuta mountain chain (Nacimiento)	0.0 (NE)^a	1	90,7 (9.4)^a	2	6.2 (6.2)^a	3	3.8 (±6.9) a	5
Pre-andean sites (Qiilleco)	45.8 (17.5)^a	5	41,3 (19.4)^{ab}	5	8.3 (4.8)^a	3	-7.1 (±7.1) ab	3

Values in parentheses indicate standard error. The different letters indicate statistically significant differences between the mean from each location within each annual increase (p≤0.05).

2.4.5.-Effect of *D. siricidicola* parasitism on *S. noctilio* populations and *P. radiata* infestation.

The average population of *S. noctilio* for the four localities during five seasons between 2012 and 2016 was 408.7, 476.5, 881.5, 544.4, and 394.5 specimens/m^3 (Table 2.5). The *S. noctilio* population increased from 2012 to 2014. By 2016, the population fell below initial levels, representing an overall 3.4% decrease in the pest density (Table 2.5). During the third year of assessment (2014), the highest emergence of *S. noctilio* was observed during the entire study period. During the same season we observed an increase in *D. siricidicola* parasitism levels which then stabilized at 90% in all four localities (Table 2.2). Between 2015 and 2016, the *S. noctilio* population decreased (Fig 2.3).
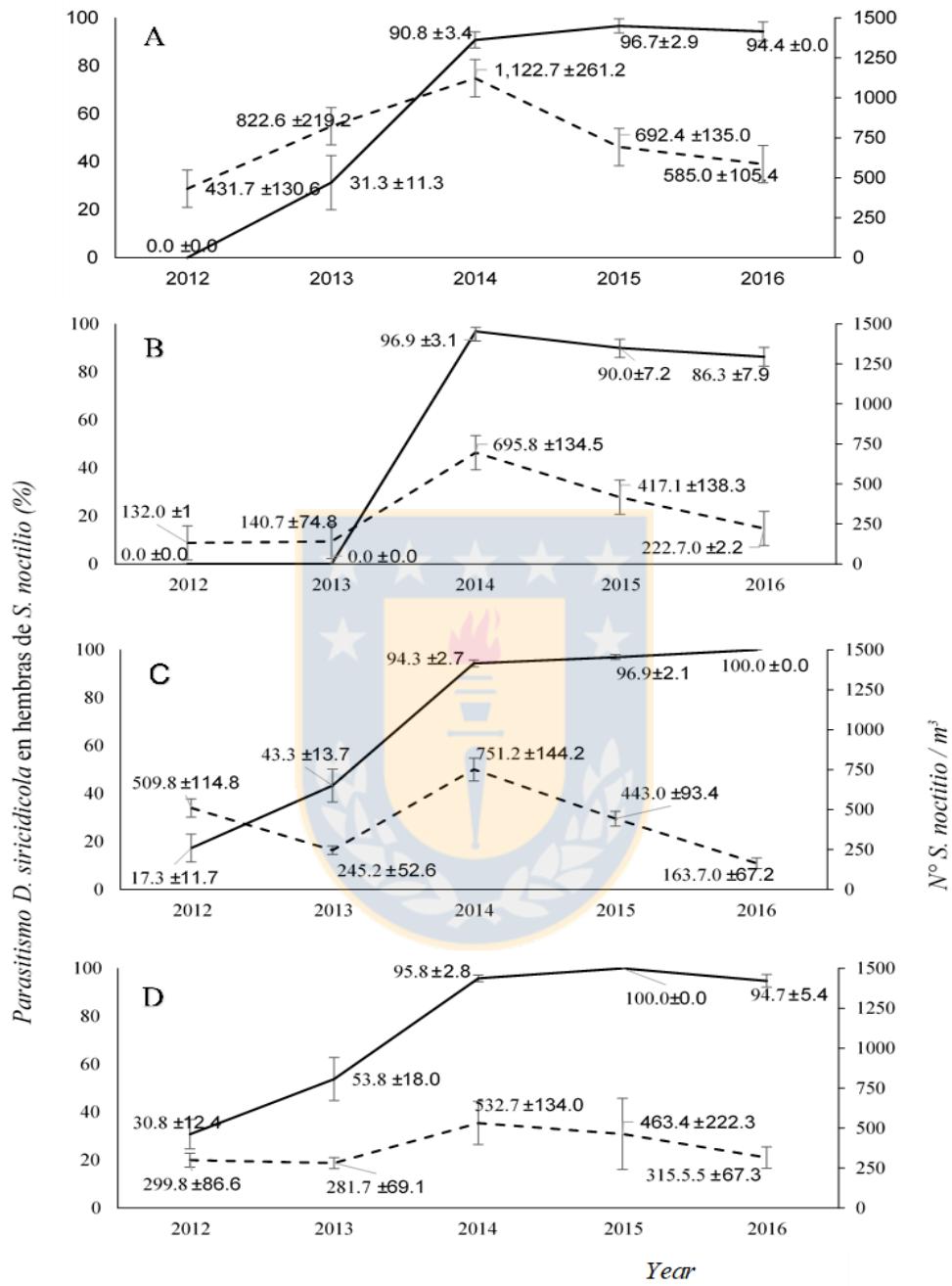
Figure 2.3. *D. siricidicola* parasitism levels and *S. noctilio* populations levels between years 2012 to 2016 in A) Los Ángeles, B) Nacimiento,C) Mulchén y D) Quilleco

Table 2.5. *Sirex noctilio* adult emergence obtained for the SUs from Los Ángeles, Mulchén, Nacimiento, and Quilleco for five seasons.

Locality	*S. noctilio*/m^3 by season				
	2012	2013	2014	2015	2016
Los Ángeles	431.7(±130.6)a	822.6(±216.2)a	1122.7(±236.7)a	692.4(±135.0)a	565.0(±105.5)a
Mulchén	509.8(±114.8)a	245.3(±52.6)b	751.2(±144.2)a	443.0(±93.4)a	163.7(±67.2)b
Nacimiento	132.0(NE)	140.7(±74.8)b	695.8(±134.5)a	417.1(±138.3)a	222.7(±22.2)ab
Quilleco	299.8(±86.6)a	281.7(±69.1)b	532.1(±134.0)a	463.4(±222.3)a	315.5(±67.3)ab
Average	408.7 (21.5)	476.5 (45.6)	881.5 (33.3)	544.4 (17.8)	394.5 (35.3)

Values in parentheses indicate standard error. NE: indicated not evaluated standard error.The different letters indicate statistically significant differences between the mean from each location within each season (p≤0.05).

Total *S. noctilio* infestation of *P. radiata* ranged from 0.3% to 5.9% between the first and last year of assessment (Table 2.6). For all four localities, *S. noctilio* infestation peaked during 2015 coinciding with the season in which the woodwasp population levels started to decrease (Fig 2.3, Table 2.5). In each subsequent year, infestation levels decreased (Table 2.6). *S. noctilio* infestation levels started to decrease during 2016, two years after *D. siricidicola* parasitism levels stabilized in 90% and one season after of the initial *S. noctilio* population decrease (Table 2.6).

Table 2.6. *Sirex noctilio* infestation of *P. radiata* determined in the Los Ángeles, Mulchén, Nacimiento and Quilleco localities during six seasons.

Locality	Sirex associated damage (%)					
	2012	**2013**	**2014**	**2015**	**2016**	**2017**
	(%)	**(%)**	**(%)**	**(%)**	**(%)**	**(%)**
Los Ángeles	**0.0** (±0.0)[a]	**2.9** (±0.6)[a]	**3.7** (±0.4)[a]	**13.5** (±1.5)[a]	**11.6** (±1.4)[a]	**8.9** (±1.9)[a]
Mulchen	**0.4** (±0.3)[a]	**3.7** (±1.3)[a]	**2.5** (±0.5)[ab]	**12.6** (±3.3)a	**3.9** (±1.0)[b]	2.1(±0.6)[b]
Nacimiento		**1.6** (±1.1)[a]	**1.9** (±0.4)[ab]	**9.7** (±1.4)[a]	**8.4** (±1.2)[ab]	4.3(±1.2)[ab]
Quilleco	**0.4** (±0.4)[a]	**5.8** (±3.4)[a]	**0.7** (±0.4)[b]	**9.3**(±1.1)[a]	**8.7** (±3.8)[ab]	**3.1** (±0.9)[b]
Mean plus	0.3	3.2	3.0	11.6	**9.2 (±0.2)**	5.9
SEM	**(±0.1)**	**(±0.2)**	**(±0.3)**	**(±0.2)**		**(±0.4)**

Values in parentheses indicate standard error. NE: indicated not evaluated standard error. The different letters indicate statistically significant differences between the mean from each location within each season (p≤0.05)

2.5.-DISCUSSION.

Evaluating *D. siricidicola* parasitism of *S. noctilio* revealed a continuous increase in the frequency of *D. siricidicola* detection and parasitism, reaching 100% and 93.6%, respectively, from 2014 onward. *D. siricidicola* nematode samples where obtained from naturally infested trees suggesting establishment of the nematode in the area as well as the probable natural dispersion through flight periods of the females as suggested by Taylor (1981) [20]. The mean parasitism levels reported during the last three seasons of evaluation (93.6%, 96.5%, and 93.1%) are closer to those reported in South Africa (96% in Cape Locality) and Brazil (90 % in Encruzilhada Do Sul) than

to the 100% parasitism levels reported for Victoria, Australia two years after inoculation (Bedding, 2009)[8]. The parasitism levels observed in this study were also higher than those reported in the US by Williams and Hajek (2017) [19], which ranged from 20.5-28.1% and 13.6-17.6%.

The wide fluctuation in parasitism levels between locations during the first two seasons of assessment (0% to 89%) suggests that *D. siricidicola* was in the middle of the colonization process. This situation changed in 2014 when parasitism levels were higher (up to 100%) and more homogeneous. The apparent absence of *D. siricidicola* during 2012 and 2013 could be due to several factors. Consistent with the density dependence question raised by Bedding (2009) [8], the low levels of inoculation after one year coupled with low *Sirex* populations could have rendered *D. siricidicola* parasitism undetectable. *D. siricidicola* parasitism was determined under a dissecting scope, a sensitive method to detect the presence of a nematode. Assuming that parasitism existed in the field, the number of samples may have been an insufficient representation of a population with low nematode presence. Moreover, *D. siricidicola* inoculum may vary in their parasitic ability as reported by Yu et al (2009) [7] in Canada, Williams and Hajek (2017) [19] in the US, and Bedding (2009) [8] in Australia. However, the viability of

inoculum as well as the inoculation technique was similar to those reported in successful cases.

The interaction between nematodes and the symbiotic fungus *A. areolatum* within *S. noctilio* was not analyzed in the present study. It¨s has been reported that nematode reproduction can be negatively affected by a unique mechanism of parasitism the adults and eggs by fungus *A. aerrolatum* (Morris & Hajek, 2014) (26). This discovery provide a possible explanation why *Deladenus* does not survivein culture when *Amylostereum* is fast growing or when the ratio of nematodes to fungus isinordinately biased toward the fungus (Morris et al., 2014) (26). This fungus has been shown to interfere with biological control of wood wasp, but in this work, the presence of *A. aerolatum* was not observed (or studied) and must be studied in the future. The insect *Ips grandicollis* has also been reported to effect biocontrol of wood wasps (Yousul et al., 2014) [27] but to our knowledge, this insect has not been reported in Chile.

Overall, the estimated population of *S. noctilio* decreased by 3.4% from 2012 to 2016. The population increased and peaked in the third season, then progressively decreased from the fourth season until the fifth, reaching population numbers lower than initial levels in 2012 (Table 2.5). This is

consistent with the results of Williams & Hajek (2017) [18] in the US, where the densities of *S. noctilio* were 1,000 Sirex/m^3 and 300 Sirex/m^3 in 2007 and 2012, respectively.

Infestation increased from 0.3 to 11,6% of trees between 2012 and 2015 followed by decreases in subsequent years reaching as low as 5.9%. The decline in infestation occurred one year after the pest population decreased. The inflection points in the curves of population density and infestation occurred after one season and two seasons, respectively, following the stabilization of *D. siricidicola* parasitism levels of *S. noctilio* at 90% in each of the four localities (Figure 2.3, Table 2.5, 2.6).

The flight period was detected between November and April in this study consistent with the range of flight period from October to May reported by Ruiz (2006) [28] in the southern hemisphere and with the October to April period indicated by Iede et al. (1993) [29] in Brazil. The 2:1 male to female emergence ratio reported during the first two seasons of the study indicated that the wood wasps were in the process of colonization (Ruiz, 2006) [28] and reached stability during the last season analyzed. In this study no effect of the zone on the level of parasitism was determined. The similarity in levels of parasitism observed between Nahuelbuta, the Central Valley (north and

south), and the Andean Precordillera as of 2014 was unexpected. *D. siricidicola* parasitism was to increase more slowly in the central valley as the product of a larger pest population; however, parasitism in the Central Valley (Los Angeles) was comparable to that of the other zones. This is due to the high parasitic capacity of *Deladenus* at high densities of the pest (Bedding, 2009; Williams & Hajek, 2017; Kroll et al., 2013) [8, 19, 30] Notably, climatic variations between zones were not significant, with average temperatures for the period under study of 14.1 ° C, 15 ° C, and 14 ° C for the Precordillera, Central Valley, and Nahuelbuta, respectively.

Catastrophic levels of damage caused by *S. noctilio* were expected in the study area, particularly in the Bio Bio region. However, economic losses reported to date have not significantly compromised the forest industry in Chile (personal communication). These results are promising in terms of controlling *S. noctilio* populations, especially given the observed penetrance of the nematode *D. siricidicola*. Nonetheless, long-term monitoring of *S. noctilio* must be considered to detect and prevent potential outbreaks. During the last year of the study, the general level of parasitism decreased from 96.5 ($\pm$ 0.4) in 2015 to 93.1 ($\pm$ 0.9) in 2016.

This observation is critical to understanding the dynamics of the nematode and wood wasp relationship and the long-term biocontrol of the pest. Achieving high levels of parasitism may come at the cost of reducing nematode presence in the environment due to reduction of the wood wasp host and its role as a dispersion medium for the nematode. Given that the success of nematodes as a biocontrol measure is density-dependent (Bedding, 2009) [8], drastically reducing the wood wasp population would likely limit the range and propagation of the nematode, which could affect the sustainability and long-term success of the biocontrol program.

2.6.-CONCLUSIONS.

The nematode *D. siricidicola*, parasite of the woodwasp *S. noctilio*, has been successfully established in the study area of the Biobio region in southern Chile, dispersing naturally into pine plantations where the nematode was not actively inoculated. Parasitism levels of *S. noctilio* by the nematode *D. siricidicola* increased progressively from inoculation until the third year following inoculation to eventually stabilize around 90% of parasitism. Three years after inoculation, *D. siricidicola* was established in similar levels in all three regions including Pre-Andean sites, the Central Valley, and the Nahuelbuta mountain range in the Biobio region. Our work demonstrates the

reduction of *S. noctilio* populations and associated decrease in *P. radiata* infestation after parasitism by the nematode *D. siricidicola* reached 90% in all localities and serves as a useful example of the scale and timeframe over which biological control of *S. noctilio* is possible.

2.7.-Acknowledgment

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CAPITULO III. Determination of *Deladenus siricidicola* Bedding (Tylenchida: Neotylenchidae) parasitizing *Sirex noctilio* Fabricius (hymenoptera: siricidae) in southern Chile.

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3.1.-ABSTRACT

The woodwasp *Sirex noctilio* is one of the most threatening pests of *Pinus* species in the southern hemisphere. The Kamona strain of the female sterilizing nematode *Deladenus siricidicola* is the most important control agent of this wasp. A "non-sterilizing" (NS) strain of *D. siricidicola* has been reported in Europe, Japan, New Zealand, and North America. *S. noctilio* was detected in 2009 in the Biobío region of Chile, and implementation of an intense biocontrol program using *Deladenus siricidicola* (Encruziliada do Sul) followed in 2010. We studied *D. siricidicola* parasitism and the presence of the Kamona and NS strain in the Biobío Region of Chile eight years after implementation of the biocontrol program. Parasitism by *D. siricidicola* was determined thorugh dissection of *S. noctilio* adult females. The Kamona and NS strains were distinguished by DNA sequencing and PCR-RFLP using the restriction enzymes Acil, Rsal, and BsrGl. Parasitism of *S. noctilio* by *D. siricidicola* reached 93.1% with detection of the Kamona strain in 100% of parasitized wasps. Finally, the NS strain of *D. siricidicola* was detected for the first time in Chile, at parasitism levels of 30%.

Key words: *Pinus*, *Sirex*, *Deladenus,* Biocontrol, Chile.

3.2.-INTRODUCTION.

The woodwasp *Sirex noctilio Fabricius* (Hymenoptera: *Siricidae*), native to Eurasia, deteriorates wood quality and causes mortality in *Pinus* species (Talbot, 1977). It is considered one of the most economically important pests in countries of the southern hemisphere, where it has dispersed widely in the absence of native natural enemies (Williams and Hajek, 2017). This wasp has been reported outside its origin in New Zealand (1900), Australia (1952), Uruguay (1980), Argentina (1985), Brazil (1988), South Africa (1994), Chile (2001), United States (2004), Canada (2005) and China (2013) (Bain 2005; Bedding 2009; Hoebebeke et al., 2005; De Groot et al., 2006; Li et al., 2015). The most widely recognized natural agent for efficient control of this pest in the southern hemisphere is the nematode *Deladenus siridicola* (Bedding 2009; Bedding and Arkus, 1974). *D. siridicola* exhibits a mycetophagous life form, feeding on the fungus *Amylostereum areolatum* (Fries) Boidin present in the wood of infested trees (Bain, 2005). Additionally, the nematode demonstrates an infective form that that acquires in the vicinity of wasp, parasitizing larvae and pupae of *S. noctilio* (Bain, 2005). Once inside the parasitized wasp, female *D. siricidicola* produce juveniles which migrate to the female reproductive organs, penetrating the wasp eggs upon pupation,

rendering the females sterile, and compromising the viability of eggs (Lanfranco and Aguilar, 1990). As host development and growth are unaffected by parasitism, female *S. noctilio* wasps emerge, fly, and deposit eggs infected with the nematode in a recurrent manner, effectively dispersing the biocontrol agent (Williams and Hajek, 2017, Taylor, 1981).

The selection of *D. siricidicola* for use in biocontrol programs was developed in Australia by the Congress of Scientific and Industrial Research Organization (CSIRO) during the 1960s and 1970s (Bedding, 2009). In this program, several species of *Sirex* affecting conifers in Europe, the United States, Canada, India, Pakistan, Turkey, Morocco and Japan were collected, yielding seven distinct species of *Deladenus* (Bedding and Akhurst, 1974). *D. siricidicola* - Sopron provenance was selected for its ability to sterilize females, high levels of parasitism, and minimal impact on wasp adult size (Bedding 2009). In Australia, *D. siricidicola* – Sopron was mass produced in the laboratory in its free-living state and subsequently used with success to control *S. noctilio* populations. However, the prolonged in vitro culture time of the nematode (20 years) using only the mycetophage cycle eventually selected for a strain with depleted biocontrol capacity (Bedding, 2009). In response, the original strain was re-isolated in 1991 from the Kamona locality

of Tasmania where the Sopron strain was originally released in the 1970s (Bedding, 2009). This strain is currently recognized as *D. siricidicola* – Kamona and is widely used for woodwasp control (Bedding, 2009; Williams & Hajek, 2017).

Deladenus siricidicola – Sopron was first introduced into Brazil from Australia in 1989 followed by reintroduction of the Kamona strain in 1995. As a result, a residual population of the "defective" *D. siricidicola* strain may persist in Brazil and the surrounding region. Kamona strain nematodes isolated from *S. noctilio* in Brazil were sent to Argentina in 1995 and then to Uruguay and Chile in subsequent years (Hurley et al., 2007). In addition to the defective Australian strain, a "non-sterilizing" (NS) strain of *D. siricidicola* was first detected in New Zealand in the 1970s (Zondag, 1975) followed by similar detection in Europe, Japan (Bedding & Akhurts, 1978), Canada, and the United States (Yu et al., 2009; Kroll et al., 2013; Williams and Hajek, 2017). The NS strain of *D. siricidicola* associates with the ovaries of *S. noctilio* but does not penetrate the eggs and therefore does not cause infertility (Yu et al., 2009; Willimas et al., 2009; Williams & Hajek, 2017; Kroll et al., 2013). Together, these detection events suggest geographic

diversity of *D. siricidicola* strains with region-specific consequences for woodwasp biocontrol.

Sirex noctilio has been present in Chile since 2001 and currently affects approximately 1,400,000 ha of *Pinus radiata* between the regions of Valparaíso (33° 24'44 "S, 70° 37'12" W) and Aysén (47° 04'23 "S, 71° 57'46" W) (SAG 2012, 2017). Detection of *S. noctilio* in the Biobío Region in 2009 catalyzed an intense biocontrol program using *D. siricidicola* origin Encruziliada do Sul (Beeche et al., 2012). Parasitism in this region between 2012 and 2016 increased from 29% to 93%, resulting in a 3.4% decrease in the woodwasp population. Moreover, infestation of *Pinus radiata* decreased from 11.6% to 5.9% between 2015 and 2017, indicating positive outcomes from the biocontrol program (Castillo et al., 2018).

Despite these advances in controlling *S. noctilio* populations in Chile, little is known about *D. siricidicola* strain diversity in this region. Considering the detection NS strains elsewhere in the world and the implications for costly biocontrol programs, we aimed to evaluate the parasitism of *D. siricidicola*, verify the presence of the Kamona strain, and determine the presence of the non-sterilizing strain in Biobío region of southern Chile.

3.3.-MATERIALS AND METHODS.

3.3.1.- Field Sampling.

The study was conducted in *P. radiata* plantations in the Biobío region of Chile from October 2017 to May 2018. In the study area, 76,000 trees were inoculated with *D. siricidicola* origin Encruziliada do Sul between 2010 and 2017. Nematode inoculates were produced by the Consortium of Phytosanitary Protection SA located in Los Angeles, Biobío Region, Chile, and were applied on trees naturally attacked by the woodwasp at a dose of 200,000 to 300,000 nematodes per tree (Bedding, 2009; Goycoolea et al., 2015). Inoculation took place from April to September each year, avoiding rain and at temperatures that fluctuated between 7ºC to 24°C according to the methodology proposed by Goycoolea et al., (2015).

Nine sampling zones were established in the study region (Nacimiento, Mulchen, Santa Bárbara, Laja, Los Angeles, Quilleco, Hualqui, Cabrero-Pemuco, El Carmen). In each sampling area, three *P. radiata* stands 15-17 years in age infected with woodwasps were selected regardless of previous *D. siricidicola* inoculation status. Three trees showing obvious signs of *S. noctilio* oviposures were randomly selected from each stand.
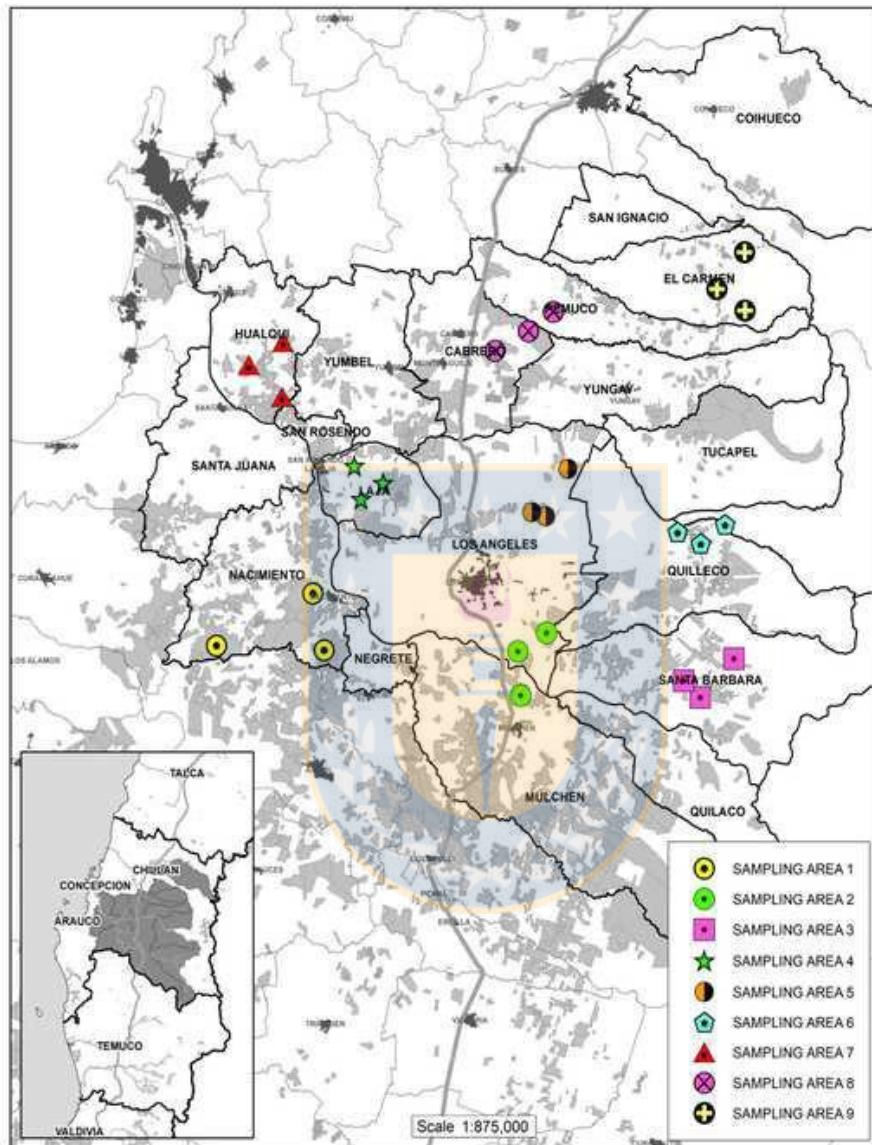
Figure 3.1. Sampling zones for evaluation of parasitism of *Sirex noctilio* by

Deladenus siricidicola. (Map was elaborated using software ArcView 3.2).

The selected trees were cut in October 2017, and a 1 m long log was extracted from the middle section of each tree. The three logs collected from the same stand were kept together in aging chambers at room temperature (14°C-25°C) for 8 months (October 2017 to May 2018). The aging chamber consisted of a cardboard drum (1.1 m high by 0.7 m wide) with a metal mesh cover (Goycoolea et al., 2015). *S. noctilio* adults were collected after emergence, registered at the level of sampling area and breeding chamber, and fixed in 70% ethanol.

3.3.2.- Determination of parasitism of *Sirex noctilio* females by *Deladenus siricidicola*, through dissection.

Sirex noctilio females collected from the breeding chambers were dissected using the methodology described by Zondang (1969). The abdomen was cut longitudinally on clock glasses containing distilled water, and the presence of nematodes in the abdomen, eggs, and ovaries was evaluated under a magnifying glass (10x). Parasitism for each stand (chamber) was calculated according to the following formula:

$$Parasitized\ S\ noctilio\ female\ (\%) = \left(\frac{N^{\underline{o}}\ of\ parasitized\ with\ Deladenus}{N^{\underline{o}}\ of\ Sirex\ dissected}\right) * 100$$

Parasitism results were aggregated at the level of sampling area.

Differences between sampling zones were tested using the MIXED procedure for 'plague incidence mean' with lsmeans ($p \leq 0.05$) (SAS-Institute 1996).

3.3.3 Molecular identification of nematode species and strain

We randomly selected 10 *S. noctilio* females from each sampling area (total n=90) parasitized with *D. siricidicola* as determined by dissection. Abdomens were extracted from the samples stored in 70% ethanol using a flamed scalpel to avoid cross contamination between samples. DNA was extracted using the QIAamp Microkit according to the manufacturer's instructions with a final elution volume of 60 µL. Nucleic acid concentration was quantified using the Thermo Scientifics Nanodrop 2000 spectrophotometer. Samples were subsequently diluted to achieve an equal concentration of 50 ng/µL.

The gene for the mitochondrial protein cytochrome oxidase I (mtCOI) is used to distinguish strains of the nematode *Deladenus siricidicola* with primers DSCOI-F (5'CCTACTATGATTGGTGGTTTTGGTAAT3') and DSCOI-R1 (5'CAGGCAGTAAAATA AGCACGAGAATCT 3') (Yu et

al., 2009; Williams & Hajek, 2017). The thermocycler program for amplification consisted of an initial denaturing stage at 94 °C for 3 minutes followed by 32 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 1 minute followed by a final extension at 72 °C for 5 minutes. Resulting PCR products were separated by electrophoresis on a 2% agarose gel. GelRed was used to visualize the DNA compared to the molecular weight marker GeneRuler 1Kb plus under excitation by UV light in a Vilber Lourmat transilluminator.

An enzymatic restriction assay was performed by PCR-RFLP (Restriction Fragment Length Polymorphism) to identify the nematode strains present in *S. noctilio* abdomen samples. To define how to differentiate between strains, we analyzed the sequences of mtCOl gene fragments available in the NCBI Genbank, including the Kamona strain [Genbank JQ241276.1], the *D. siricidicola* noc 173 strain (North American non-sterilizing strain) [Genbtnk JX104276.1] and *Deladenus proximus* [Genbank KU705689.1. Additionally, the mtCOI PCR product from the *Deladenus* sp. collected in Chile was sequenced by HiSeq 2000 (Illumina) at Macrogen Inc. (https://dna.macrogen.com) according to the manufacturer's recommendations. The sequences were analyzed *in silico* using MegAline

and Clustal W. NEBcutter (New England Biolabs) was used to determine restriction enzyme cutting sites.

The restriction enzyme AciI was used to differentiate between the Kamona and NS strain of *D. siricidicola,* as this enzyme that cuts the 5'-CCGC-3' palindromic sequence in *mtCOI* generates two bands corresponding to 433 bp and 247 bp in the NS strain but does not cut the Kamona strain (Morris et al., 2013; Williams & Hajek, 2017). The restriction enzyme RsaI was used to complement and corroborate this distinction, as it cuts the Kamona strain at two sites yielding three fragments of 323 bp, 230 bp and 127 bp while cutting the NS strain at three sites yielding four fragments of 323 bp, 127 bp, 126 bp and 104 bp (Morris et al., 2013).

Likewise, the BsrGI restriction enzyme was used to differentiate between the Kamona strain of *D. siricidicola,* which it does not cut, and both *Deladenus proximus* Bedding and the NS *D. siricidicola* strain. BsrGI cuts the NS strain of *D. siricidicola* into two fragments of 253 bp and 427 bp, banding pattern very similar to that obtained with *D. Proximus*, generating two fragments of 253 bp and 426 bp. (Williams & Hajek, 2017). PCR-RFLP was performed by incubating 10 µl of the PCR product, 1.5 µl of 10X Buffer, 5 Units of each enzyme, and 3 µl of H_2O at 37 °C for 24h. The reactions were inactivated by

subsequently incubating the mixture at the respective temperature indicated by the supplier for each restriction enzyme for 20 minutes. Digestion products were labeled with GelRed and visualized with electrophoresis in 2% agarose or 10% polyachrilamide gel in the case of the digestion with the enzyme RsaI, using UV light to reveal the banding pattern. A clone of *D. siricidicola* Kamona previously characterized by our group was used as a positive digestion control (unpublished data).

3.3.4.- Evaluation of *S. noctilio* eggs by electron microscopy.

The presence of nematodes in 40 randomly selected *S. noctilio* eggs was evaluated by scanning electron microscopy. Approximately 0.5 cm^2 segments of *S. noctilio* eggs were incubated in fixation solution (3% glutaraldehyde (v/v), Sodium cacodylate 0.268M, pH 7.2) at 4 °C for 3 hours. Samples were then rinsed with 0.268 M Sodium Cacodylate pH 7.2 and dehydrated in solutions of increasing acetone concentration (50, 70, 95 and 100%; 1 h each dipping). The fixed *S. noctilio* eggs underwent a critical drying point and were mounted on an aluminum support. A gold bath was applied using the variam/vacuum division equipment (vacuum evaporator PS 100E) courtesy of the Department of Microscopy of the Faculty of Biological Sciences of the Pontificia Universidad Católica de Chile. The observations

were made in 100X and 300X magnifications in the SEM LEO 1420VP Scanning Electron Microscope.

3.4.-RESULTS.

During the summer of 2017-2018, a total of 703 *S. noctilio* individuals emerged from the 27 breeding chambers, 347 of which (49%) were females. This 1:1 male to female sex ratio was consistent across all nine areas evaluated (Figure 3.2). Total *S. noctilio* emergence was higher in sampling areas 8 and 9 which corresponded to the northern extents of the central valley and the pre-Cordillera of the Andes, respectively (Figure 3.1 and 3.2).
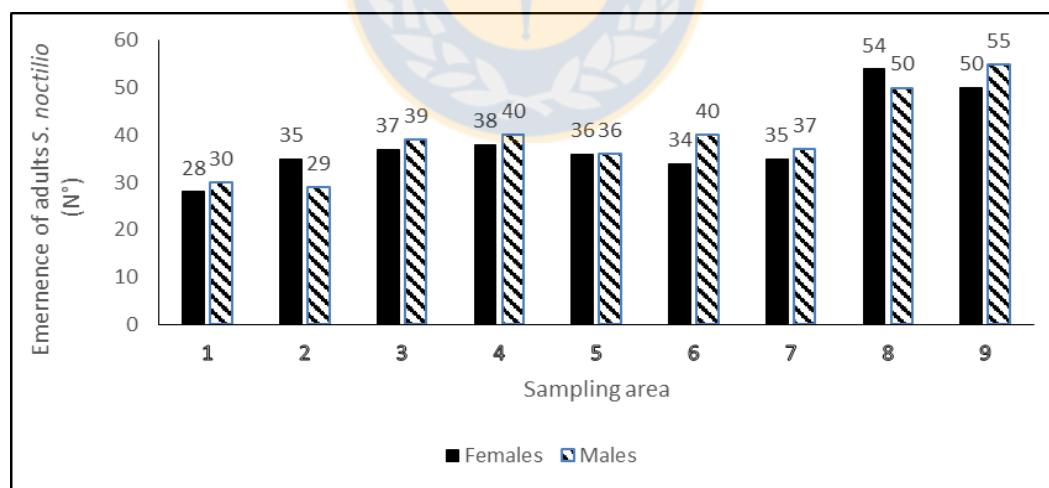
Figure 3.2. Male to female ratio of adult *Sirex noctilio* emergence from the nine sampling areas.

In all sampling areas, the presence of nematodes morphologically corresponding to *D. siricidicola* was determined by dissection of *S. noctilio* adult females under stereoscopic microscopy (Figure 3.3a). Average parasitism across all sampling areas was 93.1%, ranging from 81.5% to 100%. In three sampling zones, 100% parasitism was observed (Table 3.1).

Table 3.1. Parasitism of the *Sirex noctilio* females across nine sampling areas during the 2017-2018 summer as determined by abdominal dissection

Sampling area	Dissected females (N°)	Parasitism in females of *S. noctilio* (%)
1	28	92.9 (± 4.1)[ab]
2	35	85.9 (± 7.1)[ab]
3	37	94.4 (± 5.6)[ab]
4	38	81.9 (± 9.7)[b]
5	36	100.0 (± 0.0)[a]
6	34	100.0 (± 0.0)[a]
7	35	88.2 (± 5,9)[ab]
8	54	94.3 (± 2,.9)[ab]
9	50	100.0 (± 0.0)[a]
Total	**347**	**93.1 (± 2.2)**

Values in parentheses indicate standard error.
The different letters indicate statistically significant differences between mean parasitism levels per zone ($p \leq 0.05$).

The presence of the nematode *D. siricidicola* DNA in the *S. noctilio* abdomens was confirmed through amplification of the *mtCO1* gene. PCR-RFLP of the *mtCO1* gene with restriction enzymes Acil, Rsal and BsrGl revealed surprising results regarding *D. siricidicola* carriage within the same individual *S. noctilio* female (Table 3.2).

Table 3.2. Molecular determination of nematode species and strain in parasitized S. *noctilio* as determined by PCR-RFLP.

Sampling area	Female of *S. noctilio* analyzed (N°)	Female of *S. noctilio* with presence of specie/stain of nematode (%)	
		D. siricidicola - Kamona	*Non-sterilizing stain of D. siricidicola -*
1	10	100	40
2	10	100	40
3	10	100	40
4	10	100	60
5	10	100	20
6	10	100	10
7	10	100	10
8	10	100	20
9	10	100	30
Total	**90**	**100**	**30,0**

BsrGI has been used previously to distinguish between the Kamona and *D. proximus* strains (Williams & Hajek 2017; Bedding, 1974). Our *in-silico* analysis of the *mtCO1* gene revealed that the enzyme would cut the same site in the NS strain of *D. siricidicola* (Figure 3.3e) generating two

restriction fragments. Digestion with the restriction enzyme BsrGI revealed single fragments of length 680 bp in 100% of individuals (Figure 3.3b), indicating all *Sirex* samples to be infected with the Kamona strain of *D. siricidicola*. Among these individuals, an additional 23.3% of infected females also yielded bands of length 253 bp and 427 bp (Figure 3.3b lines 5, 6, 8 and 3.3e) corresponding to the NS or *D. proximus* strains. Digestion with restriction enzyme AciI, which distinguishes between the Kamona and NS strain (Morris, 2013), revealed bands of 680 bp in all ten individuals in each sampling area, confirming the presence of the Kamona strain. Among these females, 30% of infected individuals across all sampling zones yielded additional bands of 433 bp and 247 bp corresponding to the NS strain (Figure 3.3c Table 3.2). Finally, the restriction enzyme RsaI, which cuts the Kamona strain at two sites, yielded the characteristic banding pattern previously reported (Morris, 2013) with three fragments of length 323 bp, 230 bp, and 127 bp in all infected females, confirming the ubiquitous presence of *D. siricidicola* Kamona (Figure 3.3c and 3.3e). Moreover, 28-30% of the samples yielded two additional bands of length 104 bp and 126 bp, corresponding to the third cutting site of RsaI in the NS strain. The above was corroborated by

sequencing analysis of the strains that showed the restriction sites by similar restriction pattern whose sequence was included in figure 3.3 (e) as *Deladenus* non-sterilizing. Finally, digestion with the restriction enzyme BsrGl yielded banding patterns associated with the Kamona and NS strains *D. siricidicola* rather than the two bands of 426 bp and 253 bp characteristic of *D. proximus*. Therefore, the fragments observed above for digestion with the restriction enzyme BsrGI can be attributed to the NS strain since *D. proximus* was otherwise not observed in this study (Figure 3.3 d, Table 3.2). Taken together, these results indicate universal infestation with *D. siricidicola* Kamona paired with co-infestation of the NS in 30.0% of *S. noctilio* females.

a.

b.

M 1 2 3 4 5 6 7 8

680 pb →
428 pb →
252pb →

c.

M 1 2 3 4 5 6 7 8

680 pb →
433 pb →
247 pb →

3e

d.

1 M 2 3 7 4

323 pb →
230 pb →
129, 127, 126 pb →
104 pb →

```
D.siricidicola(Kamona)      AGTTTTTGGCTATTGCCAACTGCTTTAGTTTTGGTCTTATCTTCAGGTTTTGTTGATATA   120
                            **  ************** **  ********** **  *** * *****************

D.proximus                  GGTGGGGGGACTAGCTGGACTGTGTATCCTCCATTAAGAACTTTTGGACATTTGGGTGCT   180
Deladenus-Chile             GGTGGGGGTACTAGTTGAACGGTTTACCCACCTTTAAGAACTTTTGGTCATTTGGGTGCT   180
Deladenus(non-sterilizing)  GGTGGGGGTACTAGTTGAACGGTTTACCCACCTTTAAGAACTTTTGGTCATTTGGGTGCT   180
D.siricidicola(Kamona)      GGTGGGGGTACTAGTTGAACGGTTTACCCACCTTTAAGAACTTTTGGTCATTTGGGTGCT   180
                            *******  *** **  **  **  **  **  ** *************  **********

D.proximus                  AGGGTTGATTTAGTTATTTTTAGGTTACATTGTGCTGGGGATTAGTTCTATTTTAGGTGGT   240
Deladenus-Chile             AGTATTGATTTAGTTATTTTTAGTTTGCATTGTGCTGGGGATTAGTTCTATTTTGGGTGGT   240
Deladenus(non-sterilizing)  AGTATTGATTTAGTTATTTTTAGTTTGCATTGTGCTGGGGATTAGTTCTATTTTGGGTGGT   240
D.siricidicola(Kamona)      AGTATTGATTTAGTTATTTTTAGTTTGCATTGTGCTGGGGATTAGTTCTATTTTGGGTGGT   240
                            **  *****************  **  *****************************  ******

D.proximus                  ATTAATTTTATGCGTACAGTAAAGAATTTGCGTAGTAGTTCCTTGTCGCTAGAACATATG   300
Deladenus-Chile             ATTAATTTTATATGCCACAGTAAAGAATTTGCGTAGTAGATCTTTATCTTTGGAACATATA   300
Deladenus(non-sterilizing)  ATTAATTTTATATGTACATAAAGAATTTGCGTAGTAGATCTTTATCTTTGGAACATATA   300
D.siricidicola(Kamona)      ATTAATTTTATATGCACAGTAAAGAATTTGCGTAGTAGATCTTTATCTTTGGAACATATA   300
                            ***********  **  ******************* **  ** **  * ********

D.proximus                  AGTTTGTTGTTTGGAGTGTTTTTGTTACTGTTTTTTTTATTGGTCTTATCTCTTCCTGTG   360
Deladenus-Chile             AGTTTATTTGTTTGAAGTGTTTTTTGTAACAGTTTTTTTGTTGGTTTTGTCTTTACCAGTA   360
Deladenus(non-sterilizing)  AGTTTATTTGTTTGAAGTGTTTTTTGTAACAGTTTTTTTGTTGGTTTTGTCTTTACCAGTA   360
D.siricidicola(Kamona)      AGTTTATTTGTTTGAAGTGTTTTTTGTAACAGTTTTTTTGTTGGTTTTGTCTTTACCAGTA   360
                            ***** *******  *********** **  ***** *  ** ***  *  ** **

D.proximus                  TTGGCAGGGGCTATCACTATGTTGTTGACAGATCGTAATTTTAATACTTCTTTTTTTGAT   420
Deladenus-Chile             CTAGCTGGTGCTATTACTATGTTATTGACAGATCGTAATTTTAATACTTCTTTTTTTGAT   420
Deladenus(non-sterilizing)  CTAGCTGGTGCTATTACTATGTTATTGACAGATCGTAATTTTAATACTTCTTTTTTTGAT   420
D.siricidicola(Kamona)      CTAGCTGGTGCTATTACTATGTTATTGACAGATCGTAATTTTAATACTTCTTTTTTTGAT   420
                            *  ** **  *****  ***** **  ****************************

D.proximus                  CCAAGTTTGGGGGTAACCCTTTGATTTATCAGCATTGTTTTGGTTTTTGGTCATCCT   480
Deladenus-Chile             CCTAGTTTTGGTGGTAACCCTTTGATTTATCAACACTGTTTTGATTTTTTGGTCATCCG   480
Deladenus(non-sterilizing)  CCTAGTTTTGGCGGTAACCCTTTGATTTATCAACACTGTTTTGATTTTTTGGTCATCCG   480
D.siricidicola(Kamona)      CCTAGTTTTGGTGGTAACCCTTTGATTTATCAACACTGTTTTGATTTTTTGGTCATCCG   480
                            ** *******  *******************  **  ********  **************

D.proximus                  GAAGTTTATATTTTAATTTTACCTGCTTTTGGAATCTTGAGTCATAGTGCTTTAAGACTT   540
Deladenus-Chile             GAGGTTTATATTTTGATTTTGCCTGCTTTTGGTATTTTAAGTCATAGTGCCTTGAGACTT   540
Deladenus(non-sterilizing)  GAGGTTTATATTTTTGATTTTGCCTGCTTTTGGTATTTTAAGTCATAGTGCCTTGAGACTT   540
D.siricidicola(Kamona)      GAGGTTTATATTTTTGATTTTGCCTGCTTTTGGTATTTTAAGTCATAGTGCCTTGAGACTT   540
                            ** ********** *****  *********** **  **  ********** **  ******

D.proximus                  ACTGGTAAAAAAGAGAGATTTTTGGTAGTTTGGGTATAATTTATGCTATTTTAAGTATTGGG   600
Deladenus-Chile             ACAGGTAAAAAAGAGATTTTTTGGTAGTTTAGGAATAATTTATGCTATTTTAAGTATTGGT   600
Deladenus(non-sterilizing)  ACAGGTAAAAAAGAGATTTTTTGGTAGTTTAGGAATAATTTATGCTATTTTAAGTATTGGT   600
D.siricidicola(Kamona)      ACAGGTAAAAAAGAGATTTTTTGGTAGTTTAGGAATAATTTATGCTATTTTAAGTATTGGT   600
                            **  ***********************  ** **  **  ***********************

D.proximus                  TTAATTGGTTGTGTTGTTGGGCACACCATATATATACTGTTGGGATAGATTTAGATTCT   660
Deladenus-Chile             CTTATTGGTTGTGTTGTTGAGCTCATCACATGTATACTGTAGGTATAGATTTAGATTCT   660
Deladenus(non-sterilizing)  CTTATTGGTTGTGTTGTTGAGCTCATCACATGTATACTGTAGGTATAGATTTAGATTCT   660
D.siricidicola(Kamona)      CTTATTGGTTGTGTTGTTGAGCTCATCACATGTATACTGTAGGTATAGATTTAGATTCT   660
                            *  ****************** **  ** **  ** ******  ** ***************

D.proximus                  CGTGCTTATTTTACTGCCT-       679    □ AciI
Deladenus-Chile             CGTGCTTATTTTACTGCCTG       680    □ RsaI
Deladenus(non-sterilizing)  CGTGCTTATTTTACTGCCTG       680    □ BsrGI
D.siricidicola(Kamona)      CGTGCTTATTTTACTGCCTG       680    — Variation site: Deladenus Non-Sterilizing vs
                            *******************                 D. Chile and kamona
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Figure 3.3. PCR-RFLP analysis with the enzymes RsaI, BsrGI and AciI of the *mtCO1* gene amplified from the DNA of *S. noctilio* abdomens. (a) SEM image of *S. noctilio* eggs. (b) Representative restriction with RsaI enzyme in 5 sampling zones. (c) Representative restriction with the BsrGI enzyme in 8 sampling zones. (d) Representative restriction with AciI enzyme in 8 sampling zones. Lanes 1-5 or 1-8 show the banding pattern of PCR products obtained from *S. noctilio* egg samples, M: GeneRuler molecular

weight marker 1Kb, (e) Analysis of DNA sequences by restriction sites across species of *Deladenus.*

Analysis of *S. noctilio* eggs by scanning electron microscopy showed the presence of juvenile nematodes in 90% of the eggs analyzed (Figure 3.4a). Parasitized and non-parasitized eggs coexisted within the same *S. noctilio* female (Figure 3.4b).
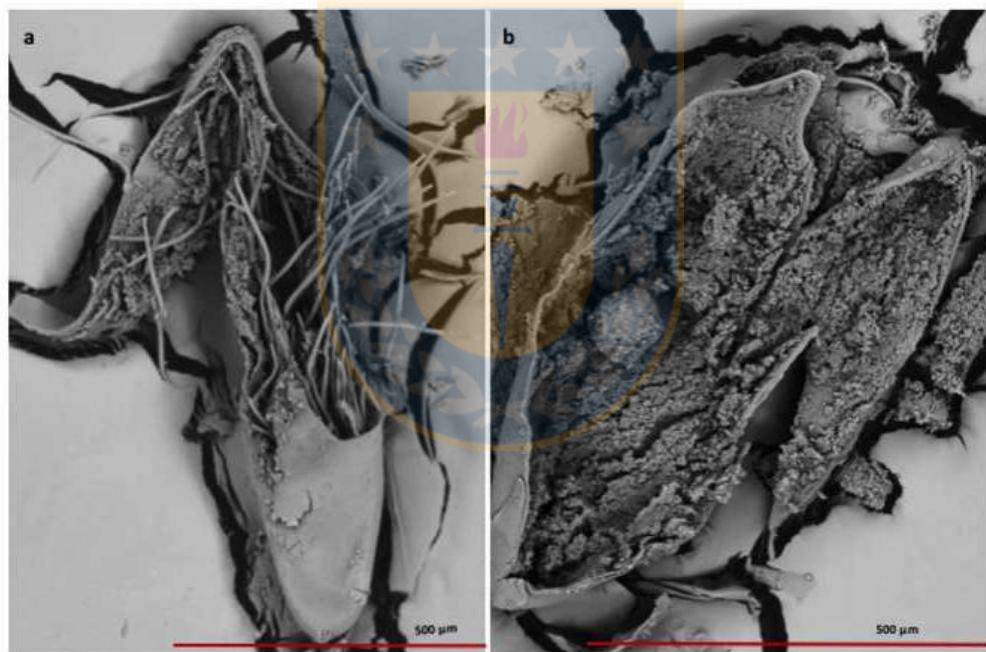

Figure 3.4. Scanning electron microscopy of *S. noctilio* eggs at 300x magnification. (a) Parasitized egg with *D. siricidicola* juveniles (b) Egg without parasitism by *D. siricidicola* juveniles.

3.5.-DISCUSSION.

The principal purpose of this study was to survey a portion of the genetic diversity of *D. siricidicola* nematode strains parasitizing *S. noctilio* woodwasps in the Biobío region of Chile. Parasitism of *S. noctilio* by *D. siricidicola* was detected in all nine sampling areas evaluated, at levels ranging from 81 to 100% with an average parasitism value of 93% (93.1 ± 2.2). This level of parasitism is consistent with levels of 93% reported for this same region in 2016 by Castillo et al. (2018) and 90% in 1994 in Encruzilhado do Sul in Brazil (Bedding, 2009) with nematodes of the same origin as in the Chilean case.

Molecular characterization of the *D. siricidicola* strain type revealed 100% of infected *S. noctilio* females to be parasitized by *D. siricidicola* Kamona. This finding confirms the establishment of the Kamona strain in the Biobío region of Chile and aligns with the history of biocontrol using *D. siricidicola* in Chile: *D. siricidicola* was introduced to Chile from Encruzilhada do Sul, Brazil (Beeche et al., 2012) which in turn originated from the Kamona strain introduced to Brazil from Australia (Bedding, 2009, Hurley et al., 2007). The establishment of the Kamona strain in the Biobío region with high levels of parasitism comparable to other countries

in the southern hemisphere where this strategy has been incorporated (Bedding, 2009) supports its suitability in woodwasp biocontrol.

In addition to confirming the universal presence of *D. siricidicola* Kamona in the nine sampling areas, this study also detected for the first time the presence of the non-sterilizing (NS) strain of *D. siricidicola* parasitizing *S. noctilio* in Chile. Importantly, the NS *D. siricidicola* strain was always found in conjunction with the Kamona strain (Figure 3.3 and Table 3.2). NS *D. siricidicola* was previously described to parasitize *S. noctilio* females in Canada and the United States without penetrating eggs, therefore preserving viability (Schields, 2009; Williams et al., 2009; Yu et al., 2009; Williams & Hajek 2017). In contrast, the success of *D. siricidicola* sterilizing strains (e.g., Sopron and Kamona) lies primarily in their ability to invade and destroy the eggs, thus sterilizing female *Sirex* (Bedding & Iede, 2005; Bedding et al., 2009; Kroll et al., 2013). According to Kroll et al., (2013) the NS strain of *D. siricidicola* would affect the fecundity of *S. noctilio* females only by decreasing the number of eggs. The present study could not evaluate if this *D. siricidicola* strain was in fact non-sterilizing, as it was found exclusively in co-occurrence with the Kamona strain. Considering the behavior of the NS strain reported in North

America (Yu et al., 2009, Williams et al, 2009, Kroll et al., 2013), the detection of this *D. siricidicola* variant for the first time in Chile could represent a risk for the sustainability of this country's biocontrol program using the nematode. Williams & Hajek (2017) suggested the possibility of hybridization between the NS and Kamona strains of *D. siricidicola*, which might produce less virulent variants. A case of hybridization with positive results was carried out in Australia, where six crosses between the "defective" strain and the Kamona strain yielded fully infective hybrids (Bedding & Iede, 2005; Bedding, 2009).

Determining *D. siricidicola* strain type in *S. noctilio* females naturally attacking *P. radiata* trees served to reveal key information about the establishment and natural dispersion of the nematode. According to Kroll et al., (2013) dispersion of the NS *D. siricidicola* strain occurs through infected *S. noctilio* females, which inject the nematodes to new trees during oviposition. Since these females remain fertile despite infection, they pass the parasitic nematode onto their offspring. In contrast, the dispersion of sterilizing strains depends on oviposition by healthy females on trees previously attacked by an infected female wasp. This requirement would slow the dispersion of sterilizing strains (Williams & Hajek, 2017),

opening a competitive advantage to the NS *D. siricidicola* strain in conditions of low woodwasp population density. Moreover, our observation that not all eggs within an *S. noctilio* female were parasitized by *D. siricidicola* Kamona suggests incomplete sterilization. This may in part be explained by the co-occurrence of the non-sterilizing strain, but could also be due to other factors such as temperature, as described by Yousuf et al., (2014a). The extent of sterilization could also depend on the synchronization between the release of juvenile nematodes in the hemocoel and the development of the insect egg. If juvenile nematodes are released when the egg is in advanced stages of development, they cannot penetrate the egg (Bedding, 1972; Williams & Hajek, 2017). In an evolutionary context, the NS condition may be a useful adaptation for *D. siricidicola*, especially considering its persistence in other parts of the world (Bedding, 1972; Nuttall, 1980; Williams & Hajek 2017). However, additional studies are needed to understand the specific biological role of the NS strain detected in this work and its implications for biocontrol of the woodwasp in Chile.

Based on the PCR-RFLP results, in which two bands were observed following digestion with BsrGI but not RsaI, this work points to the

apparent absence of *D. proximus* in Chile. This finding was corroborated by the absence of the RsaI cutting site in the DNA sequence of the Chilean strain. However, considering that parasitism of *S. noctilio* by *D. proximus* was previously reported by Morris et al., (2013, 2014) in the United States paired with the observation that *D. proximus* can grow in *Amylostereum aerolatum*, the fungus associated with *S. noctilio* (Morris et al., 2014), additional work is needed to rule out the presence of this nematode variant in other regions of Chile. *D. proximus* could be a possible agent of biological control against the wood wasp, as it sterilizes *Sirex nogricolis* females without affecting the size of the adult body or the dispersal capacity and that additionally. This characteristic could prove advantageous in efforts to control woodwasp populations in Chile.

In the seventeen years since the detection of *S. nocitlio* in Chile and implementation of a successful biocontrol program with *D. siricidicola,* important questions remain regarding the nematode and its interactions. Most notably, the origin of the NS strain of *D. siricidicola* in Chile is unknown. With the exception of *S. noctilio,* Chile has no native *Sirex* species, and no other species of the genus have been reported. Considering the intensity of commercial exchange with several countries, we propose that trade may have

introduced *S. noctilio* individuals infected with NS *D. siricidicola.* Collectively, the results in this work constitute a concrete contribution to our understanding of the genetic diversity and natural dispersion of *D. siricidicola* in Chile and highlight multiple lines of research that remain to be addressed.

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DISCUSIÓN GENERAL.

A cuatro años de haber iniciado el programa de control biológico de *S. noctilio* en el área de estudio en la región del Biobío-Chile, *Deladenus siricidicola* cepa Kamona, fue determinado parasitando en todas las zonas de mustreo evaluadas; tanto la frecuencia de detección del nematodo, como su nivel de parasitismo, incrementaron sostenida y consistentemente entre temporadas de evaluación, alcanzando 100% de presencia y 93,6% de parasitismo promedio a partir del año 2014. Considerando que la presencia del nemátodo *D. siricidicola* Kamona - procedencia Encruzilhado do Sul, se basó en muestras obtenidas a partir de árboles naturalmente atacados, este resultado sugiere su establecimiento en el área evaluada e incluso la ocurrencia de dispersión natural del biocontrolador ejercida por las hembras durante su período de vuelo y ovipostura, coincidiendo con lo planteado por Williams & Hajek (2017) y Taylor, (1981) respecto del rol de la hembra de la avispa en la dispersión del nemátodo. La determinación molecular de la cepa Kamona participando del 100% de este parasitismo era el resultado esperado, ya que el nemátodo usado en la implementación del programa de control biológico en Chile, correspondió a *D. siricidicola* procedencia Encruzilhada Do sul, que fue introducido desde Brasil (Beeche et al., 2012)

y que a su vez, correspondería a la cepa Kamona incorporada a Brasil desde Australia (Beding, 2009; Hurley et al., 2007).

El establecimiento de la cepa Kamona con altos niveles de parasitismo promedios, determinados en la región del Biobío durante las últimas temporadas de evaluación, 93,6%, 96,5% y 93,1% son consistentes con la alta virulencia del nematodo a altas densidades de la plaga (Bedding, 2009; Kroll, 2013, Williams & Hajek, 2017) y sus valores serían próximos a los niveles de parasitismo de 90% reportados en Brasil en Encruzilhado Do sul desde donde procede el nematodo usado en Chile, de 96% señalado en Sudáfrica (Cape Town) y por debajo del 100% de parasitismo reportado para Australia, en Victoria, después de dos años de realizada la inoculación (Bedding, 2009) y serían superiores al rango de infección reportados en los Estados Unidos de Norteamérica por Williams y Hajek, (2017) de 28,1-20,5% y de 17,6-13,6%, usando la cepa australiana Kamona.

El período de vuelo de adultos de *S. noctilio*, ocurrió en este estudio, entre los meses de noviembre a abril del año siguiente. Las cuatro primeras temporadas la emergencia de adultos se registró entre los meses de diciembre a abril y en la quinta temporada, el vuelo ocurrió entre noviembre a marzo del año siguiente (Figura 3.2). Esta determinación sería coincidente con

estaría dentro del rango del período de vuelo de entre octubre a mayo, señalado por Ruiz (2006) en el hemisferio sur; y semejante al período octubre a abril que señala Iede et al. (1993) para Brasil. La razón sexual de dos machos cada hembra de *S. noctilio* emergida, obtenido en las primeras temporadas de este estudio, indicaría que el insecto se encuentra en general en proceso de colonización (Ruíz, 2006) y la alcanzada en la última temporada en etapa de control.
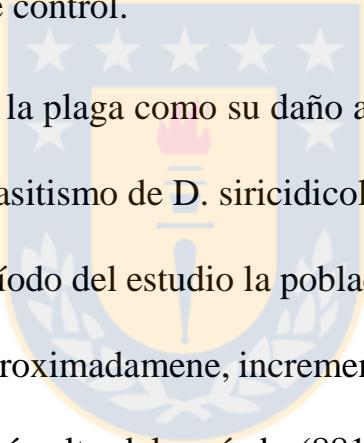
Tanto la población de la plaga como su daño asociado disminuyeron espués que los niveles de parasitismo de D. siricidicola alcanzaran niveles cercanos al 90%. Durante elperíodo del estudio la poblacional de *S. noctilio* estimada, disminuyó en 3,4% aproximadamene, incrementó hasta la tercera temporada, alcanzando el valor más alto del período (881,5 individuos/m^3), para luego disminuir progresivamente a partir de la cuarta temporada, hasta situarse por debajo del nivel inicial en un valor de (394,5 individuos/m^3). Un resultado similar fue reportado por Williams and Hajek, (2017) en EEUU, donde se registró una disminución en la población de *S. noctilo* de 1.000 Sirex/m^{-3} hasta alcanzar valores cercanos a 300 Sirex/m^{-3}, entre los año 2007 a 2012. Esta disminución en la densidad poblacional de la plaga, sería atribuible a la acción esterilizante de *D. siricidicola*, lograda a través de su alta capacidad

para penetrar y destruir los huevos de las hembras de la avispa parasitadas (Bedding & Iede, 2005; Yu et al., 2009; Kroll et al., 2013). El nivel de ataque observado en el estudio, incrementó de 0,3 a 12,0% hasta el año 2015 y luego disminuyó en los años siguientes hasta un valor de 5,9% de árboles afectados. El punto de inflexión en las curvas, del nivel poblacional y de ataque de la plaga, ocurrieron, una temporada y dos temporadas, respectivamente; después que el parasitismo de *D. siricidicola* alcanzara y se estabilizara en valores cercanos al 90%, en cada una de las cuatro comunas consideradas para este estudio específico (Figura 2.3, Tabla 2.5, 2.6). Los escasos resultados descritos en detalle de control de ataque de *S. noctilio*, señalan importantes niveles de control de la plaga, hasta niveles imperceptibles, después de dos a tres años que el parasitismo de *D. siricidicola* ha alcanzado niveles superiores a 90%, coincidiendo con los resultados de este estudio. En Australia se reportó disminución desde 80% de árboles muertos en 1987 a nivel de difícil detección en 1989, con parasitismo de 100% (Beddding, 2009); en Brasil se registró 30% de árboles atacados en 1991 que se redujeron a niveles de imperceptible en 1995 después de haber alcanzado parasitismo de 92% (Bedding & Iede, 2005) y en Sudáfrica se reportó mermas de ataque de 3,2% a difícil detección con parasitismo de 96,1% (Tribe y Cillié, 2004,

Bedding 2009). En el valle central (Los Ángeles) se observó un mayor nivel de población y ataque de la avispa, en relación a Precordillera de Los Ándes (Quilleco) y Nahuelbuta (Nacimiento) (Figura 2.3; Tabla 2.5, 2.6), lo que podría deberse a que en general los sitios del valle central de la Región del Biobío son menos productivos, presentando árboles disminuidos en su vigor, condición que incrementaría su susceptibilidad a la plaga, según lo señalado por diversos autores en relación a que árboles suprimidos, como los que se encuentran en rodales con alta competencia o estresados por sequía, son más susceptibles al ataque de *S. noctilio* (Haugen et al. 1990; Newmann et al., 1987; Aguilar y Lanfranco, 1988, Neumann y Minko 1981).

La detección por primera vez en Chile de la cepa de *D. siricidicola* NE parasitando *S. noctilio,* podría representar un riesgo para mantener los buenos resultados hasta aquí obtenidos en el programa de control de la plaga. La cepa NE de *D. siricidicola* se asocia a los ovarios, aunque no penetra los huevos, por lo que estos permanecen viables (Schields, 2009; Williams et al., 2009; Yu et al., 2009); en contraste, la cepa esterilizantes de *D. siricidicola* (por ej. Sopron y Kamona), radica fundamentalmente en su capacidad de entrar y destruir los huevos, esterilizando con ello a la hembra de *Sirex* (Bedding & Iede, 2005; Yu et al., 2009; Kroll et al., 2013). El presente

estudio no permite discriminar si esta cepa de *D. siricidicola* se comporta como no esterilizante, tampoco si ha mermedo los buenos resultados en el control de la plaga alcanzados con *D. siricidicola* en la región, ya que siempre fue encontrada en hembras de *S. noctilio* acompañada de la cepa Kamona. De comprobarse su rol no esterilizante, de acuerdo a lo observado en Norte América (Yu et al., 2009; Williams et al, 2009; Kroll et al., 2013), su presencia podría representar un riesgo para la sustentabilidad del programa de control biológico con el nemátodo en Chile, pues la cepa NE de *D. siricidicola*, podría hibridizarse con la cepa Kamona, lo que tendría insospechadas implicancias desde el punto de vista del traspaso de características de una cepa u otra a las nuevas generaciones híbridas, pudiendo en el peor de los escenarios significar una pérdida de virulencia para la cepa Kamona de *D. siricidicola*.

La cepa no esterilizante de *D. siricidicola*, según Kroll et al., (2013) afecta la fecundidad de la hembra de *S. noctilio* al disminuir su número de huevos, la dispersión de *D. siricidicola* cepa NE, ocurriría a través de la propia hembras de *S. noctilio* infectada, la que inyecta los nemátodos a nuevos árboles durante su proceso de ovipostura, por lo que puede ocurrir dispersión de estos nemátodos a su propia descendencia. De este modo, esta condición de no

esterilizante, en un contexto evolutivo podría ser no malo para la población de *D. siricidicola*, incluso su persistencia en otras partes del mundo, sugiere que podría ser una adaptación útil (Bedding, 1972; Nuttall, 1980; Williams & Hajek, 2017), incluso para controlar la plaga en condiciones de baja densidad poblacional de Sirex al afectar su potencial reproductivo al disminuir el número de huevos.

Los resultados obtenidos en este estudio son promisorios en términos de control de la población de *S. noctilio.* Se estima que contribuyeron al logro de los altos niveles de parasitismo, el uso de una de procedencia del nematodo que mantiene su habilidad parasítica, a su aplicación correcta (Goycoolea et al., 2015) y oportuna,en la misma temporada o a lo sumo a la siguiente en que la plaga era detectada en las distintas localidades, según lo recomendado por Lanfranco & Aguilar, (1990) y Bedding, (2009). En este contexto el caso de Chile sería similar al australiano, donde se cuenta con bosques cultivados y se usa una cepa del nematodo y su simbionte (Williams & Hajek, 2017), en EEUU de Norteamérica en cambio el sistema de control biológico de *S. noctilio* es complejo, debido a que se tiene diversas especies nativas de *Pinus* y *Sirex*, asociados a cepas de nematodos y hongos (Williams & Hajek, 2017).

Pese a lo anterior, dado a que el nematodo es denso dependiente (Bedding, 2009), y que se ha detectado la cepa no esterilizante del nematodo, debe considerarse un permanente monitoreo de la plaga que permitan detectar y control potenciales irrupciones poblacionales, de igual modo debe necesariamente, mantenerse poblaciones puras de kamona para ser usado en los procesos de masificación del nematodo en laboratorio y su consiguiente aplicación en plantaciones afectadas por la plaga.

Este trabajo representa un aporte, primero en términos deconstatar el establecimiento de *D. siricidicola* en la región del Biobío Chile y su efecto en el control de la plaga con mediciones de daño en el bosque, información que es eacasa por su alto costo (Bedding 2009) y segundo por la determinación de la cepa no esterilizante lo que permitirá tomar acciones de mitigación necesarias en aras a mantener el éxito del programa de control biológico de *S. noctilio* en el país.

CONCLUSIONES GENERAÑES

a) El nemátodo *D. siricidicola,* parásito de *S. noctilio,* se ha establecido exitosamente en el área de estudio de la región del Biobío, dispersándose naturalmente aún en zonas donde no se había inoculado.

b) El nivel de parasitismo del nemátodo *D. siricidicola* incrementa progresivamente respecto de la fecha de inicio del proceso de inoculación hasta el tercer año post inoculación y después se estabiliza a valores cercanos a 90%.

c) El parasitismo de *D. siricidicola* se relaciona en forma directa con la población de *S. noctilio* y su daño, afectándolos uno y dos años después de alcanzar valores de parasitismo cercanos al 90%.

d) *D. siricidicola* cepa Kamona se encuentra presente en el área de estudio–Región del Biobío, Chile, participando a nivel de 100% en el parasitismo de hembras de *S. noctilio*.

e) La cepa no esterilizante de *Deladenus siricidicola* se encuentra participando en el parasitismo de *S. noctilio* en la el área de estudio, a nivel de 30%, coparasitando el mismo individuo junto a la cepa Kamona.

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