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ECOLOGÍA MOLECULAR DE INSECTOS BARRENADORES: interacciones físicas y bioquímicas en el árbol *Spondias purpurea*

T E S I S

QUE PARA OBTENER EL GRADO ACADÉMICO DE

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P R E S E N T A

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COORDINACIÓN

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Por medio de la presente me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día 9 de agosto del 2010, se acordó poner a su consideración el siguiente jurado para el examen de DOCTORA EN CIENCIAS de la alumna **CALDERÓN CORTÉS NANCY** con número de cuenta **505017002,** con la tesis titulada: **"ECOLOGÍA MOLECULAR DE INSECTOS BARRENADORES: interacciones físicas y bioquímicas en el árbol *Spondias purpurea*"**, bajo la dirección del **Dr. Mauricio R. Quesada Avendaño.**

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A t e n t a m e n t e
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Nature … invites us to lay our eye level with her smallest leaf, and take an insect view of its plain.

Henry David Thoreau

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RESUMEN

Uno de los temas de mayor interés en Ecología es el entendimiento de los procesos involucrados en el origen y mantenimiento de la biodiversidad. Los insectos constituyen aproximadamente la mitad de la diversidad del planeta, con 400,000 especies de herbívoros. Estos insectos se alimentan exclusivamente de tejidos vegetales y enfrentan la digestión de moléculas complejas como la celulosa. Un grupo de insectos herbívoros poco estudiado está constituido por los insectos barrenadores, que se desarrollan y alimentan de tejidos vegetales como la madera. Entre los insectos barrenadores, algunas especies tienen la capacidad de crear un ambiente nuevo y/o proporcionar recursos para otras especies, generando así interacciones ecológicas complejas. El punto clave en el establecimiento de estas interacciones es la modificación estructural del tejido hospedero, que al parecer depende en cierto grado de la capacidad para degradar y digerir el tejido vegetal. Por lo anterior, en este trabajo se estudiaron los genes de celulasas y las enzimas digestivas del escarabajo barrenador *Oncideres albomarginata chamela* (Cerambycidae) que están involucradas en el proceso de degradación de madera del árbol tropical *Spondias purpurea* (Anacardiaceae). Así mismo, se realizó un estudio ecológico-molecular para evaluar el papel de este insecto como ingeniero natural del ecosistema (tanto físico como bioquímico). Los resultados demostraron que *O. albomarginata chamela* posee tres genes endógenos que codifican celulasas, de los cuales se clonaron y caracterizaron dos, Oa-EGase I y Oa-EGase II. La actividad total de estas celulasas fue 208.13 U/g de larva. La secuencia de aminoácidos deducida y la estructura tridimensional de las enzimas indicaron que las celulasas de *O. albomarginata chamela* poseen la arquitectura general y los dominios catalíticos representativos de las familias Glicosil Hidrolasa (GH) 5 y 45, así como altos niveles de homología con otras celulasas de escarabajos. *O. albomarginata chamela* también demostró desempeñar un papel ecológico importante como ingeniero natural del ecosistema, debido a que el proceso de transformación que realiza sobre las ramas de su árbol hospedero tuvo efectos positivos significativos en la frecuencia de colonización de ramas, abundancia, riqueza de especies y composición de la comunidad de colonizadores secundarios, que

incluye otros insectos barrenadores, depredadores y parasitoides. La ingeniería de ecosistemas realizada por *O. albomarginata chamela* fue responsable del 95% de la abundancia de los colonizadores secundarios y el 82% de la riqueza de especies. Estos efectos positivos estuvieron relacionados principalmente a la creación de hábitats con una mayor disponibilidad de sitios de oviposición para los colonizadores secundarios (ingeniería física), y a la presencia del ingeniero, que podría estar a su vez relacionada con la capacidad metabólica de degradación de madera (ingeniería bioquímica). El análisis de la capacidad de digestión de los principales constituyentes de la madera, indicó diferencias significativas en la actividad de celulasas y xilanasas entre las especies analizadas, siendo *O. albomarginata chamela* la especie que presentó la mayor actividad de ambas enzimas. Los insectos oportunistas *Lissonotus flavocinctus, Ataxia alpha, Estoloides chamelae,* Cerambycinae sp., y Lamiinae sp. presentaron actividades enzimáticas marginales. Estos resultados demostraron que *O. albomarginata chamela* es la única especie que posee la capacidad de transformar los polímeros estructurales de la madera en azúcares simples. Cierta porción de estos azúcares es eliminada en las heces, incrementando así la disponibilidad de recursos para los barrenadores oportunistas, lo cual podría explicar el efecto positivo de la presencia de *O. albomarginata chamela* sobre la riqueza de especies y composición de la comunidad de artrópodos asociada a las ramas de *S. purpurea*. Analizados desde una perspectiva evolutiva los resultados de este estudio mostraron que las familias de los genes clonados incluyen genes ancestrales para los organismos metazoarios, y que al menos un gen de la familia GH5 estuvo presente en el ancestro común de los animales invertebrados. La presencia de genes y enzimas para la degradación de tejidos vegetales en insectos de 16 ordenes incluyendo los insectos vivos más ancestrales (Zygentoma), así como los parientes más cercanos de los insectos (colémbolos y crustáceos), aunado a una reconstrucción de la evolución de caracteres ancestrales, indicaron que el mecanismo endógeno (genes de los insectos) para degradación de tejidos vegetales podría ser el mecanismo ancestral para los insectos, y probablemente uno de los factores relacionados con la evolución y diversificación de los insectos.

ABSTRACT

One of the major issues in Ecology is the understanding of processes involved in the origin and maintenance of diversity. Insects constitute almost half of the animal diversity on the planet, including 400,000 herbivore species. Insect herbivores feed exclusively on vegetal tissues, and face the digestion of complex molecules such as cellulose. One group of insect herbivores that has been understudied is constituted by borer insects. These insects develop and feed on vegetal tissues such as wood. Among borer insects, some species have the ability to create new habitats and supply resources to other species, generating complex ecological interactions. The key of the establishment of these interactions is the structural modification of the host tissue, which can depend to some extent on the ability to digest vegetal tissues. Therefore, we studied the genes and digestive enzymes of the borer beetle *Oncideres albomarginata chamela* (Cerambycidae) that are involved in the degradation process of wood of the tropical tree *Spondias purpurea* (Anacardiaceae). In addition, we carried out an ecological-molecular study to evaluate the role of this insect as natural ecosystem engineer (physical and biochemical). The results demonstrated that *O. albomarginata chamela* has three endogenous genes encoding cellulases. Two of these genes, Oa-EGase I and Oa-EGase II, were cloned and characterized. Total cellulase activity in the digestive tract of *O. albomarginata chamela* was 208.13 U/g larvae. The deduced amino acid sequence and the tridimensional structure of *O. albomarginata chamela* cellulases share the general architecture and conserved catalytic domains of Glycosyl Hydrolase Families (GHF) 5 and 45, and showed high homology levels with other beetle cellulases. *O. albomarginata chamela* showed to play an important ecological role as a natural ecosystem engineer, since the physical and nutritional modification of host plant branches made by this species, had significant positive effects on the frequency of colonization, abundance, species richness and composition of an associated arthropod community. This community includes other borer insects, predators and parasitoids. *O. albomarginata chamela* ecosystem engineering was responsible for 95% of the abundance of secondary colonizers, and 82% of the species richness. These positive effects were related to the

creation of habitats with higher availability of oviposition sites for secondary colonizers (physical engineering), and to the presence of the engineer which can be related to the metabolic ability for degrading wood (biochemical engineering). The analysis of the ability to digest the main wood polymers indicated significant differences in cellulase and xylanase activities between species, being *O. albomarginata chamela* the species with the highest activity of both enzymes. *Lissonotus flavocinctus, Ataxia alpha, Estoloides chamelae,* Lamiinae sp. and Cerambycinae sp. showed negligible enzymatic activities. These results demonstrated that *O. albomarginata chamela* was the only species that has the ability of transform structural polymers of wood into simple sugars. A proportion of these sugars can be eliminated through frass, increasing the availability of more digestible food for opportunistic borers. This can explain the positive effect of the presence of *O. albomarginata chamela* on species richness and community composition.

From an evolutionary perspective the results of this study showed that the families of the cloned cellulase genes include ancestral genes for metazoan organisms, and that at least one gene of the family GH5 was present in the common ancestor of invertebrate animals. The presence of genes and enzymes for degrading vegetal tissues in insects from 16 orders including the most ancestral living insects (Zygentoma), as well as the most closely related arthropod groups to insects (Collembola and Crustacea), jointly with the reconstruction of the evolution of ancestral characters, indicated that the endogenous (genes from insects) mechanism for degrading vegetal tissues might be the ancestral mechanism for insects, and probably one of the factors related to the evolution and diversification of insects.

Introducción general

1. INTRODUCCIÓN

Uno de los temas de mayor interés en Ecología está representado por el entendimiento de los procesos involucrados en el origen y mantenimiento de la biodiversidad. Dado que las especies existen en la naturaleza como miembros de ensamblajes interactivos, las interacciones bióticas juegan un papel fundamental en el funcionamiento de los ecosistemas, afectando así los patrones de biodiversidad (Grimm 1995). Las interacciones entre las especies incluyen interacciones antagonistas como depredación o parasitismo, interacciones neutrales como comensalismos, e interacciones benéficas como mutualismos, simbiosis y facilitación (Morin 1999). Dentro de las interacciones bióticas, las interacciones antagónicas, así como las interacciones que involucran facilitación ecológica, han sido ampliamente estudiadas (ej. Paine 1969, Tilman 1982, Hairston 1989, Bruno et al. 2003). Otra clase de interacciones bióticas comparativamente menos estudiadas, incluyen las interacciones que resultan de la influencia de cambios físicos causados por algunos organismos, proceso conocido como ingeniería natural del ecosistema (Jones et al. 1994, Lawton y Jones, 1995, Jones et al. 1997). Los ingenieros naturales del ecosistema son organismos que controlan directa o indirectamente la disponibilidad de recursos para otras especies, al modificar el estado físico de los materiales bióticos o abióticos, con lo cual mantienen y crean nuevos hábitats (Jones et al. 1994, Lawton y Jones 1995, Jones et al. 1997). Los ingenieros autogénicos (ej. corales, árboles) modifican el ambiente vía sus propias estructuras físicas, mientras que los ingenieros alogénicos (ej. castores, lombrices) modifican el ambiente por medio de la transformación de materiales. Entre estos últimos, se ha reconocido como ingenieros de ecosistemas al grupo de organismos que perforan los troncos de árboles o ramas (ej. carpinteros) y los que realizan galerías, en el que se incluyen los insectos barrenadores (Lawton y Jones 1995, Marquis y Lill 2007).

Los insectos barrenadores son organismos que se desarrollan (al menos en alguna etapa de su ciclo de vida) en la madera, corteza o ramas leñosas de plantas (Lieutier et al. 2004). Los insectos barrenadores están representados por un grupo diverso que

incluye los órdenes: Lepidoptera (mariposas nocturnas), Hymenoptera (moscas de la sierra), Isoptera (termitas), y Coleoptera (Lieutier et al. 2004). El orden Coleoptera a su vez, incluye al menos siete familias: Cerambycidae, Buprestidae, Curculionidae (p.ej. Scolytinae, Platypodinae), Bostrichidae, Lyctidae, Anobiidae y Brentidae (Paine 2009), de las cuales la familia Cerambycidae presenta la mayor diversidad (Nielsen 1981).

Entre los cerambícidos, las hembras ovipositan bajo la madera y las larvas se alimentan del tejido vegetal (Nielsen 1981). Como consecuencia de este proceso, algunos cerambícidos crean un ambiente físico nuevo que puede ser utilizado por otros organismos (colonizadores secundarios). Es así como estos insectos proporcionan recursos a numerosas especies promoviendo interacciones bióticas complejas al modificar el ambiente (Linsley 1959, 1961; Feller y Mathis 1997, Hanks 1999). El punto clave en el establecimiento de estas interacciones es el proceso de transformación de las ramas (Pollock et al. 1995), que al parecer depende de la capacidad de los cerambícidos para degradar y digerir el tejido vegetal.

La celulosa es el polisacárido más abundante y la principal fuente de carbono presente en los tejidos vegetales (Rowell et al. 2000). La explotación de celulosa como recurso alimenticio está restringida por la capacidad de degradar la pared celular vegetal (Lee et al., 2004). La degradación de la pared celular de las plantas es un proceso complejo que requiere la producción de una gran variedad enzimas líticas que originalmente se consideraron exclusivas de bacterias, hongos y protistas (Beguin y Aubert 1994). Durante más de un siglo, se ha creído que los animales no pueden digerir la celulosa sin el establecimiento de relaciones simbióticas con estos microorganismos, debido a su incapacidad para producir sus propias enzimas líticas (Cleveland 1924). No obstante, este paradigma en la biología está cambiando, debido a que se ha comprobado a nivel genético que algunos de los genes que codifican estas enzimas están presentes en el genoma de diversos animales invertebrados, como nematodos, anélidos, moluscos y artrópodos (Smant et al. 1998, Watanabe et al. 1998, Byrne et al. 1999, Rosso et al. 1999, Tokuda et al. 1999, Lo et al. 2000, Xu et al. 2000, Watanabe y Tokuda 2001,

Tokuda et al. 2002, Genta et al. 2003), incluidas dos especies de escarabajos cerambícidos, *Psacothea hilaris* (Sugimura et al. 2003) y *Apriona germari* (Lee et al. 2004).

El insecto barrenador *Oncideres albomarginata chamela* (Coleoptera: Cerambycidae) remueve, oviposita y desarrolla su progenie (i.e. larvas) en la madera de las ramas del árbol tropical *Spondias purpurea* (Noguera 1993). Los estudios preliminares sugieren que este insecto depende exclusivamente de la degradación de madera para sobrevivir y desarrollarse hasta adulto (Uribe-Mú y Quesada 2006). Además, se ha encontrado que otras especies de coleópteros (colonizadores secundarios), aprovechan las ramas originalmente cortadas por especies del género *Oncideres* (Baucke 1962, Hovore y Penrose 1982). Por lo tanto, *O. albomarginata chamela* representa un excelente modelo en el que es posible estudiar: la expresión endógena de genes de celulasas animales, específicamente de coleópteros para los cuales existen pocos reportes; el efecto del trabajo mecánico de remoción de ramas (i.e. ingeniería natural de los ecosistemas); y el efecto de la transformación metabólica de la madera mediado por la capacidad de degradación enzimática del insecto *per se*.

2. MARCO TEÓRICO

2.1 PARED CELULAR VEGETAL

La pared celular vegetal, es una capa de material estructural que se encuentra en la parte externa de la célula. Usualmente tiene un grosor de 0.1-10 μm, es una estructura semirrígida, semipermeable, dinámica y metabólicamente activa que proporciona soporte físico y mecánico a la planta, mantiene la forma de las células, controla la expansión celular, regula el transporte de iones, los procesos de señalización y comunicación intercelular (Bidlack et al. 1992, Cosgrove 1997, Pennell 1998, Fry 2000). Es además, una de las principales fuentes de carbono en la biósfera al ser utilizada por varios organismos y actúa como la principal barrera contra patógenos y herbívoros (Smart 1991, Bayer et al. 1998, Fry 2000)

En una pared celular bien desarrollada se pueden distinguir tres componentes estructurales: lámina media, pared celular primaria y pared celular secundaria (Fig. 2.1.1). La pared celular primaria es aquella en la que la estructura de polisacáridos se deposita durante el crecimiento en la superficie de la célula, mientras que la pared celular secundaria, es aquella en la cual la estructura de polisacáridos se deposita una vez que ha cesado el crecimiento de la célula, por lo cual es interna a la pared primaria (Smart 1991, Fry 2000).

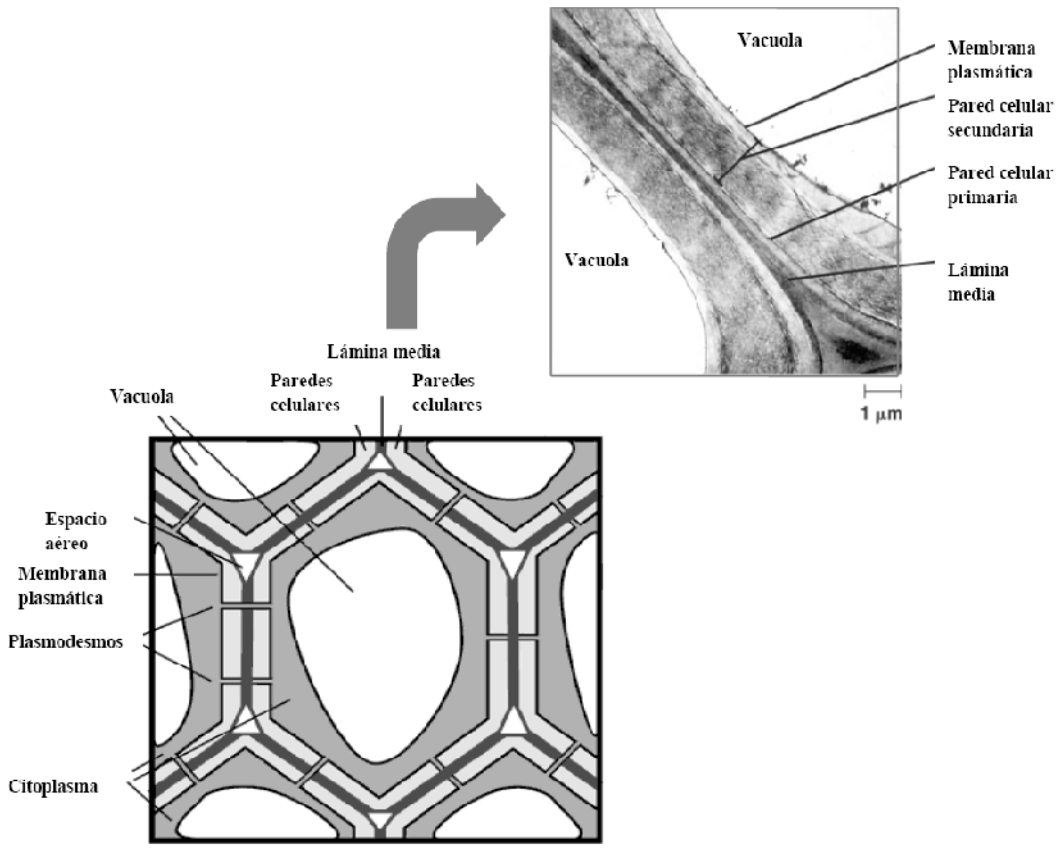

Figura 2.1.1 Representación esquemática de una célula vegetal mostrando la estructura de una pared celular vegetal bien desarrollada.

La pared celular vegetal está compuesta por una mezcla de celulosa, pectina, hemicelulosa, lignina y proteínas, que se arreglan en una estructura compleja y altamente ordenada. En esta estructura, la celulosa y pequeñas cantidades de proteínas se encuentran embebidas en una matriz de polisacáridos compuesta por pectina altamente hidratada (para paredes primarias: Fig. 2.1.2A) o por lignina (para paredes secundarias: Fig. 2.1.2B) y por hemicelulosas que unen la celulosa mediante enlaces entrecruzados (Smant 1991, Bidlack et al. 1992 Cosgrove 1997, Fry 2000, Rowell et al. 2000).

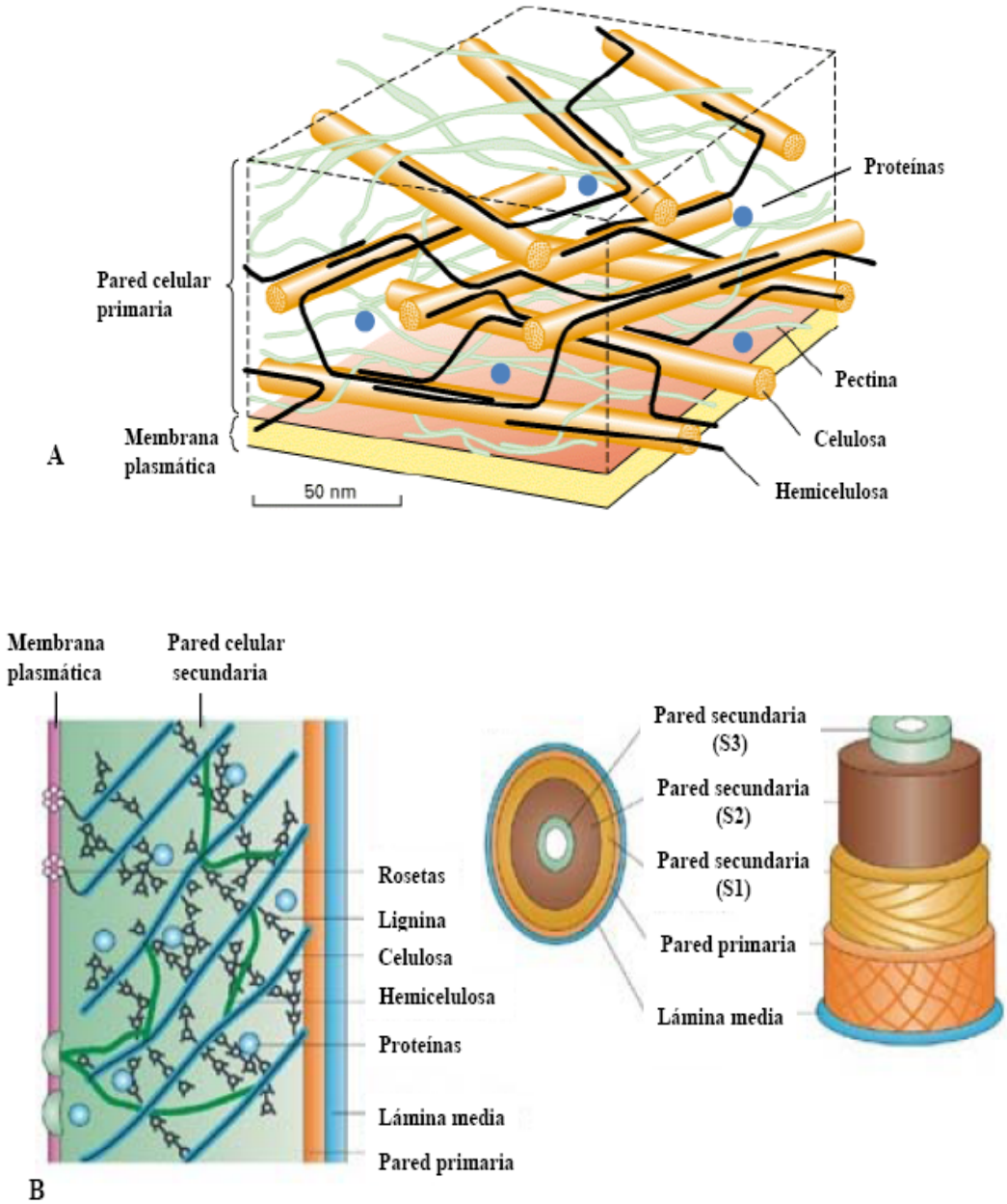

Figura 2.1.2. Estructura y composición de la pared celular vegetal. A) Pared celular primaria, B) pared celular secundaria: la lignificación ocurre en las capas S1, S2 y S3 de la pared celular. Modificado de Sticklen (2008).

La celulosa es el principal componente estructural de la pared celular vegetal. Este es un polímero lineal de 8,000-15,000 residuos de D-glucosa unidos por enlaces β-1,4-glucosídicos que forman una cadena. Entre 35-200 cadenas se unen por puentes de hidrógeno formando microfibrillas, que a su vez se agrupan para formar macrofibrillas. Finalmente la unión de estas macrofibrillas da lugar a las fibras de celulosa (Cosgrove 1997, Fry 2000, Rowell et al. 2000). Aún cuando la celulosa es un homopolímero compuesto sólo por unidades de glucosa, la celulosa posee una subestructura que consiste de dominios cristalinos altamente organizados y de regiones amorfas menos organizadas (Fig. 2.1.3). La celulosa está involucrada principalmente en el desarrollo de la planta, representa un sitio de deposición de carbohidratos que sirve como templado para ensamblar la lignina, además dirige los patrones de arreglo de las microfibrillas en la pared celular que son responsables del tipo de crecimiento de la pared celular (al azar, isodiamétrico o direccional (Cosgrove 1997, Emons y Mulder 1998, Smith 1999).

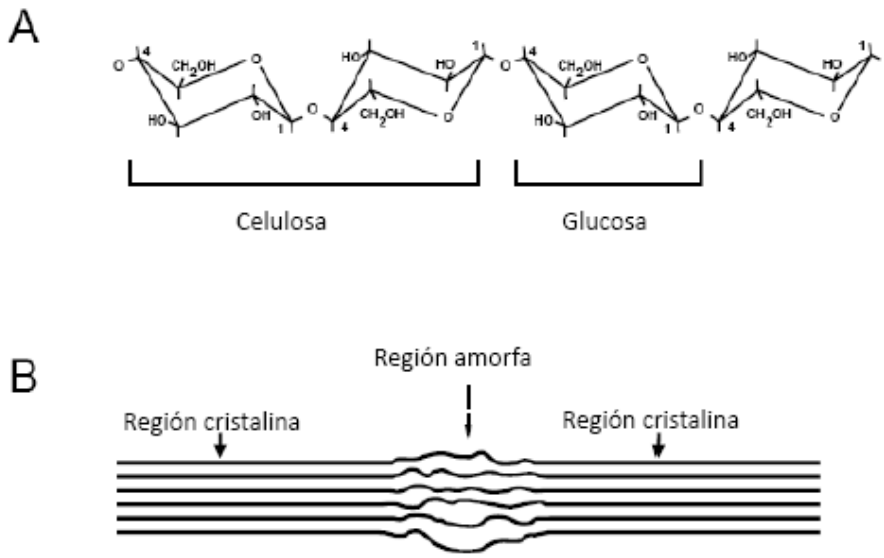

Figura 2.1.3. Estructura de celulosa. (A) Enlaces ß- 1,4-glucosídicos. (B) Principales regiones de una fibra de celulosa. Tomado de Beguin y Aubert (1994).

Las hemicelulosas, después de la celulosa son los polisacáridos más abundantes de la pared celular vegetal y se les considera como uno de los polímeros más heterogéneos. El componente principal de la hemicelulosa lo constituye la xilana, la cual consiste de unidades de D-xilosa unidas mediante enlaces β-1,4 que pueden ser sustituidos por diferentes grupos laterales tales como L-arabinosa, D-galactosa, D-mananosa, acetilo, residuos de ácido glucorónico, etc. (Fig. 2.1.4). Sin embargo, otros enlaces como: β-1,2, β-1,3 y β-1,6, también pueden estar presentes en las hemicelulosas. En esta estructura, las cadenas laterales no interfieren con la geometría del enlace glucosídico. La función principal de las hemicelulasas reside al igual que para la celulosa, en la morfogénesis de la planta. Sin embargo, las funciones precisas de dichos polisacáridos no han sido completamente entendidas (Cosgrove 1997, Fry 2000, Rowell et al. 2000).

Los polímeros pécticos son extraordinariamente complejos en su composición y predominan en la región de la lámina media, confiriéndole a esta las características propias para la formación de un gel amorfo. La pectina incluye polisacáridos diversos, ricos en ácido galacturónico con distintos dominios estructurales. La homogalacturonana, es un homopolímero de aproximadamente 200 residuos de ácido poligalacturónico unidos mediante enlaces α-1,4, los cuales pueden estar metil-esterificados en el C-6 ó presentar grupos acetilo en O-2 y O-3. La ramnogalacturonana I, se forma por cadenas largas de ácido galacturónico unidas mediante enlaces α-1,4 entremezclados con ramnosa mediante enlaces α-1,2. El ramnogalacturonano II, es un polímero de ácido galacturónico unido mediante enlaces α-1,4 altamente sustituido con al menos 11 azúcares diferentes. Estos dominios pueden estar o no unidos covalentemente a la red péctica (Fig. 2.1.5). Los polisacáridos pécticos que proporcionan un ambiente dinámico para la deposición y extensión de la red celulósica, están implicados en la regulación del estado iónico, en la porosidad de la pared celular y constituyen el principal material adhesivo entre las células, así como en las respuestas de defensa (Cosgrove 1997, Fry 2000, Rowell et al 2000).

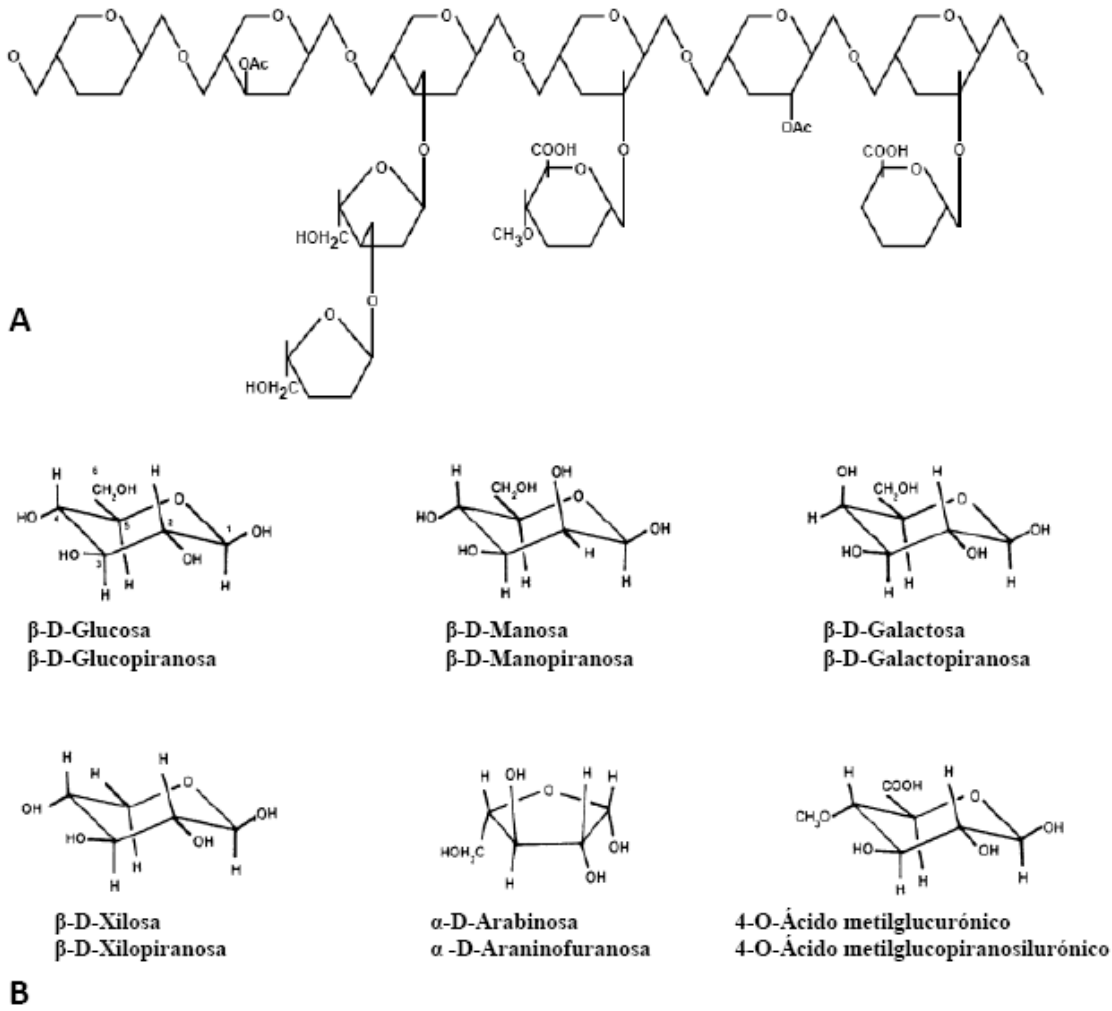

Figura 2.1.4. Estructura y composición de hemicelulosas. A) Parte de una molécula hipotética de xilana; B) Estructura de los principales monómeros que constituyen las hemicelulosas. Modificado de Thomson (1993) y Rowell et al. (2000).

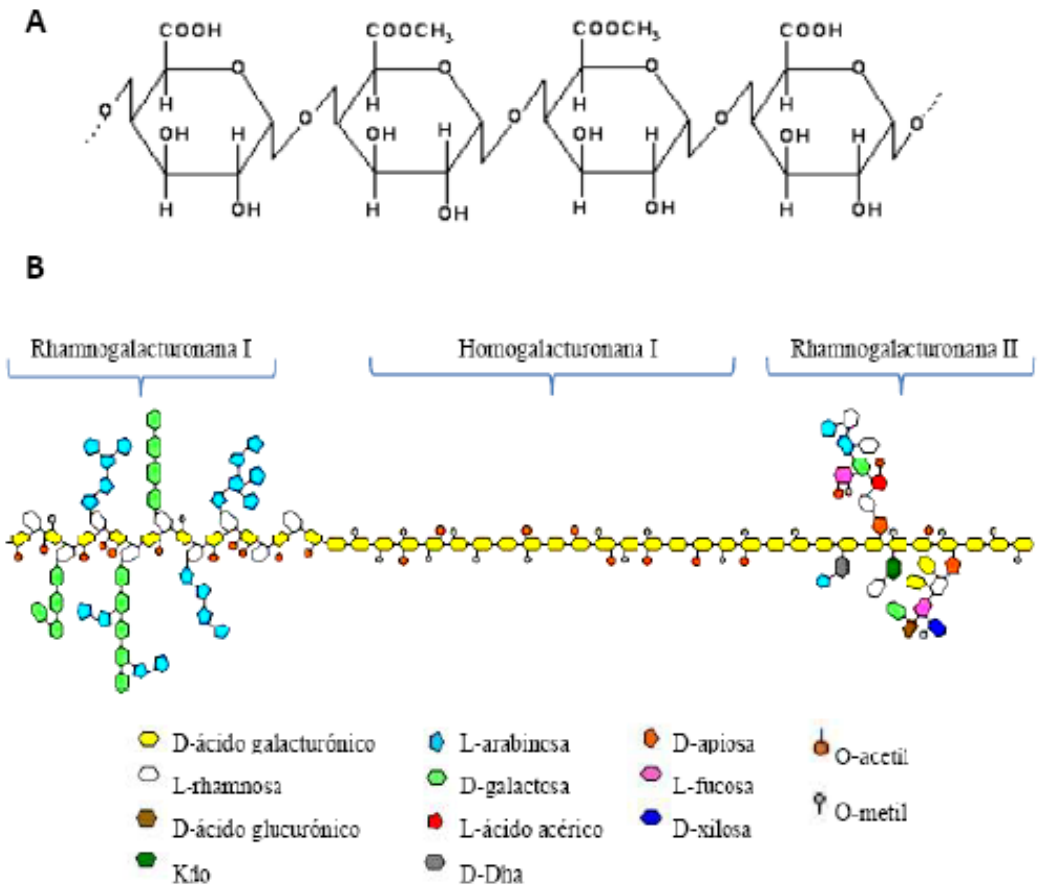

Figura 2.1.5. Estructura esquemática y composición de la pectina. A) Estructura del homopolímero que constituye la pectina. B) Estructura general de la pectina. Modificado de Soriano (2004).

Además de los carbohidratos, la pared celular vegetal está constituida por derivados del ácido cinámico, de los cuales, la forma más común es la lignina. La lignina es un polímero tridimensional muy complejo constituido por enlaces C-O-C y C-C. Los monómeros de lignina: *ρ*- ácido coumarico (*ρ*CA), ácid ferúlico (FA), ácido diferúlico (DFA), ácido sinápico (SA), ácido cinámico (CA), y *ρ*-ácido hidroxibenzoico (BA), son sintetizados a partir de la vía del ácido shikímico y el metabolismo de los fenilpropanoides. Posteriormente, estos monómeros son transformados a tres alcoholes: p-coumaril, coniferil, y sinapil, que interactúan con los polisacáridos de la pared celular

y se polimerizan para formar la lignina en las paredes secundarias (Fig. 2.1.6). La lignina es el componente estructural más importante de las paredes celulares de plantas vasculares (Smart 1991, Bidlack et al. 1992, Rowell et al. 2000). Se ha demostrado que los tejidos lignificados obstaculizan la degradación enzimática, por lo que la lignificación de los tejidos, representa un mecanismo inducido de resistencia a herbívoros y patógenos (Smart 1991, Fry 2000).

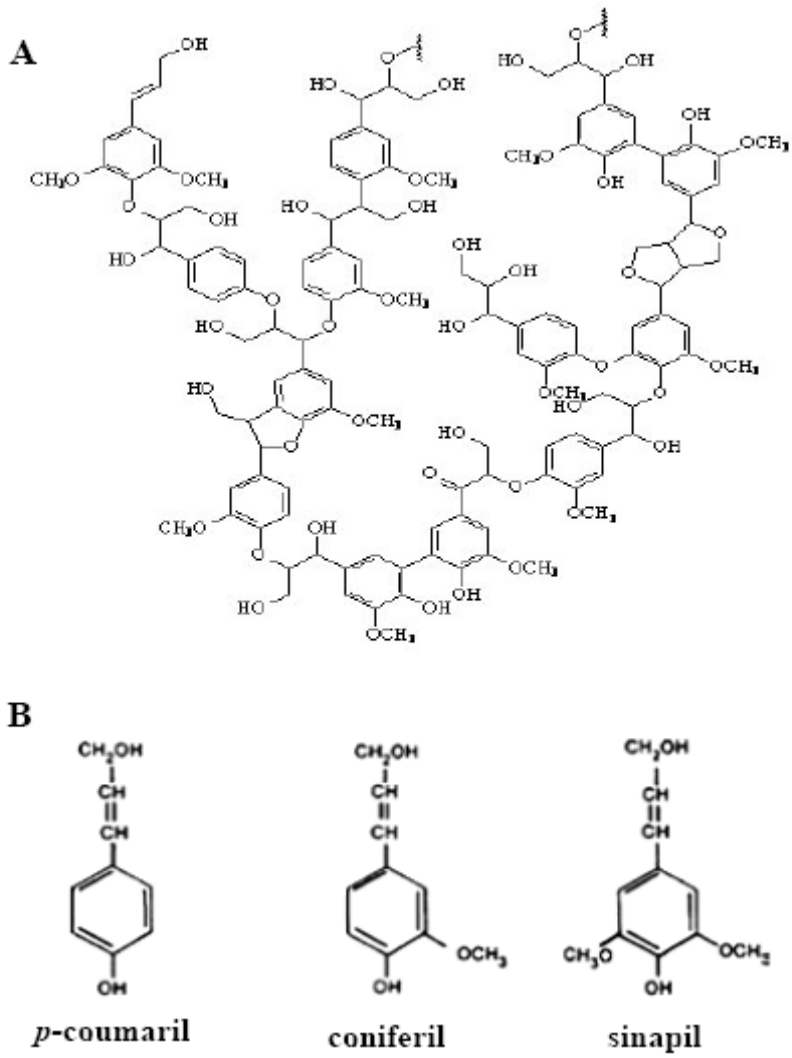

Figura 2.1.6. Estructura de la lignina. A) representación de una molécula hipotética de lignina. B) estructura de los tres principales alcoholes que conforman la lignina. Modificado de Rowell et al. (2000).

Finalmente, la pared celular vegetal está compuesta por un porcentaje variable de proteínas en un arreglo complejo y poco estudiado. Las proteínas pueden dividirse en tres clases con base en su función: a) extensinas, las cuales son glicoproteínas altamente insolubles que contienen hidroxiprolina y residuos de ditirosina, y que al parecer están involucradas en la resistencia de la pared celular vegetal al ingreso de hongos patógenos, ya que su concentración aumenta durante el ataque de patógenos; b) proteínas arabinogalactanas (AGP´s), que también son glicoproteínas que contienen hidroxiprolina pero no contienen ditirosina, son solubles en su estado libre y están involucradas en el crecimiento, nutrición y guía de tubos polínicos en tejidos del estilo, así como en otros procesos de desarrollo de la planta; y c) peroxidasas con múltiples isozimas, las cuales están involucradas en la lignificación, en la formación de residuos de ditirosina responsables de la insolubilidad de las extensinas, en la formación de enlaces diferuloril en arabinoxilanas de pastos y en el metabolismo de auxinas (Smart 1991, Cosgrove 1997, Showalter 2001).

Composición de diferentes tipos de pared celular vegetal

Una pared celular primaria típica, está compuesta de microfibrillas de celulosa (9-30%), una matriz de hemicelulosas (25-50%), pectinas (10-35%) y proteínas (10%). En términos generales, el 90% de la pared celular primaria consiste de carbohidratos y el 10% restante de proteínas. Los principales monosacáridos presentes en la pared celular primaria son tres hexosas: D-glucosa, D-Galactosa y D-manosa, dos pentosas (L-arabinosa y D-xilosa), dos ácidos urónicos (D-ácido glucurónico y D-ácido galacturónico) y dos deoxihexosas (L-ramnosa y L-fucosa). Una característica particular de la pared celular primaria es su alto grado de hidratación, ya que el agua llega a constituir hasta dos tercios de la masa total. El agua se localiza principalmente en la matriz de polisacáridos, en la cual se encuentran embebidas las microfibrillas de celulosa y pequeñas cantidades de proteínas.

Por su parte, la pared celular secundaria que representa el grueso en las plantas maduras, contiene celulosa (40-80%), hemicelulosa (10-40%) y lignina (5-25%). Los monómeros presentes en las paredes celulares secundarias, incluyen los monosacáridos: D-glucosa, D-xilopiranosa, L-arabinofuranosa, L-arabinoxilosa; y los monómeros de lignina: ácido ρ-coumárico, ácido ferúlico, ácido diferúlico, ácido cinámico y ácido ρ-hidroxibenzoico. El arreglo de estos componentes permite que las microfibrillas de celulosa estén embebidas en la lignina. Tanto la celulosa como la hemicelulosa parecen estar estructuralmente más organizadas en la pared secundaria que en la pared primaria. La celulosa se une a la hemicelulosa mediante puentes de hidrógeno, así como enlaces éster y éter son responsables de la unión de la hemicelulosa a la lignina (Bidlack et al.1992, Fry 2000). La composición de la pared celular vegetal también difiere entre diferentes tejidos de una misma planta y entre plantas (Tabla 2.1.1).

Tabla 2.1.1. Composición de paredes celulares vegetales de diferentes tejidos.
Modificado de Gong et al. (1981)

Tipo de material	% Hemicelulosa	% Celulosa	% Lignina
Monocotiledóneas			
Tallos	25-50	25-40	10-30
Hojas	80-85	15-20	-
Fibras	5-20	80-95	-
Maderas			
Angiospermas	24-40	40-55	18-25
Gimnospermas	25-35	45-50	25-35

2.2 DEGRADACIÓN DE LA PARED CELULAR VEGETAL

La pared celular vegetal presenta dos atributos físicos importantes: su resistencia mecánica y permeabilidad al agua, iones y otras moléculas como enzimas y estimuladores. La pared celular es impresionantemente fuerte bajo estrés de tensión, y por su lignificación es resistente a fuerzas compresivas. Estas demandas mecánicas impuestas por el ambiente físico y la morfología de la planta hacen más difícil su degradación (Smart 1991, Cosgrove 1997). Sin embargo, la pared celular vegetal puede ser degradada por sistemas biológicos en diferentes situaciones: a) los microorganismos patogénicos para penetrar su planta hospedera y nutrirse del contenido de las células que atacan, requieren de la digestión de la pared celular; b) la pared celular vegetal también es degradada por los organismos detritívoros (en los que destacan los microorganismos del suelo) una vez que la planta ha muerto; y c) las células vegetales también son digeridas en el tracto digestivo de animales que derivan gran parte de sus requerimientos de carbono de los polímeros de la pared celular vegetal (herbívoros).

Debido a que la pared celular vegetal está compuesta en su mayor parte de polisacáridos, las principales enzimas involucradas en su desdoblamiento son enzimas hidrolíticas de polisacáridos, entre estas enzimas destacan las celulasas y hemicelulasas. Sin embargo, para un desdoblamiento completo de la pared celular vegetal, en especial de paredes primarias también es necesaria la participación de otras proteínas como las pectinasas o enzimas pectolíticas; y en el caso de paredes secundarias, de las enzimas involucradas en la degradación de la lignina, conocidas como "ligninasas" (lacasas y peroxidasas) (Fry 2000).

Celulasas

En general, este sistema consiste de tres clases de enzimas: endo-β-1,4-D-glucanasas (endoglucanasas), exo-β-1,4-D-glucanasas (celobiohidrolasas o exoglucanasas) y β-1,4-D-glucosidasas (ß-glucosidasas o celobiasas). Las endoglucanasas son las enzimas que comienzan el rompimiento de la celulosa, estas enzimas hidrolizan al azar los enlaces glucosídicos internos de la celulosa (sólo en las regiones amorfas) produciendo una mezcla de oligosacáridos con diferente grado de polimerización y con extremos no reductores. Las celobiohidrolasas, actúan sobre estos oligosacáridos a partir de los extremos no reductores y producen unidades de celobiosa. Finalmente, las celobiasas convierten la celobiosa a unidades de glucosa cristalina (Beguin y Aubert 1994) (Fig. 2.2.1).

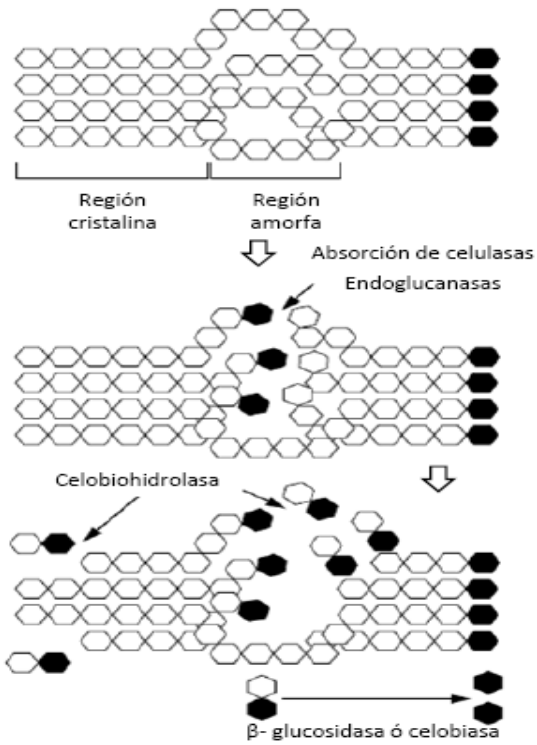

Figura 2.2.1. Degradación de celulosa mediante el complejo enzimático de hongos. Los monómeros de glucosa están representados por hexágonos, y los extremos no reductores se muestran en negro. Tomado de Beguin y Aubert (1994).

Hemicelulasas

Debido a que la hemicelulosa está constituida principalmente por xilana, la cual contiene diferentes azúcares (L-arabinosa, D-galactosa, etc.) en los grupos laterales, una hidrólisis enzimática eficiente de este heteroplímero requiere de la acción de un sistema de enzimas con diferente especificidad y modos de acción (Beguin y Aubert 1994). Algunas de las enzimas involucradas en la degradación de xilana son: endo-β-1,4-xilanasas que actúan sobre xilanas produciendo principalmente mezclas de xilooligosacáridos; β-D-xilosidasas que hidrolizan los xylooligasacáridos producidos por la acción de las endoxilanasas hasta residuos D-xilosa; α-glucuronidasas y α-L-arabinofuranosidasas que hidrolizan las ramificaciones laterales de la xilana; y xilano-acetilesterasas y arilesterasas que remueven los grupos laterales acetilo y fenilo de la xilana (Thomson 1993, Aro et al. 2001) (Fig. 2.2.2).

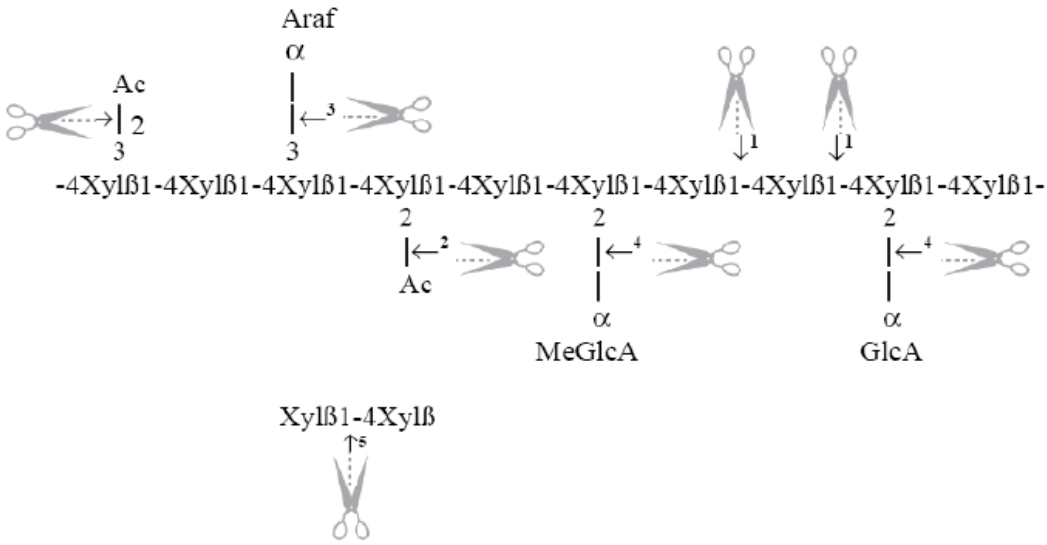

Figura 2.2.2. Degradación de una molécula hipotética de xilana. Las tijeras muestran los sitios de hidrólisis de enzimas xilanolíticas de complejos microbianos. 1=endo-1,4-ß-xilanasas; 2=acetil esterasas; 3=α-L-arabinofuranosidasas; 4=α-glucuronidasas; 5=ß-xilosidasas. Modificado de Thomson (1993).

Pectinasas o enzimas pectolíticas

El proceso de degradación de pectina, es un proceso complejo, que involucra la acción combinada de varias enzimas pectolíticas, las cuales destruyen la lámina media que se encuentra entre las paredes celulares ocasionando la maceración del tejido, lisis celular y modificación de la estructura de la pared celular. Con base en su modo de acción, dichas enzimas se clasifican en dos grupos: esterasas, que incluyen a las pectin esterasas, las cuales remueven grupos metoxil de la pectina y catalizan la liberación de metanol; y depolimerasas (hidrolasas y liasas) que degradan la cadena medular de la pectina. En este segundo grupo se encuentran: las poligalaturonasas, enzimas que hidrolizan los enlaces glicosídicos α-1,4 entre dos residuos de ácido galacturónico no esterificados; las pectin liasas, que catalizan la β-eliminación entre dos residuos de ácido poligalacturónico esterificados, y originan como productos finales ésteres urónicos insaturados; y las pectato liasas las cuales son específicas para poligalacturonato o pectato no metilado, pero que también pueden actuar sobre pectina con bajo grado de metil-esterificación (Nedjma et al. 2001, Soriano 2004) (Fig. 2.2.3).

Ligninasas

La degradación biológica de lignina es un proceso oxidativo que requiere de la acción de tres tipos de enzimas: lacasas (polifenoloxidasas), lignina-peroxidasas y manganeso-peroxidasas. Las lignina-peroxidasas catalizan la oxidación de varios compuestos aromáticos y producen radicales aril cargados positivamente, mientras que las manganeso-oxidasas oxidan el Mn(II) a Mn(III), el cual se difunde a partir de la enzima y oxida varios compuestos fenólicos. Estas enzimas requieren de peróxido de hidrógeno para sus actividades. Las lacasas también catalizan la oxidación de varios compuestos fenólicos y aminas aromáticas, pero esta reacción está acoplada a la reducción de oxígeno molecular a agua (Ahmed et al. 2001).

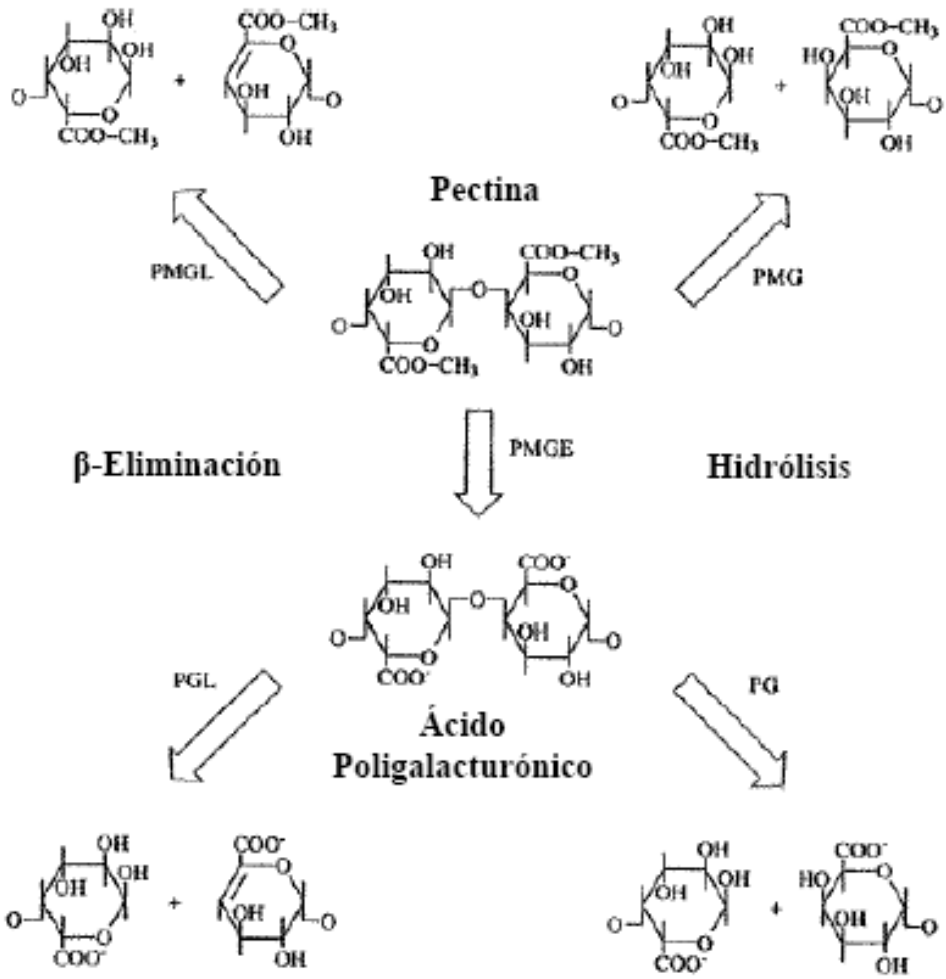

Figura 2.2.3. Representación esquemática de la degradación de pectina. PMGE= polimetilgalacturonato esterasa (pectinesterasa); PMGL=polimetilgalacturonato liasa (pectina liasa); PMG= polimetilgalacturonasa (pectina hidrolasa); PGL: poligalacturonato liasa (pectato liasa); PG= poligalacturonasa (pectato hidrolasa). Tomado de Soriano 2004.

2.3 TRACTO DIGESTIVO DE INSECTOS

De manera general, el tracto digestivo de los insectos se compone de intestino anterior, intestino medio e intestino posterior (Fig. 2.3.1). El intestino anterior incluye la cavidad bucal (en la cual se encuentran las glándulas salivales), la faringe, el esófago y el buche. El buche es un órgano de almacenaje en muchos insectos y sirve como sitio de digestión para otros. El intestino anterior está recubierto por una cutícula y en algunos insectos esta reducido a un tubo delgado. El proventrículo es un órgano de trituración en algunos insectos y en la mayoría de las especies está provisto de válvulas que controlan la entrada de alimento al intestino medio. El intestino medio es el principal sitio para la digestión y absorción de nutrientes, consiste de un solo tubo (ventrículo) a partir del cual, los sacos del ciego gástrico se ramifican a partir del extremo posterior del intestino anterior. En la mayoría de los insectos, el intestino medio está recubierto por una estructura quitinosa, llamada membrana peritrófica la cual separa los contenidos del lumen en dos compartimentos: el espacio endoperitrófico (dentro de la membrana) y el espacio ectoperitrófico (fuera de la membrana). El píloro o esfínter separa el intestino medio del posterior, y es el sitio donde se ramifican los túbulos de Malpigio hacia el exterior a partir del intestino. Los túbulos de Malpigio son órganos de excreción. El intestino posterior incluye el íleum y el recto, y termina en el ano. El intestino posterior también está recubierto por una cutícula. En algunos insectos, el intestino posterior está reducido a un tubo recto, mientras que en otros, está modificado como una cámara de fermentación y/o en un estómago; ambas estructuras alojan microorganismos que pueden asistir en la digestión de materiales celulósicos (Fig. 2.3.2) (Terra y Ferreira 1994, Chapman 1998, Gullan y Cranston 2005).

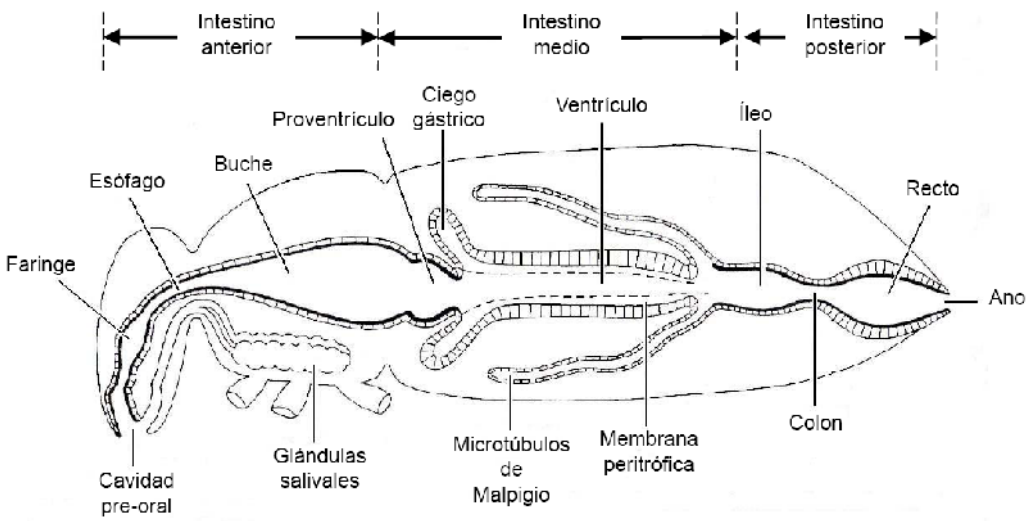

Figura 2.3.1. Tracto digestivo de insectos generalizado.

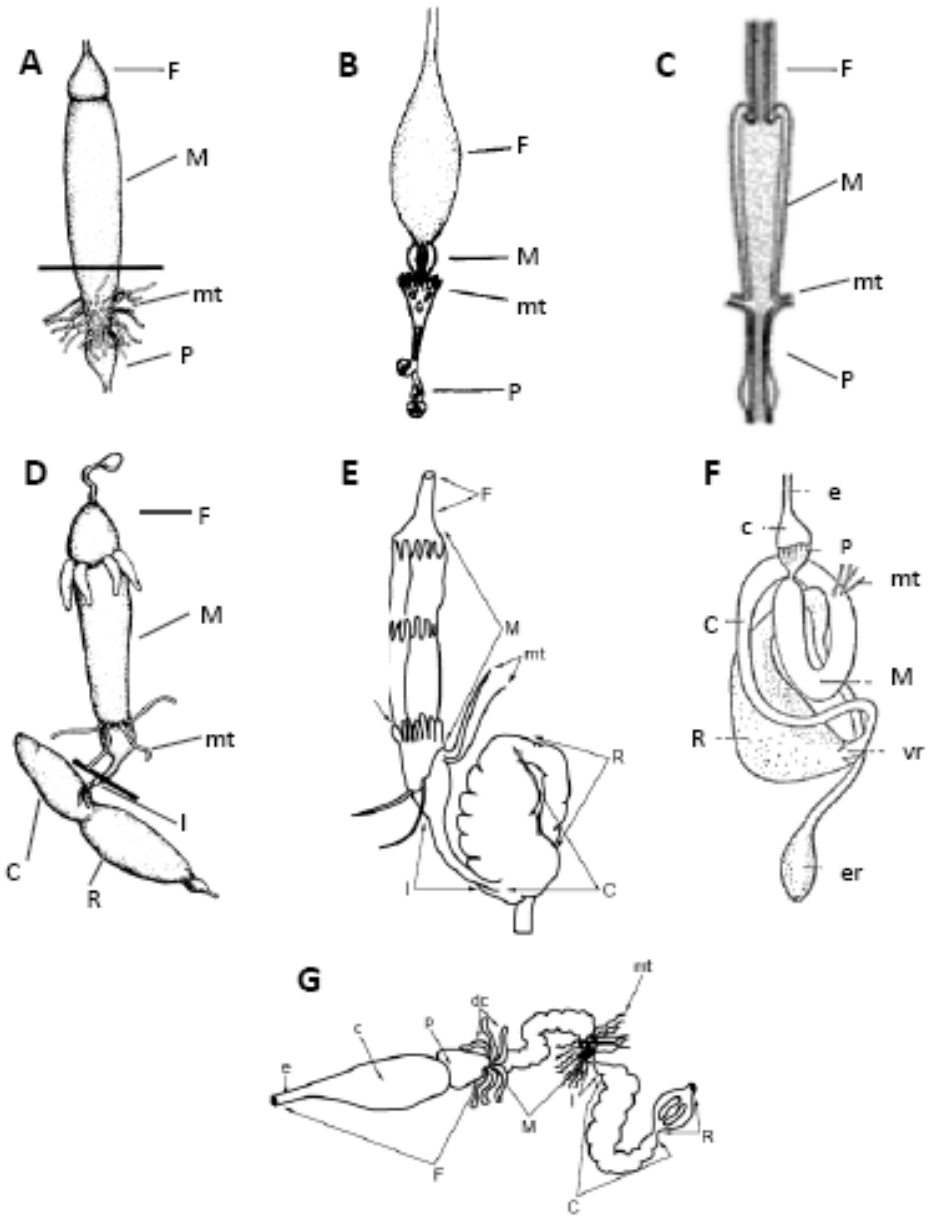

Figura 2.3.2. Diversidad en la morfología del tracto digestivo en insectos. A) Plecoptera, B) Zygentoma, C) Coleoptera: Cerambycidae, D) Diptera: Tipulidae, E) Coleoptera: Scarabaeidae, F) Isoptera (termitas "inferiores", G) Blattaria. F=intestino anterior [e= esófago, p: proventrículo, dc=ciego digestivo], M=intestino medio, mt=túbulos de Malpigio, H=intestino posterior [I= íleo, C= colón, R= recto, vr=válvula rectal, er=recto terminal]. Tomado de Wiedemann (1930), Wigglesworth (1984), Bracke et al. (1979), Nation (1983), Sinsabaugh et al. (1985), Zinkler y Götze (1987).

El alimento entra al intestino anterior, después pasa al intestino medio donde se lleva a cabo la mayor parte de la digestión, y finalmente al intestino posterior (Fig. 2.3.1). El intestino medio desempeña varias funciones como: absorción y secreción de agua, producción de enzimas digestivas libres y asociadas a la membrana microvillar, absorción de nutrientes en solución, producción de células regenerativas y células con función endócrina, y transporte activo de iones. En el intestino posterior también se lleva a cabo en menor grado la absorción de nutrientes. Sin embargo, su función principal está relacionada con la fermentación de los componentes vegetales del alimento por microorganismos simbiontes. Una válvula entérica previene el reflujo del contenido del intestino posterior al intestino medio, de manera que los productos de digestión son finalmente expuestos al ataque microbiano en el intestino posterior (Terra 1990, Prins y Kreulen 1991, Chapman 1998**)**.

Todos los insectos, independientemente de sus hábitos alimenticios, poseen una diversidad de enzimas en el intestino medio, pero cuando la dieta es especializada, las enzimas presentes comúnmente están adaptadas a esta. Las enzimas solo exhiben su máxima actividad bajo ciertas condiciones, en las cuales el pH, el potencial redox y la temperatura juegan un papel importante (Chapman 1998). Las enzimas presentan una mayor actividad dentro de un rango limitado de pH, de manera que un sistema de amortiguamiento es necesario en el intestino para garantizar una digestión eficiente. El pH del intestino anterior está fuertemente influenciado por el alimento, y varía con la dieta, por lo que el amortiguamiento en esta parte del tracto digestivo no es apreciable. Usualmente, el intestino medio es amortiguado y el pH se mantiene relativamente constante. En el intestino medio, el pH es 6.0-8.0, pero en larvas de lepidópteros el pH común es 8.0-10.0. Un pH alcalino es común en insectos fitófagos, mientras que un pH ácido es común en insectos carnívoros. Generalmente, el intestino posterior es ligeramente más ácido que el intestino medio, en parte debido a las secreciones del los túbulos de Malpigi (Terra y Ferreira 1994, Chapman 1998).

2.4 INSECTOS XILÓFAGOS

Muchos insectos han evolucionado para vivir y alimentarse en ambientes leñosos. Estos insectos pueden habitar: árboles vivos, árboles recientemente muertos o en descomposición, o madera procesada y muebles. Algunas especies son xilomicetófagos porque se alimentan principalmente de hongos simbióticos que crecen en la madera de árboles (Tabla 2.4.1) (Haack y Slansky 1987).

Tabla 2.4.1. Familias principales de insectos xilófagos

Orden Familia	No. aprox. de especies	Plantas hospederas	Principales tejidos utilizados	Condición del material habitado[1]
Coleoptera				
Anobiidae	911	A, G	S, D	mu, m
Bostrichidae	455	A	F, Al	v, mu, m
Buprestidae	11, 391	A, G	F, Al, D	v, mu
Cerambycidae	30,000	A, G	F, Al, D	v, mu, m
Lyctidae	65	A	Al	ma
Curculionidae				
Platypodinae*	1,000	A, G	Al, D	v, mu
Scolytinae*	6, 000	A, G	F, Al, D	v, mu
Hymenoptera				
Siricidae*	85	A, G	F, D	v, mu
Xiphydriidae*	82	A	Al	mu
Lepidoptera				
Cossidae	650	A	F, Al, D	v
Sesiidae	1063	A, G	F, Al	v

*Incluye especies xilomicetófagos. A=angiospermas, B=gimnospermas, F= floema, Al= albura, D= duramen. [a] v= árboles vivos: sanos, debilitados /estresados o moribundos; muerto= árboles recientemente muertos o caídos, así como árboles en cualquier grado de descomposición; m= madera parcial o completamente seca, ma= madera en cualquier estado de procesamiento. Tomado de Haack y Slansky (1987).

Los árboles generalmente se clasifican como “maderas duras” (angiospermas) o “maderas suaves” (gimnospermas o coníferas). Al proceder hacia dentro a través de un corte transversal en un árbol típico, primero se encuentra la corteza exterior para protección, seguida de una corteza interior o floema relativamente delgado que conduce sustancias nutritivas. En seguida se encuentra el cambium, una capa de células

meristemáticas que producen floema al exterior y xilema al interior. El xilema comprende la mayor parte del tejido leñoso y se divide en una parte externa o "albura" que conduce agua, y que usualmente representa la porción clara del tronco; y una parte interna o "duramen" fisiológicamente inactiva, que usualmente representa la porción obscura del tronco. En el centro del tronco se encuentra una medula suave (Fig. 2.4.1). Asimismo, las plantas leñosas agregan capas consecutivas de xilema y floema (anillos de crecimiento) a lo largo de su vida; el xilema se acumula a lo largo del tiempo, mientras que el floema viejo eventualmente se elimina. Dentro de un solo anillo de crecimiento de xilema, se presenta una transición desde células con paredes delgadas producidas al inicio de la temporada de crecimiento (madera temprana) hasta células con paredes gruesas producidas posteriormente (madera tardía) (Fig. 2.4.1) (Haack y Slansky 1987).

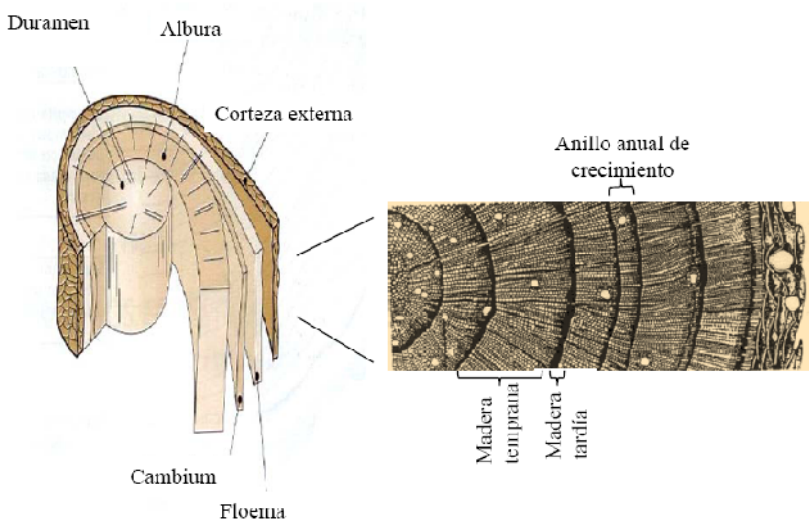

Figura 2.4.1. Estructura del tallo de una planta leñosa (madera). Modificado de Fritts (1976).

En términos de calidad nutricional, el patrón general entre los diferentes tejidos del tallo de las plantas leñosas es: cambium>floema>albura>duramen. La corteza (externa) es tejido muerto, que generalmente no es consumido por los insectos debido a que contiene bajas concentraciones de la mayoría de los nutrientes y agua, además es un tejido altamente suberizado (ceroso) y puede contener altos niveles de compuestos

fenólicos y alcaloides. Si bien, el cambium es el tejido más nutritivo para los insectos que se alimentan de madera, este comprende la fracción más pequeña de los tejidos del tallo. Otros tejidos moderadamente nutritivos como el floema y la albura pueden contener taninos y resinas en altas concentraciones. El duramen es el tejido más pobre en nutrientes (Tabla 2.4.2), con niveles de nitrógeno (N) < 0.3% y altas concentraciones de compuestos secundarios como resinas, polifenoles (taninos, glicósidos y lignina), tropolones (substancias similares a fenoles con propiedades fungidas y bactericidas) y alcaloides. Sin embargo, los insectos que se alimentan (en alguna etapa de su vida) del duramen de ramas y ramificaciones vivas, pueden eventualmente acceder a la médula que es un tejido rico en nutrientes. Además, la región central del duramen (cerca de la médula) presenta altos niveles de Nitrógeno en comparación con las porciones externas. Si bien, la información nutricional sobre la médula es limitada, su calidad nutricional podría ser mayor que la de la albura, pero más baja que en el floema (Haack y Slansky 1987).

Tabla 2.4.2. Características químicas, nutricionales y físicas de la mayoría de los tejidos del tallo de árboles de zonas templadas.

Compuesto	Corteza (%)	Floema (%)	Cambium (%)	Albura (%)	Duramen (%)
Compuestos de Pared celular					
Celulosa[a]	20-30	21-37	20-43	40-45	43-44
Hemicelulosa[a]	sin datos	9-30	30-45	23-35	25-34
Lignina[a]	27-58	4-53	2-20	21-30	23-30
Pectina[a]	sin datos	3-18	3-22	1-4	0.3-1.0
Suberina[b]	2-40	–	–	–	–
Compuestos nutritivos					
almidón + azúcares[b]	sin datos	4-23	3-37	1-5	–
Nitrogeno[b]	0.2-0.6	0.2-2	1-5	0.05-0.3	0.03-0.1
Lípidos[b]	1-38	0.3-4	sin datos	0.1-7	0.2-20
Cenizas (minerals)[b]	0.2-3.0	1-10	3-22	0.2-0.7	0.2-0.8
Agua[c]	17-28	38-71	84-94	28-71	23-62
Densidad[d]	0.4-0.7	0.3-0.5	sin datos	0.3-0.8	0.3-0.8

[a] Los valores se calcularon con base en peso seco libre de extractivos; [b] Los valores se calcularon con base en peso seco sin proceso de extracción; [c] El contenido de agua presente se calculó con base en el peso fresco; [d] Densidad= sobre peso seco de una muestra/peso del agua desplazada por muestra respecto a la condición de tejido fresco.

En resumen, el ambiente leñoso presenta muchos obstáculos físicos y nutricionales para los insectos xilófagos. Entre estos están: (1) restricción de tejidos del tallo que son fisiológicamente activos (y por lo tanto los más nutritivos) en una capa delgada de floema y albura en ambos lados del cambium; (2) protección de esta capa altamente nutritiva con tejidos densos, ricos en fibra, pobres en nutrientes y ricos en aleloquímicos (corteza y madera); (3) construcción de paredes celulares utilizando polímeros complejos y altamente resistentes a la digestión. No obstante, los insectos xilófagos presentan adaptaciones a ambientes leñosos como: la forma del cuerpo, tegumento, partes bucales y tracto digestivo. Entre estas, la talla y el tiempo de desarrollo son importantes, por ejemplo, el tamaño del cuerpo tiende a incrementarse cuando decrece la calidad del alimento (Tabla 2.4.3). Esta tendencia en el tamaño del cuerpo en los insectos xilófagos pudo haber evolucionado para permitir una mejor digestión de alimentos de baja calidad, debido a que el tracto digestivo de los insectos xilófagos varía de acuerdo al tejido leñoso consumido. Por ejemplo: los sistemas digestivos de los comedores de xilema son más largos y complejos que en los comedores de floema; comedores de madera seca o duramen poseen una "molleja" que fragmenta la madera ingerida, exponiendo así una mayor superficie de contacto para las enzimas digestivas. El tracto digestivo, especialmente el intestino medio, es muy largo en algunos cerambícidos habitantes de madera seca y duramen, lo que probablemente permite un rompimiento eficiente de alimento así como su absorción.

Tabla 2.4.3. Largo del cuerpo y tiempo de desarrollo de las familias de insectos xilófagos que habitan varios tejidos del tallo de árboles de zonas templadas.

Orden Familia	Zona de alimentación	No. de especies	Largo del cuerpo (mm)[a] media (rango)		Tiempo de desarrollo (años) media (rango)	
Coleoptera						
Anobiidae	Al, D	6	5	(4-7)	1.8	(1-3)
Bostrichidae	Al	9	7	(3-16)	1.7	(0.5-3.0)
Buprestidae	F	6	9	(8-12)	1.2	(1-2)
	F, Al	10	10	(6-18)	1.9	(1-2)
	Al, D	10	19	(12-30)	3.0	(2-4)
Cerambycidae	C	2	19	(17-20)	2.3	(2-3)
	F	11	14	(6-25)	1.1	(1-2)
	F, Al	24	17	(11-30)	1.6	(1-2)
	F, Al, D	6	20	(14-24)	2.2	(1-3)
	Al, D	3	27	(15-37)	3.1	(2-4)
	D	20	30	(14-53)	3.6	(2-5)
Lyctidae	Al	7	4	(3-5)	1.0	(0.5-2)
Curculionidae						
Platypodinae*	Al, D	4	5	(4-6)	1.0	(0.5-2)
Scolytinae	F	32	4	(2-7)	1.0	(0.5-2)
	Al, D*	10	3	(2-4)	0.6	(0.3-1)
Hymenoptera*						
Siricidae	Al, D	9	25	(16-44)	1.8	(1-3)
Xiphydriidae	Al	8	15	(11-17)	1.2	(1-2)
Lepidoptera						
Cossidae	F, Al, D	5	47	(37-60)	2.9	(2-4)
Sesiidae	F	11	18	(13-27)	1.0	(1-2)
	F, Al	10	29	(22-40)	2.3	(2-4)

*Insectos xilomicetófagos; C=corteza; F= floema; Al=albura; D=duramen; [a] Longitud del cuerpo de adultos para Coleoptera e Hymenoptera, y longitud del cuerpo de larvas (último estadio) para Lepidoptera.

3. SISTEMA DE ESTUDIO

Oncideres albomarginata chamela Chemsak y Gisbert es un escarabajo longicornio que corta ramas reproductivas de 2-3 cm (Uribe-Mú y Quesada 2006) del árbol tropical *Spondias purpurea* (Fig. 3.1) para ovipositar y desarrollar su progenie en ellas. Plantas hospederas alternativas pero menos colonizadas que se han reportado para esta especie incluyen: *Comocladia engleriana* Loes (Anacardiaceae), *Manguifera indica* L. (Anacardiaceae), *Amphipterygium adstringens* Schide ex Schlecht (Rubiaceae), *Bursera* Jacq. ex L. spp. (Burseraceae), *Ceiba pentandra* (L.) Gaertn (Bombacaceae), *Urera* (L.) Gaud. sp. (Urticacaceae) y *Delonix regia* (Bojer ex Hook) Raf. (Fabaceae) (Chemsak y Noguera 1993). *O. albomarginata chamela* se distribuye exclusivamente en México en los estados de Jalisco, Nayarit, Guerrero, Oaxaca, Chiapas y Veracruz (Noguera 1993), mientras que la especie *O. albomarginata* Thomson además de distribuirse en México, también se distribuye en Centroamerica (Nicaragua) y Sudamérica (Guayana Francesa y Británica, Venezuela) (Duffy 1960). *O. albomarginata chamela* mide de 17 a 31 mm de longitud, y de 6.5 a 12 mm de ancho (Noguera 1993). El período reproductivo de esta especie comienza en Octubre y termina en Abril (Uribe-Mú y Quesada 2006). El ciclo comienza cuando los huevos son depositados bajo la corteza de las ramas removidas y termina con la emergencia de los adultos, lo cual requiere de un período de aproximadamente 6-8 meses (Fig. 3.2); las poblaciones de adultos de esta especie en general presentan bajas densidades (Noguera, 1993). Las adultos se alimentan de la corteza fresca de ramificaciones laterales (Fig. 3.3) de las ramas que remueven (*observaciones personales*). Las hembras adultas de *O. albomarginata chamela* son las únicas que poseen la capacidad de cortar las ramas reproductivas del árbol *S. purpurea* para ovipositar en ellas (Uribe-Mú y Quesada 2006), pero después de cierto tiempo (pocos días) otras especies de insectos barrenadores que no tienen la capacidad de remover ramas toman ventaja de las ramas removidas por *O. albomarginata chamela* y también ovipositan en estas ramas.

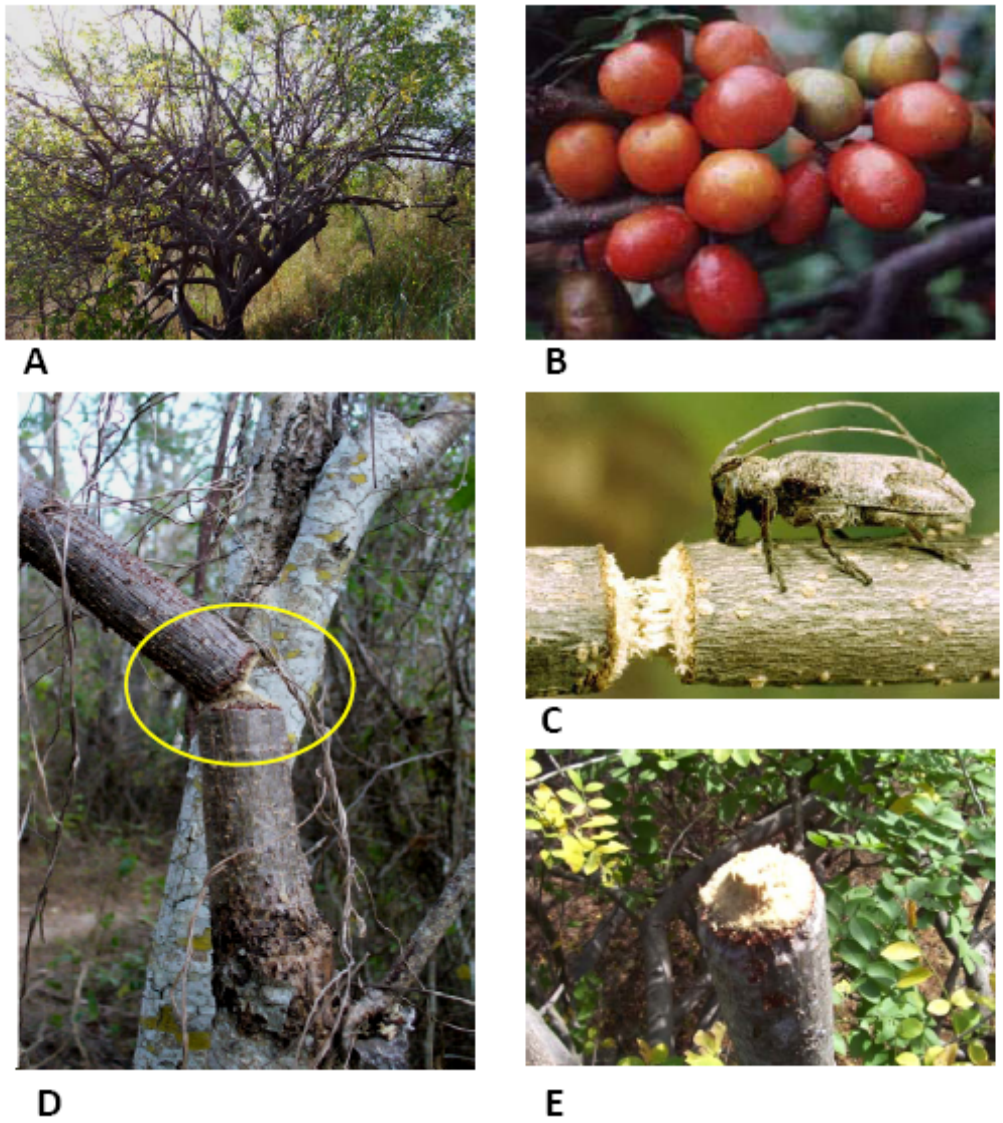

Figura 3.1. Interacción entre el escarabajo "corta palo" ***Oncideres albomarginata chamela*** **y su planta hospedera** ***Spondias purpurea.*** A) árbol del ciruelo *S. purpurea;* B) frutos de *S. purpurea;* C) hembra *O. albomarginata chamela* realizando el corte circular a una rama de *S. purpurea;* D) rama removida después del corte realizado*:* E) cicatriz del corte en el árbol.

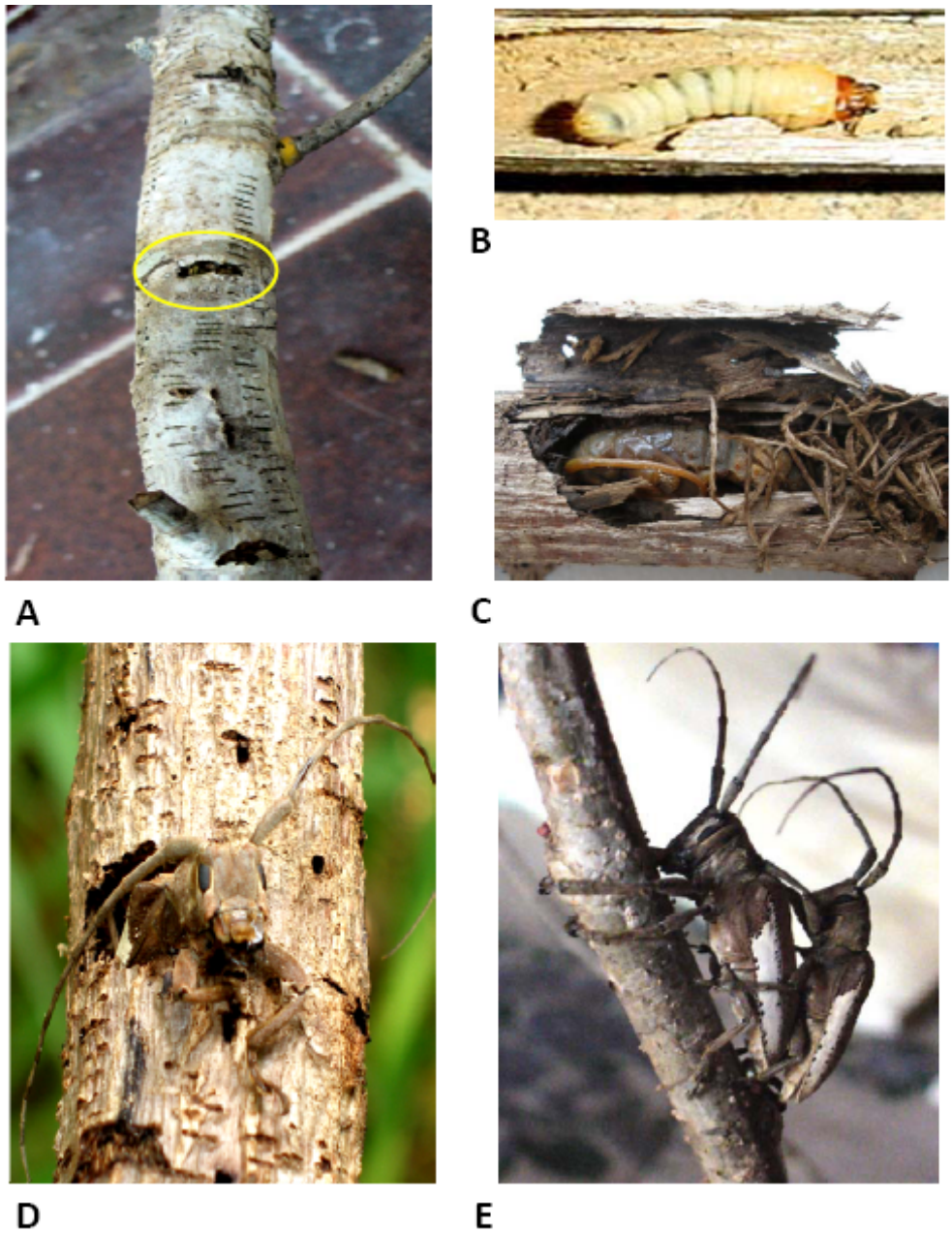

Figura 3.2. Ciclo de vida de *Oncideres albomarginata chamela.* A) Sitios de oviposición; B) estadio larvario; C) pupa; D) emergencia de insecto adulto; E) reproducción.

Figura 3.3. *Oncideres albomarginata chamela* adultos alimentándose de la corteza y tejido joven de las ramificaciones laterales de ramas del árbol *Spondias purpurea.*

Spondias purpurea L. (Anacardiaceae) (Bullock y Solís-Magallanes 1990) es un árbol dioico deciduo común en el bosque tropical seco de México (Fig. 13A) (Bullock 1992), con una proporción de machos y hembras 1:1 en el sitio de estudio (Uribe-Mú y Quesada 2006). Esta especie alcanza hasta los 15 m de altura y hasta 80 cm de diámetro en la base. Tiene hojas compuestas con 5-12 foliolos elípticos u ovalados de 2 a 4 cm de longitud dispuestos de manera opuesta o alterna (Martínez 1979). El período de floración se presenta de Enero a Marzo (Bullock y Solís-Magallanes 1990). Las flores son pequeñas (6-7 mm de diámetro), rojas, sésiles, dimórficas y se encuentran en panículas cortas de 1 a 2.5 cm de largo (Bullock 1992). La polinización de esta especie se lleva cabo por abejas y avispas pequeñas (Bullock 1994). La producción de frutos (drupas ovaladas de color rojo; Fig. 13B) se presenta de Febrero a Julio (Bullock y Solís-Magallanes 1990). Los frutos son comestibles y representan una de las principales fuentes de agua y alimento para mamíferos y aves en la época seca del bosque tropical seco (Mandujano 2002).

4. ÁREA DE ESTUDIO

El estudio se realizó en la Reserva de la Biósfera Chamela-Cuixmala, localizada en la Costa del Pacífico, Jalisco, México (Fig. 4.1). Las poblaciones de *S. purpurea* usadas para este estudio se localizan dentro de la Estación de Biología Chamela del Instituto de Biología, UNAM (19°30'N, 105°03'W) y en los alrededores (Fig. 4.1). La vegetación en la reserva es bosque tropical seco, con una precipitación anual de 707 mm que se concentra en los meses de Junio a Octubre y una época seca que se extiende desde Noviembre hasta Junio (Bullock 1988).

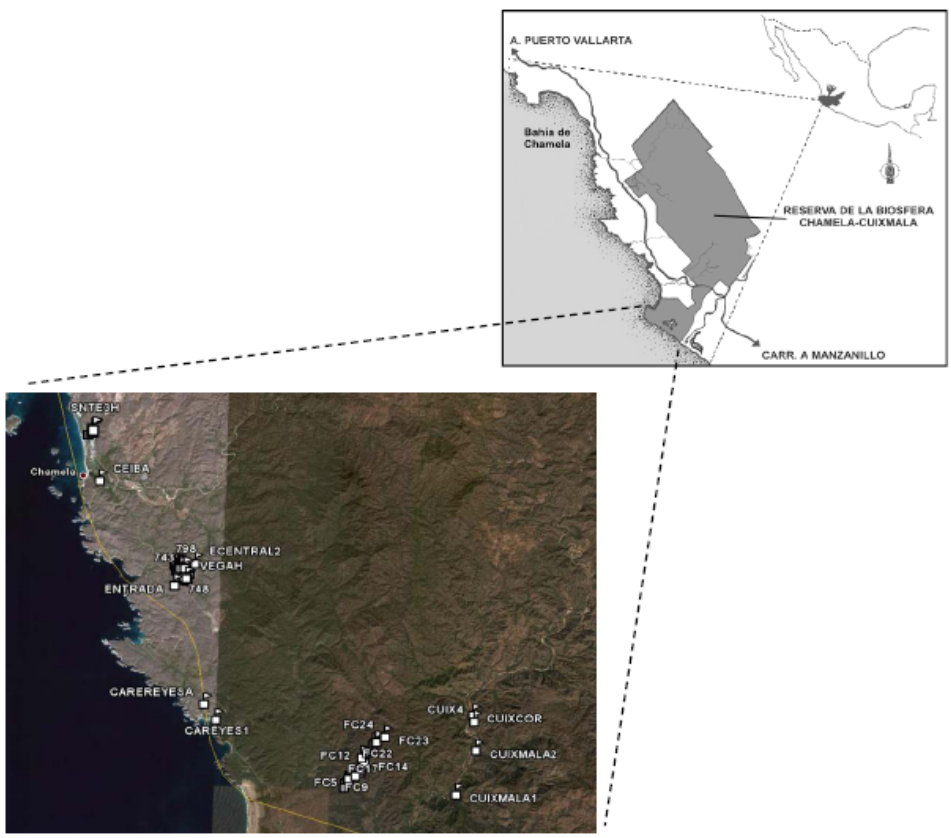

Figura 4.1. Localización del área de estudio. Las poblaciones de *Spondias purpurea* que se utilizaron en este estudio se muestran en el mapa.

5. HIPOTÉSIS

Oncideres albomarginata chamela remueve ramas de su árbol huésped y expresa enzimas líticas para la degradación de madera, lo cual representa un proceso de transformación física y bioquímica de la madera que proporciona recursos y facilita la colonización de otras especies de insectos. Sin embargo, los efectos que *O. albomarginata chamela* puede tener como especie clave o ingeniero del ecosistema no han sido evaluados aún.

6. OBJETIVOS GENERALES

- Estudiar los genes y las enzimas de *O. albomarginata chamela* que están involucradas en el proceso de degradación de madera.

- Estudiar las interacciones bióticas que resultan del proceso de ingeniería natural del ecosistema y de la degradación de madera por el escarabajo barrenador *Oncideres albomarginata chamela* en el árbol *Spondias purpurea.*

- Analizar la evolución de los mecanismos involucrados en la degradación de pared celular vegetal en insectos.

7. ESTRUCTURA DE LA TESIS

En el capítulo 1 se analiza la capacidad de *O. albomarginata chamela* para degradar celulosa mediante sus propias enzimas celulíticas, para lo cual se clonaron dos genes de celulasas. En este capítulo también se realizaron análisis filogenéticos para el estudio de la evolución de celulasas en animales.

En el capítulo 2 se analiza el efecto de *O. albomarginata chamela* en el establecimiento y diversidad de la comunidad de artrópodos asociados al proceso de ingeniería natural del ecosistema.

En el capítulo 3 se examina la capacidad de degradar los principales constituyentes de la madera (celulosa y hemicelulosa) de *O. albomarginata chamela* y cinco especies de escarabajos barrenadores secundarios (Cerambycidae) que utilizan las ramas removidas y colonizadas por *O. albomarginata chamela* (ingeniería bioquímica del ecosistema).

En el capítulo cuatro se revisó la literatura actual de los mecanismos de degradación de pared de celular vegetal, a partir de la cual se realizó un análisis de la evolución de estos mecanismos en los insectos.

En esta tesis también se identificaron a nivel de especie los estadios larvarios de tres especies de colonizadores secundarios mediante la secuenciación de la región universal del "Barcode" del gen mitocondrial citocromo oxidasa I; está información se presenta en el Apéndice A2. Finalmente, en el Apéndice A3 se describe una técnica de extracción de DNA de insectos xilófagos.

Capítulo 1

cDNA CLONING, HOMOLOGY MODELLING AND EVOLUTIONARY INSIGHTS INTO NOVEL ENDOGENOUS CELLULASES OF THE BORER BEETLE *Oncideres albomarginata chamela* (CERAMBYCIDAE)*

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cDNA cloning, homology modelling and evolutionary insights into novel endogenous cellulases of the borer beetle *Oncideres albomarginata chamela* (Cerambycidae)

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Abstract

Novel endogenous cDNAs of β-1, 4-endoglucanases (Oa-EGase I and Oa-EGase II) were cloned from the cerambycid beetle *Oncideres albomarginata chamela*. Oa-EGase I- and Oa-EGase II-deduced proteins and three-dimensional structures possess all features, including general architecture, signature motifs and catalytic domains, of glycosyl hydrolase families 5 and 45 (GHF5 and GHF45) and also share high levels of homology with other beetle cellulases. Total carboxymethylcellulase activity of *O. a. chamela* was 208.13 U/g of larvae. Phylogenetic analyses suggest that insect GHF5 and GHF45 are very ancient gene families and indicate, at least in the case of GHF5, that this family likely evolved from a common ancestor rather than, as is often reported, via horizontal gene transfer. Beetle GHF45 cellulases did not cluster with other metazoan cellulases. However, the presence of GHF45 cellulases in ancient molluscan taxa puts into question the hypothesis of horizontal gene transfer for the evolution of cellulases in animals.

Keywords: *Oncideres albomarginata chamela*, cellulase, protein three-dimensional structure, phylogenetic analysis, glycosyl hydrolase evolution.

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Introduction

It is striking that in a green world dominated by cellulose so few animals are able to exploit this nutritive resource. Undoubtedly this molecule is the most abundant polysaccharide in nature and the major energy resource present in plant tissue (Bayer *et al.*, 1998). Cellulose digestion is a complex process that involves many enzymes with a wide spectrum of substrate specificities, and in some cases macro-structures bundling different enzymes on the cell surface (Beguin & Aubert, 1994; Tomme *et al.*, 1995; Beguin & Lemaire, 1996; Teeri, 1997). Originally, these enzymes were exclusively considered to be part of the metabolic activity of cellulolytic bacteria, fungi and protozoa (Bayer *et al.*, 1998). Furthermore, it was believed that the presence of these microorganisms was essential in the tract of xylophagous and phytophagous animals that lack cellulases (Cleveland, 1924). Recent studies have demonstrated that some metazoans, including nematodes, molluscans and arthropods, are able to produce endogenous cellulases (Smant *et al.*, 1998; Watanabe *et al.*, 1998; Byrne *et al.*, 1999; Girard & Jouanin, 1999; Rosso *et al.*, 1999; Tokuda *et al.*, 1999; Lo *et al.*, 2000; Xu *et al.*, 2000; Watanabe & Tokuda, 2001; Nakashima *et al.*, 2002; Genta *et al.*, 2003; Kim *et al.*, 2008). However, it is still believed that types of cellulolytic enzymes of most metazoans are much more limited than those produced by fungi and bacteria (Watanabe & Tokuda, 2001).

Gene cloning and enzymatic activity detection of endogenous insect cellulases have been focused mainly on termites (Tokuda *et al.*, 1997; Watanabe *et al.*, 1998;

Tokuda *et al*., 1999; Nakashima *et al*., 2002) and cockroaches (Scrivener & Slaytor, 1994; Lo *et al*., 2000). The order Coleoptera includes a great number of phytophagous and xylophagous species (Farrel, 1998). However, few studies have described the presence of enzymes involved in wood digestion in phytophagous and xylophagous beetles (Chararas & Chipoulet, 1982; Chararas *et al*., 1983; Chipoulet & Chararas, 1984; Kukor & Martin, 1986; Martin, 1991; Scrivener *et al*., 1997). Endogenous endoglucanase activity has been demonstrated only for three species of beetles: *Phaedon cochleariae* (Chrysomelidae: Girard & Jouanin, 1999), *Psacothea hilaris* (Cerambycidae: Sugimura *et al*., 2003) and *Apriona germari* (Cerambycidae: Lee *et al*., 2004, 2005; Wei *et al*., 2006a).

Known insect cellulases are classified in three glycosyl hydrolase families (GHFs) (Cantarel *et al*., 2009; http://www.cazy.org/fam/acc_GH.html): GHF5 (*P. hilaris* and *A. germari* bettles), GHF9 (termites, cockroaches and orthopterans), and GHF45 (*Ph. cochleariae* and *A. germari* beetles). These GHFs are structurally and phylogenetically unrelated, indicating that cellulase-containing GHFs evolved independently several times (Henrissat *et al*., 1995). There is strong evidence suggesting that GHF9 cellulases have been inherited vertically from a eukaryote ancestor (Lo *et al*., 2003; Davison & Blaxter, 2005). The evolutionary origin of GHF5 and GHF45 have also been investigated, showing reasonable phylogenetic evidence that both GHF5 and GHF45 cellulases are inherited by horizontal gene transfer (HGT) (Yan *et al*., 1998; Davis *et al*., 2000; Scholl *et al*., 2003; Kikuchi *et al*., 2004). However, only a few available cellulase sequences were included in these phylogenetic analyses, and some animal cellulases were not considered. Given that phylogenetic analyses to test for an ancient origin of GHF5 and GHF45 have been questioned because of the lack of data (Lo *et al*., 2003), a new phylogenetic analysis with the addition of more recently discovered animal cellulases could give improved insights into GHF5 and GHF45 evolution. This study, therefore, describes the cloning of two novel cDNAs of GHF5 and GHF45 encoding cellulases (β-1, 4-endoglucanases, EC 3.2.1.4) and shows evidence for the presence of an additional GHF45 cellulase from the xylophagous beetle *Oncideres albomarginata chamela* (Cerambycidae: Laminae). We also present three-dimensional structures of the protein sequences encoded by those cDNAs, generated with the homology modelling approach. In addition, we investigate the evolutionary origin of GHF5 and GHF45 cellulases for which phylogenetic comparisons, including new animal cellulases of GHF5 and GHF45, were performed. This is the fourth coleopteran species for which molecular evidence of cellulases is reported, and the second case in which two cellulase genes (GHF5 and GHF45) of different phylogenetic origins are simultaneously present in an insect genome.

Results

Enzyme assay

Carboxymethylcellulase (CMCase) assays showed that enzyme activity was 208.13 U/g of larvae (±48.63 SE), and 14.36 U/mg of protein (±2.29 SE).

GHF5 β-1, 4-endoglucanase cDNA cloning and structural analysis

A degenerate primer was designed based on the consensus amino acid region that includes the catalytic proton donor site of the reported endoglucanases of GHF5, and a ≈ 600 bp 3′-fragment was amplified. The flanking region of the 5′-end of cDNA was obtained by 5′RACE with a gene specific primer based on the sequence of the first fragment. A full length of *O. a. chamela* cDNA clone encoding a cellulase (β-1, 4-endoglucanase) gene was obtained from the midgut and then sequenced. The nucleotide sequence was deposited in GenBank (accession number is GU001941). DNA sequence analysis revealed a cDNA that included part of the 3′ untranslated region (nucleotides 673-1050). The 3′ untranslated region contains one putative polyadenylation signal (AATAAA), 19 nucleotides upstream from the poly (A) tail, suggesting that the 3′-untranslated region is complete. The complete Oa-EGase I cDNA sequence shows an ORF of 972 bp long, corresponding to 323 amino acids. An N-terminal secretion signal sequence of 21 amino acids could clearly be identified with the SignalP V1.1 World Wide Web Server (Nielsen *et al*., 1997). Protein molecular mass and pI were calculated to be 36.16 KDa, 4.71, respectively (ExPASy Proteomics Server, Swiss Institute of Bioinformatics http://www.expasy.org/). *O. a. chamela* Oa-EGase I cellulase consisted of a single catalytic module. One putative N-glycosylation site (NGTI) at position 258-261 was found along the sequence. BLAST searches with the deduced amino acid sequence and the sequence alignment of the deduced protein sequence of *O. a. chamela* gene (Fig. 1) indicated that this cellulase was closely related to other cellulases of the beetles that belong to GHF5. Deduced protein sequence of the *O. a. chamela* cellulase showed 57% and 55% identity and 72% homologies with cellulases of the beetles *P. hilaris* (Sugimura *et al*., 2003) and *A. germari* (Wei *et al*., 2006a), respectively. However, high amino acid identity is also observed with other cellulases belonging to GHF5: 41–49% for nematode cellulases, 41–42% for insect symbiotic protist cellulases and 39–43% for bacterial cellulases.

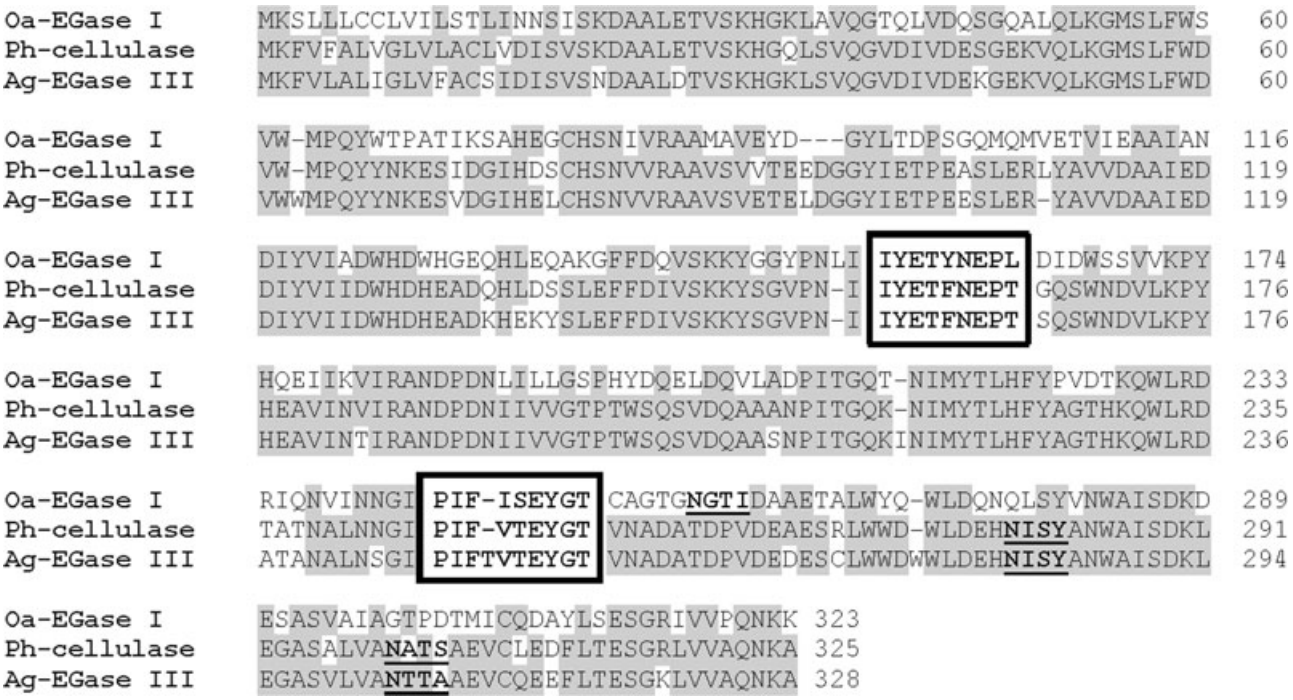

Figure 1. Alignment of the amino acid sequence of Oa-EGase I cDNA with known GHF5 beetle cellulases. The identical residues are shaded. Dashes represent gaps introduced to preserve alignment. The potential catalytic proton donor (at site 155-163) and nucleophile (at site 244-252) amino acids are shown in solid boxes. Potential N-glycosylation sites are underlined below the alignment.

GHF45 β-1, 4-endoglucanases detection and cloning

A cDNA library was constructed using the midgut of *O. a. chamela* larvae. Harbouring cDNA inserts were randomly selected and sequenced to generate expressed sequence tags (ESTs). Of these ESTs, one exhibited similarity to previously reported cellulases. The deduced protein sequence from this EST: TRYWDCCKPSLCPWRRT-TGRHG (GenBank accession number is GU001943) corresponds to the catalytic domain of the cellulases that belong to GHF45. The first ten amino acid residues that represent the center of the catalytic domain of GHF45 cellulases, showed 90–100% identities to those catalytic domains [TRYWDCCKPSC] of the cellulases of *Ph. cochleariae* and *A. germari*.

In search of *O. a. chamela* ESTs, one cDNA was identified with high homology to previously reported cellulase (β-1, 4-endoglucanase) genes of GHF45. The cDNA clone, including the full-length open reading frame (ORF), was sequenced and characterized. The nucleotide sequence was deposited in GenBank (accession number is GU001942). The Oa-EGase II cDNA contains an ORF of 708 nucleotides capable of encoding a 235 amino acid polypeptide, including an N-terminal secretion signal sequence of 17 amino acids identified with the SignalP V1.1 World Wide Web Server (Nielsen *et al.*, 1997). The ORF had both start (ATG) and stop codons (TGA), indicating that Oa-EGase II sequence contains the complete coding region. A putative polyadenylation signal, AATAAA, was located 19 nucleotides upstream from the poly (A) tail. Protein molecular mass and pI were calculated to be 24.22 KDa, 4.51, respectively (ExPASy Proteomics Server, Swiss Institute of Bioinformatics http://www.expasy.org/). This Oa-EGase II cDNA consists of a single catalytic module. Two putative N-glycosylation sites (NPSG and NSSF) at positions 54–57 and 96–99 were found along the sequence, as well as 14 conserved cysteine residues (at positions 41, 42, 46, 63, 79, 87, 117, 118, 120, 166, 190, 201, 223 and 233; Fig. 2). A multiple sequence alignment of the deduced protein sequence of Oa-EGase II cDNA with available GHF45 cellulase sequences is shown in Fig. 2. Alignment with the deduced amino acid sequence, indicated that Oa-EGase II sequence was closely related to the cellulases of the beetles, *A. germari* (67% identity and 79 and 78% homologies; Lee *et al.*, 2004, 2005) and *Ph. cochleariae* (52% identity and 66% homologies; Girard & Jouanin, 1999). The deduced protein sequence of *O. a. chamela* OaEGase-II cellulase showed high amino acid identity with other cellulases of GHF45: 52% with the *Bursaphelenchus xilophilus* nematode cellulase, 48–60% with the insect symbiotic protist cellulases, and 47–56% with fungal cellulases.

Figure 2. Alignment of the amino acid sequence of Oa-EGase II cDNA with known GHF45 beetle cellulases. The identical residues are shaded. Dashes represent gaps introduced to preserve alignment. The conserved catalytic site (at site 36-46) is shown in a solid box. Potential N-glycosylation sites are underlined below the alignment. Arrows indicates conserved cysteine residues.

Endogenous origin of Oncideres albomarginata chamela *endoglucanases*

Genomic PCR amplifications with primers designed from the sequences for Oa-EGase I and Oa-EGase II cDNAs, resulted in the amplification of one ~500 bp fragment of each cDNA. The sequences of the amplified fragments obtained from the legs of *O. a. chamela* adults matched the sequences of the cellulase cDNAs obtained from the midgut tissue. Given that legs should be free of symbionts from internal organs; these results confirm that Oa-EGase I and Oa-EGase II genes are encoded by the *O. a. chamela* genome.

To discover the origin of the two GHF45 cellulase ESTs, a Southern blot assay was performed using the ~500 bp genomic amplified fragment of Oa-EGase II. The Southern blot pattern suggests the presence of four hybridization signals (Fig. 3). A restriction map of the probe Oa-EGase II used shows no restriction sites for endonuclease Xho I, suggesting the presence of at least three genes encoding GHF45 cellulases in *O. a. chamela.* These results confirm the presence of the EST that exhibited similarity to previously reported GHF45 cellulases in the *O. a. chamela* genome, and suggest the presence of an additional GHF45 gene.

Protein homology modelling

The fold of Oa-EGase I and II obtained by homology modelling coincided with the fold of enzymes belonging to GHF5 and GHF45, respectively. After energy minimization in vacua, energy values for the *O. a. chamela* cellulases models were −1221 kJ/mol for Oa-Egase I and −7659 kJ/mol for Oa-EGase II. The quality of the models generated was assessed by plotting dihedrals Φ and Ψ onto Ramachandran plots (SPDBV vs. 4.01, Guex & Peitsch, 1997). The results are in agreement with the requirements for preferred and allowed regions, except for six non-glycine residues (2.28%) for Oa-EGase I, and one non-glycine residue (0.56%) for Oa-EGase II.

Oa-EGase I is a globular protein that appears to share a general architecture of α/β barrel of eight α-helices and eight β-strands with other GHF5 cellulases (Fig. 4); while Oa-EGase II is a globular protein that shows a general architecture of six-stranded β-barrel (Fig. 5). The general architecture for each GHF was shared by the *A. germari* cellulases as well (Figs 4, 5). Superposition of α-carbon skeleton of the template endoglucanase models (1EGZ and 2ENG) and Oa-EGase I and II theoretical models obtained, showed root mean square deviation (rms deviation) of 0.35 Å for Oa-EGase I, and 0.39 Å for Oa-EGase II. When beetle endoglucanases were compared, the values of rms deviation for Cα-atoms were: 0.33 Å between *O. a. chamela* Oa-EGase I and *A. germari* Ag-EGase III, 0.47 Å between *O. a. chamela* Oa-EGase II and *A. germari* Ag-EGase I, and 0.53 Å between *O. a. chamela* Oa-EGase II and *A. germari* Ag-EGase II. These results demonstrate a remarkable similarity of the structural conformation between the *O. a. chamela* endogluca-

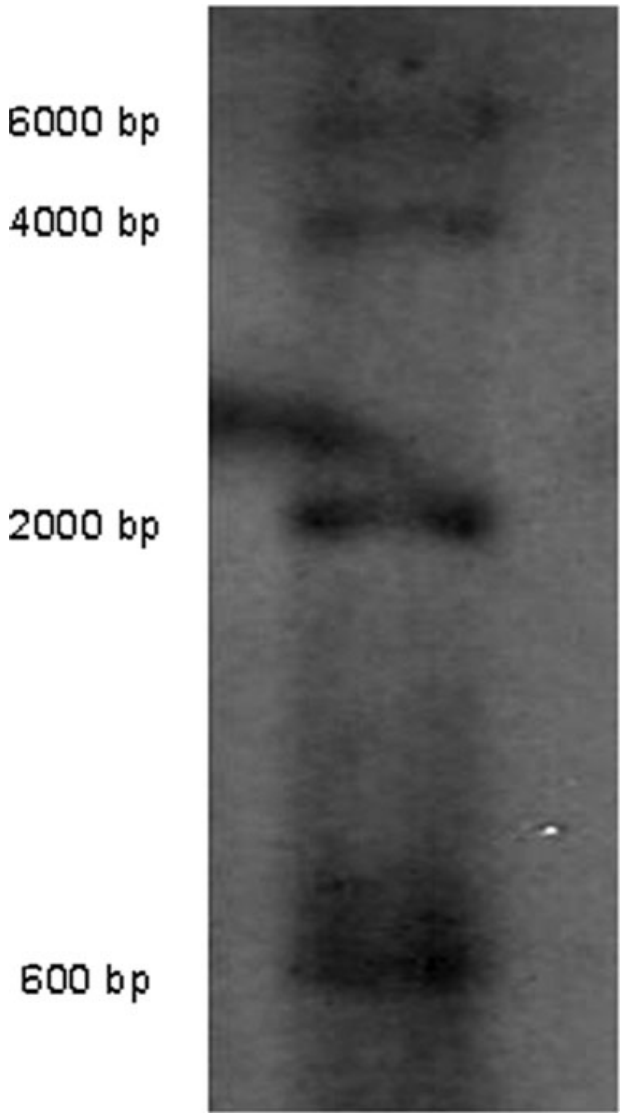

Figure 3. Southern blot analysis of *Oncideres albomarginata chamela* genomic DNA for GHF45 cellulases. 10 μg of genomic DNA was digested with Xho I and hybridized with [Digoxigenin (DIG)-dUTP] 500-bp Oa-EGase II cDNA. Left, molecular weight marker for hybridization signals.

nase theoretical models with previous experimental models reported for the bacteria *Erwinia chrysanthemi* (1EGZ for Oa-EGase I) and for the fungus *Humicola insolens* (2ENG for Oa-EGase II), suggesting that Oa-EGase I and II models have the correct fold for GHF5 and GHF45, respectively.

Phylogenetic analyses

Phylogenetic relationships of GHF5 and GHF45 cellulase genes were investigated using deduced amino acid sequences of representatives from bacteria, fungi and metazoan organisms including beetles, using Bayesian and maximum parsimony (MP) methods. Trees inferred from MP analysis for both GHF5 (Fig. 6) and GHF45 (Fig. 7) cellulases showed less resolution than those inferred from Bayesian analysis, since they had a number of unresolved branches. Bayesian analysis (Fig. 6) revealed that GHF5 cellulases are divided in two groups; the first one is mainly represented by metazoan organisms such as beetles, plant-parasitic nematodes and insect symbiotic protists, while the second one is mainly represented by fungi. Notably, cellulases of beetles, plant-parasitic nematodes and insect symbiotic protists are clustered together with 97% posterior probability, with the exception of the cellulase of the symbiotic protist of the termite *Mastotermes darwiniensis.* Nevertheless, MP bootstrap analysis does not support this cluster. Metazoan and protozoan sequences were found to be most closely related to sequences of bacteria and the rumen fungus *Orpinomyces joyonii* sequences (100% posterior probability for Bayesian analysis, and 60% bootstrap support for MP analysis). Ciliate protist sequences are clustered with some bacterial and fungal cellulases (88% posterior probability for Bayesian analysis, but no bootstrap support for MP analysis) and were related to the major group of fungal sequences according to Bayesian analysis, with 58% of posterior probability.

Contrary to the results of the GHF5 phylogenetic analysis, animal cellulases of GHF45 do not form a monophyletical group (Fig. 7). Cellulases of the nematode *B. xylophilus*, insect symbiotic protists and beetles are clustered in the fungal paraphyletic group. This cluster is well supported for both MP and Bayesian analyses (99% posterior probability and 100% of bootstrap support). However, molluscan cellulases are not clustered in this group, and they seem to be the most divergent GHF45 cellulases.

Discussion

Adult females of longicorn beetle *O. a. chamela* use their mandibles to girdle and detach living tree branches to lay their eggs inside of them. After oviposition, larvae hatch and begin feeding on sapwood beneath the bark, and continue their growth and development by ingesting the solid wood until their emergence as adults (Uribe-Mú & Quesada, 2006). Solid wood of branches usually contains a high proportion of cellulose (Rowell *et al*., 2005). Hence, cellulase activity is expected in this wood-feeding organism. Total cellulase activity in the gut of larvae of *O. a. chamela* was assayed, and the results demonstrated that the larvae are able to hydrolyse amorphous cellulose. Total CMCase activity of *O. a. chamela* larvae (208.13 U/g larvae/min and 14.36 U/mg protein/min) corresponds to the range of endoglucanase activity reported for xylophagous insects such as termites. For example, total endog-

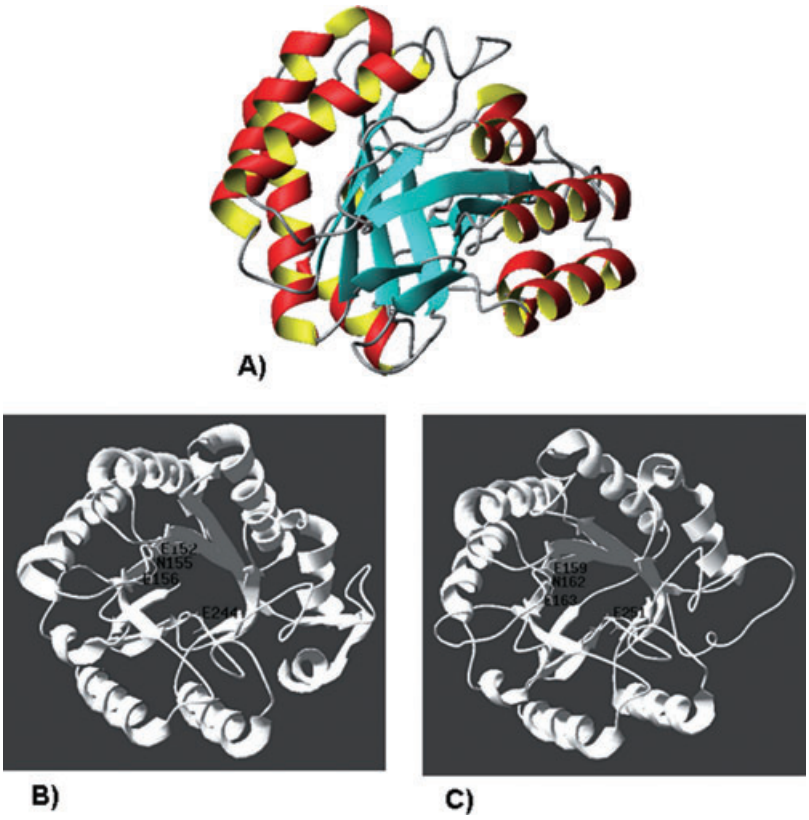

Figure 4. Three-dimensional structures of *Oncideres albomarginata chamela* and *Apriona germari* cellulases of GHF5. (A) the ribbon backbone of *O. a. chamela* Oa-EGase I; (B) alternative orientation of the ribbon backbone *O. a. chamela* Oa-EGase I showing highly conserved residues involved in catalysis; and (C) the ribbon backbone of *A. germari* Ag-EGase III (AAX18655). Catalytic site of both enzymes resides in the same motif and contains highly conserved residues which have active role in catalysis.

enous endoglucanase activity in the salivary glands (132.2 U/g termite/min), foregut (4.4 U/g termite/min) and midgut (47.1 U/g termite/min) of *Coptotermes formosanus* reaches a value of 183.7 U/g termite/min (Tokuda *et al*., 2004).

The results indicate that *O. a. chamela* possesses a family consisting of three endogenous cellulases: one GHF5 endoglucanase and two GHF45 endoglucanases. Endogenous endoglucanases have only been reported for two cerambycid beetles, *P. hilaris* and *A. germari*, and the chrysomelid *Ph. cochleariae*. Of these, three endoglucanases, one belonging to GHF5 and two belonging to GHF45, have been cloned for *A. germari* (Lee *et al*., 2004, 2005; Wei *et al*., 2006a), while only one endoglucanase has been cloned for *P. hilaris* (Sugimura *et al*., 2003) and *Ph. cochleariae* (Girard & Jouanin, 1999), belonging to GHF5 and GHF45, respectively. Multigene families appear to be a common feature of cellulases (Rosso *et al*., 1999; Gao *et al*., 2004; Kikuchi *et al*., 2004; Ledger *et al*., 2006).

Oa-EGase I (Fig. 4), showed a general (β/α)8 barrel structure (Henrissat *et al*., 1995), with four highly conserved residues (E152, N155, E156, E244; Fig. 4) critical for the activity of GHF5 cellulases (Ducros *et al*., 1995). One potential N-glycosylation site was found in the Oa-EGase I amino acid deduced sequence (Fig. 1). However, as N-glycosylation does not have an essential role for the enzyme activity of Ag-EGase III (Wei *et al*., 2006a), N-glycosylation might not be necessary for enzyme activity of *O. a. chamela* Oa-EGase I. On the other hand, the Oa-EGase II three-dimensional theoretical model consists of six β-barrels, three α-helixes and inter-domain regions (Fig. 5) with two highly conserved catalytic centers, a proton donor (D40) and a nucleophile center (D151) (Fig. 5; Davies *et al*., 1993). Oa-EGase II also has 14 conserved cysteine residues (Fig. 2), common in most GHF45 cellulases (Davies *et al*., 1993; Girard & Jouanin, 1999; Xu *et al*., 2000, 2001; Lee *et al*., 2004, 2005; Kikuchi *et al*., 2004), and two putative N-glycosylation sites (Fig. 2). Cysteine residues contribute to protein thermal stability (Xu *et al*., 2001), while the N-glycosylation site (N-S-T/S-F/L; Fig. 2) probably plays an important role in the enzymatic activity of *O. a. chamela* Oa-EGase II, as has been shown for *A. germari* Ag-Egase I and II cellulases (Wei *et al*., 2005, 2006b). The high conservancy of these structural features and the functional similarity between the endoglucanases of the beetle *A. germari* and the cDNA-deduced amino acid sequences of the endoglucanases of *O. a. chamela*, suggest that Oa-EGase I and Oa-Egase II encode functional proteins.

The phylogenetic analyses for GHF5 indicate that beetle cellulases form a clade under a superclade with plant-parasitic nematodes and with most of the insect symbiotic protists (Fig. 6). Similar results were observed in

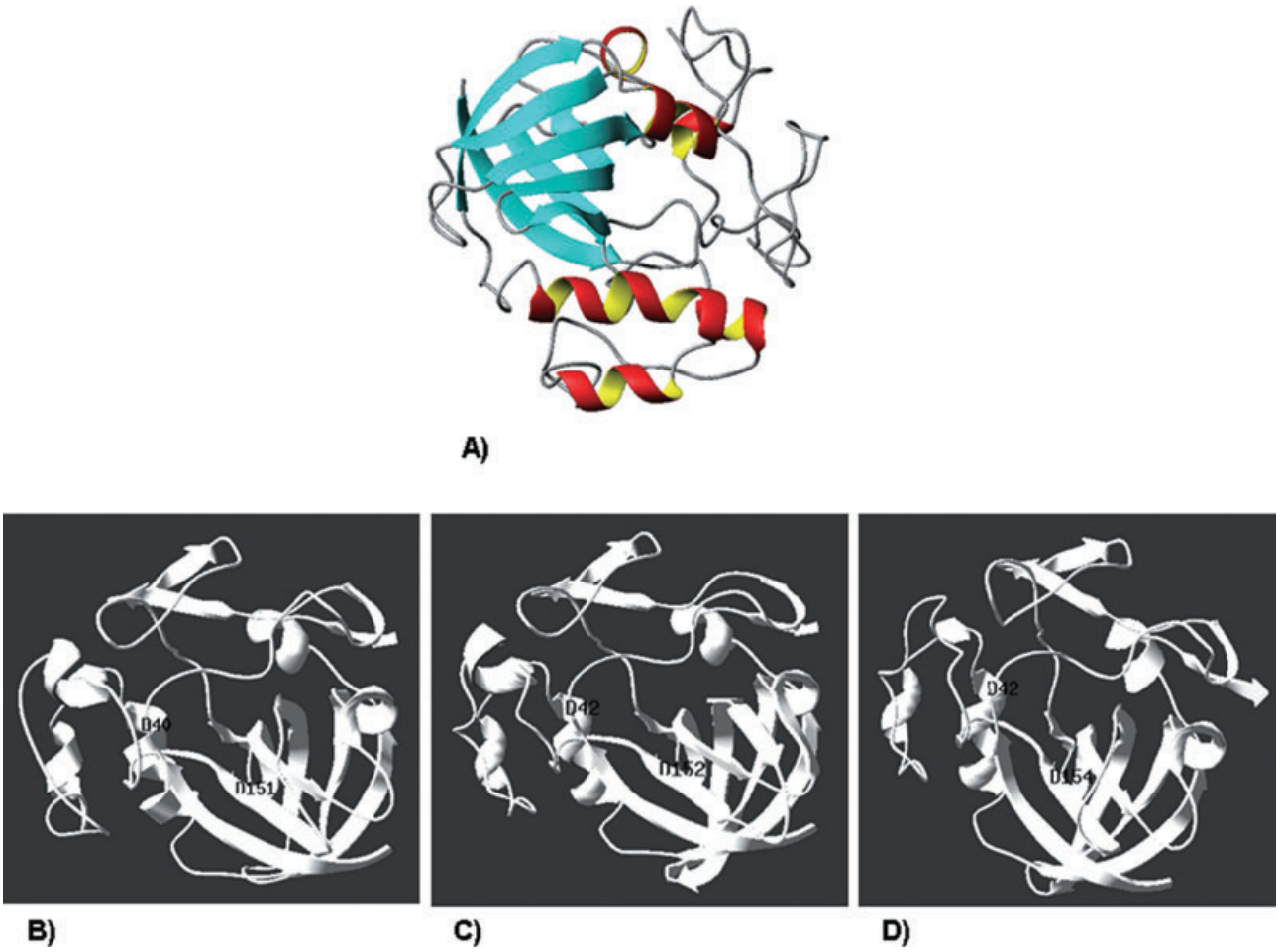

Figure 5. Three-dimensional structures of Oncideres albomarginata chamela and Apriona germari cellulases of GHF45. (A) the ribbon backbone of *O. a. chamela* Oa-EGase II; (B) alternative orientation of the ribbon backbone of *O. a. chamela* Oa-EGase II showing highly conserved residues which have active role in catalysis; (C) the ribbon backbone of *A. germari* Ag-EGase I (AAN78326); and (D) the ribbon backbone of *A. germari* Ag-EGase II (AAR22385). The three cellulases show the same general architecture in which catalytic site is well conserved.

the phylogeny of GHF5 amino acid sequences that includes some nematode cellulases and the beetle *P. hilaris* cellulase (Lo *et al.*, 2003). However, in a recent phylogenetic study of the evolution of GHF5 cellulases in plant-parasitic nematodes, nematode and beetle cellulases are clustered together, but these animal cellulases cluster separately from the cellulases of insect symbiotic protists (Kyndt *et al.*, 2008). Given that fungi are among the closest relatives of animals, we might expect a closer relationship between animal and fungal cellulases, than that between animal and symbiotic protist cellulases. However, a closer relationship between animal and fungal cellulases of GHF5 has not been found in previous studies (Lo *et al.*, 2003; Kyndt *et al.*, 2008). This could be explained by the very ancient divergences observed in the phylogeny of GHF5 cellulases, particularly those corresponding to divergences between prokaryotic cellulases and early eukaryotic cellulases (ciliate and parabasalian cellulases). Remarkably, the apparently close relationship between animal and prokaryotic cellulases and the absence of cellulases in most animal taxa, have led to the hypothesis that animals acquired these genes by horizontal gene transfer (HGT) from bacteria (Yan *et al.*, 1998; Davis *et al.*, 2000; Scholl *et al.*, 2003). However, the role of the HGT hypothesis in the evolution of animal cellulases has been questioned because these animal genes possess several eukaryotic elements (Watanabe & Tokuda, 2001; Lo *et al.*, 2003; Davison & Blaxter, 2005 but see Kyndt *et al.*, 2008).

In contrast to the animal GHF5 members, the beetle GHF45 members did not form a clade with the nematode *B. xilophilus* cellulase or molluscan cellulases (Fig. 7). This suggests that GHF45 cellulases could have evolved independently several times in animals, probably by HGT from fungi, as suggested in a previous phylogenetic study of the nematode *B. xilophilus* cellulase (Kikuchi *et al.*, 2004) and the molluscan *Corbicula japonica* cellulase (Sakamoto & Toyohara, 2009).

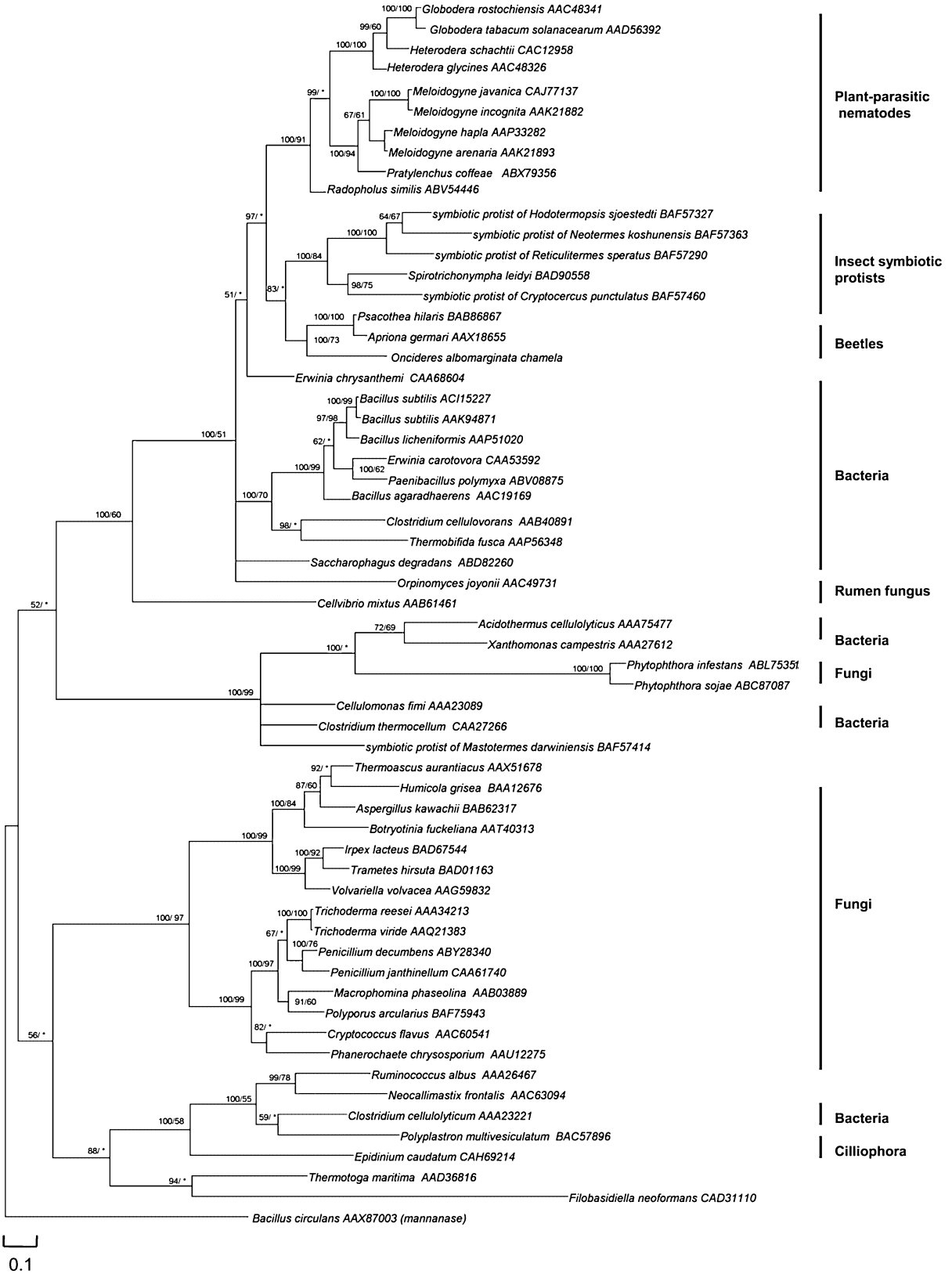

Figure 6. Phylogeny of GHF5 cellulase enzymes. The phylogeny shown is the Bayesian topology and branch lengths inferred using MrBayes vs. 3.1.2, with the WAG + I + G model. Numbers above the diagonal indicate posterior probability values from Bayesian analysis. Numbers below the diagonal indicate bootstrap percentage values from a bootstrap analysis inferred using the same alignment with PAUP*4.0. *Bacillus circulans* mannanase was used as outgroup. The asterisks represent branches that were not supported in 50% or more of bootstraps. The scale bar represents the number of substitutions per site. GenBank accession numbers are shown adjacent to each enzyme. Phylogenetic tree was edited using Dendroscope software (Huson *et al.*, 2007).

HGT is recognized as a significant feature of genome evolution in prokaryotes, and recently its relevance in eukaryotic evolution has also been recognized (Keeling & Palmer, 2008). However, HGT seems to be less likely in the genes encoding proteins involved in large complexes (Jain *et al.*, 1999), just as in the case of carbohydrate active enzymes. For example, carbohydrate active enzymes belonging to different GHFs have been reported for several eukaryotic organisms such as protists (seven GHFs), fungi (ten GHFs), plants (at least two GHFs), molluscans (five GHFs) and beetles (two GHFs) (Cantarel *et al.*, 2009; CAZy Web Server at http://www.cazy.org/fam/acc_GH.html). Then, it seems unlikely that different HGT events have occurred several times to explain the presence of at least two gene families (GHF5 and GHF45) in all eukaryotic lineages before mentioned.

Indeed, recent evidence indicates that GHF9 genes of five metazoan phyla (including insects) are derived from an ancestral gene in the last common ancestor of protostomes and deuterostomes that has been vertically inherited over several hundred million years from a primitive metazoan ancestor (Lo *et al.*, 2003; Davison & Blaxter, 2005). The monophyly of animal (nematodes and beetles) GHF5 cellulases that we found suggests a single ancient origin for animal cellulases that resulted from vertical descent from a common ancestor. Moreover, GHF5 is represented for more than 1500 genes that, besides cellulases, comprise: mannanases of different metazoan organisms such as Mollusca and Viridiplantae; and endoglycoceramidases of cnidarian *Hydra magnipapillata* and *Cyanea nozakii* (Cantarel *et al.*, 2009; CAZy Web Server at http://www.cazy.org/fam/acc_GH.html). The presence of GHF5 and GHF45 genes in ancient taxa such as Cnidaria and Mollusca, suggests that GHF5 and GHF45 genes could be present in early metazoans, as reported for GHF9 (Lo *et al.*, 2003; Davison & Blaxter, 2005). However, a broader and systematic search for metazoan cellulases is necessary to clarify the origin and evolution of GHF5 and GHF45 genes.

Experimental procedures

Insects

O. a. chamela (Coleoptera: Cerambycidae) is a borer beetle that removes entire twigs or branches of the tropical tree *Spondias purpurea* (Anacardiaceae). Female beetles oviposit the removed branches where their eggs pupate and develop inside until adults emerge (Uribe-Mú & Quesada, 2006). In our study, oviposited branches of the tree *S. purpurea* were collected to obtain larvae of the beetle *O. a. chamela* within the Chamela-Cuixmala Biosphere Reserve in the central Pacific coast of Mexico (*c.* 19°30′N, 105°03′W). Larvae were collected and stored for later DNA extraction. Species identification was carried out using reported literature (Böving & Craighead, 1931; Duffy, 1960).

Enzyme assay

Gut and its content of *O. a. chamela* larvae was removed by dissection and transferred into 1.5 ml plastic centrifuge tubes. Samples were homogenized in 200-μl protease inhibitor cocktail (complete Mini EDTA free; Roche Diagnostics, Basel, Switzerland) using a glass homogenizer and centrifuged at 10 000 *g* for 10 min (4°C). Insoluble materials were discarded. Prior to enzymatic assays, pH value of the recovered supernatant was measured with a pen-type pH meter (model B-212; Horiba, Kyoto, Japan). The pH value estimated in the gut juice of *O. a. chamela* larvae was 6.5. Hence, a CMCase assay was performed by measuring the amount of reducing sugars after incubation of 100 μl 1% (w/v) CMC (standard molecular mass, 250 KDa; degree of carboxymethyl substitution, 0.7; Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M sodium phosphate (pH 6.5) with a 25-μl sample diluted 200 times at 37°C for an appropriate time period. Reducing sugars were measured with tetrazolium blue (Sigma-Aldrich) as a chromogenic reagent with glucose as a standard (Jue & Lipke, 1985). Controls without enzyme or without substrate were included. One unit (U) of cellulase activity is defined as the amount of enzyme which produced 1 μmol of reducing sugar (glucose equivalents) per minute from CMC. Specific activity is defined as U/mg protein. Protein concentration was determined by using a protein assay kit (Coomasie Plus Protein Assay Reagent, Pierce, Biotechnology, Rockford, IL, USA) with BSA as standard.

GHF5 β-1, 4-endoglucanase cDNA cloning and sequencing

A degenerate oligonucleotide primer was designed based on a consensus amino acid region [IYETFNEP] that includes the catalytic proton donor site for the reported GHF5 endoglucanases of *P. hilaris* (BAB86867), *Globodera rostochiensis* (GR-ENG1, AAC48325, and GR-ENG2, AAC48341), *Heterodera glycines* (HG-ENG1, AAC48327, and HG-ENG2, AAC48326) and *Meloidogyne incognita* ENG1 (MI-ENG1, AAD45868). This degenerate primer corresponds to the amino acid positions I157-P164 of the *P. hilaris* cellulase.

The digestive tract of phytophagous insects is usually divided into three main regions: foregut (stomodeum), midgut (mesenteron) and hindgut (proctodeum). The midgut is the region where epithelial cells produce and secrete digestive enzymes, and it is usually free of endosymbionts (Chown & Nicolson, 2004). Hence, we dissected and used the midgut of *O. a. chamela* larvae for isolation of mRNA using A QuickPrep *Micro* mRNA Purification Kit (Amersham Bioscience, Little Chalfont, UK). First-strand cDNA synthesis from isolated mRNA and the following

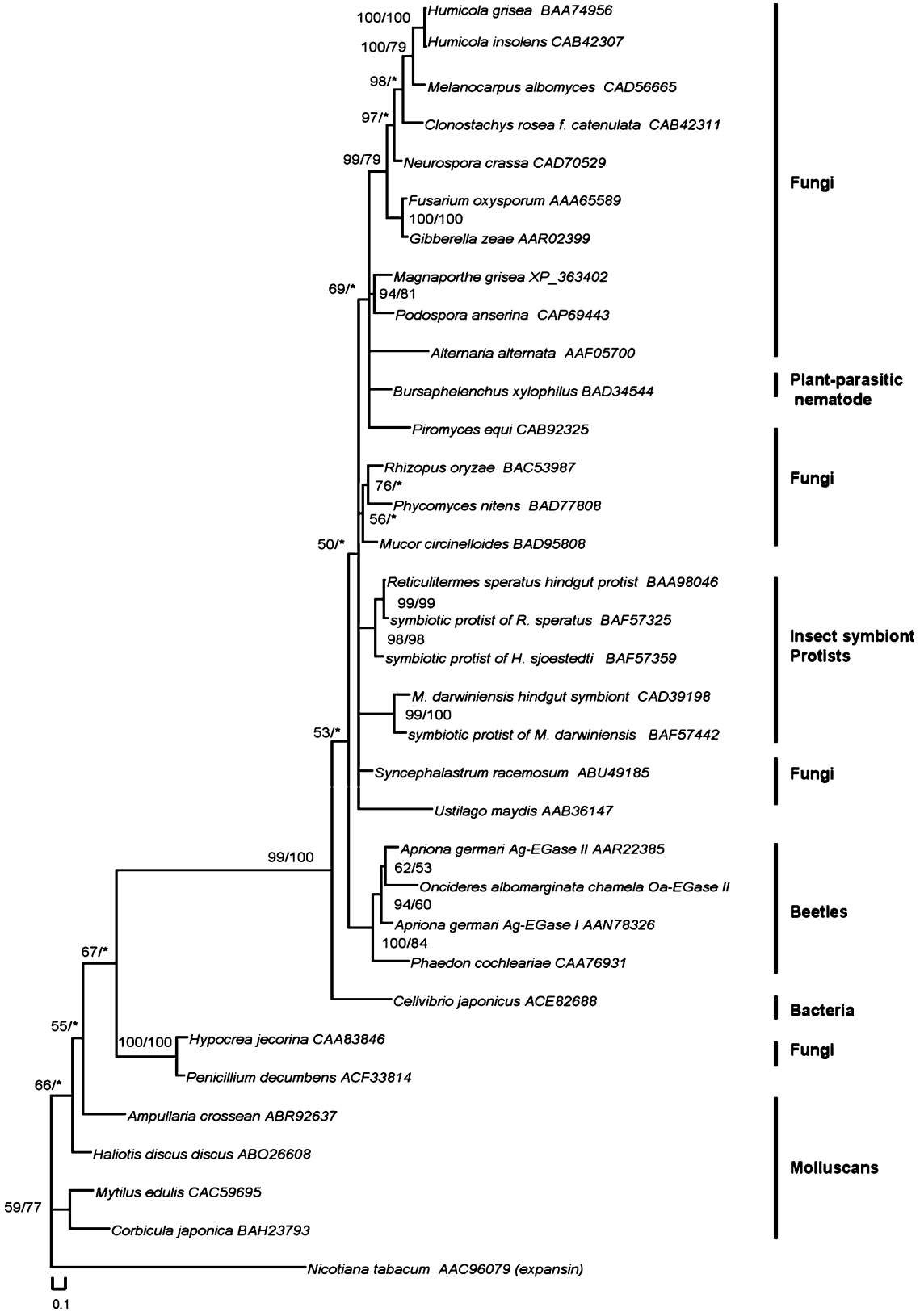

Figure 7. Phylogeny of GHF45 cellulase enzymes. The shown topology and branch lengths were inferred using MrBayes vs. 3.1.2 with the WAG + G model. *Nicotiana tabacum* expansin was included as outgroup. Numbers above diagonal indicate posterior probability values from Bayesian analysis. Numbers below the diagonal indicate bootstrap percentage values from a bootstrap analysis inferred using the same alignment with PAUP*4.0. The asterisks represent branches that were not supported in 50% or more of bootstraps. The scale bar represents the number of substitutions per site. GenBank accession numbers are shown adjacent to each enzyme. Phylogenetic tree was edited using Dendroscope software (Huson *et al*., 2007).

amplification of the target cDNA were performed with a SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to manufacturer's instruction using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). An annealing gradient and ramping-down of the annealing temperature were employed as previously reported (Tokuda *et al*., 2002) using a PCR machine with a temperature gradient function (iCycler, Bio-Rad, Hercules, CA, USA). The fragment obtained was ligated into the pGEM-T plasmid vector (Promega, Madison, WI, USA), transformed into DH5α bacterial host strain (Takara, Tokyo, Japan), and the nucleotide sequence was determined. Sequencing reaction was carried out using the BigDye Terminator v3.1 cycle sequencing kit, with an Automated DNA sequencer (B13700 Applied Biosystems, Foster City, CA, USA). Sequence similarities were determined by a BLAST research (http://www.ncbi.nlm.nih.gov/BLAST/). Flanking region for the 5′-end of cDNA was obtained by 5′RACE (SMART™ RACE cDNA Amplification Kit, Clontech) with a gene specific primer (5′-GTCTGGCCTGTGATT GGGTCAGC-3′) based on the sequence of the first fragment, and cloned and sequenced as described above.

GHF45 endo-β-1, 4-glucanases cloning and sequencing

For a cDNA library construction, an enriched full-length double-stranded (ds) cDNA was prepared from mRNA using a Creator SMART cDNA library construction kit (Clontech). The ds cDNA molecules were inserted into the pGEM-T plasmid vector (Promega) and subsequently electrotransformed into the JM109 bacterial host strain (Takara) using a MicroPulser electroporation apparatus (Bio-Rad). Individual colonies were picked at random. The presence of an insert was checked by PCR using M13 reverse and forward primers. To generate expressed sequence tags (ESTs), PCR fragments from positive clones were sequenced (B13700 Applied Biosystems) in both orientations with SP6 and T7 primers. Sequence similarities to cellulases reported in GenBank were obtained by a BLAST research (http://www.ncbi.nlm.nih.gov/BLAST/).

For cloning the full-length GHF45 cDNAs, a pair of primers (C7d3 5′-ACGAGGTATTGGGACTGCTGCAA-3′ and C7p3 5′-AGGAGTCCAGCCAGGAGCAAGT-3′) were synthesized based on the sequences of cellulases obtained from the cDNA library. 3′ RACE amplifications with the primers were performed using a SMART™ RACE cDNA Amplification Kit (Clontech). Only one primer (EST C7d3) produced a successful amplification. The fragment obtained was cloned in pCR 2.1-TOPO plasmid vector and transformed into *Escherichia coli* chemically competent cells of TOPO TA cloning system (Invitrogen). Flanking region for the 5′-end of the cDNA of GHF45 was obtained by 5′RACE (SMART™ RACE cDNA Amplification Kit, Clontech) with a gene specific primer (5′-GCGCAACACATGCTAGTGTC TGCACCGCC-3′) based on the sequence of the first fragment. This primer amplified a fragment with an overlapping region of 250 pb, ensuring that a full-length cDNA sequence could be determined. Multiple sequence alignments were performed with Clustal W (Thompson *et al*., 1994) with subsequent manual optimization.

Genomic DNA isolation, genomic PCR and Southern blot analysis

As mentioned above legs are free of endosymbionts, therefore, legs of *O. a. chamela* adults were dissected and used to isolate DNA (Fet *et al*., 2000). Tissue was disrupted in liquid nitrogen, and tissue powder was extracted with a buffer [100 mM Tris-HCl (pH 9.2), 200 mM sucrose, 50 mM EDTA, 0.5% SDS, 150 μg Proteinase K (Sigma-Aldrich)]; a final step of polysaccharide removal with Ethylene Glycol Monobutyl Ether was included (Chung *et al*., 1996). Primers used for amplification of the genomic DNA encoding Oa-EGase I and Oa-EGase II were: 5′GTACGCGGGGGTAAATTGGCC-3′and 5′GTCTGGCCTGTGA TTGGGTCAGC-3′ based on the Oa-EGase I cDNA cloned in this study; and 5′-CACGAGGTATTGGGACTGCT-3′ and 5′-CCGG TAAGATCTCCAGGACA-3′ based on the Oa-Egase II cDNA cloned in this study as well. PCR was performed using Platinum Taq DNA Polymerase (Invitrogen) following manufacturer's recommendations. To obtain optimal PCR conditions, a ramping-down of the annealing temperature ('touchdown'; −3°C for every three cycles) was employed. PCR amplification was started using the following settings: 4°C/30 s (denaturing), 70°C/30 s (base annealing temperature of first three cycles) and 72°C/4 min (extension), and was continued until the base annealing temperature reached the final condition of 55°C. Under the final conditions, the amplification was continued for 18 cycles. Amplified fragments were cloned using TOPO TA cloning system (Invitrogen), and sequenced as previously described.

Since DNA purified from legs of *O. a. chamela* adult was obtained in low quantity, DNA purified from fat tissue of larvae (assumed to be free of endosymbionts) as previously described, was digested with Xho I restriction endonuclease, separated by agarose gel electrophoresis, and then transferred and cross-linked to a nitrocellulose membrane. The membrane was treated with hybridization solution (5X SSC, 0.1% w/v N-lauroyl sarcosine, 0.02% w/v SDS, 1:20 blocking reagent, DNA, 50% formamide) at 42°C for 6 h. The 5′fragment of ~500 bp corresponding to Oa-EGase II cDNA, was [Digoxigenin (DIG)-dUTP]-labelled according to the manufacture's instruction (DIG-System, Boehringer Mannheim). The membrane was hybridized overnight at 42°C. Following hybridization the membrane was washed twice for 10 min in 2X SSC and 0.1% SDS at room temperature and twice for 15 min in 0.5X SSC and 0.1% SDS at 65°C. Hybridization signals were visualized on X-ray film with a chemiluminescence reaction of an alkaline phosphatase–conjugated antidigoxigenin-Fab fragment (dilution 1:10 000) with the luminescence reagent CSPD (dilution 1:200; Boehringer Mannheim).

Protein modelling

cDNAs encoding cellulases of the beetle *A. germari* were expressed in baculovirus-infected insect cells and the recombi-

nant enzymes demonstrated to be active (Lee *et al*., 2004, 2005; Wei *et al*., 2006a). In order to investigate if Oa-EGase I and II cDNAs were encoding functional proteins, structural models for the β-1, 4-endoglucanases (Oa-EGase I and II, Ag-EGase I, II and III) of the beetles *O. a. chamela* and *A. germari* were generated. Tertiary structure of these β-1, 4-endoglucanases were predicted by a homology modelling approach. Oa-EGase I, 3D model was generated using the ModWeb Comparative Modeling Server version SVN.r665 (http://modbase.compbio.ucsf.edu/ModWeb20-html/modweb.html). The best template of Oa-EGase I was 1EGZ of *E. chrysanthemi*, showing 43% of amino acid identity. Homology models of Oa-EGase II, Ag-EGase I and Ag-EGase II were generated using the Swiss-Model Server (Bordoli *et al*., 2006; htpp://www.expasy.ch/swissmod/SWISS-MODEL.html) based on crystal structure of the β-1, 4-endoglucanase V of *Humicola insolens* (2ENG) as template; the alignment of Oa-EGase II and β-1, 4-endoglucanase V was obtained with Clustal W (Thompson *et al*., 1994). A homology model of Ag-EGase III was generated using the Swiss-Model Server as well, based on the crystal structure of the β-1, 4-endoglucanase Z of the bacteria *E. chrysanthemi* (1EGZ CHAIN A). Evaluation of structural parameters and prediction quality of the modelled structures were done using the programs SPDBV vs. 4.01 (Guex & Peitsch, 1997) and MOLMOL (Koradi *et al*., 1996). Energy minimization of models Oa-EGase I and II was carried out by GROMOS96 (van Gunsteren *et al*., 1996) provided by SPDBV program using the steepest descent method of 200 steps. MOLMOL and SPDBV vs. 4.01 were used for visualization of molecular structures. Superposition of coordinates of Cα-atoms of protein models, which share 35–50% sequence identity with their templates, will generally deviate by 1.0–1.5 Å from their experimental counter parts (Bordoli *et al*., 2006). Cα-atoms of theoretical models of *A. germari* and *O. a. chamela* cellulases were carried out using the software SPDBV vs. 4.01.

Phylogenetic analyses

A phylogenetic analysis was performed on *O. a. chamela* cellulase sequences and GHF 5 and GHF45 cellulases, previously reported in the GenBank (http://www.ncbi.nlm.nih.gov/) and CAZY glycosyl hydrolase (http://www.cazy.org/fam/acc_GH.html) databases. All nucleotide sequences were translated into amino acid sequences and aligned using Clustal W (Thompson *et al*., 1994), using default parameters and adjusted by eye. Prior to phylogenetic analyses, signal peptide sequences, N-terminal and C-terminal extensions peculiar to individual taxa, and other ancillary domains such as cellulose binding domains were excluded, since not all sequences contain these accessory domains. Gap-prone segments (with cut value of 50%) were also removed. Phylogenetic analyses were performed under maximum parsimony and Bayesian criteria, using the programs PAUP*v 4b10 (Swofford, 2000) and MrBayes vs. 3.1.2 (Huelsenbeck & Ronquist, 2001). For maximum parsimony analyses, the most parsimonious trees were estimated using the heuristic search option [TBR branch swapping, saving only a single tree in each case] with random sequence addition (five random replicates). Support for the different clusters was evaluated by bootstrap analysis using the full heuristic search option with 1000 replicates. Gaps were treated as the 21st amino acid. For Bayesian analyses, an appropriate model of nucleotide substitution for each GHF analysis was determined using the program ProtTest vs. 1.4 (Abascal *et al*., 2005). The best fit model of amino acid evolution was a WAG + I model with gamma correction (Yang, 1993; Whelan & Goldman, 2001) for GHF5 and a WAG model with gamma correction (Reeves, 1992; Whelan & Goldman, 2001) for GHF45. For Bayesian criteria, the analyses were performed using the selected model of substitution. A total of 10 000 trees were obtained based on the settings ngen = 1000 000, samplefreq = 100. Prior to estimating the support of the topologies founded, we checked the convergence overall chains (4) when the log likelihood values reached the stationary distribution. The first 2000 trees were 'burn in' and discarded, and a 50% majority rule consensus tree of the remaining trees was generated. We used the *Bacillus circulans* mannanase (AAX87003) as outgroup for GHF5 analyses, and the *Nicotiana tabacum* expansin (AAC96079) as outgroup for GHF45 analyses.

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Capítulo 2

INSECTS AS STEM ENGINEERS: LONG-TERM INTERACTIONS MEDIATED BY THE TWIG-GIRDLER *Oncideres albomarginata chamela* ENHANCE ARTHROPOD DIVERSITY*

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*Sometido a: Oikos (2010)

ABSTRACT

Indirect interactions such as ecosystem engineering influence the communities and biodiversity by controlling the availability of resources and/or habitats used by other organisms. Insect herbivores may act as ecosystem engineers. However, we still have a poor understanding of the effects of insect ecosystem engineering on arthropod communities. In this study, we evaluated the effect of ecosystem engineering by the stem-borer *Oncideres albomarginata chamela* on the arthropod community of a tropical dry forest across three consecutive years. The results showed that *O. albomarginata chamela* ecosystem engineering has strong positive effects on the colonization, abundance, species richness and composition of its associated arthropod community, mainly through the creation of a habitat with high availability of oviposition sites for secondary colonizers. These effects cascade upward to higher trophic levels including predators and parasitoids. Overall, *O. albomarginata chamela* ecosystem engineering was responsible for nearly 95% of the abundance of secondary colonizers and 82% of the species richness, suggesting that ecosystem engineering by *O. albomarginata chamela* is a keystone process. To our knowledge, this is the first empirical study that evaluates the effect of the ecosystem engineering by stem-borer insects on arthropod community, and the first one that provides evidence of positive effects of biochemical ecosystem engineering.

Keywords: habitat modification, herbivore communities, keystone process, indirect interactions, positive effects.

INTRODUCTION

Species occur in nature as members of interactive assemblages, affecting the diversity of biological communities and ecosystem functioning. Even though direct pairwise interactions (e.g. exploitative competition and predation) play a major role in explaining the structure of many biological communities (e.g. Tilman 1982, Hairston 1989), indirect interactions mediated by a third species, are also recognized as important interactions structuring biological communities (Morin 1999, Ohgushi 2005). One indirect interaction with important consequences on biological communities and biodiversity is the relationship between organisms that modify or create new habitats with those organisms that use these new habitats, a process called "ecosystem engineering" (Jones et al. 1994, 1997), or habitat modification (Stachowicz 2001). Ecosystem engineers are particular species that control the availability of resources for other species by causing physical state changes in biotic or abiotic materials (Jones et al. 1994, 1997). Because some ecosystem engineers create habitats on which an entire community is built in, they are also named "foundation species" (Stachowicz 2001) and/or "keystone engineers" (Power et al. 1996).

Several insect herbivores manipulate their host-plants to build a variety of structures, which are secondarily occupied by organisms other than the original constructor. Hence, the former species can act as ecosystem engineers (Marquis and Lill 2007). The role of insects as ecosystem engineers has been experimentally evaluated for some guilds, including leaf-rollers (Martinsen et al. 2000, Lill and Marquis 2003), gall makers (Bailey and Whitman 2003) and leaf miners (Johnson et al. 2002, Kagata and Ohgushi 2004). These studies indicate that ecosystem engineering by insect herbivores can influence overall abundance, species richness, and composition of arthropod communities by providing new habitats for other herbivores that are used for shelter and for the food they contain. Ecosystem engineering effects can propagate to higher trophic levels, triggering cascades of other interactions including trophic, antagonistic and mutualistic interactions (Ohgushi 2005).

One insect guild comparatively less studied is represented by stem-borers, which are insects that develop (at least part of their life cycle) in wood, bark or woody stems of plants. Many of them begin their life cycle as eggs laid under bark by free-living adult females; larvae feed on the wood of stems upon hatching, and eventually emerge from a stem boring hole as adults to repeat the cycle (Lieutier et al. 2004), producing quite complex systems of cavities that can be secondarily occupied by other arthropods (Marquis and Lill 2007). Studies analyzing stem-borer insects have reported secondary-opportunistic insects occupying the host-plants originally colonized by the stem-borers (Polk & Ueckert 1973; Hovore & Penrose 1982; Di Iorio 1996; Feller and Mathis 1997; Aukema et al. 2004). These studies suggest that the guild of stem-borers includes several species that can act as ecosystem engineers (Marquis and Lill 2007). However, empirical studies evaluating the effects of stem-borer engineering on arthropod communities are currently lacking.

Here we describe a field experiment designed to evaluate the effect of the ecosystem engineering by the stem-borer beetle *Oncideres albomarginata chamela* (Cerambycidae: Lamiinae) on the arthropod community associated to *Spondias purpurea* (Anacardiaceae) detached-branches for/across three consecutive years. *O. albomarginata chamela* actively manipulates its host plant through a process consisting of two steps. First, adult females of *O. albomarginata chamela* preferentially girdle and detach reproductive branches of *S. purpurea* in which they make incisions and gnaw egg niches along the branches to oviposit in them (Uribe-Mú and Quesada 2006). This is made before the reproductive season of the tree (Uribe-Mú and Quesada 2006), after reproductive branches have accumulated the maximum concentration of non-structural carbohydrates (Bullock 1992) and nitrogen (Uribe-Mú and Quesada 2006). Therefore, *O. albomarginata chamela* females provide a high quality environment for the offspring development (Forcella 1982), but incidentally also render a suitable environment for insect secondary colonization (Hanks 1999), particularly for many insects that oviposit opportunistically in cracks and crevices present into the bark or cortex of plants (Hanks 1999, Lieutier et al. 2004). Second, *O. albomarginata chamela* larvae have the ability to

digest and assimilate nutrients available in the wood of *S. purpurea* branches, particularly structural carbohydrates such as cellulose (Calderón-Cortés et al. 2010). Hence, *O. albomarginata chamela* larvae can transform complex structural carbohydrates (e.g. cellulose) into simple sugars (e.g. glucose) which can be available for secondary colonizers.

Based on this evidence, we hypothesized that the manipulation of tree branches by *O. albomarginata chamela* plays a key role in the establishment of a new arthropod community and promotes indirect interactions with positive effects on arthropod abundance and diversity. To test this hypothesis, we simulated *O. albomarginata chamela* manipulation on *S. purpurea* branches and compared the community composition, frequency of colonization, abundance and species richness between non-engineered branches and engineered branches (both artificially and naturally engineered branches).

MATERIALS AND METHODS

Study system

Oncideres albomarginata chamela Chemsak and Gisbert is a longhorn beetle that detaches branches from 2-3 cm in diameter (Uribe-Mú and Quesada 2006) of the tropical tree *Spondias purpurea* L. (Anacardiaceae), and oviposits within them. Alternative but less used host plants of *O. albomarginata chamela* include: *Comocladia engleriana* Loes (Anacardiaceae), *Manguifera indica* L. (Anacardiaceae), *Amphipterygium adstringens* Schide ex Schlecht (Rubiaceae), *Bursera* Jacq. ex L. spp. (Burseraceae), *Ceiba pentandra* (L.) Gaertn (Bombacaceae), *Urera* (L.) Gaud. sp. (Urticacaceae) and *Delonix regia* (Bojer ex Hook) Raf. (Fabaceae) (Chemsak and Noguera 1993). *O. albomarginata chamela* is distributed in México in the states of Jalisco, Nayarit, Guerrero, Oaxaca, Chiapas and Veracruz (Noguera 1993), but *O.*

albomarginata Thomson is distributed in México, Central America (Nicaragua) and South America (British and Fresh Guiana, Venezuela) (Duffy 1960). The body length of *O. albomarginata chamela* is 17 to 31 mm and 6.5 to 12 mm wide (Noguera 1993). The reproductive period of this species begins in October and finishes in February; eggs pupate and develop inside detached branches until the adults emerge after 6-8 months later in low densities. Adult females of *O. albomarginata chamela* are the only that detach and immediately oviposit the branches of *S. purpurea* in the study site (Uribe-Mú and Quesada 2006); but after a certain time period other species of wood-borer beetles (mainly non-girdling species) take advantage of the branches detached by *O. albomarginata chamela* and oviposit on them as well.

S. purpurea is a common dioecious tree of the tropical dry forest of México (Bullock 1992). Gender ratio of male and female trees of *S. purpurea* in the population at the study site is 1:1 (Uribe-Mú and Quesada 2006). This species can reach 15 m in height and almost 80 cm in diameter at the base and leaves are compound with 5 to 12 elliptic-acute leaflets of 2 to 4 cm in length (Uribe-Mú and Quesada 2006). Flowers are red, sessile, unisexual and dimorphic between males and females (Bullock 1992). Trees are deciduous with flowering and fruiting occurring from December to May and leaves are maintained from June to November (Bullock and Solís-Magallanes 1990).

Study site

The study was conducted in the Chamela-Cuixmala Biosphere Reserve at Chamela Biological Station, UNAM (19°30'N, 105°03'W) located on the Pacific coast of Jalisco, Mexico, from December 2006 to January 2010. The vegetation is tropical dry forest with a mean annual rainfall of 707 mm and a dry season that extends from November to June (Bullock 1988).

Experimental design

In order to evaluate the effect of *O. albomarginata chamela* on the arthropod community associated to ecosystem engineering, during Dec-2006 to Jan-2007, we developed a field experiment consisting of three treatments ($N \approx 50$ branches/treatment): *O. albomarginata chamela* engineered and colonized-branches (OE), artificially simulated engineered branches (SE) and non-engineered branches (NE). For treatment OE, we collected branches of *S. purpurea* naturally detached and colonized by *O. albomarginata chamela* on Dec-2006. This treatment was used as control to provide baseline data on the arthropod community in *S. purpurea* branches, and to analyze the effects of the ecosystem engineer presence. For treatment SE, branches exhibiting similar characteristics (reproductive branches from 2-3 cm in diameter) to those detached and colonized by *O. albomarginata chamela* were artificially cut off from *S. purpurea* trees. We artificially simulated the structural modification of branches made by adult females of *O. albomarginata chamela*, by making numerous incisions (every 5 mm) with scissors on the bark of these detached branches. Treatment NE consisted of simply artificially detached reproductive branches of 2-3 cm in diameter of *S. purpurea* with no manipulation. We call this treatment "non-engineered branches" because mechanical factors, such as wind, water stress, mechanical branch damage, among others, detach a great proportion of branches and twigs from trees in the study site. Specifically, broken branches (2-20 cm in circumference) constitute the most important component (43%) of the forest total above-ground dead phytomass in Chamela tropical dry forest (Maass et al. 2002). Thus, broken branches can represent non-engineered but available habitats. All branches were marked, and they were left hanging on the source-tree during 45 days (Dec-2006 to Feb-2007) to allow the colonization of secondary opportunistic species. Our preliminary analysis indicated that 30-45 days (during that period of the year) is when most insect borers colonize *S. purpurea* detached branches. Gender of each source-tree was registered. To control for the size of the branches used for each treatment, we measured the diameter at the point of branch cutting with an electronic caliper (Mytutoyo Inc). To control for adult female host selection, we cut off two branches for treatments NE and

SE from the same tree where *O. albomarginata chamela* had previously detached and colonized branches. Additionally, the treatments were conducted in the same host plants to control for any related chemical attractive signals emitted by them, as well as to control for any other selective factors associated to the nutritional value of host trees (Uribe-Mú and Quesada 2006). Therefore, the branches of the three treatments had the same probability to be located by the secondary colonizers. After 45 days, all branches were enclosed in mesh bags (< 0.5 mm of aperture) to prevent any further colonization and escape. Branches collected in mesh bags were placed in a protected exclosure at the study site, and maintained at local environmental conditions. Emerging arthropods from each branch enclosed in a mesh bag were collected monthly from Mar-2007 to Jan-2008. We measured the total length of 20-40 adult insects for each species to estimate the size of the secondary colonizers. The exact same experiment was repeated for two more consecutive years: Dec-2007 to Jan-2009, and Dec-2008 to Jan-2010. Taxonomic identification of species that emerged was carried out by the beetle specialist Dr. Felipe A. Noguera and using reported literature (Gerberg 1957, Arnett 1963, Binda and Joly 1991).

Data analysis

First, we compared the diameter (at the point of branch cutting) of detached branches to determine differences in sizes between treatments, through one way Analysis of Variance (ANOVA) using PROC ANOVA (SAS, 2002). Our results indicated that branch diameter did not differ significantly between treatments (2007: $F_{2, 154} = 1.564$, $P = 0.213$; 2008: $F_{2, 155} = 1.068$, $P = 0.346$; 2009: $F_{2, 178} = 0.263$, $P=0.769$). In a previous study, Uribe-Mú and Quesada (2006) found that branch gender had no effect on *O. albomarginata chamela* larval performance. Therefore we expected that branch gender has no effect on the number of secondary insects emerged from *S. purpurea* branches. This was confirmed when, we analyzed the variation associated with branch gender thorough a Generalized Linear Model using a GENMOD procedure (SAS 2002), in which the number of secondary colonizers that emerged from *S. purpurea* branches was

used as the response variable, and the gender of the branch as the independent variable. We used a Poisson distribution with logarithmic link function in the analysis. Branch gender had no significant effect (2007: $\chi^2_{1, 154}$= 0.37, P = 0.5428; 2008: $\chi^2_{1, 155}$ = 1.42, P = 0.2327; 2009: $\chi^2_{1, 178}$= 2.09, P = 0.1479) and was not included in further analyses.

Non-metric multidimensional scaling (NMDS) was used as an ordination procedure to illustrate differences in community composition among/between OE, SE and NE branches. The NMDS analysis is based on ranked Bray-Curtis dissimilarity distances (Faith et al. 1987), and is not susceptible to problems associated with zero truncation (Minchin 1987). Differences in community composition between treatments were tested using an analysis of similarity (ANOSIM), which uses 1000 random reassignments of species to groups and determines whether the group assignments were significantly different from those generated by chance. NMDS and ANOSIM analyses were performed with the software PRIMER 5.2.9 for windows (PRIMER-Ltd, Plymouth, U.K.). Multiple comparisons in ANOSIM were made using a sequential Bonferroni correction (Rice 1989).

To evaluate the effect of *O. albomarginata chamela* on the frequency of colonization of the secondary colonizers of *S. purpurea* branches, each species was quantified as being present or absent. Data were analyzed using a Generalized Linear Model with a binomial distribution for the dependent variable, and a logit link function using a GENMOD procedure (SAS, 2002). Branch condition (colonized *vs*. non-colonized) was the response variable, while treatment effects were independent variables. We used Least Square Means (LSM) for comparisons of means between treatments (α= 0.05).

Secondary xylovores showed two general traits in size and developmental time. These traits are considered key life-history traits that have important implications for different aspects of insects such as: fitness, habitat selection, oviposition strategies,

response to natural enemies, among others (Nylin and Gotthard 1998). Therefore, we used them to define two putative life forms: a) species with small body size and short developmental time (life form I); and b) species with large body size and longer developmental time (life form II). Natural enemies were analyzed separately. A Generalized Linear Model was used to evaluate the effect of *O. albomarginata chamela* on the abundance of the secondary colonizers. This model used the number of adult secondary colonizers that emerged from branches as the response variable and treatment as the independent variable. We used a Poisson distribution with a logarithmic link function in the analysis, applying a GENMOD procedure (SAS, 2002). LSM were used for comparisons of means between treatments ($\alpha = 0.05$).

To determine the impact of ecosystem engineers on species richness of engineered-habitats, we used a Generalized Linear Model (GENMOD procedure, SAS 2002), in which the number of species that emerged from *S. purpurea* branches was the response variable and treatment was the independent variable. We used a Poisson distribution with a logarithmic link function in the analysis, and LSM for comparisons of means between treatments ($\alpha = 0.05$). Increased species number is expected as a random consequence of larger pools of individuals (Gotelli and Colwell 2001). Therefore to examine whether treatment differences in the species richness of secondary colonizers were driven by differences in the abundance of secondary colonizers, we constructed rarefaction curves for each treatment. We used cumulative species per branch including all branches sampled along the three years (EcoSim 7.0, 10,000 iterations; Gotelli and Entsminger 2001).

RESULTS

Overall, 28 301 secondary colonizers emerged from a total of 487 detached branches of *S. purpurea*. These included at least 25 species from eight families (Table1), of which Bostrichidae was the most abundant, comprising 76 % (± 10 SD) of the overall natural arthropod community (Table1), and Cerambycidae was the more diverse (9 spp.) (Table1). The natural arthropod community consisted of xylovore and predator beetles, and parasitic wasps (Table 1). Additional to secondary colonizers that use *S. purpurea* branches for oviposition and development of offspring, other "inquiline" species (which eventually arrived to *S. purpurea* branches, but not oviposited on them) were recorded. This species included: termites, ants, pseudoscorpions, spiders, crickets and silverfish. However, given that inquilines emerged in very low numbers and were not present every year, we did not consider them in further analyses.

Secondary xylovores of life form I (Table1) began to emerge one month after branches were enclosed in mesh bags, with a maximum peak of emergence recorded in May. Secondary xylovores of life form II and natural enemies (Table 1) emerged throughout the year, but their maximum peaks of emergence were observed in September and July, respectively. *O. albomarginata chamela,* the species with the greater size (23.58 mm, ±2.24), was the latest species to emerge (September to December). These patterns of emergence were consistent across years.

Table 1. Secondary colonizers emerged from *Spondias purpurea* branches detached and colonized by *Oncideres albomarginata chamela*

Family	Abundance (%)	Species	Size (mm)
XYLOVORE BEETLES			
Bostrichidae	75.76 (±9.8)	*Amphicerus* (LeConte) sp. ‡	11.13 (±1.43)
		Bostrychopsis (Lesne) sp. †	3.58 (±0.23)
		Dendrobiella (Casey) sp. *†	5.49 (±0.25)
		Melalgus (Dejean) sp. ‡	11.56 (±1.42)
		Micrapate (Casey) sp. †	3.56 (±0.18)
		Prostephanus truncatus (Horn) †	3.40 (±0.22)
		Xylobiops (Casey) sp. †	3.71 (±0.24)
Curculionidae (Scolytinae)	4.10 (±3.1)	*Hypothenemus* (Weswoot) spp. †	1.59 (±0.27)
		Pityophthorus (Eichhoff) sp. †	2.00 (±0.25)
Lyctidae	7.07 (±3.7)	*Lyctus* (Fabricius) sp. †	3.11 (±0.38)
Buprestidae	1.45 (±1.2)	*Acmaeodera* (Eschscholtz) sp. †	6.27 (±0.54)
		Agrilus (Curtis) sp. †	4.43 (±0.35)
Cerambycidae	3.25 (±2.6)	*Ataxia alpha* (Chemsak and Noguera)* §	14.30 (±1.44)
		Estoloides chamelae (Chemsak and Noguera)* ‡§	12.47 (±1.17)
		Eutrichillus comus (Bartes) ‡	8.08 (±0.47)
		Lagocheirus obsoletus (Thomson) ‡	13.52 (±1.53)
		Lissonotus flavocinctus (Dupont)* ‡§	13.53 (±2.63)
		Poliaenus hesperus (Chemsak and Noguera) ‡	8.69 (±0.60)
		Sphaenothecus maccartyi (Chemsak and Noguera) ‡§	14.61 (±1.46)
		Sphaenothecus trilineatus (Dupont) ‡	21.42 (±1.52)
		Trachyderes mandibularis (Serville)* ‡§	21.97 (±0.79)
NATURAL ENEMIES			
Predator beetles			
Histeridae	7.58 (±3.2)	*Teretriosoma nigrescens* (Lewis) †	2.26 (±0.16)
Cleridae	0.30 (±0.2)	*Enoclerus quadrisignatus* (Say.) ‡	10.40 (±0.64)
Parasitic waps			
Hymenoptera	0.48 (±0.06)	ND §	ND

* Not recorded in 2007; † Life form I; ‡ Life form II; § Not recorded in non-engineered branches (NE); ND =not determined. Abundance values are means across the three years (±SD).

Effect of habitat engineering on the colonization frequency of secondary colonizers

Data analyses were performed separately by families and years, with the exception of Bostrichidae, which were analyzed in two species groups because they exhibit two different life forms (I and II, Table 1). The results indicated a highly significant effect of treatment for all families across years (Table 2). All secondary colonizers significantly colonized more frequently engineered branches (treatments OE and SE) than non-engineered branches (NE) (Fig. 1), with the exception of Buprestidae beetles for which significant differences were found only between *O. albomarginata chamela*-colonized branches (OE) and non-engineered branches (NE) in 2007 and 2008 (Fig. 1). To determine if the presence of *O. albomarginata chamela* had an effect on secondary colonization of *S. purpurea* detached branches, we compared between treatments OE *vs.* SE. This comparison showed variation across years and groups of secondary colonizers (Fig. 1), but in general there was not a significant effect related to the presence of *O. albomarginata chamela.*

Table 2. Effect of habitat engineering on the frequency of colonization of secondary colonizers. Data analyses were performed through a linear model with a binomial distribution and a logit link function using a GENMOD procedure.

Family	2007		2008		2009	
	$\chi^2_{2, 154}$	*P*	$\chi^2_{2, 155}$	*P*	$\chi^2_{2, 178}$	*P*
Bostrichidae (Life form I)	45.45	<0.0001	32.06	<0.0001	145.47	<0.0001
Bostrichidae (Life form II)	9.24	0.0099	28.35	<0.0001	85.56	<0.0001
Curculionidae (Scolytinae)	19.81	<0.0001	18.07	<0.0001	82.76	<0.0001
Lyctidae	48.70	<0.0001	68.26	<0.0001	127.73	<0.0001
Buprestidae	17.09	0.0002	38.30	<0.0001	12.53	<0.0001
Cerambycidae	12.66	0.0018	55.23	<0.0001	101.41	<0.0001
Histeridae	17.26	0.0002	72.98	<0.0001	126.50	<0.0001
Cleridae	10.75	0.0046	28.48	<0.0001	9.56	0.0084
Hymenoptera	21.55	<0.0001	22.25	<0.0001	25.06	<0.0001

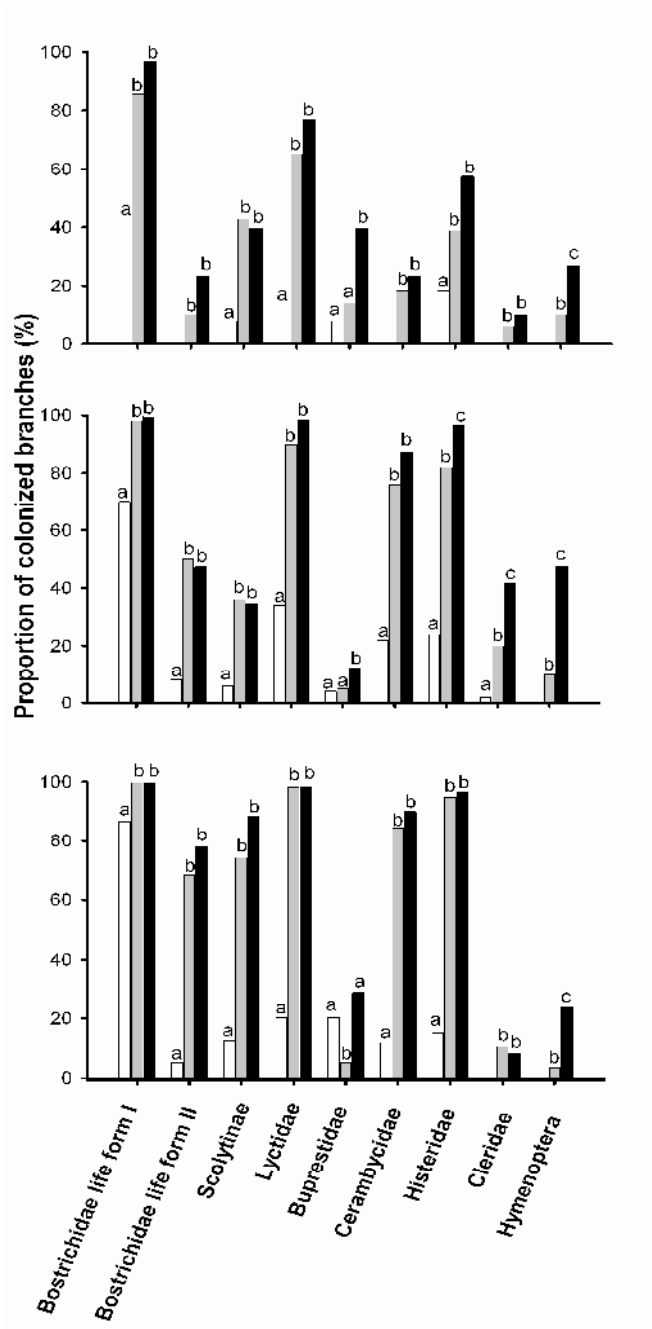

Figure 1. Frequency of colonization of detached *Spondias purpurea* branches by secondary colonizers. A) 2007, B) 2008 and C) 2009. Non-engineered branches are represented by white bars (NE); simulated-engineered branches by gray bars (SE); and *O. albomarginata chamela*- engineered branches by black bars (OE). Values are the percentage of branches colonized. Different letters indicate significant differences ($P < 0.05$) between treatments found in multiple comparisons using LSM.

Effect of habitat engineering on the community composition of secondary colonizers

There were significant differences in composition of the community of secondary colonizers between treatments (2007: $R = 0.425$, $n = 446$, $P < 0.001$; Fig. 2). However, the strongest differences were between the community composition of non-engineered (NE) and engineered (SE and OE) branches (NE *vs.* SE: $R = 0.574$, $P < 0.001$; NE *vs.* OE: $R = 0.691$, $P < 0.001$; SE *vs.* OE: $R = 0.098$, $P < 0.001$).

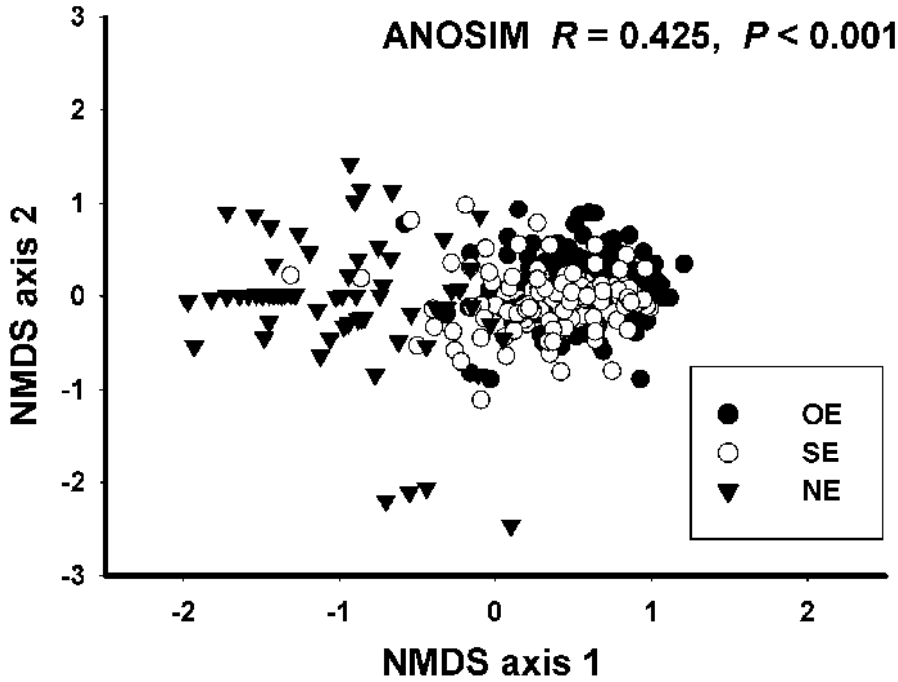

Figure 2. Arthropod community composition in detached *Spondias purpurea* branches. OE = *O. albomarginata chamela*-engineered branches; SE = simulated-engineered branches; and NE = Non-engineered branches. Each point is a two-dimensional (axis 1 and axis 2) representation of the arthropod community composition on an individual branch based on global non-metric multidimensional scaling (NMDS) analysis (stress = 0.19). ANOSIM analysis showed that arthropod community composition was significantly different among treatments ($P < 0.001$).

Effect of habitat engineering on the abundance of secondary colonizers

The results showed that there was a highly significant effect of treatment on the abundance of all groups of secondary colonizers and this pattern was consistent across years: life form I xylovores (2007: $\chi^2_{1, 154} = 62.75$, $P < 0.0001$; 2008: $\chi^2_{2, 155} = 62.75$, $P < 0.0001$; 2009: $\chi^2_{2, 178} = 235.26$, $P < 0.0001$); life form II xylovores (2007: not applicable because there was no colonization of NE branches; 2008: $\chi^2_{2, 155} = 32.88$, $P < 0.0001$; 2009: $\chi^2_{2, 178} = 145.47$, $P < 0.0001$); and natural enemies (2007: $\chi^2_{2, 154} = 27.97$, $P < 0.0001$; 2008: $\chi^2_{2, 155} = 66.08$, $P < 0.0001$; 2009: $\chi^2_{2, 178} = 155.95$, $P < 0.0001$). The three groups of secondary colonizers showed the following pattern of abundance: OE>SE>NE, where engineered *vs.* non-engineered branches (OE and SE *vs.* NE) showed significant differences for the three groups of secondary colonizers (Fig. 3). However, the OE *vs*. SE comparison only showed a significant difference for life form I xylovores in 2007, 2008 years; and for life form II xylovores in 2009 year (Fig. 3). Interestingly, the abundance of all secondary colonizers in non-engineered branches (NE) was 97% (2007), 93% (2008) and 94% (2009) lower than the abundance of secondary colonizers in *O. albomarginata chamela*-colonized branches (OE) (Fig. 3).

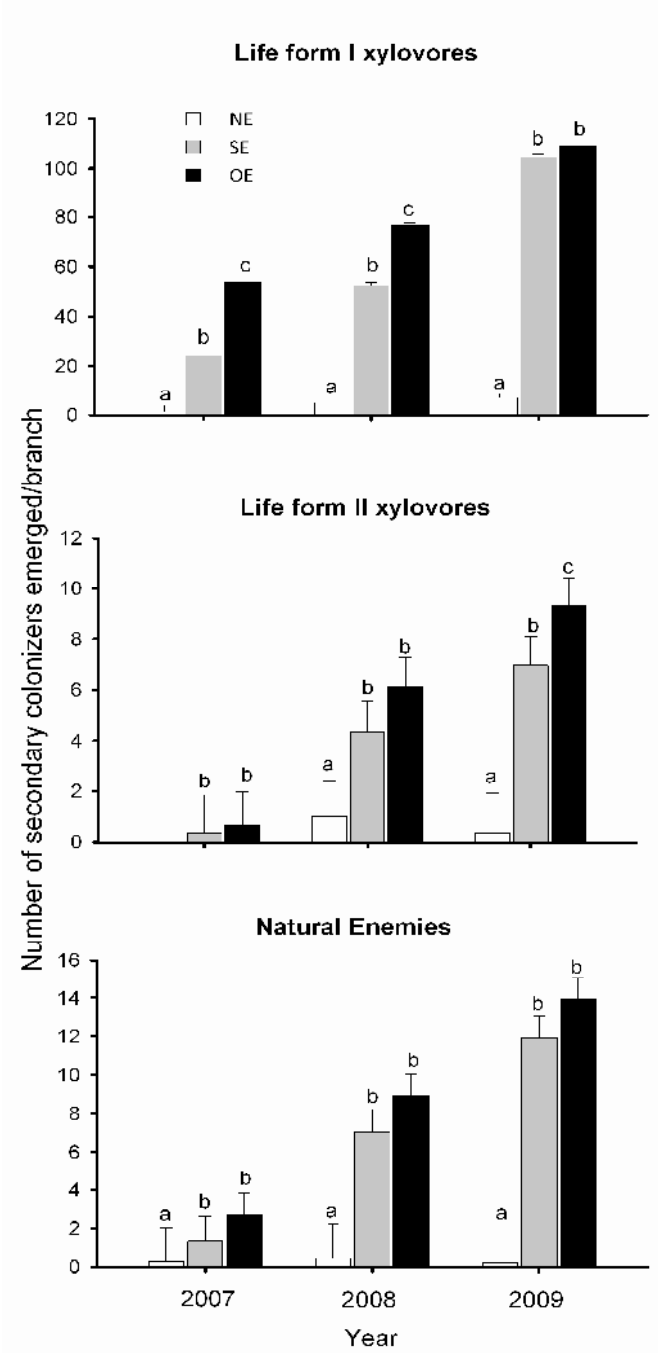

Figure 3. Abundance of the three main groups of secondary colonizers emerged from *Spondias purpurea* detached branches. Bars indicate the mean (±SE) of the number of secondary colonizers that emerged per *S. purpurea* branch along three years. White bars indicate non-engineered branches (NE); gray bars indicate simulated-engineered branches (SE); and black bars indicate *O. albomarginata chamela*-colonized branches (OE). Different letters indicate significant differences ($P < 0.05$) between treatments found in multiple comparisons using LSM.

Effect of habitat engineering on the diversity of secondary colonizers

There was a strong effect of treatment (2007: $\chi^2_{1, 154}$= 233.71, $P < 0.0001$; 2008: $\chi^2_{2, 155}$ = 343.37, $P < 0.0001$; 2009: $\chi^2_{2, 178}$= 872.51, $P < 0.0001$), habitat engineering (NE *vs.* SE and NE *vs.* OE) and the presence of the ecosystem engineer (OE *vs.* SE) on the species richness of secondary colonizers that emerged from *S. purpurea* branches (Fig. 4). The results showed the OE>SE>NE pattern of species richness, consistent across years (Fig. 4). NE branches showed 85% (2007), 80% (2008) and 82% (2009) fewer species than OE branches (Fig. 4). Rarefaction curves showed that the observed differences in cumulative species richness persisted even when samples were rarefied to similar abundances of individuals (Fig. 5).

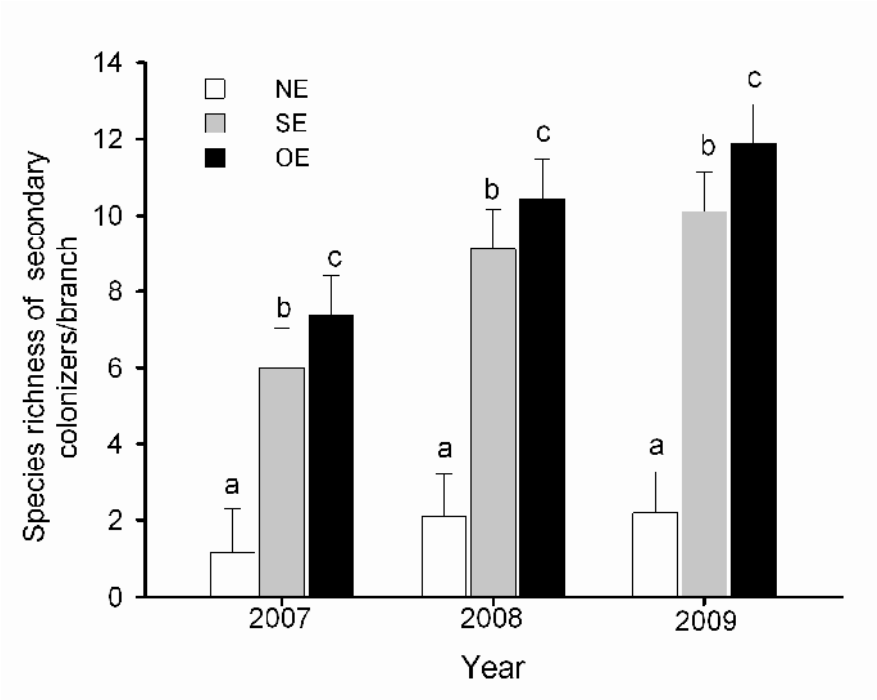

Figure 4. Species richness of secondary colonizers emerged from *Spondias purpurea* branches. The mean (±SE) of the number of species per *S. purpurea* branch along three years are show by bars; white bars indicate non-engineered branches (NE); gray bars indicate simulated-engineered branches (SE); and black bars indicate *O. albomarginata chamela*-engineered branches (OE). Different letters indicate significant differences ($P < 0.05$) between treatments found in multiple comparisons using LSM.

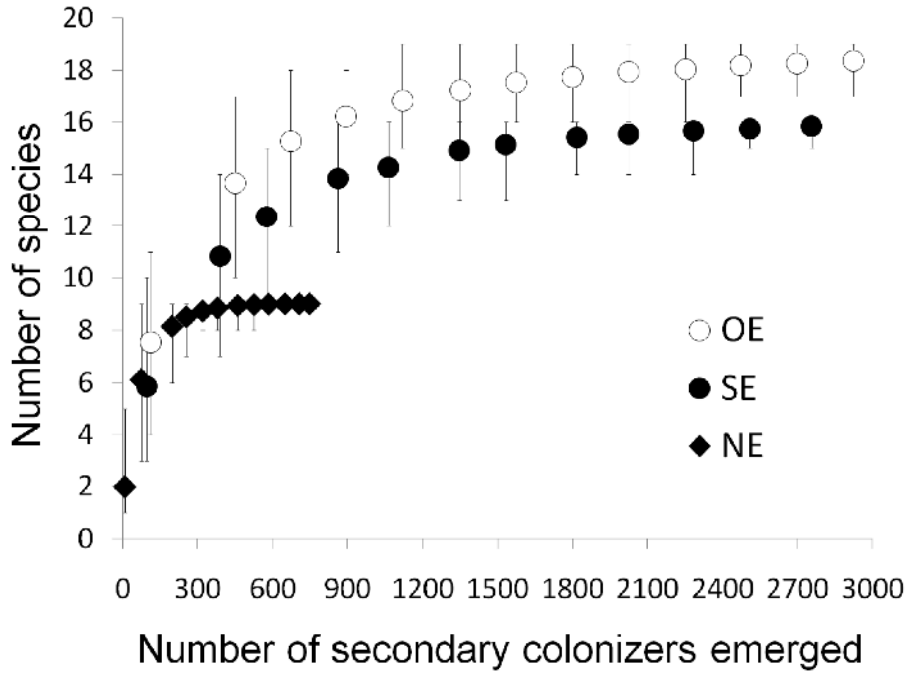

Figure 5. Rarefaction curves plotting the number of secondary colonizers *vs.* the number of individuals sampled in detached *Spondias purpurea* branches. NE = Non-engineered branches; SE = simulated-engineered branches; and OE = *O. albomarginata chamela*- engineered branches. Bars represent 95% confidence intervals obtained from 10 000 re-sampling iterations. Bars that overlap the mean for alternate treatments indicate that treatments were not significantly different ($P > 0.05$).

DISCUSSION

Several insect herbivores can create new habitats and alter habitat resource availability for other organisms, by modifying the structural and/or nutritional properties of plant tissues (Jonhson et al. 2002, Kagata and Ohgushi 2004, Ohgushi 2005, Marquis and Lill 2007)**.** *O. albomarginata chamela* actively manipulates its host plant by: (i) girdling and gnawing eggs niches and incisions into the bark or stems, and (ii) digesting wood polymers. These modifications were key factors for the establishment and development of an arthropod community composed by xylovores (Bostrichidae, Scolytinae, Buprestidae, Lyctidae and Cerambycidae) and natural enemies (Histeridae, Cleridae and Hymenoptera).

Benefits of stem-boring engineering to secondary colonizers

The reported benefits of insect ecosystem engineering to secondary colonizers include: shelter from harsh abiotic factors, avoidance of natural enemies, and modification of resource quality (Damman 1993, Ohgushi 2005, Marquis and Lill 2007). However, the importance of each benefit differs among insect guilds. For example: leaf rolls and mines are colonized for shelter rather than for the food they contain (Martinsen et al. 2000, Kagata and Ohgushi 2004); whereas galls provide shelter, protection from natural enemies and high quality food resources (review in Marquis and Lill 2007).

Stem-boring engineering by *O. albomarginata chamela* provides shelter for secondary colonizers, although the main benefits to secondary colonizers were related to the creation of a habitat with high availability of oviposition sites, because branches without incisions (non-engineered) were almost not /poorly colonized. Moreover, artificially-engineered branches were colonized by a similar arthropod community that colonized *O. albomarginata chamela* naturally detached branches. This confirms that incisions made by *O. albomarginata chamela* adult females along the detached *S.*

purpurea branches are used by other arthropod species as oviposition sites. Availability of oviposition sites (i.e. recruitment mechanism) offers three benefits to secondary colonizers because they can: (i) save costs of searching for suitable oviposition sites; (ii) diminish the "excavation costs" of the initial stem penetration (Marquis and Lill 2007); and (iii) reduce exophytic predation during the oviposition period (*sensu* Aukema and Raffa 2002).

The metabolic activities of engineers may also affect the structure of communities by increasing nutrient availability (Jones et al. 1994). The presence of *O. albomarginata chamela* had a significant effect on species richness and composition of the arthropod community. *O. albomarginata chamela* digests cellulose *(*Calderón-Cortés et al. 2010) and hemicelluloses (Calderón-Cortés, unpub. work) of which secondary colonizers have little or no ability to digest (Calderón-Cortés, unpub. work). Then, it is possible that *O. albomarginata chamela* supplies more digestible food to secondary colonizers. Indeed, previous studies suggested that secondary xylovores that are not capable to digest polymers available in the freshly cut woody tissue, depend to some extent on primary xylovores (which are able to digest and assimilate these polymers) to modify their food material (Graham 1923, Hanks 1999). This suggests that besides physical engineering, *O. albomarginata chamela* also plays a role as biochemical ecosystem engineer through its metabolic activities. Lawton and Jones (1995) recognize this type of ecosystem engineering, but studies analyzing biochemical engineering are absent.

Effects of stem-boring engineering on arthropod community

The results of this study demonstrated that ecosystem engineering by *O. albomarginata chamela* had strong positive effects on its associated arthropod community. These results are consistent with the positive effects on arthropod diversity reported for other insect ecosystem engineers such as leaf-roller caterpillars, gall-makers and leaf-miners

(Martinsen et al. 2000, Bailey and Whitham 2003, Lill and Marquis 2003, Kagata and Ohgushi 2004).

The abundance and species richness of xylovore insects were higher in engineered-branches than in non-engineered branches. The increases in the overall abundance and species richness may result from the increase in the quantity and quality of habitat and food resources (Abrams 1993, 1995). In engineered-branches, a higher/greater availability of oviposition sites leads to an increase of habitat colonization by xylovore secondary species, and thus to an increase of overall abundance of xylovores. This is consistent with previous studies reporting that higher colonization and performance, following the improvement of resource quality, increase the abundance of insect herbivores (Martinsen et al. 1998, Awmack and Leather 2002, Utsumi and Ohgushi 2009).

In addition, the improvement of resource quality can increased species richness by increasing the colonization and persistence of rare species, and decreasing interspecific competition (Abrams 1995, Srivastava and Lawton 1998, Utsumi and Ohgushi 2009). Differences in species richness and community composition between engineered and non-engineered branches can be explained by the absence of colonization of five cerambycid species in non-engineered branches (Table 1). These species appear to selectively oviposit in branches with oviposition sites (engineered branches). Cerambycidae (life form II) was one the xylovore families with low abundance. These findings suggest that the increased species richness in engineered-branches is a consequence of the enhanced colonization of rare species.

Interestingly, xylovore species occupied different parts of the *S. purpurea* detached branches (Calderón-Cortés, pers. obs.) and emerged at different times during the season: Bostrichidae, Scolytinae and Lyctidae beetles (life form I) associated to subcortical tissues were the first species emerging; whereas Cerambycidae beetles (life form II) associated to sapwood and heartwood, were the latest species emerging. This

indicates that there was both temporal and spatial resource partitioning. Resource partitioning might potentially reduce the exploitative competition, because colonizers may not be competing directly for shared resources in time or space (Tilman 1982, Damman 1993). Interference competition is more likely to occur because the first and more abundant species emerging (life form I species) can deplete resource availability for late-active species (life form II species) (Damman 1993). However, considering that *O. albomarginata chamela* has the ability to increase nutrient availability, interference competition can also be reduced. Low-moderate levels of competition allow coexistence of species (Tilman 1982), and can be related to an increase in species richness.

The increase in abundance and number of secondary xylovores that represent potential preys/hosts for natural enemies, in turn may influence the abundance and species richness of natural enemies and results in bottom-up effects (Morin 1999, Ohgushi 2005). In this study, the overall abundance and species richness of natural enemies was higher in engineered branches than in non-engineered branches. Specifically, only one (Histeridae: *Teretriosoma nigrescens*) of the three natural enemies was consistently recorded in non-engineered branches (Fig. 2). *T. nigrescens* preys upon some bostrichid beetles (Helbig and Schulz 1996), which were the main species that colonized non-engineered branches. However, there were five cerambycid species that did not colonize non-engineered branches. Cerambycid beetles were one of main preys/hosts of clerid beetles and parasitic wasps associated to engineered-branches (Kenis and Hilszczanski 2004, Calderon-Cortés, per. obs.). Therefore, the absence of colonization of clerid beetles and parasitic wasps in non-engineered branches can be explained by the reduced colonization by cerambycid beetles. These results confirm that changes in the composition of xylovores community cascade upward to higher trophic levels through bottom-up effects.

Implications of stem-boring ecosystem engineering for biodiversity

On average *O. albomarginata chamela* ecosystem engineering was responsible for nearly 95% of the abundance of secondary colonizers and 82% of species richness. Moreover, ecosystem engineering by this species allowed the establishment of an entire arthropod community, and regulated the structure of this community. Therefore, based on Paine´s (1966) "keystone" concept, *O. albomarginata chamela* ecosystem engineering can be considered a "keystone" process (sensu Power et al. 1996).

There are two explanations for this "keystone process": the existence of a highly structured community, and the degree of specialization (i.e. interaction strength) between the secondary colonizers and the engineered habitat (Paine 1966). The arthropod community associated to engineered branches by *O. albomarginata chamela* is a highly structured community, because it consists of organisms with different life history traits, trophic position and diet specializations. In addition, several lines of evidence suggest that xylovore species in the arthropod community of *S. purpurea* branches might be specialists to branches girdled and detached by *O. albomarginata chamela* and other Lamiinae beetle species. For example, the known host plants for the Cerambycidae and Scolytinae species emerging from *S. purpurea* branches completely correspond to the alternative host plants of *O. albomarginata chamela,* and to the host plants of other girdling-beetles in the study site, such as *Oncideres rubra* and *Taricanus zaragozai* (Equihua and Atkinson 1986, Chemsak and Noguera 1993). Some of these cerambycid species, as well as most species of the families Bostrichidae and Buprestidae in the *S. purpurea* branches, also use branches girdled by other beetle species in different tropical and subtropical regions (Polk and Ueckert 1973, Hovore and Penrose 1982, Ramírez-Martínez et al. 1994, Feller and Mathis 1997). These data suggests a high degree of adaptation between engineered branches and secondary colonizers, and hence they can explain the strong impact of *O. albomarginata chamela* ecosystem engineering on arthropod diversity.

Insect herbivore communities are some of the most diverse ecological communities, although the progress made in understanding the dynamics of communities is slower compared to other animal communities (Van Veen et al. 2008). The results of this study can be extrapolated to other stem-borers, particularly to girdling insects, because its co-occurrence with secondary opportunistic colonizers has been widely reported (Polk and Ueckert 1973, Hovore and Penrose 1982, Di Iorio 1996, Feller and Mathis 1997, Hanks 1999, Aukema et al. 2004). In sum, this study provides strong evidence that positive interactions mediated by ecosystem engineering may be common and widespread factors structuring insect herbivore communities.

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Capítulo 3

DIGESTION OF WOODY TISSUES BY SIX SYMPATRIC SPECIES OF LONGHORN BEETLES (CERAMBYCIDAE)

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ABSTRACT

Many organisms influence their environment by modifying the physical properties of their habitats, or by increasing availability of food resources through their metabolic activities. This type of habitat modification, which can be referred as biochemical ecosystem engineering, might have important effects on the diversity and structure of biological communities. However, studies analyzing biochemical ecosystem engineering are currently lacking. In this study, we analyzed the digestive enzymes involved in the breakdown of the main structural polysaccharides of *Spondias purpurea* wood (cellulases and xylanases) of six sympatric Cerambycidae species: *Oncideres albomarginata chamela, Ataxia alpha, Estoloides chamelae, Lissonotus flavocinctus,* Cerambycinae sp. and Lamiinae sp. The results showed that *O. albomarginata chamela* larvae have higher cellulase and xylanase activities than the other sympatric species. *L. flavocinctus* and Cerambycinae sp. showed a moderate ability to digest the cellulose, but negligible ability to digest xylan, while *A. alpha, E. chamelae* and Lamiinae sp. had no cellulase and xylanase activities. These results indicated that *O. albomarginata chamela* was the only species that possesses the ability to digest woody tissues, and thus it can potentially alter the composition and properties of wood. Thus, it is possible that the other sympatric species which do not have the ability to digest woody tissues benefit from the chemical and physical changes in the wood resulting from *O. albomarginata chamela* metabolic activities, specifically from the process of biochemical engineering.

Keywords: ecosystem engineering, xylanases, cellulases, xylophagous insects, borer beetles.

INTRODUCTION

Woody tissues are produced by each of the approximately 44,000 species of trees, shrubs and woody vines found worldwide (Hickin 1975). Woody tissues (constituted mainly by secondary cell walls) are composed of cellulose [40-80%], hemicellulose [10-40%] and lignin [5-35%] (Gong et al. 1981, Haack and Slansky 1987, Bidlack et al. 1992). These tissues are structural and chemically complex and represent harsh environments for wood feeding insects. However, many insects have evolved to live and feed in woody environments (Haack and Slansky 1987).

Feeding on woody tissues requires a complex breakdown process for its digestion, involving a suite of numerous enzymes with diverse modes of action and substrates; these enzymes include cellulases, hemicellulases and lignases (Fry 2000). In general, wood degradation process consists of three steps: depolymerization of lignin, hemicellulose degradation, and finally cellulose degradation; each step is important to enable the degradation of the next wood polymer (Fry 2000). Insects degrade woody tissues in different ways: i) many insects synthesize their own enzymes for wood degradation (Watanabe and Tokuda 2010); ii) others have been established symbiotic associations with protozoan, fungal and/or bacterial species that degrade wood (Martin 1983, 1991, Prins and Kreulen 1991, Douglas 2009); iii) and some others exploit relatively soft and nutritionally rich woody tissues such as the inner bark (phloem) (Haack and Slansky 1987).

One of the most important groups of xylophagous insects is represented by beetles (Coleoptera) (Böving and Craighead 1931, Ross et al. 1982, Haack and Slansky 1987, Lieutier *et al*. 2004). Cerambycids are among the most diverse beetles with more than 35,000 species in about 4,000 genera (Hanks 1991), and the highest diversity ways of feeding on wood (e.g. feeding on different parts of the wood such as outer and inner bark, sapwood, heartwood; Haack and Slansky 1987, Hanks 1999). Cerambycidae beetles can be categorized in: primary xylovores (particularly heartwood-feeding

species) that colonize living and healthy host plants, secondary xylovores that colonize stressed, moribund or recently dead host plants, and necrophagous species living only in dead host plants (Girs and Yanovsky 1991, Hanks 1999). It has been suggested that primary xylovores are able to digest and assimilate the polymers available in woody tissue, whereas secondary xylovores are not able to digest these polymers and depend to some extent on primary xylovores to modify their food material (Graham 1923, Girs and Yanovsky 1991, Hanks 1999). Therefore, primary xylovores could play important roles in structuring xylophagous communities by modifying the chemical and structural properties of wood, for example by increasing the availability of sugars that can be used by secondary xylovores. Studies analyzing digestive enzyme activities of xylophagous beetles are limited (Mansour and Mansour-Bek 1934, Parkin 1940, Haack and Slansky 1987, Cazemier et al. 1997, Scrivener et al. 1997, Lee et al. 2004, 2005, Wei et al. 2006, Calderón-Cortés et al. 2010, Oppert et al. 2010), and studies of digestive enzyme activities for wood digestion in sympatric xylovore insects are absent.

Currently, it is recognized that many organisms have a marked influence on their environment by modifying physical properties of their habitats, or by increasing availability of food resources (Jones et al. 1994, Bouma et al. 2005, Wright and Jones 2004, Hastings et al. 2007, Marquis and Lill 2007). This biological process is known as physical ecosystem engineering (Jones et al. 1994, 1997). In addition to this process, other types of ecosystem engineering have been recognized (e.g. chemical and transport engineering; Lawton and Jones 1995). Among these other types of ecosystem engineering, the metabolic activities of certain organisms, such as the wood modification carried out by primary xylovores, may represent a case of "biochemical engineering". However, studies analyzing this biochemical process are lacking.

In a previous study, Calderón-Cortés et al. (*submitted manuscript:* chapter 2 of this thesis) demonstrated the role of the twig-girdler beetle *Oncideres albomarginata chamela* as a physical ecosystem engineer. The ecosystem engineering by this beetle can be divided into a process that is merely physical and a putative biochemical process

(Calderon-Cortés et al. *submitted manuscript:* chapter 2 of this thesis). The contribution of each type of process to the species richness in the arthropod community associated with the *O. albomarginata chamela* ecosystem engineering was 67% and 15 %, respectively. Given that *O. albomarginata chamela* larvae have the ability to transform the cellulose of wood into simple sugars (e.g. glucose) with the action of their own cellulases (Calderón-Cortés et al. 2010: chapter 1 of this thesis), it is possible that through its metabolic activities (wood degradation) *O. albomarginata chamela* supply digestible sugars to the secondary xylovore colonizers, and plays a role as a biochemical ecosystem engineer.

In this study we analyze the ability of six sympatric species of Cerambycidae to digest woody tissues. From a dietary perspective, cellulase and hemicellulase activities are indicators of the ability to digest woody tissues. Therefore, we searched for activities of cellulases and xylanases (hemicellulases) in the digestive tracts of the ecosystem engineer *O. albomarginata chamela,* and in the secondary xylophagous beetles of the family Cerambycidae: *Ataxia alpha, Estoloides chamelae*, *Lissonotus flavocinctus,* Lamiinae sp and Cerambycinae sp. Our hypothesis was that as a biochemical ecosystem engineer (primary xylovore) *O. albomarginata chamela* is the only species that has the ability to degrade the structural polysaccharides present in the wood of its host plant *Spondias purpurea*.

MATERIALS AND METHODS

Insects

O. albomarginata chamela (Coleoptera: Cerambycidae) is a borer beetle that develops inside wood of branches of the tropical tree *Spondias purpurea.* These branches are secondarily colonized by other opportunistic endophytic insects that develop on them as well (Calderón-Cortés et al. 2010 *submitted manuscript:* chapter 2). Therefore, to obtain insect samples we collected detached branches of the tree *S. purpurea* colonized by *O.*

albomarginata chamela within the Chamela-Cuixmala Biosphere Reserve in the central Pacific coast of Mexico (*c*. 19°30′N, 105°03′W). The collected branches were transported to the laboratory. Branches were dissected and larvae inside them were harvested and classified to the subfamily level using reported literature (Böving & Craighead, 1931; Duffy, 1960). The identification of cerambycid beetles to the species level was carried out by sequencing the "universal barcoding region" of the mitochondrial gene cytochrome oxidase I (COI) (Calderón-Cortés et al., *unpublished data:* Appendix A2 of this thesis).

Preparation of gut extracts

Larvae were kept on ice and sterilized in 70% ethanol. Then, the guts of five larvae per species (*O. albomarginata chamela, Ataxia alpha, Estoloides chamelae, Lissonotus flavocinctus,* Lamiinae sp. and Cerambycinae sp.) were removed by dissection and transferred into 1.5 ml plastic centrifuge tubes. Samples (one per gut) were homogenized in 200-ml protease inhibitor cocktail (complete Mini EDTA free; Roche Diagnostics, Basel, Switzerland) using a glass homogenizer. 500 µl of molecular biology-grade water were added to samples. The samples were centrifuged at 10 000 *g* for 10 min (4°C). Insoluble materials were discarded.

Enzymatic assays

Assays for cellulase activity (CMCase assay) and xylanase activity were performed by measuring the amount of reducing sugars after incubation of 100 ml 1% (w/v) CMC (standard molecular mass, 250 KDa; degree of carboxymethyl substitution, 0.7; Sigma-Aldrich, St. Louis, MO, USA) and 1% (w/v) birchwood xylan (Sigma-Aldrich, St. Louis, MO, USA), respectively, in 0.1 M sodium phosphate (pH 6) with 25 µl of gut extract samples at 37°C during 30 min. Reducing sugars were measured with tetrazolium blue (Sigma-Aldrich) as a chromogenic reagent with glucose as a standard (Jue & Lipke,

1985). Controls without enzyme or without substrate were included. Enzymatic activities were expressed in units of enzymatic activity (U) per mg protein (specific activity). Five individuals were used to determine the enzymatic activities of each species. Enzymatic assays for each individual were carried out in triplicate, and the mean of the replicates was used for data analysis. One U is defined as the amount of enzyme which produced 1 mmol of reducing sugar (glucose and xylose equivalents) per minute from its corresponding substrate. Protein concentration was determined by using a protein assay kit (Coomasie Plus Protein Assay Reagent, Pierce, Biotechnology, Rockford, IL, USA) with BSA as standard.

Data analysis

Data were ln-transformed (Underwood 1997). Comparisons between enzymatic activities were made using ANOVA, with Tukey´s method for post hoc tests ($\alpha = 0.05$). Statistical analyses were performed using SigmaStat v. 11.0 (Systat Software Inc.). Data are presented as means (± standard error).

RESULTS

Cellulase activity

Cellulase activity was present in all cerambycid species studied (Fig. 1). However, there were significant differences in cellulase activities ($F_{5,\ 30} = 45.643$, $P < 0.001$) between species (Fig. 1). The primary xylovore, *O. albomarginata chamela,* was the species with the highest cellulase activity. Among the secondary xylovores the species of the subfamily Cerambycinae, *L. flavocinctus* and Cerambycinae sp., had moderate and low cellulase activity, respectively; while species of the subfamily Lamiinae, *A. alpha, E. chamelae* and Lamiinae sp., had weak cellulase activity (Fig. 1).

Xylanase activity

Xylanase activity in *O. albomarginata chamela* was 2.48 (±0.49) U/mg protein. The secondary xylovore cerambycids did not show xylanase activity.

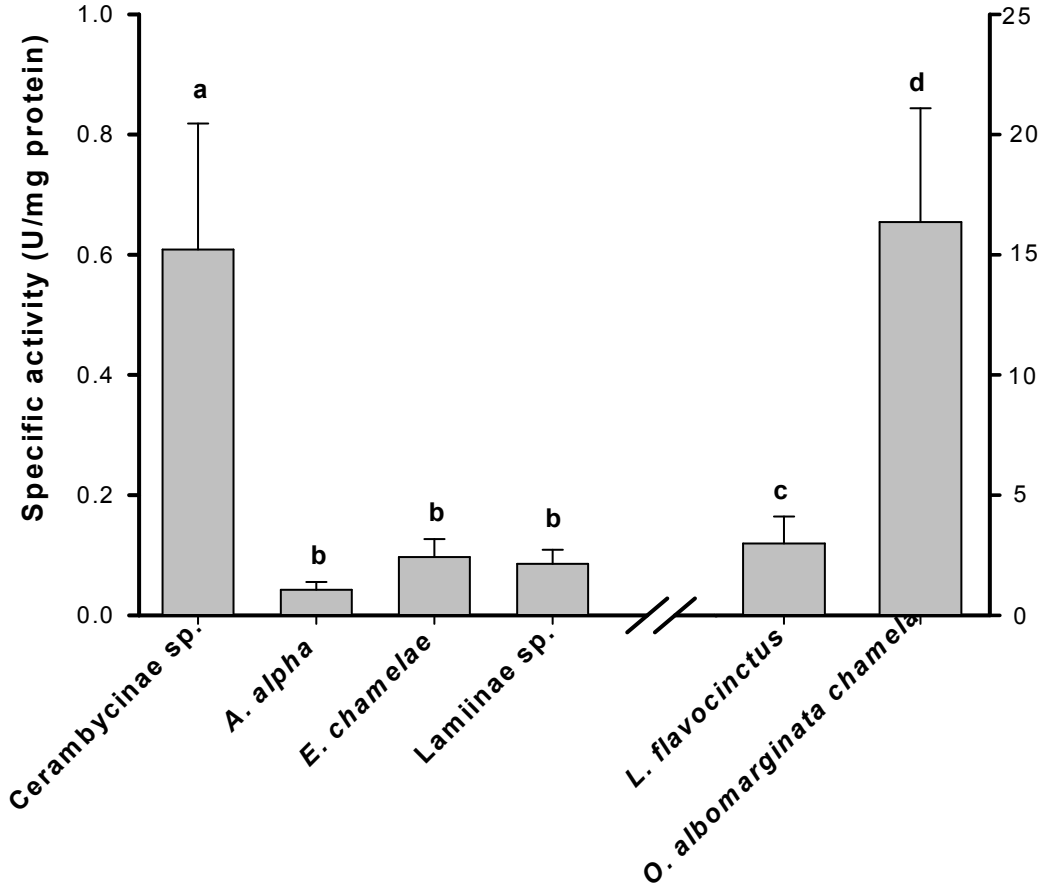

Figure 1. Comparison of cellulase activities among primary and secondary xylovore cerambycid larvae that co-occur in *Spondias purpurea* branches. Primary xylovore: *Oncideres albomarginata chamela;* Lamiinae secondary xylovores: *Ataxia alpha, Estoloides chamelae* and Lamiinae sp.; Cerambycinae secondary xylovores: *Lissonotus flavocinctus* and Cerambycinae sp. Bars indicate the mean (±SE) of the enzymatic activity. One U is defined as the amount of enzyme which produced 1 mmol of reducing sugar (glucose equivalents) per minute. Different letters indicate significant differences ($P < 0.05$) between species.

DISCUSSION

In this study, we investigated the ability of six sympatric species of the family Cerambycidae to digest woody tissues using an analysis of the digestive enzymes involved in the breakdown of the main structural polysaccharides of wood (i.e. cellulases and xylanases). Digestive enzyme activities are generally adapted to the diet on which a species feeds (Ishaaya 1986, Colepicolo-Neto et al. 1987, Terra and Ferreira 1994), hence they are tools for identifying particular components of an animal´s diet that are effectively metabolized (Ishaaya 1986, Brêthes et al. 1994, Johnston and Freeman 2005). High cellulase and xylanase activities reflect a diet consisting of woody tissues. According to our hypothesis, *O. albomarginata chamela* was the only species that has the ability to digest woody tissues, since this species showed both, cellulase and xylanase activities. *O. albomarginata chamela* cellulase activity (14.36 U/mg protein/min or 208.13 U/g larvae/min) corresponds to the range of cellulase activity reported for xylophagous insects such as termites (101.1-183.7 U/g termite/min; Tokuda *et al.* 2004), and the cerambycid borer *Psacothea hilaris* (11.16 U/mg protein/min or 670 U/mg protein/h; Scrivener et al. 1997). Xylanase activity in *O. albomarginata chamela* larvae was slightly lower than the xylanase activity reported for *P. hilaris* (4 U/mg protein/min or 240 U/mg protein/h; Scrivener et al. 1997), but it cannot be directly compared with the enzymatic activities reported for other xylophagous species (due to differences among the reported enzymatic units). However, in qualitative terms *O. albomarginata chamela* larvae showed a moderate xylanase activity similar to the wood-feeding cockroaches C*ryptocercus clevelandi* (Cazemier et al. 1997) and *Panesthia cribata* (Zhang et al. 1993).

On the basis of the enzymatic activities that we found, the secondary xylovores associated with *S. purpurea* branches can be divided into two groups, one group consisting of the Lamiinae species, *A. alpha, E. chamelae* and Lamiinae sp., which had no significant cellulase and xylanase activities; and the other one consisting of the Cerambycinae species, *L. flavocinctus* and Cerambycinae sp., which had a moderate

ability to digest the cellulose, but no ability to digest xylan. Similar to our results, a wide range of variation in the percentage of digestibility of cellulose (14-58%) and hemicelluloses (0-54%) has been reported for Cerambycidae (Haack and Slansky 1987). Then, how can these species survive in woody tissues if they do not digest efficiently the cellulose and hemicellulose present in wood? The answer is related to the fact that tissues of woody plants vary in their nutritional quality for wood-boring insects: a) bark is generally very low in nutrients (carbohydrates, nitrogen); b) subcortical tissues (inner bark, cambium, immature xylem) have many times higher concentrations of nutrients such sugars, starch and proteins; c) the much thicker sapwood is low in nutritional quality; d) the quality of heartwood is lower still; e) pith has a somewhat higher nitrogen content than heartwood (Haack and Slansky 1987, Hanks 1999).

Reflecting the relative nutritional quality of wood tissues, most wood-feeding insects confine their feeding to subcortical tissues and few species feed within the wood (Graham 1923, Hanks 1999). All species that we studied feed subcortically at the beginning of their life cycle, but in latter developmental stages there is a spatial partitioning for feeding (*personal observations*): the Cerambycinae species, *L. flavocinctus* and Cerambycinae sp. (probably *Sphenothecus*), are subcortical species; whereas the Lamiinae species, *A. alpha, E. chamelae* and the Lamiinae sp. (probably *Lagocheirus araeniformis*), are sapwood species, but can move to the heartwood when sapwood has declined. The insects that feed on subcortical tissues (i.e. inner bark, cambium) limit the length of the feeding period and develop rapidly while food remains suitable for them, and before other organisms alter the chemical and physical nature of the tissues (Graham 1923, Haack and Slansky 1987). These species are not expected to have the ability to digest cellulose and hemicellulose efficiently. Hence, the moderate enzymatic activities that we found for these species might be related to the use of cellulose present in subcortical tissues as a supplementary food resource, given that these tissues are rich in sugars and starch (3-37%; Haack and Slansky 1987).

For sapwood and heartwood feeders, an efficient digestion of cellulose and hemicellulose is needed, given that these tissues have a low content of sugars and starch (1-5%; Haack and Slansky 1987). However, the three sapwood and heartwood feeders studied did not digest hemicelluloses, and probably did not digest cellulose either. Other sapwood-feeding species, such as bostrichids and lyctids that lack the ability to degrade hemicelluloses and cellulose, derive nourishment from parenchyma, which is the most nutritious tissue of sapwood (Haack and Slansky 1987). In addition, in twigs and young branches, pith cells have high concentrations of soluble sugars (Haack and Slansky 1987). Therefore, it is possible that the sapwood and heartwood species analyzed in this study, use these nutritious tissues as food. However, these species might also benefit from the chemical and physical changes to the wood resulting from *O. albomarginata chamela* (the primary xylovore) metabolic activities, likely from the sugars eliminated with the frass (feces) and from bacteria growing on frass (Weiss 2006). This can explain at least partially why the presence of *O. albomarginata chamela* is responsible for about 15% of the species richness of the arthropod community associated with *S. purpurea* branches, because without *O. albomarginata chamela* the food materials within the xylem (sapwood and heartwood) remain securely locked up and the development of the some xylem species would not be possible.

This study provides evidence for the existence of a process of biochemical ecosystem engineering that might have effects on the diversity and structure of biological communities. These effects can be represented in a conceptual model of the potential interactions (Fig. 2). Biochemical engineers alter the composition and structure of their environment (wood, in the case of borer insects) through their metabolic activities (enzymatic activities). As a result of this physiological activity, partially-digested food and metabolic waste products are eliminated as feces. The localized deposition of feces can generate resource subsidies for other species, such as obligate and facultative coprophages. Feces also represent a substrate for microbial colonization. Microorganisms growing on feces, in turn, can be used as food organisms feeding on feces and detritivores. Therefore, biochemical engineers can indirectly regulate the

availability of resources (food) used by other species, thereby affecting the biological communities.

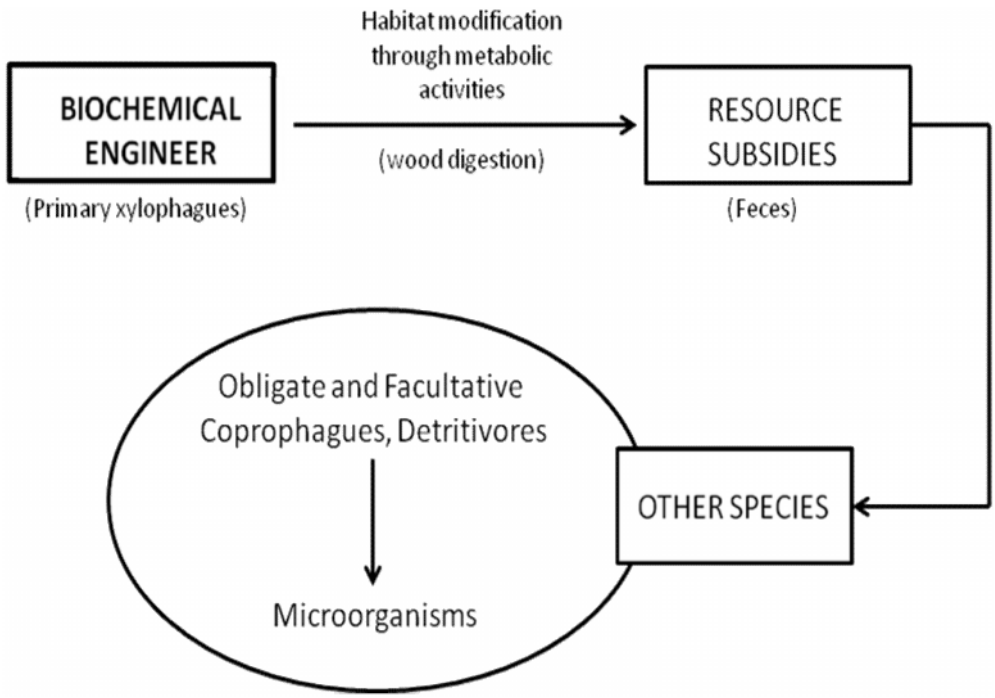

Figure 2. A conceptual model of biochemical engineering. Biochemical engineers alter the composition and structure of their environment through their metabolic activities and supply resources for other species.

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Capítulo 4

EVOLUTIONARY AND ECOLOGICAL CONSEQUENCES OF INSECT LIGNOCELLULOLYTIC ENZYMES ON PLANT-INSECT INTERACTIONS*

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*Sometido a: Annual Review in Ecology, Evolution and Systematics

ABSTRACT

The prevailing view that insects lack endogenous genes and enzymes for plant cell wall (PCW) digestion, had led to the hypothesis that PCW digestion evolved independently in different insect taxa through the establishment of symbiotic relationships with microorganisms. However, recent studies reporting endogenous lignocellulolytic genes for several insect and arthropod groups, including the more ancestral living insects and the most closely related arthropod groups to insects, challenge this hypothesis. In this review, we summarized the molecular and biochemical evidence on the mechanisms of PCW digestion in insects in order to analyze its evolutionary pathways. Our analysis reveals that symbiotic-independent mechanism for PCW digestion evolved once in the Insecta class and was lost several times in some insect orders. We discuss the implications of this alternative hyphotesis in the evolution of plant-insect interactions, and suggest that changes in the composition of lignocellulolytic complexes were involved in the evolution of feeding habits and diet specializations in insects, playing important roles in the evolution of plant-insect interactions. We propose new areas for the study of plant-insect interactions that can improve our understanding of the evolution and diversification of insects.

Keywords: plant cell wall digestion, lignocellulolytic symbionts, endogenous cellulases, endogenous hemicellulases, endogenous pectinases, insect diversification.

INTRODUCTION

Most studies on plant-herbivore interactions have focused on the chemical "arms race" between plants and insects (Fraenkel 1959, Erlich and Raven 1964). This hypothesis proposes that secondary compounds produced by plants are the result of defensive responses to the attack of herbivores, and the herbivores in turn escape, metabolize or use these chemicals for their own benefit. Therefore, there are constant reciprocal selective pressures of plants on insects and viceversa, affecting their fitness. Alternative explanations suggest that several other selective pressures (e.g., natural enemies) play a prominent role in the evolution of plant-insect interactions (i.e., Abe and Higashi 1991, Bernays 1991, Jermy 1993, Hochuli 1996, Agrawal 2006). A comparatively understudied plant-insect interaction is the ability of insects to feed on plant tissues by digesting complex structural molecules such as lignocellulose. Undoubtedly this molecule, present in plant cell walls (PCW), is the most abundant polysaccharide in nature and the major source of energy (via carbon supply) in the biosphere (Bayer et al. 1998. Interestingly, PCW is an important food source for a great number of insects that thrive on wood, foliage and detritus. However, due to it has proven that some of the microorganisms that are associated with insects participate in PCW digestion (reviewed in Martin 1983, Prins and Kreulen 1991), the hypothesis widely accepted in the scientific literature is that insects only can digest PCW through the establishment of symbiotic relationships with microorganisms residing in their digestive tracts (Cleveland 1924).

This traditional view of PCW digestion in animals was challenged by the symbiont-independent cellulase activity in some species of termites and cockroaches (reviewed in Slaytor 1992), the first two reports of endogenous animal cellulase genes from plant-parasitic nematodes and a termites (Smant et al. 1998, Watanabe et al. 1998), and more recent studies on animal cellulase genes (see review Watanabe and Tokuda 2001). Lignocellulolytic genes have been reported for different insects of the major

insect lineages and for insects with diets other than xylophagy. Moreover, the use of molecular biology techniques has led to new insights into the role of symbiotic microorganisms in the process of PCW digestion in insect guts. Therefore, studies analyzing this new information in a synthetic perspective are needed.

In this review we analyze the studies that have reported symbiotic-independent mechanisms of PCW digestion in insects while analyzing the studies that have found symbiotic-dependent mechanisms. We summarized the molecular and biochemical evidence on each mechanism in order to know its evolutionary pathways and its implications in the evolution of plant-insect interactions. We propose that symbiotic-independent mechanism for PCW digestion might be the ancestral mechanism in insects, and that insect lignocellulolytic enzymes were involved in the evolution of different feeding habits and diet specializations, playing important ecological roles in insect-plant interactions. With this alternative hypothesis we do not deny the importance of symbiotic-dependent mechanisms in PCW digestion and evolution of plant-insect interactions, but rather we draw the attention to the possibility that insect lignocellulolytic enzymes can have remarkable but, thus far, neglected ecological and evolutionary implications, such as the evolution of feeding habits and diet specializations.

This review is divided in two sections. In the first section we briefly discuss the mechanisms of PCW digestion in insects and summarized the molecular and biochemical evidence currently available. In the second section, we use this information as evidence to analyze and discuss the evolution of PCW digestion in insects and its implications in the evolution of plant-insect interactions.

1. PCW DIGESTION IN INSECTS

In insect herbivores, the digestive tract is usually divided into three main regions: the foregut, concerned with ingestion, storage, grinding and transport of food to the next regions; the midgut, where the ephitelial cells produce and secrete digestive enzymes and also absorb the resultant food breakdown products; and hindgut, where absorption of water, salts and other valuable molecules occurs before the elimination of feces. Some digestion may occur in the foregut as a result of insect salivary enzymes or midgut enzymes moving anteriorly, but most biochemical transformation occurs in the midgut (review in Terra 1990, Terra and Ferreira 1994, Watanabe and Tokuda 2010). However, in some insects such as termites (Isoptera), wood-feeding cockroaches (Blattaria), Scarabaeidae beetles (Coleoptera), and Tipulidae flies (Diptera), digestion can also occurs in specialized hindgut structures by the action of digestive enzymes of symbiotic microorganisms (Terra 1990).

PCW is a complex and dynamic cellular layer that varies in its composition not only between plant species, but also between tissues (detailed information about the structure and composition of PCW can be found in Bidlack et al. 1992, Cosgrove 1997, Rowell et al. 2000). In general, PCW is made of an interconnected network of cellulose and hemicelluloses, which is embedded in a complex pectin or lignin matrix (for primary or secondary PCW, respectively) (Bidlack et al. 1992, Rowell et al. 2000). Hence, PCW digestion requires an extremely complex breakdown process for its digestion, involving a suite of numerous enzymes with diverse modes of action and substrates; these enzymes include cellulases, hemicellulases, pectinases and lignases, collectively named lignocellulolytic enzymes (Fry 2000). Biochemical and structural information about lignocellulolytic enzymes have been extensively reviewed (Beguin and Aubert 1994, Ahmed et al. 2001, Shallom and Shoham 2003, Jayani et al. 2005).

Cellulases include three major classes of hydrolytic enzymes: endoglucanases (cellulases), exoglucanases (cellobiohydrolases), and β-glucosidases (cellobiases) (Beguin and Aubert 1994). Hemicellulases include all the enzymes which hydrolyze hemicellulose polysaccharides; the most representative are xylanases and xylooligosaccharidases, but they also include α-glucuronidase, α-L-arabinofuranosidase, esterases such as acetyl- and xylan-acetylesterases and arylesterases, laminarinases, liquenases, among others (Shallom and Shoham 2003). Pectinases include pectinesterases, pectin and pectate lyases and polygalacturonases (Jayani et al. 2005). Lignases group consists mainly of phenoloxidases (laccases) and peroxidases (lignin and manganese peroxidades) (Ahmed et al. 2001). In general, the PCW degradation process consists of three steps: depolymerization of lignin or pectin (for primary and secondary PCW, respectively), hemicellulose degradation, and finally cellulose degradation; each step is important to enable the degradation of the next PCW polymer (Fry 2000).

2. MECHANISMS FOR PCW DIGESTION

Different mechanisms have been proposed to explain PCW digestion by insects: (I) lignocellulolytic capacity of hindgut protozoan symbionts; (II) lignocellulolytic capacity of hindgut bacteria (III) lignocellulolytic capacity of midgut symbotic yeasts and bacteria; (IV) ectosymbiosis with fungi and reliance upon fungal enzymes originating in the food, and remaining active in the gut following ingestion; and (V) a more controversial mechanism of secretion of enzymes by an endogenous lignocellulolytic system in insects (Martin 1983, 1991).

Despite the prevailing view that symbionts (protozoans, bacteria and fungi) are responsible for PCW digestion in insects (review in Breznak, 1982; Martin, 1983, 1991; Prins and Kreulen, 1991; Brauman et al., 2001, Ohkuma 2003), more recent evidence has resulted in a debate on the role of symbionts in the PCW digestion (discussed in

Slaytor 1992, Bignell 2000, Douglas 2009, Watanabe and Tokuda 2010). Most of the past studies supporting this hypothesis are based on the presence of cellulolytic microorganisms in the insect gut, and on the possible effect that the removal of these microorganisms may have on the survival and digestive efficiency of aposymbiotic (symbiont-free) insects thriving on cellulose-diets. In some cases, the removal of protozoans resulted in the death of the aposymbiotic insects (i.e. Cleveland 1924). However, the methods for removing the protozoans have been questioned (see Slaytor 1992), because the defaunation process can affect a broad spectrum of non-cellulolytic bacteria, which can play other important roles in the survival of insects. Furthermore, the symbiotic lignocellulolytic system is poorly understood because the isolation and characterization of the symbiotic genes encoding the enzymes involved in PCW digestion have not been carried out. Theferore, it is important to isolate, characterize and identify the origin of the genes and enzymes involved in the PCW digestion process. Recent research on the PCW digestion in insects has been focused on the cloning and characterization of the genes encoding lignocellulolytic enzymes from both insects and symbionts. We summarized this evidence and discuss the contribution of each mechanism to the process of PCW digestion in insects.

Hindgut symbiotic protozoans

Termites possess a great diversity of symbiotic microorganisms in their specialized hindguts, including protozoans, bacteria, and Archaea (Ohkuma 2003). Anaerobic symbiotic protozoans (Parabasalia: Trichomonadida, Hypermastigida and Oxymonadida) are restricted to "lower" termites (Mastotermitidae, Kalotermitidae, Termopsidae, Hodotermitidae, Rhinotermitidae, Serritermitidae) and wood-feeding cockroaches (Ohkuma 2003). These protozoans possess several lignocellulolytic genes (Table 1) encoding cellulases (endoglucanases, cellobiohydrolases and β-glucosidases) and hemicellulases (xylanases, mannanases, etc.). The presence of these lignocellulolytic enzymes indicates that symbiotic protozoans have the ability to digest PCW, although

their contribution and specific role in termite digestion is poorly understood. Total contribution of symbiotic endoglucanases in the hindgut of lower termites varies between 12- 40% (Veivers et al. 1982, Tokuda et al. 2004, Zhou et al. 2007), but the contribution of symbiotic cellobiohydrolases (~62%) and xylanases (88-98%) in the termites *Coptotermes formosanus* (Nakashima et al. 2002a, Arakawa et al. 2009) and *Reticulitermes flavipes* (Zhou et al. 2007) is higher. Tartar et al. (2009) suggest that hemicellulose was exclusively digested by symbiotic hemicellulases in *R. flavipes*. Additionally, recent metatranscriptome analyses indicate that symbiotic cellobiohydrolases and xylanases are the major expressed enzymes in some species of lower termites and the wood-feeding cockroach *Cryptocercus punctulatus* (Todaka et al. 2007, 2010, Tartar et al. 2009). Hence, it appears that the main contribution of hindgut symbiotic protozoans is related to the digestion of highly polymerized cellulose and hemicellulose. However, it is possible that not all protozoan species contribute equally in this process; for example, among the main protozoan symbionts, *P. grassii* is the only one involved in the degradation of highly polymerized cellulose, whereas *H. mirabile* and *S. leiydi* only utilize low molecular weight cellulose (Inoue et al. 2005, Tanaka et al. 2006). Cellobiohydrolases and xylanases have only been reported for *P. grassii* (Nakashima et al. 2002b, Watanabe et al. 2002) and *H. mirabile* (Arakawa et al. 2009), respectively. The presence of cellobiohydrolases in *P. grasii* explains its ability to digest highly polymerized cellulose, while xylanases of *H. mirabile* indicate the importance of this species in hemicellulose digestion. Considering that cellobiohydrolases and xylanases represent key enzymes that act together to make an effective degradation of lignocellulose (Beguin and Aubert 1994, Arakawa et al. 2009), the overall protozoan lignocellulolytic system could explain the specialization of lower termites on diets consisting mainly of wood.

Hindgut symbiotic bacteria

Higher termites (Termitidae) comprising 75 % of all termite species, usually lack the cellulolytic protozoa typical of the lower termites (Martin et al. 1983). Instead of protozoan symbionts, Termitidae species have diverse symbiotic bacterial communities, including Archaea (methanogens), Proteobacteria, Bacteroides and Spirochaetes (Brauman et al. 2001, Friedich et al. 2001, Okhuma 2003). Given the lack of protozoans, it was often assumed that PCW digestion (including lignin digestion; Prins and Kreulen 1991, Brune et al. 1995) by higher termites is accomplished by these hindgut bacteria. However, without isolation of lignocellulolytic bacteria, the significance of such symbionts resided in their provision of vitamins, sterols, lipids and essential amino acids (Breznak 1982, Martin 1983, Prins and Kreulen 1991). Recently it was reported that cellulase activity against highly polymerized cellulose (involving a complete cellulolytic system) in the bacterial insoluble fraction on the hindgut of *Nasutitermes takasagoensis* and *N. walkeri* contributed up to 59% of total cellulase activity on crystalline cellulose (Tokuda and Watanabe 2007). Additionally, in a metagenomic analysis of the hindgut bacteria of *Nasutitermes* sp. more than 100 cellulases (endoglucanases, cellobiohydrolases and β-glucosidases), xylanases and pectinases sequences were identified (Warnecke et al. 2007). Hence is very likely that hindgut symbiotic bacteria contribute significantly to PCW-polysaccharide digestion in higher termites.

Cellulolysis by cultivated hindgut bacteria has also been reported for some Orthoptera, Coleoptera and Diptera species (Table 1), but the isolation of lignocellulolytic genes has been reported only for two bacteria species, *Cellulomonas pachnodae* and *Cellulosimicrobium* sp. HY-12 isolated from a scarabaeid beetle and a cricket, respectively (Table1). Interestingly, Scarabaeidae (Coleoptera) and Tipulidae (Diptera) species have a differentiated and highly specialized hindgut (i.e. fermentation chamber) harboring numerous microorganisms, like termites and wood-feeding cockroaches (Terra 1990). Despite that Gryllidae and Gryllotalpidae (Orthoptera)

species do not have a fermentation chamber, their hindguts contain 3-4 rows of projecting papillae with long setae densely populated by microorganisms (Nation 1983). This suggests that insects with these particular morphological adaptations in their hindguts are the only insects for which hindgut bacterial symbionts can contribute to PCW digestion. However, the bacterial symbiotic system and its contribution in the digestion of these insects is poorly understood yet.

Midgut symbiotic yeasts and bacteria

Symbiotic yeasts and bacteria housed in specialized cells called mycetocytes represent a widespread insect-microbial interaction (Douglas 2009). Mycetocytes are located in the haemocoel and fat body in some insects (e.g., hemipterans, cockroaches), but they also can be located in the ephithelium of the midgut caeca in other insects (e.g., cerambycid beetles) and in specialized organs (mycetomes) of the gut (e.g., hemipterans) (Douglas 2006, 2009). Microbial symbionts in mycetocytes and mycetomes are represented by yeasts (Fungi) and bacteria (Douglas 2009). Most fungal symbionts associated with mycetocytes have both intracellular and extracellular phases, when yeast cells are released into the gut lumen (Berkov et al. 2007, Grünwald et al. 2010). Furthermore, free-living yeasts can also be found in the gut content (Grünwald et al. 2010).

Since most of the insects having mycetocytes feed on plant tissues, it was believed that such symbionts might also be involved in PCW digestion (Martin 1983), but evidence supporting this idea is debated. Yeasts have been isolated from the gut contents of 27 beetle families (Suh et al. 2005, 2008, Berkov et al. 2007, Grünwald et al. 2010) and from species of six additional insect orders (Orthoptera, Blattaria, Dermaptera, Hymenoptera, Neuroptera and Megaloptera) (Suh et al. 2008), but the majority of these yeast endosymbionts are classified as species of true yeasts in the genus *Candida* (Saccharomycetales) that do not show lignocellulolytic capacity (Suh et al. 2005, 2008, Berkov et al. 2007, Grünwald et al. 2010). Nevertheless, *Pichia stipitis*

and closely related yeast-like symbionts (YLS) have been reported to ferment xylose, and these YLS were found in gut contents of some insects (Table 1). Cellulolytic and hemicelullolytic genes have been identified in the genome of a strain of *P. stipitis* that has been isolated from the gut of a passalid beetle (Jeffries et al. 2007). On the other hand, evidence for the presence of lignocellulolytic bacteria in epithelial cells of the midgut is available for orders Lepidoptera, Coleoptera and higher termites (Termitidae) (Table 1). Bacterial lignocellulolytic genes have been isolated from at least one species of these insect orders (Table 1). This evidence confirms that some insects harbor midgut microorganisms that can metabolize polysaccharides, although it is not clear if these microorganisms contribute significantly to insect nutrient gain, since the number of microorganisms present in this region of the gut is low or even absent (compared with the number of hindgut microorganisms) in some insects (Slaytor 1992, Egert et al. 2005), and/or because the composition of midgut communities often comprises a significant fraction of transient microorganisms derived from food (Douglas 1998, Broderick et al. 2004, DelaLibera et al. 2005, Schloss et al. 2006, Berkov et al. 2007, Grünwald et al. 2010).

Ectosymbiosis and acquired fungal enzymes

Ectosymbiotic relationships that best represent this mechanism for PCW digestion in insects are those between ants (Myrmicine: Attini) and the basidiomycete fungi *Leucoagaricus* spp. and *Leucocoprinus* spp. (Mueller et al. 2001), and between Macrotermitidae termites and the basidiomycete fungi *Termytomyces* spp. (Martin and Martin, 1978). Several roles for fungi in this kind of symbiosis have been proposed: i) fungi serve as nitrogen-rich food, ii) fungi degrade plant polysaccharides and provide oligosaccharides that can be digested by insect enzymes (complementary enzymatic system), iii) fungi degrade plant lignin to improve cellulose digestion, and iv) fungi supply cellulases and hemicellulases (that remain intact throughout the insect gut and are active in faecal residues) to act synergistically with the enzymes produced by the insect

(D´Ettore et al. 2002, Hyodo et al. 2003, Ohkuma 2003, Taprab et al. 2005). Even when there is variation in the importance of each role across species, in the termite-fungus mutualism the major role of fungi appears to be the degradation of lignin contained in fungus combs, which enables termites to use cellulose more efficiently (Hyodo et al. 2000, Taprab et al. 2005, but see Johjima et al. 2006); while in the ant-fungus mutualism, fungi degrade cellulose and other polysaccharides such as hemicellulose (mainly xylan), and ants hydrolyze oligosaccarides such as β-glucosides, β-xylosides, β-galactosides which are degraded products of cultured plant material (D´Ettore et al. 2002, Richard et al. 2005). Fungi also appear to be an essential food source for fungus growing-insects (Mueller and Gerardo 2002, Hyodo et al. 2003, Richard et al. 2005). However, much attention has been devoted to the last role, also known as the "acquired enzyme hypothesis". Strong evidence supporting this hypothesis exists only for three species of fungus-growing termites, *Macrotermes natalensis* (Martin and Martin 1978), *M. mulleri* (Rouland et al. 1988a, 1988b) and *M. bellicosus* (Matoub and Rouland 1995) and one cerambycid beetle *Monochamus marmorator* (Kukor and Martin 1986), in which enzymes from the gut of the insect host and from fungi were independently isolated, and demonstrated to be identical. Based on these results, it was proposed that acquired fungal lignocellulolytic enzymes that degrade PCW components are widespread among xylophagous and detritivorous insects (Kukor and Martin 1983, 1986, Kukor et al. 1988). However, this generalization has been questioned because for some insect species, acquired fungal enzymes play little, if any, role in cellulose digestion (Veivers et al. 1991, Slaytor 1992, Scrivener and Slaytor 1994).

Table 1. Biochemical and molecular evidence of symbiotic-mediated mechanisms for plant cell wall digestion in insects

Insect Order Family Species	Symbiont	Type of symbiont	Tissue used	Type of enzymes	Biochemical evidence	Molecular evidence	References
Orthoptera							
Gryllotalpidae							
Gryllotalpa orinetalis	*Cellulosimicrobium* sp. HY-12	B	H	XYL	-	GHF10 genes	[1]
Blattaria							
Cryptocercidae							
Cryptocercus punctulatus	uncultured symbiotic protists	P	G	CEL, XYL	-	GHF5, GHF7, GHF10, GHF45 cDNAs	[2]
Isoptera							
Mastotermitidae							
Mastotermes darwiniensis	*Koruga bonita, Deltotrichonympa nana*	P	H	CEL	-	GHF45	[3]
	uncultured symbiotic protists	P	G	CEL, XYL		GHF5, GHF7, GHF10 ,GHF45 cDNAs	[2]
Termopsidae	*Cellulosimicrobium variable* sp. nov	B	H	CEL, XYL	-	-	[4]
Hodotermopsis sjoestedti				CEL, XYL		GHF5, GHF7, GHF10, GHF11, GHF45	
	uncultured symbiotic protists	P	G		-	cDNAs	[2]
Zootermopsis angusticollis	*Cellulomonas* sp., *Microbacterium* sp.	B	G	CEL	low-high EA	-	[5]
	Bacillus spp., Paenibacillus sp., Sphingomonas sp.	B	G	CEL	low-high EA	-	[5]
Kalotermitidae							
Neotermes castaneus	*Spirochaeta coccoides* sp. nov.	B	H	XYL	AP	-	[6]
N. koshuensis	uncultured symbiotic protists	P	G	CEL, XYL	-	GHF5, GHF7, GHF10 cDNAs	[2]
Rhinotermitidae							
Coptotermes formosanus	*Pseudotrichonympha grasii,*	P	H	CEL[a], XYL	AP	GHF7 genes	[7] [8]
	Spirotrichonympha leidyi	P	H	CEL	0.7 U	GHF5	[9]
	Holomastigotoides mirabile	P	H	CEL, XYL[a]	0.37 U/mg	GHF7, GHF11 genes	[8] [10]
C. lacteus	*P. grasii, H. mirabile*	P	H	CEL	0.7 U	GHF7 genes	[8]
Reticulitermes flavipes	uncultured symbiotic protists	P	G	CEL, XYL	-	cDNAs of 18 GHFs	[11][12]
R. speratus	*Trychonympha agilis, Teranympha miabilis*	P	H	CEL	-	GHF45	[13]
	uncultured symbiotic protists	P	G	CEL, XYL	-	GHF5, GHF7, GHF11, GHF45 cDNAs	[2][14]
Termitidae							
Macrotermes bellicosus	*Termitomyces* sp.	F	G	XYL	EP 1.4-1.6 U/mg	-	[15]
M. mülleri	*Termitomyces* sp.	F	G	CEL[a], XYL[b]	EP, [b]750 U/mg	-	[16][17]
M. natalensis	*Termitomyces* sp.	F	G	CEL	EP		[18]
Nasutitermes sp.	Spirochaetes and Fibrobacter spp.	B	H	CEL, XYL, PEC	-	>100 genes of different GHFs	[19]
N. walkeri	*Clostridium*termitidis sp. nov	B	M-H	CEL	-	-	[20]
N. takasagoensis	SR *Clostridium thermocellum, B. subtilis*	B	M-H	CEL	-	-	[21]
Coleoptera							
Scarabaeidae							
Melolontha melolontha	Clostridiales and Bacteriodetes spp.	B	H	CEL, XYL	-	-	[22]
Pachnoda ephippiata	*Clostridium* spp [ab], *Staphylococcus* sp[a].,						
	Promicromonospora sp.b, Arthrobacter spb.	B	H[a], M[b]	CEL, XYL	-	-	[23]
P. marginata	*C. pachnodae*[a] *Promicromonospora pachnodae*[b]	B	H[a], M[b]	CEL, XYL	Z[a], 6U/mg[b]	GHF6, GHF10, GHF11 genes	[24] [25] [26]
Passalidae							
Odontotaenius disjunctus	*Pichia stipitis*-like yeast	F	G	XYL	-	-	[27]

Verres sternbergianus	*Pichia stipitis*-like yeast	F	G	XYL	-	-	[27]
Cerambycidae							
Batocera horsfieldi	*Sphingobacterium* sp. TN19	B	G	XYL	EP 196U/mg	GHF10 gene	[28]
Corymbia rubra	Gammaproteobacteria	B	G	XYL, PEC	high EA	-	[29]
Psacothea hilaris	Actinobacteria, Gammaproteobacteria	B	G	XYL[a], PEC[b]	[a]moderate, [b]low EA	-	[29]
Massicus raddei	Actinobacteria, Gammaproteobacteria	B	G	XYL[a], PEC[b]	[a]high, [b]low EA	-	[29]
Mesosa hirsute	Actinobacteria, Gammaproteobacteria	B	G	XYL[a], PEC[b]	[a]moderate, [b]low EA	-	[29]
Moechotypa diphysis	Actinobacteria, Gammaproteobacteria	B	G	XYL[a], PEC[b]	[a]high, [b]low EA	-	[29]
	Paenibacillus sp. Hy-8	B	G	XYL	EP 147 U/mg	GHF11 gene	[30]
Monochamus alternatus	Gammaproteobacteria	B	G	XYL	low EA	-	[29]
M. marmorator	*Trichoderma harzianum*	F	G	CEL	EP	-	[31]
Olenecamptus clarus	Gammaproteobacteria	B	G	XYL	low EA	-	[29]
Prionus insularis	Actinobacteria	B	G	XYL	low EA	-	[29]
Saperda vestita	*Sphingobium yanoikuyae*	B	G	CEL (CBH)	weak EA	-	[32]
	SR *Nectria haematococca*	F	G	CEL	AP	-	[32]
	SR *Fusarium culmorum, Penicillium*	F	G	CEL	AP	-	[32]
Curculionidae							
Dendroctonus frontalis	SR *Penicillium*	F	G	CEL	AP	-	[32]
Ips pini	SR *Penicillium*	F	G	CEL	AP	-	[32]
Hymenoptera							
Siricidae sp.	*Amylostereum chailletii*	F	G	CEL, XYL	EP	-	[33]
Xiphydriidae sp.	*Amylostereum chailletii*	F	G	CEL, XYL	EP	-	[33]
Lepidoptera							
Saturniidae							
likely *Rotschildia lebaeu*	SR rumen fungi and bacteria	NI	G	XYL	Z	GHF8, GHF11 genes	[34]
Lasiocampidae							
Samia cynthia pryeri	*Aeromonas* sp.	B	G	XYL	EP 88.45 U/mg	-	[35]
Diptera							
Tipulidae							
Tipula abdominalis	Proteobacteria spp.	B	H	CEL, XYL, PEC	AP	-	[36]
	Actinobacteria spp.	B	H	CEL, XYL, PEC	AP	-	[36]
	Firmicutes spp.	B	H	CEL, XYL, PEC	AP	-	[36]

Type of symbiont: SR= strain related to B=Bacteria, P=Protozoa, F=Fungi, NI=non-identified. **Localization:** G=whole gut, H=hindgut, M=midgut. **Type of enzyme:** CEL= cellulases, XYL=xylanases, PEC=pectinases. **Biochemical evidence:** EA= enzymatic activity, AP= enzymatic activity presence, EP=enzyme purification, Z=zymogram, U=unit of enzymatic activity expressed as μm of reducing sugar/min. **Molecular evidence:** GHF= glycosyl hydrolase family. **References:** [1] Oh et al. 2008; [2] Todaka et al. 2010; [3] Li et al. 2003; [4] Bakalidou et al. 2002; [5] Wenzel et al. 2002; [6] Dröge et al. 2006; [7] Nakashima et al. 2002b; [8] Watanabe et al. 2002; [9] Inoue et al. 2005; [10] Arakawa et al. 2009; [11] Zhou et al. 2007; [12] Tartar et al. 2009; [13] Ohtoko et al 2000; [14] Todaka et al. 2007; [15] Matoub and Rouland 1995; [16] Rouland et al. 1988a; [17] Rouland et al .1988 b; [18] Martin and Martin 1978; [19] Warnecke et al. 2007, [20] Hethener et al. 1992; [21] Tokuda et al. 2000; [22] Egert et al. 2005; [23] Egert et al. 2003; [24] Cazemier et al. 1999a; [25] Cazemier et al. 1999b; [26] Cazemier et al. 2003; [27] Suh et al. 2003; [28] Zhou et al. 2009; [29] Park et al. 2007; [30] Sunyeon et al. 2006; [31] Kukor and Martin 1986; [32] DelaLibera et al. 2005; [33] Kukor and Martin 1983; [34] Brennan et al. 2004; [35] Roy et al. 2003; [36] Cook et al. 2007.

Symbiotic-independent mechanism

Because insects are almost universally associated with microorganisms, it has been difficult to refute the long-standing hypothesis of symbiotic and mutualistic interactions for PCW digestion (Chown and Nicolson 2004). Nevertheless, studies suggesting endogenous lignocellulolytic enzymes without the isolation of genes are abundant (Review in Watanabe and Tokuda 2001). In some of these studies the authors were able to dissect the gut of insects and isolate the insect enzymes present in salivary glands and midgut, from those of their symbionts present mainly in the hindgut (e.g. Hori 1975, Laurema et al. 1985, Zinkler and Götze 1987, Tokuda et al. 1997). Cloning and characterization of genes encoding lignocellulolytic enzymes have finally demonstrated symbiont-independent digestion of PCW digestion in insects.

In insects, as well as other animals, carbohydrates are generally absorbed as monosaccharides, thus PCW polysaccharides must be broken down by different enzymes to produce monosaccharides before they are absorbed (Chapman 1982). Lignocellulolytic systems of insects consist of cellulases, hemicellulases, pectinases and lignases (Table 2).

Cellulases

Insect cellulase complex (studied in xylophagous insects) consists of endoglucanases (EC 3.2.1.4) which act along the cellulose chains and β-glucosidases (EC 3.2.1.21) which hydrolyze cellobiose to glucose, although this complex appears to lack cellobiohydrolases (EC 3.2.1.91) that are active against crystalline cellulose, and that are common in other organisms such as bacteria, protozoans and fungi (Martin 1983, 1991, Scrivener and Slaytor 1994). Different morphological and physiological adaptations have been proposed to explain the lack of cellobiohydrolases in xylophagous insects: (i) the presence of grinding organs, such as mandibles which physically crush wood into small particles ranging from 20 to 50 µm, allowing

cellulolytic enzymes to effectively access substrates due to the increase in accessible surface area (Nakashima et al. 2002a); (ii) the presence of a remarkably long digestive tract, allowing a slow transit which is presumed to gives the cellulases enough time to hydrolyze the cellulose (Watanabe and Tokuda 2010); and (iii) the presence of numerous and large amounts of endoglucanases to compensate for their inefficiency against crystalline cellulose (Scrivener and Slaytor 1994).

In most insects, cellulose digestion is a process mediated by the action of endoglucanases expressed mainly in midgut cell glands (Sugimura et al. 2003, Lee et al. 2004, 2005, Byeon et al. 2005, Wei et al. 2006), and in salivary glands in lower termites (Tokuda et al. 2004), honey bees (Kunieda et al. 2006) and likely in lepidopterans (Oppert et al. 2010). Endoglucanases appear to be enzymes widespread among insects, since molecular and biochemical evidence for the presence of these enzymes exists for 16 insect orders (Table 2). All feeding habits (i.e., detritivory, omnivory, xylophagy, herbivory, carnivory, parasitism) are represented by insects possessing endoglucanase genes and/or enzymes (Table 2). However, for some carnivorous insects no gene is reported yet, and for the body louse (Phthiraptera) and the fruit fly *Drosophila melanogaster,* the reported sequences homologous to cellulases have not been proven to encode active enzymes for digesting cellulose, and their presence in these insect genomes need to be explained.

Insect cellulases have been shown to be more important (based on enzymatic activity data) for omnivorous, detritivorous and xylophagous insects than for most insect herbivores, for which lower cellulase activities are reported (Table 2). In most insects β-glucosidases hydrolyze oligo- and di-saccharaides and play roles other than PCW digestion that are discussed in the next section. Nevertheless, these enzymes are commonly present in animals. Hence, we have not shown information about reported sequences in table 2.

Hemicellulases

Nutritional studies reporting hemicellulase activities (i.e. xylanase, licheninase and laminarinase activities) have shown that hemicelluloses may be utilized by insects (Martin et al. 1981, Chipoulet and Chararas 1984, 1985, Scrivener and Slaytor 1994, Terra and Ferreira 1994, Scrivener et al. 1997). The presence of xylanases (EC 3.2.1.8) and laminarinases (EC 3.2.1.6) has only been reported for two cerambycid beetle species (Table 2). Xylanases are the main enzymes involved in hemicellulose degradation (Arakawa et al. 2009). However, several lines of evidence suggest that xylanases might be enzymes rarely present in insects since: i) it was shown that xylanases enzymes and genes isolated and characterized from termite guts originated from fungus ingested by termites (Martin and Martin 1978, Rouland et al. 1988a, 1988b, Matoub and Rouland 1995) and/or from symbiotic protozoans harbored in the hindgut (Arakawa et al. 2009, Tartar et al. 2009); and ii) no gene encoding xylanases were found in the reported insect herbivore genomes (the hymenopteran *Apis mellifera,* the lepidopteran *Bombix mori* and the beetle *Tribolium castaneum*), and in transcriptome analyses of the beetle *Chrysomela tremulae* (Chrysomelidae; Pauchet et al. 2009a). However, xylan is also hydrolyzed by endoglucanases (Scrivener et al. 1997). Endoglucanases exhibiting high activity towards xyloglucan, but low activity towards β-glucan, are named xyloglucanases (EC 3.2.1.115) (Grishutin et al. 2004). A xyloglucanase showed high activity in the gut of the cerambycid beetle *Psacothea hilaris* (Scrivener et al. 1997). Therefore, it is likely that these enzymes could be involved in the xylan hydrolysis reported for most herbivorous insects instead of xylanases, although they merit further study.

β-1,3-glucanases encoding genes have been reported for several insect species (Table 2). β-1,3-glucanases (EC 3.2.1.39) are enzymes that hydrolyze exclusively β-1,3-linkages, and they are recognized as components of the laminarinase complex (Fry 2000, Terra and Ferreira 1994, Pauchet et al. 2009b). β-1,3-glucans are found in cell walls of plants (i.e., callose), algae (i.e., laminarin), phytoplankton (i.e., chrysolaminarin) and fungi (i.e., lentinan, pleuran, zymosan) (Fry 2000, Terra and

Ferreira 1994). Hence β-1,3-glucanases can be key enzymes for hemicelluloses digestion, particularly in omnivorous and detritivorous insects.

Pectinases

The insect pectinase complex comprises endopolygalacturonases and pectin methylesterases, and they seem to occur mainly in Hemiptera and Coleoptera (Table 2). However they are apparently not present in termites (Tartar et al. 2009). In piercing-sucking insects such as aphids and other hemipterans, salivary pectinases have been suggested to be involved in the penetration of plants (Shen et al. 1996, Cherqui and Tjallingii 2000, Boyd et al. 2002). Within the order Coleoptera, pectinases might be related to the digestion of young plant tissues (such as leaves) and grains, since pectin is abundant in tissues with high content of primary PCW (Bidlack et al. 1992, Rowell et al. 2000).

Lignases

Laccases (phenoloxidases) and peroxidases can be found in insects: laccases in the epidermis of insects (Dittmer et al. 2004, Arakane et al. 2005) and other phenoloxidases and peroxidases in aphid salivary secretions (Table 2), but the functions of these enzymes are not digestive (Urbanska et al. 1998, Cherqui and Tjallingii 2000). For example, insect laccases are involved in the sclerotization of cuticle (Arakane et al. 2005), while phenol oxidizing enzymes in aphid saliva presumably play an important role in chemical stabilization of the stylet sheath and to enable phytophagous insects to overcome plant defenses by neutralizing toxic phenolics (Urbanska et al. 1998, Cherqui and Tjallingii 2000). Laccases have also been found in the midgut of some insects (Table 2), but their function in this internal organ is not yet understood (Nakamura et al. 2005). However, some authors have suggested that they are involved in lignin degradation (Tartar et al. 2009). Thus, it is possible that some insects (particularly wood- and humus- feeding insects) can degrade lignin in their guts.

Table 2. Biochemical and molecular evidence of symbiotic-independent (endogenous mechanism) for plant cell wall digestion in insects

Insect Order Family Species (common name)	Feeding habit (hervibore guild)	Enzyme	Biochemical evidence	Enzymatic activity	Molecular evidence	GenBank No.	References
ZYGENTOMA							
Lepismatidae							
Thermobia domestica (firebrat)	D	CEL, CBH	FA	86.4, 89 U/g weight/h	-	-	[1] [2]
EPHEMENOPTERA							
Heptageniidae							
Heptagenia sulphurea (yellow may)	D	CEL, CBH	GA	slight activity	-	-	[3]
Ecdyonurus dispar (autumn dun)	D	CEL, CBH	GA	slight activity	-	-	[3]
Caenidae							
Caenis horaria (mayfly)	D	CEL, CBH	GA	slight activity	-	-	[3]
PLECOPTERA							
Pteronarcyidae							
Pteronarcys proteus (giant stonefly)	D	CEL	FA + MA	4600 mg /g weight	-	-	[4]
ORTHOPTERA							
Gryllidae							
Allonemobius sp. (ground cricket)	O	CEL	HA	0.1-0.6 U/mg pr/h	-	-	[5]
Teleogryllus emma (emma field cricket)	O	CEL	-	-	GHF9 gene	EU126927	[6]
Acrididae							
Chortophaga viridifasciata (green stripped grasshopper)	H (LC)	CEL	HA	0.3 U/mg pr/h	-	-	[5]
Hippiscus ocelote (wrinkled grasshopper)	H (LC)	CEL	HA	0.1-0.6 U/mg pr/h	-	-	[5]
Melanoplus differentialis (differential grasshopper)	H (LC)	CEL	HA	0.25 U/mg pr/h	-	-	[5]
M. femurrubrum (redlegged grasshopper)	H (LC)	CEL	HA	0.15-0.7 U/mg pr/h	-	-	[5]
Schistocerca americana (american bird grasshopper)	H (LC)	CEL	HA, FA, MA	0.1 U/mg pr/h	-	-	[5]
S. gregaria (desert locust)	H (LC)	CEL	FA, MA	160, 81 U/ml gut	-	-	[7]
		XYL	FA, MA	5.2, 5.3 U/ml gut	-	-	[7]
Syrbula admirabilis (admirable grasshopper)	H (LC)	CEL	HA	0.15-0.6 U/mg pr/h	-	-	[5]
PHASMATODEA							
Phasmatidae							
Eurycantha calcarata (giant spiny stick insect)	H (LC)	CEL	FA, MA	48, 57 U/ml gut	-	-	[7]
BLATTARIA		XYL	FA, MA	0.7, 1.0 U/ml gut	-	-	[7]
Polyphagidae							
Polyphaga aegyptiaca (egyptian desert cockroach)	O	CEL	-	-	GHF9 gene	-	[8]
Blattellidae							
Blatella germanica (german cockroach)	O	CEL	-	-	GHF9 gene	-	[8]

Blaberidae							
Blaberus fusca (dwarf cave roach)	O	CEL	FA, MA	1.1, 75 U/ml gut	-	-	[7]
		XYL	FA, MA	0.4, 0.9 U/ml 20 guts	-	-	[7]
Calolampra elegans (cockroach)	D	CEL	GA	531 U/g/h	-	-	[9]
		XYL	FA, MA	380, 397.7 mg glu/mg pr/h	-	-	[9]
Geoscapheus dilatatus (cockroach)	D	CEL	GA	533 U/g/h	-	-	[9]
		XYL	FA, MA	2.1, 1.8 mg glu/mg pr/h	-	-	[9]
Panesthia cribata (australian wood-feeding cockroach)	D	CEL	GA	247 U/mg pr	GHF9 genes	AF220583-AF220585	[10]
		XYL	FA, MA	1.9, 3.38 mg glu/mg pr/h	-	-	[9]
P. angustipennis spadica (cockroach)	D	CEL	-	-	GHF9 genes	AB438950-AB438952	[11]
Pycnoscelus surinamensis (surinam cockroach)	O	CEL	FA, MA	17, 87 U/ml gut		-	[7]
Salganea esakii (cockroach)		CEL	-	-	GHF9 genes	AB438946-AB438948	[11]
Blattidae							
Gromphadorhina portentosa (hissing roach)	D	CEL	FA, MA	116, 220 U/ml gut	-	-	[7]
		XYL	FA, MA	0.3, 0.3 U/ml gut	-	-	[7]
Periplaneta americana (american cockroach)	O	CEL	SGA, FA, MA	5.3, 28.8, 44.1 mU/mg pr	GHF9 genes	AF220586, AF220587	[12] [8]
		LIQ	SGA, FA, MA	13.0, 20.9, 48.6 mU/mg pr	-	-	[12]
		LAM	SGA, FA, MA	20.1, 17.6, 52.4 mU/mg pr	-	-	[12]
		G-LAM	-	-	GHF16 gene	ABR28480	GenBank
		XYL	FA, MA	1, 0.8 U/ml gut	-	-	[7]
P. australasia (australian cockroach)	O	CEL	FA, MA	57, 171 U/ml gut	-	-	[7]
		XYL	FA, MA	1.3, 5.2 U/ml gut	-	-	[7]
Cryptocercidae							
Cryptocercus clevelandi (wood roach)	H (XY)	CEL	-	-	GHF9 genes	AF220588-AF220590	[8]
		XYL	FA, MA	0.4,2.2 U/ml gut	-	-	[7]
ISOPTERA							
Mastotermitidae							
Mastotermes darwiniensis (giant northern termite)	D (XY)	CEL	MA	52 U/ml gut	GHF9 genes	AF220593, AF220594	[13] [8]
		XYL	MA	4.3 U/ml gut	-	-	[7]
Termopsidae							
Hodotermopsis sjoestedti (damp wood termite)	D (XY)	CEL	GA	-	GHF9 genes	AB118794-AB118796	[13]
Kalotermitidae				-			
Neotermes koshuensis (termite)	D (XY)	CEL	EP		GHF9 gene	BAB91145	[14] [13]
Rhinotermitidae							
Coptotermes formosanus (formosan subterranean termite)	D (XY)	CEL	EP	-	GHF9 genes	AY572862,BAB40693-BAB40697	[15]
		XYL	SGA, FA, MA	0.83, 0.95, 21 U/min	-		[16]
Reticulitermes speratus (japanese termite)	D (XY)	CEL	EP	-	GHF9 genes	AB118797-AB118799	[17] [18] [19]
R. flavipes (eastern subterranean termite)	D (XY)	CEL	-	-	GHF9 genes	AB008778, AB019095	[20] [21]
		LAC	SGA+FA	20nm/min/mg pr	-	FL635524, FL639514	[21]
Termitidae							
Odontotermes formosanus (black-winged subterranean t.)	D (XY)	CEL	GA	-	GHF9 genes	AB118800-AB118802	[13]
Nasutitermes takasagoensis (termite)	D (XY)	CEL	EP	-	GHF9 genes	AB013272, AB118803, AB019585	[22] [19] [13]

N. walkeri (niggerhead termite)	D (XY)	CEL			GHF9 gene	AB013273	[19]
Sinocapritermes mushae	D	CEL	GA	-	GHF9 genes	AB118804-AB118806	[13]
DERMAPTERA							
Forficulidae							
Forficula auricularia (european earwig)	O	CEL	GA	0.1 U/mg pr/h	-	-	[5]
HEMIPTERA							
Heteroptera							
Miridae							
Deraeocoris nebulosus (plant bug)	C	PG	SGA, MA	0.74, 0.18 (1/ck)/mg pr	-	-	[23]
Lygus disponsi (plant bug)	H (SS)	PG	SGA, MA	weak and strong	-	-	[24]
		CEL	SGA, MA	<0.35 mg glu/ bug	-	-	[24]
L. saundersi (plant bug)	H (SS)	PG	SGA, MA	weak	-	-	[24]
		CEL	SGA, MA	<0.35 mg glu/ bug	-	-	[24]
L. rugulipennis (plant bug)	H (SS)	PG	SGA, MA	21.33 µm rg/h/2glands	-	-	[25]
		PHO	SGA, MA	-	-	-	[25]
Adelphocoris suturalis (plant bug)	H (SS)	PG	SGA, MA	strong and weak	-	-	[24]
		CEL	SGA, MA	<0.35 mg glu/ bug	-	-	[24]
Orthocephalus funestus (plant bug)	H (SS)	PG	SGA, MA	weak and strong	-	-	[24]
		CEL	SGA, MA	<0.35 mg glu/ bug	-	-	[24]
Pentatomidae							
Palomena angulosa (stink bug)	H (SS)	PG	SGA, MA	strong and slight	-	-	[24]
		CEL	SGA, MA	0.02-0.07 mg glu/ bug	-	-	[24]
Eurydema rugosum (cabbage bug)	H (SS)	PG	MA	slight activity	-	-	[24]
		CEL	SGA, MA	<0.35 mg glu/ bug)	-	-	[24]
Coreidae							
Coreus marginatus (dock bug)	H (LC)	CEL	SGA, MA	0.01-0.14 mg glu/ bug	-	-	[24]
Sternorrhyncha							
Aphididae							
Acyrthosiphon pisum (pea aphid)	H (SS)	CEL	-	-	GHF9 homologous	XM_001944739	Genome
		PG, PME	AP	-	-	-	[26]
		PHO, PO	AP	-	-	-	[26]
Myzas persicae (green peach aphid)	H (SS)	PG, PME	AP	-	-	-	[26]
		PHO, PO	AP	-	-	-	[26]
Schizaphis graminum (greenbug)	H (SS)	PG, PME	AP	-	-	-	[26]
		PHO	AP	-	-	-	[26]
PHTHIRAPTERA							
Pediculidae							
Pediculus humanus humanus (body louse)	P	CEL	-	-	GHF9 homologous	XM_002426420	Genome
COLEOPTERA							
Elateridae							
Pyrophorus divergens (brazilian luminescent click beetle)	H (SE, RO)	CEL	EP	-	-	-	[27]
Tenebronidae							

Tenebrio molitor (mealworm beetle)	H (SP)	G-LAM	-	-	GHF16 gene	ACS36221	GenBank
Tribolium castaneum (red flour beetle)	H (SP)	CEL	-	-	GHF9 homologous	XM_001810641	Genome
Bruchidae							
Callosobruchus maculatus (cowpea weevil)	H (SE)	PG	-	-	GHF28 homologous	CmaE1-37_P02_T3	[28]
Chrysomelidae							
Chrysomela tremulae (poplar leaf beetle)	H (LC)	CEL	-	-	GHF45 homologous	-	[29]
		PG	-	-	GHF28 homologous	FJ654710	[29]
Diabrotica virgifera (western corn rootworm)	H (RO)	EG	-	-	GHF45 homologous	CN498076	[30]
		PG	-	-	GHF28 homologous	EW772675, EW773294, EW774154	[30]
Leptinotarsa decemlineata (Colorado potato beetle)	H (LC)	CEL	-	-	GHF45 homologous	EB756191, EB759034	GenBank
		PG	-	-	GHF28 homologous	EB755504	GenBank
Phaedon cochlearieae (mustard beetle)	H (LC)	CEL	Z	-	GHF45 gene	Y17907	[31]
		PG	Z	-	GHF28 gene	Y17906	[31]
		XYL	Z	-	GHF11 gene	Y17908	[31]
Cerambycidae							
Psacothea hilaris (yellow-spotted longicorn beetle)	H (B-XY, LC)	CEL	EP	150 U/mg pr	GHF5 gene	AB080266	[32] [33]
		PG	GA	11600 U/hr/g weight	-	-	[32]
		XYL	GA	720 U/hr/g weight	-	-	[32]
Hylotrupes bajulus (european house borer)	H (B-XY)	CEL	FA, MA	298, 43 U/ml gut	-	-	[7]
		XYL	FA, MA	4, 1.1 U/ml gut	-	-	[7]
Apriona germari (mulberry longhorn beetle)	H (B-XY, LC)	CEL	EP	992, 812, 1037 U/mg	GHF5, GHF45 genes	AY771358, AY162317, AY451326	[34] [35] [36]
Oncideres albomarginata chamela (twig girdle beetle)	H (B-XY)	CEL	GA	208.13 U/g weight	GHF5, GHF45 genes	GU001941- GU001943	[37]
Rhagium inquisitor (ribbed pine borer)	D (XY)	CEL	GA	380 µg glu/mg pr/h	-	-	[38]
		XYL	GA	1600 µg glu/mg pr/h	-	-	[38]
		LAM	GA, EP	2250 µg glu/mg pr/h	-	-	[39] [38]
		LIQ	GA	1720 µg glu/mg pr/h	-	-	[38]
		PG	GA	4430 µg glu/mg pr/h	-	-	[38]
Ergates faber (longhorn beetle)	D (XY)	CEL	EP	30-157 pU/g gut/ 30 min	-	-	[40]
Curculionidae							
Dendrotocnus ponderosa (mountain pine beetle)	H (B-XY)	CEL	-	-	GHF45 homologous	EZ115483, EZ115796	[41]
		PG	-	-	GHF28 homologous	EZ115239, EZ115279, EZ115849	[41]
					CE8 homologous	EZ115093	[40]
Diaprepes abbreviatus (root weevil)	H (RO)	CEL	-	-	GHF45 homologous	DN200030, DN200477	GenBank
		PG	EP	0.48 U/min/mg pr	GHF28 homologous	DN200807, DN200833	[42] GenBank
Sitophylus oryzae (rice weevil)	H (SP)	PG	EP	-	GHF28 gene	AF207068	[43] [44]
		PME	EP	-	CE8 gene	AY41894	[45] [46]
Ips pini (pine engraver)	H (B-XY)	EG	-	-	GHF45 homologous	CB407674, CB407893, CB408817	[47]
		PG	-	-	GHF28 homologous	CB408126, CB408155, CB409031	[47]
Hypothenemus hampei (coffee berry borer)	H (B-XY)	CEL	-	-	GHF45 homologous	FD662946, FD662948-FD662950	GenBank
		PG	-	-	GHF28 homologous	FD663237	GenBank

MEGALOPTERA							
Sialidae							
Sialis lutaria (alderfly)	C	CEL, CBH	GA	slight activity	-	-	[4]
HYMENOPTERA							
Apidae							
Apis mellifera (honey bee)	H (PO-N)	CEL	-	-	GHF9 homologous	XM_396791	Genome
Pteromalidae							
Nasonia vitripennis (jewel wasp)	P	CEL	-	-	GHF9 homologous	XM_001606404	Genome
LEPIDOPTERA							
Plutellidae							
Plutella xylostella (diamondback moth)	H (LC)	G-LAM	-	-	GHF16 homologous	ACI32820	[48]
Amphisbatidae							
Psilocorsis cryptolechiella (beech leaftier)	H (LC)	CEL, CBH	GA	0.1, 0.1-0.2 U/mg pr/h	-	-	[5]
Sesiidae							
Melittia satyriniformis (squash vine borer moth)	H (B)	CEL	HA	0.05-0.4 U/mg pr/h	-	-	[5]
Synanthedon scitula (dogwood borer)	H (B)	CEL	HA	0.9-2 U/mg pr/h	-	-	[5]
Crambidae							
Diatrea saccharalis (sugarcane borer)	H (B)	G-LAM	-	-	GHF16 homologous	ABR28479	GenBank
Ostrinia nubilalis (european corn borer)	H (B)	G-LAM	-	-	GHF16 homologous	ACI32836	[48]
Saucrobotys futilalis (dogbane Saucrobotys moth)	H (LC)	CEL	GA	0.1 U/mg pr/h	-	-	[5]
Pyralidae							
Galleria mellonella (wax moth)		G-LAM	-	-	GHF16 homologous	CAK22401	GenBank
Lasiocampidae							
Malacosoma americana (eastern tent caterpillar)	H (LC)	CEL	GA	0.15 U/mg pr/h	-	-	[5]
Bombycidae							
Bombyx mori (silkmoth)	H (LC)	G-LAM	-	-	GHF16 gene	ACU57045	[48]
Saturniidae							
Philosamia ricini (eri silkworm)	H (LC)	CEL	GA	2 mg gluc/g tis	-	-	[49]
Arctiidae							
Halysidota tessellaris (banded tussock moth)	H (LC)	CEL	GA	0.1-0.2 U/mg pr/h	-	-	[5]
Noctuidae							
Helicoverpa armigera (cotton bollworm)	H (LC)	G-LAM	-	-	GHF16 gene	ABU98621	[48]
Spodoptera frugiperda (fall armyworm)	H (LC)	G-LAM	-	-	GHF16 homologous	ACS74832, ABR2847	GenBank
S. littoralis (Mediterranean brocade)	H (LC)	G-LAM	-	-	GHF16 homologous	ACI32818	GenBank
Pieridae							
Anthocharis cardamines (orange tip)	H (LC)	G-LAM	-	-	GHF16 homologous	ACI32832	[48]
Delias nigrina (common jezebel)	H (LC)	G-LAM	-	-	GHF16 homologous	ACI32830	[48]
Pieris rapae (cabbage white)	H (LC)	G-LAM	-	-	GHF16 homologous	ACI32824	[48]
TRICHOPTERA							
Polycentropodidae							
Polycentropus flavomaculatus (net-spinning caddis)	D	CEL, CBH	GA	slight activity	-	-	[4]
Limnephilidae							

Halesus sp. (northern casemaker caddisfly)	D	CEL, CBH	GA	slight activity	-	-	[4]
Potamophylax sp. (northern casemaker caddisfly)	H	CEL, CBH	GA	slight activity	-	-	[4]
Pycnopsyche guttifer (northern casemaker caddisfly)	D	XYL, LAM	MA	5.5, 72-149 U/min/mg weight	-	-	[50]
Phryganeidae							
Agrypnia vestita (giant caddisfly)	D	CEL, XYL, LAM	MA	6-9, 5-13, 13-19 U/min/mg weight	-	-	[50]
Phryganea sp. (giant caddisfly)	O	XYL, LAM	MA	3.6, 2-32 U/min/mg weight	-	-	[50]
DIPTERA							
Drosophilidae							
Drosophila melanogaster (fruit fly)	D	CEL	-	-	GHF45 homologous	EC068056, CO334668	GenBank
		PG	-	-	GHF28 homologous	CO335003	GenBank

Feeding habits and herbiviore guilds: D=detritivorous, O=omnivorous, H=herbivore, C=carnivorous, P=parasite; LC=leaf-chewing, XY=xylophagous, SS=sap sucking, SE=seed feeder, RO=root feeder, SP=stored products (e.g. grains) feeder, B=borer, B-XY= wood borer, PO-N= pollen and nectar feeder. **Enzymes:** CEL= celullase (endoglucanase), CBH=cellobiohydrolase, XYL=xylanase, LAM=laminarinase, LIQ=liquenase, G-LAM= β-1, 3-glucanase-laminarinase, PG=endopolygalacturonase, PME=pectin methylesterase, PHO=phenol oxidase, PO=peroxidase. **Biochemical evidence:** GA= enzymatic activity in gut, SGA= enzymatic activity in salivary glands, FA= enzymatic activity in foregut, MA=enzymatic activity in midgut, HA=enzymatic activity in head, EP=enzyme purification, Z=zymogram, U=unit of enzymatic activity expressed as µm of reducing sugars. **Molecular evidence:** GHF=glycosyl hydrolase family. **References:** [1] Zinkler and Götze 1987; [2] Treves and Martin 1994; [3] Monk 1976; [4] Sinsabaugh et al. 1985; [5] Oppert et al. 2010; [6] Kim et al. 2008; [7] Cazemier et al. 1997; [8] Lo et al. 2000; [9] Zhang et al. 1993; [10] Scrivener and Slaytor 1994; [11] Shimada and Maekawa 2008; [12] Genta et al. 2003; [13] Tokuda et al. 2004; [14] Tokuda et al. 2002; [15] Nakashima et al. 2002a; [16] Arakawa et al. 2009; [17] Watanabe et al. 1997, [18] Watanabe et al. 1998; [19] Tokuda et al. 1999; [20] Scharf et al. 2005; [21] Tartar et al. 2009; [22] Tokuda et al. 1997; [23] Boyd et al. 2002; [24] Hori 1975; [25] Laurema et al. 1985; [26] Cherqui and Tjallingii 2000; [27] Colepicolo-Neto et al. 1987; [28] Pedra et al. 2003; [29] Pauchet et al. 2009a; [30] Siegfried et al. 2005; [31] Girard and Jouanin 1999; [32] Scrivener et al. 1997; [33] Sugimura et al. 2003; [34] Lee et al. 2004; [35] Lee et al. 2005; [36] Wei et al. 2006; [37] Calderón-Cortés et al. 2010; [38] Chipoulet and Chararas 1985; [39] Chipoulet and Chararas 1984; [40] Chararas et al 1983; [41] Aw et al. 2010; [42] Doostdar et al. 1997; [43] Shen et al. 1999; [44] Shen et al. 2005; [45] Shen et al. 1996, [46] Shen et al. 2003; [47] Eigenheer et al. 2003; [48] Pauchet et al. 2009b; [49] Pant and Ramana 1989; [50] Martin et al. 1981.

PCW digestion: symbiotic, endogenous or both?

PCW digestion by termite hindgut symbionts has been extensively studied and used as a model to explain PCW digestion in other insects. However, the evidence presented here suggests that there are some insect orders whose PCW digestion has not been studied (e.g., Thysanoptera, Raphidioptera, Mecoptera), and others such as Coleoptera and Lepidoptera that present a high diversity of diets and feeding habits which are not represented by the species studied. Therefore, these results must be cautiously generalized.

The balance of current evidence favors a combination of endogenous and symbiotic enzymes for PCW digestion in termites, wood-feeding cockroaches, Scarabaeidae beetles, Tipulidae flies and likely some Orthoptera species which possess specialized hindguts to maintain populations of symbiotic organisms, as well as those fungus-growing insects such as ants and termites (Macrotermitidae). The participation of enzymes of multiple sources (i.e., insect, protozoans, bacteria and fungi), and with different enzymatic properties which are complementary (endoglucanases from insects, cellobiohydrolases and xylanases from symbionts) seems to be important for the successful feeding on wood and other cellulose-rich diets, due to termites are the insects with the greatest efficiency (74-99%) of cellulose digestion (Prins and Kreulen 1991). However, most insects do not have specialized hindguts (Terra 1990). How can these insects digest PCW present in their diets without the contribution of permanent hindgut symbionts?

One of the explanations that has been proposed is that gut microorganisms (midgut microorganisms and transient hindgut microorganisms) and ingested fungal enzymes accomplish the PCW digestion in most insects (Martin 1983). The evidence presented here, confirms that most of these microorganisms (bacteria and fungi) have (hemi)cellulolytic enzymes. Nevertheless, as mentioned above, it is not clear if these microorganisms contribute significantly to insect nutrient gain, because a great

proportion of the gut microorganisms are ingested with the food and are voided with the feces (transient microorganisms) (Harris 1993, Douglas 1998, Grünwald et al 2010).This leads to the question of differentiation between resident and transient gut microorganisms: resident and stable microorganisms are more likely to represent symbiotic associations, while transient microorganisms may represent commensalisms (Harris 1993). With the exception of the putative symbiotic association between the yeast *P. stipitis* and Passalidae beetles, for which subsociality and proctodeal trophollaxis have an essential role in the vertical transmission of gut symbionts (Suh et al. 2003), the information reviewed here shows no evidence supporting the existence of obligate symbiosis (vertically inherited) between insects and lignocellulolytic microorganisms (e.g., Harris 1993, Broderick et al. 2004, Schloss et al. 2006, Berkov et al. 2007, Grünwald et al. 2010). Furthermore, none of the sequenced genome of the vertically inherited bacterial symbionts associated with mycetocytes showed lignocellulolytic genes (Shigenobu et al. 2000, Nardi et al. 2002, Wu et al. 2006, McCutcheon and Moran 2007, McCutcheon et al. 2009, Sabree et al. 2009). Obligate symbiotic and mutualistic associations have proven to be important to their hosts by: i) provisioning a supplement of nitrogen (e.g., synthesis of essential amino acids, conservation and upgrading of uric acid nitrogen, nitrogen fixation) and critical micronutrients (e.g., vitamins and essential fatty acids) (Genomic analyses: Shigenobu et al. 2000, Wu et al. 2006, McCutcheon and Moran 2007, McCutcheon et al. 2009, Sabree et al. 2009); ii) protecting the host against disease agents (de Vries et al. 2004, Broderick et al. 2004, Oliver et al. 2010); iii) detoxifying secondary plant compounds (Dowd 1989, Douglas 2009). Indeed, Simpson and Raubenheimer (1993) reported that the presence of mycetocyte (obligate) symbionts in insects is associated with nitrogen poor diets. Then, it is possible that the major benefit of the association between gut symbionts and insects may be to improve the nitrogen supply and critical micronutrients rather than the degradation of refractory PCW polymers (Bignell 2000, Nardi et al. 2002).

The alternative explanation lies in endogenous lignocellulolytic ability. Most insects have endogenous enzymes involved in PCW degradation (Table 2). However, insects depending to a major extent on their own enzymatic abilities also have complementary adaptations to assist PCW digestion. For example, omnivorous and detritivorous insects have broad diets (Nalepa et al. 2001), beetles feeding on living wood have long midguts and developmental periods (Haack and Slansky 1987), most herbivores such lepidopterans and grasshoppers extract cell contents (Abe and Higashi 1991, Barbehenn1992, Hochuli 1996). Therefore, these complementary adaptations can explain the widespread presence of insect lignocellulolytic enzymes in insects.

3. EVOLUTION OF PCW DIGESTION IN INSECTS

In his seminal paper, Martin (1991) argued that "independent-symbiotic cellulolytic capacity is an uncommon trait in insects rarely advantageous to possess" because insects are nitrogen rather than carbon limited. In this context, the evolution of cellulose digestion in insects was hypothesized to be through the establishment of symbiotic relationships. Insect-symbiotic interactions have evolved independently in different groups (Martin 1991, Terra and Ferreira 1994, Jones et al. 1999, Mueller and Gerardo 2002, Douglas 2009). Even though there is little evidence about the dating of the origin of most of these insect symbiotic interactions (e.g., 160-280 Mya for hemipteran-obligate symbionts; Moran et al. 1993), the appearance of most modern herbivorous and xylophagous insects (e.g., grasshoppers, cockroaches, termites, hemipterans, beetles) whit obligate symbiotic interactions, is not known until the Mesozoic (early Cretaceous) (Labandeira 2002, Grimaldi and Engel 2005). This situation raises questions such as: How were wood, leaf litter, humus, herbivore dung and other abundant plant materials processed prior to the Cretaceous (Grimaldi and Engel 2005), and what is the most ancient mechanism of PCW digestion in insects?

In searching for answers to these questions, we examined the evolution of the symbiotic-independent mechanism – based on presence/absence of biochemical and/or molecular evidence – on the analysis of a previously reported hexapod phylogeny (Gullan and Cranston 2005) by unordered parsimony character state reconstruction using the software MacClade version 4.0 (Maddison 2002). Data on the biochemical and molecular evidence used to define the "presence" character of endogenous lignocellulolytic enzymes for the analysis are those shown in Table 2. The character state of Entognatha corresponds to the "presence" of endogenous lignocellulolytic enzymes, given that three lignocellulolytic genes have been reported for the springtail *Cryptopygus antarcticus:* two hemicellulases (mannanase: Song et al. 2008; laminarinase: Song et al. 2010) and one cellulase (GenBank: FJ648735).

The results of the analysis show a parsimonious reconstruction (Fig. 1), which optimized the presence of endogenous lignocellulolytic enzymes (symbiotic-independent mechanism) as the ancestral state within Hexapoda. This reconstruction also suggests that symbiotic-independent mechanism for PCW digestion arose once in the ancestor of Insecta, and likely has been lost independently several times, particularly in carnivorous insects (Fig. 1). However, these results must be cautiously interpreted because the "absence character" in most of the cases is due to the absence of evidence more than evidence of absence. This also applies for the reconstruction character within Polyneoptera which is ambiguous (Fig. 1).

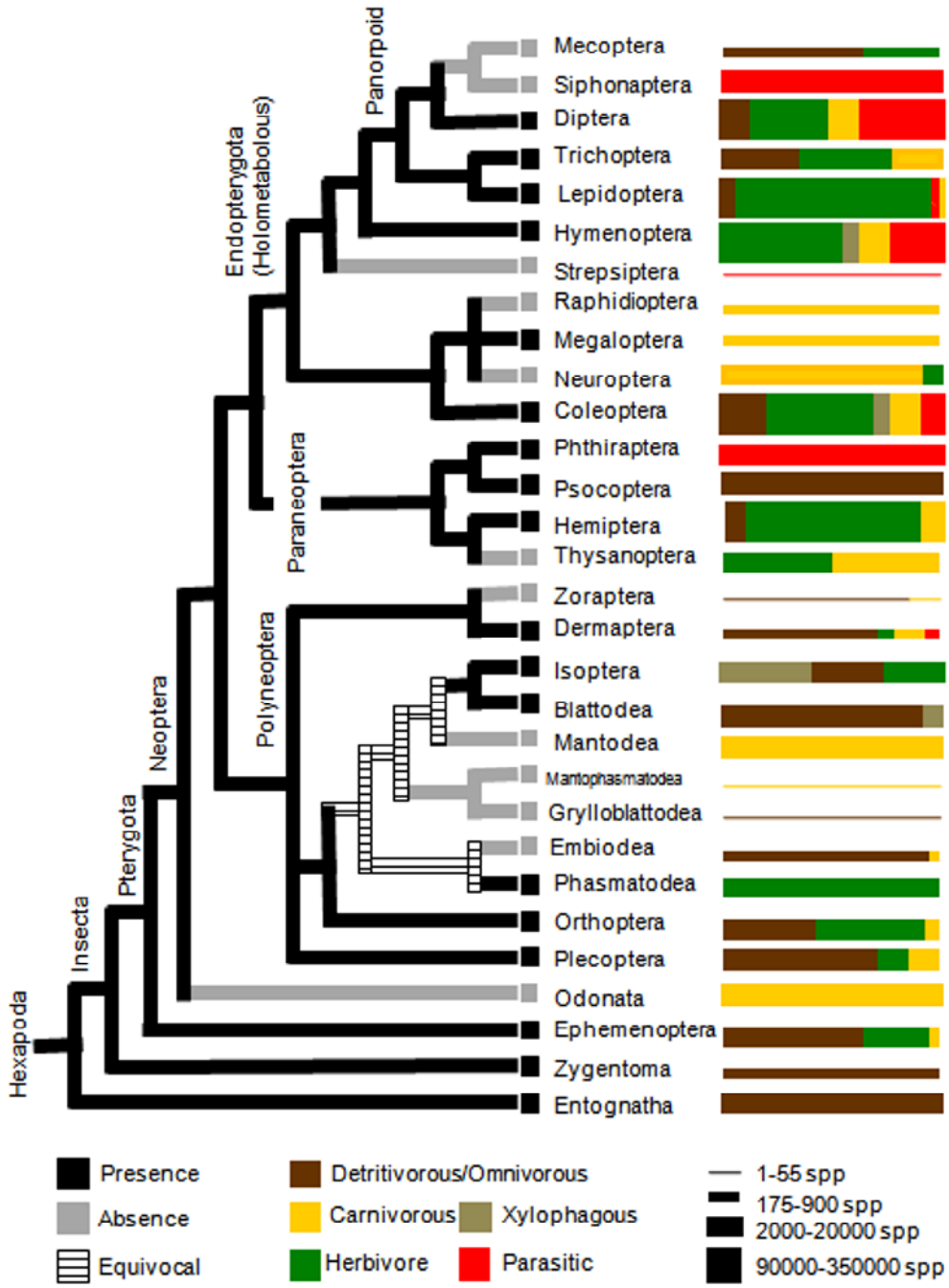

Figure 1. Reconstruction of the evolution of the symbiotic-independent mechanism for plant cell wall digestion in insects based on presence/absence of biochemical and/or molecular evidence data. The analysis was carried out on a previously reported hexapod phylogeny (Gullan and Cranston 2005) by unordered parsimony character state reconstruction using the software MacClade version 4.0 (Maddison 2002). The most parsimonious reconstruction requires nine steps.

The most striking feature in this analysis is the presence of a sequence homologous to cellulases in a member of the parasitic group Phthiraptera (Table 2, Fig. 1), as well as the cellulase and pectinase enzymatic activities in a predaceous plant bug (Hemiptera: Miridae: Table 2). Discordant results between the occurrence of digestive enzymes and feeding habits/diets have been previously reported for insects (e.g., Hori 1975, Monk 1976, Colepicolo-Neto et al. 1987, Ferreira and Terra 1989, Boyd et al. 2002). Based on this, it has been hypothesized that all insects have a full complement of ordinary genes encoding digestive enzymes (Colepicolo-Neto et al. 1987, Terra and Ferreira 1994). Our results are in agreement with this hypothesis, at least for the case of cellulases that are present in 16 insect orders (Table 2) representing all major insect lineages and feeding habits (Fig. 1), and also suggest that a cellulase gene was present in the last common ancestor of hexapods (Fig. 1). Furthermore, endogenous cellulase genes have been reported for Nematoda (GenBank accession numbers: AAC15707, AAC33848, AAC48326, AAC48341, AAC63988, AAD45868, AAD56392- AAD56393, AAK21881-AAK21895, AAK85303, AAN32884, AAN03645-AAN03647, AAM50039, AAP33282, AAR37374, ABY52965, ABZ79356, ABZ78968, ACC77826-ACC77827, ACD12136, ACJ60676, ACM44321- ACM44323, ACO55952, ACP20205, BAB68522-BAB68523, BAD34543- BAD34545), Mollusca (GenBank accession numbers: AAT76428, ABD24274-ABD24274, ABO26608-ABO26609, ABR92637-ABR92638, ACS15341-ACS15350, BAC67186, BAD44734, BAH23793-BAH23794, BAF38757, CAC59695), Annelida (GenBank accession numbers: ACE75511, AAX92641, BAH22180) and Crustacea (GenBank accession numbers: ABA87134, AAD38027, ACY70393, ADB85440-ADB85442, ADE58567-ADE58569), suggesting that cellulase genes indeed, were present in the last common ancestor of bilaterian animals (and likely in the common ancestor of metazoan organisms: see Bachman and McClay 1996, Davison and Blaxter 2005, Calderón-Cortés et al. 2010, Watanabe and Tokuda 2010). This is supported by phylogenetic analyses performed with some of these sequences (i.e., orhtology), as well as the positional identity of introns across some cellulase genes (Lo et al. 2003).

4. EVOLUTONARY TRENDS OF INSECT FEEDING HABITS: FROM DETRITIVORY TO HERBIVORY

The earliest fossil record of a hexapod represented by the collembolan, *Rhyniella praecursor* (Paleozoic: Devonian) was probably detritivorous (Chaloner et al. 1991), like modern springtails (Collembola) and phylogenetically basal insects such as Zygentoma (Fig. 1). This suggests that detritivory might be the ancestral feeding habit for insects. On adapting to omnivorous/detritus feeding, the acquisition or presence of enzymes active against a wide spectrum of substrates (e.g., cellulose, xylan, laminarin, liquenin, callose, curdlan) is certainly a key step (Genta et al. 2003). These enzymes make possible the use of the cell wall polysaccharides present in plants, algae, fungal cells and microbial biomass (usually associated with detritus) as nutrients (Sinsabaugh et al. 1985, Genta et al. 2003). According to this, our review shows that detritivorous and/or omnivorous insects (e.g., the cockroach *Periplaneta americana,* the beetle *Ergates faber,* the caddisfly *Agrypnia vestita*) possess a diverse array of enzymatic activities (cellulases, laminarinases, xylanases and liqueninases: Table 2).

Therefore, if we assume that detritivory/omnivory is the ancestral feeding habit for insects, it is then conceivable that having a full complement of digestive enzymes was an adaptive trait for early evolving insects feeding on broad diets. Moreover, given that detritivores feed mainly on rotting plant material, detritivory could be related to the success of insects living in the Carboniferous (e.g., Gryllidae, Dictyoptera and their ancestors) because: i) as long ago as the Silurian and continuing through the Carboniferous, primary production was overwhelmingly routed through detritivores; ii) detritivores could benefit from large-scale events of mortality of vegetation (e.g., the extinction of primitive vascular plants in the Devonian) and changes in plant species composition that have occurred since the Paleozoic (Nalepa et al. 2001). In this scenario, detritivory played an important role in insect evolution because it is a feeding strategy that prevailed in several modern insect orders (fig. 1),

and more important because detritivory seems to be a prerequisite for the evolution of symbiotic-dependent mechanisms for PCW digestion in insects (Martin 1991, Nalepa et al. 2001, Grimaldi and Engel 2005). For example, detritivorous insects with diverse communities of non-cellulolytic microorganisms in their guts were more likely to evolve symbiont-mediated cellulolytic processes by incidental colonization of hindgut by cellulolytic microorganisms, and consequently they could specialize on diets rich in cellulose (Martin 1991, for details see Nalepa et al. 2001).

The second and most important step in the evolution of PCW-consuming insects was the evolution of herbivory. The extinct palaeodictyopterids and several families of Protorthoptera were the first herbivores. These insects had piercing/sucking and chewing mouthparts, respectively, but it is not clear how they exploited the plant tissues (Grimaldi and Engel 2005). Modern herbivores (Acrididae, Hemiptera, Coleoptera, Hymenoptera, and Lepidoptera) show cellulase, hemicellulase and pectinase activities (Table 2). However, the range of cellulases and hemicellulases activities is lower than that for detritivorous/omnivorous (Table 2). Usually, enzymatic activities change in response to the amount of their substrates in the diet (Colepicolo-Neto et al. 1987, Terra and Ferreira 1994). This change may be the result of the adaptation of a particular diet, leading to activities of some enzymes being permanently higher than activities of others (Terra and Ferreira 1994). Low-moderate cellulase and hemicellulase activities reported for most insect herbivores (Table 2) can be correlated with the evolution of alternative feeding strategies to consume different plant organs or tissues, including behavioral, morphological and physiological adaptations such as: i) the consumption of more nutritious portions of the plants such as fruits, seeds, pollen, nectar and young tissues (Abe and Higashi 1991); ii) the consumption of large amounts of plant tissues (i.e. overcompensation; Hochuli 1996, Chown and Nicolson 2004) and mouthpart adaptations to maximize the efficiency of processing vegetal tissues particularly leaf-chewing insects (Bernays 1991, Barbehenn 1992); and iii) gut physiology adaptations such as high alkalinity to extract PCW polymers and proteins (particularly in Panorpoid insects and

Scarabaeidae beetles; Griffiths and Cheshire1987, Terra 1990, Johnson and Felton 1996).

If insect herbivores display a diverse array of feeding strategies for PCW digestion, the following questions can be addressed: Is the endogenous lignocellulolytic capacity an adaptive trait for insect herbivores? Are insect lignocellulolytic enzymes involved in the evolution of plant-insect interactions? Contrary to the traditional belief, several lines of evidence suggest that endogenous lignocellulolytic capacity is certainly an adaptive trait for herbivores. The presence and activity of pectinases correlates with the herbivorous feeding habit (Table 2), although the importance of each class of enzymes varies across insect guilds. Pectinases represent key enzymes for the adaptation to feeding on tissues rich in primary PCW such as young tissues, fruits and seeds, as well as for piercing-sucking feeding exhibited by most Hemipterans (Table 2). Similarly, cellulases and hemicellulases appear to be key enzymes for herbivorus borers (e.g., some Cerambycid and Curculionidae beetles and some Lepidopterans in Table 2, but see midgut symbiotic yeasts and bacteria section in this review). In other insect guilds such as insects feeding on pollen/nectar and leaf-chewing insects, endogenous lignocellolulytic capacity seems to be less important but still complementary to other feeding and digesting strategies. Leaf-chewing insects (e.g., Acrididae and most Lepidoptera) extract soluble carbohydrates and proteins from cell contents by crushing and snipping leaf tissues (see Bernays 1991 and Barbehenn 1992 for detailed information), and likely by the alkaline gut pH (in the case of lepidopterans) (Terra 1990, Johnson and Felton 1996). Even though, these insects have evolved efficient feeding strategies, cellulase and hemicellulase activities can assist the nutrient extraction process through partial PCW digestion. This can explains the presence of endogenous cellulases and hemicellulases in leaf-chewing insects reviewed here (Table 2). Endogenous cellulases of insects feeding on pollen/nectar, such as bees and most adult lepidopterans (Table 2), might be needed to digest the

cellulose present in the inner wall of pollen grains and improve the release of nutrients present in the pollen (Kunieda et al. 2006).

Interestingly, most of the lignocellulolytic enzymes (particularly β-glycosidases, pectinases, phenoloxidases, peroxidases and β-1, 3-glucanases) in insect herbivores, besides their digestive roles, can play other functions such as detoxifying functions (Terra and Ferreira 1994, Marana et al. 1995, 2000, Ferreira et al. 1997, 1998, Urbanska et al. 1998, Miles 1999, Cherqui and Tjallingii 2000, Pontoh and Low 2002, Byeon et al. 2005, Zagrobelny et al. 2009) and immune-defense responses (i.e. glucan recognition proteins; Fabrick et al. 2003, Jiang et al. 2004, Pauchet et al. 2009b). The appareance of new functions (i.e. neofunctionalization, subfunctionalization) in a gene family can result from gene duplication events, which are frequently associated with a particular adaptive pressure (Zhang 2006, Rastogi and Liberles 2005). Undoubtedly, secondary compounds produced by plants (i.e., "chemical arms race"; Fraenkel 1959, Erlich and Raven 1964) and natural enemies (Jermy 1993, Agrawal 2006) represent important selective pressures for insect herbivores. Therefore, lignocellulolytic genes with new functions, besides representing adaptations for diet specialization, can also represent adaptations to overcome plant toxic compounds and natural enemies. However future sturdies addressing these hypotheses are needed.

Additionally, some ecological studies have demonstrated that β-glucosidases and pectinases are important mediating factors in plant-insect interactions because some of the products of the activity of these enzymes elicit signaling pathways that activate both, direct (e.g., the synthesis of toxic β-glycosides, protein inhibitors: Orozco-Cardenas and Ryan 1999; Ma *et al.* 1998; Cherqui and Tjallingii 2000; Orozco-Cardenas et al. 2001, Jones et al. 2002, Marana et al. 2000, Zhu-Salzman et al. 2005) and indirect plant defense responses (e.g., the release of terpenoid volatile blends that attract natural enemies: Mattiacci et al. 1995, Kessler and Baldwin 2002).

Overall this evidence supports the hypothesis that insect lignocellulolytic genes could play an important role in the evolution of plant-insect interactions.

FUTURE DIRECTIONS

The last decade has seen important advances in our understanding of insect PCW digestion. The evidence summarized and the results of this study suggest that the symbiotic-independent mechanism is the ancestral mechanism for PCW digestion in insects. However, much information is still needed to confirm this evolutionary scenario. Evidence for several insect orders is available only for one species, whereas others (e.g. Odonata, Embioidea, Mecoptera) have not been studied. Some feeding habits such as mining and galling has not been studied neither. This emphasized the need for studies that systematically analyze the presence of lignocellulolytic enzymes and genes in insects. The genomic approaches such as transcriptome and metagenome projects recently adopted to explore the nutritional capabilities of symbionts and insects, have demonstrated to be powerful tools that generate a large amount of information, which will update the field of PCW digestion in insects. However, the analysis of lignocellulolytic enzymes (i.e. enzymatic and structural properties) remains as a central issue in the study of PCW digestion in insects. Other important unresolved issues are related to the putative neofuctionalization of lignocellulolytic genes and the ecological importance of lignocellulolytic genes in plant-insect interactions.

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Discusión general

En un "mundo verde" dominado por las plantas, la base de la energía está almacenada en los tejidos vegetales, esencialmente en la lignocelulosa (celulosa, hemicelulosas y lignina), la cual representa el recurso renovable más abundante del planeta (>1.3 x 10^{10} toneladas al año; Demain et al. 2005). En consecuencia, el flujo de energía en los ecosistemas depende de las interacciones entre las plantas y los organismos que las consumen (Agrawal 2006). Las plantas y los insectos herbívoros constituyen más de la mitad de todas las especies terrestres conocidas (Farrel et al. 1992). De ahí que sus interacciones podrían estar involucradas en el origen y mantenimiento de la mayor parte de la diversidad biológica terrestre (Ehrlich y Raven 1964).

Un aspecto reconocido en las últimas décadas, de las interacciones planta-insecto, es que los cambios de las propiedades estructurales y/o químicas de las plantas inducidos o llevados a cabo por un organismo herbívoro (interacciones indirectas), pueden ser de fundamental importancia en el establecimiento de interacciones ecológicas y evolutivas con otros miembros de la comunidad (revisado en Ohgushi et al. 2007). Desde esta perspectiva, la estructura y diversidad de las comunidades está regulada por "redes de interacciones bióticas", que no pueden ser predichas por el estudio de interacciones aisladas entre pares de especies (Thompson 1996, Strauss e Irwin 2004, Ohgushi 2005). Por lo que las consecuencias ecológicas y evolutivas de las interacciones, requiere del estudio de dichas redes de interacciones bióticas.

Uno de los objetivos generales del presente trabajo, fue estudiar las interacciones bióticas que resultan de la interacción *Spondias purpurea-Oncideres albomarginata chamela*, específicamente del proceso de ingeniería natural del ecosistema (física y bioquímica) por el escarabajo barrenador *O. albomarginata chamela*. Para ello, primero se analizó la interacción *O. albomaginata chamela-Spondias purpurea* desde la perspectiva del insecto con un enfoque centrado en la nutrición/alimentación, en donde se estudió el uso del tejido de la planta hospedera por parte del insecto (Cap. 1). Particularmente, se analizó la capacidad de las larvas de *O. albomarginata chamela* para digerir la celulosa (uno de los principales constituyentes de los tejidos vegetales). Los

resultados obtenidos demostraron que *O. albomarginata chamela* posee un mecanismo endógeno de degradación de celulosa, que le permite usar tejidos pobres en nutrientes por su recalcitrancia a la digestión (como el xilema de la madera) como fuente de alimento. Este mecanismo de digestión se lleva a cabo mediante la acción de enzimas líticas como las celulasas, codificadas por genes que se encuentran presentes en el genoma de *O. albomarginata chamela* y que pueden representar una adaptación especializada con importantes ventajas como el uso de tejidos (i.e. pobres en nutrientes) que se encuentran sujetos a una menor competencia (Haack y Slansky 1987).

Posteriormente, se analizó el papel de *O. albomarginata chamela* como ingeniero natural del ecosistema mediante un experimento de campo en el que se manipuló el trabajo físico que realizan las hembras adultas de esta especie (Cap. 2). Los resultados de este capítulo demostraron que *O. albomarginata chamela* desempeña un papel ecológico importante como ingeniero natural del ecosistema, debido a que el proceso de transformación que realiza sobre las ramas de su árbol hospedero tuvo efectos positivos significativos en la frecuencia de colonización de ramas, abundancia y riqueza de especies de la comunidad de colonizadores secundarios, que incluye otros insectos barrenadores, depredadores y parasitoides. Estos efectos positivos estuvieron relacionados principalmente a la creación de hábitats con una mayor disponibilidad de sitios de oviposición para los colonizadores secundarios (ingeniería física), y a la presencia del ingeniero que podría estar a su vez relacionada con la capacidad metabólica de degradación de madera (ingeniería bioquímica). Está última hipótesis se sometió a prueba con un análisis de las enzimas digestivas (celulasas y xilanasas) involucradas en la degradación de los principales polisacáridos estructurales de la madera de seis especies simpátricas de cerambicídos: *O. albomarginata chamela, Ataxia alpha, Estoloides chamelae, Lissonotus flavocinctus,* Cerambycinae sp. y Lamiinae sp. (Cap. 3). Este análisis, indicó que *O. albomarginata chamela* es la única especie que posee la capacidad de transformar los polímeros estructurales de la madera de las ramas *S. purpurea* en azúcares simples que podrían ser usados por algunas de las especies que no presentan la capacidad de degradar madera, lo cual explica el efecto positivo de la

presencia de *O. albomarginata chamela* sobre la riqueza de especies o ingeniería bioquímica del ecosistema.

En general, los resultados obtenidos en el presente trabajo (cap. 1, 2 y 3), proveen evidencia que apoya que la modificación de las propiedades físicas, estructurales y nutritivas (ingeniería física y bioquímica del ecosistema) de las ramas de *S. purpurea,* realizada por *O. albomarginata chamela* es un proceso clave que desencadena una cascada compleja de interacciones bióticas (tanto directas como indirectas), que resultan en efectos positivos sobre la diversidad de artrópodos. La integración de los resultados obtenidos en un contexto general se muestra en la figura 1 y se discute a continuación.

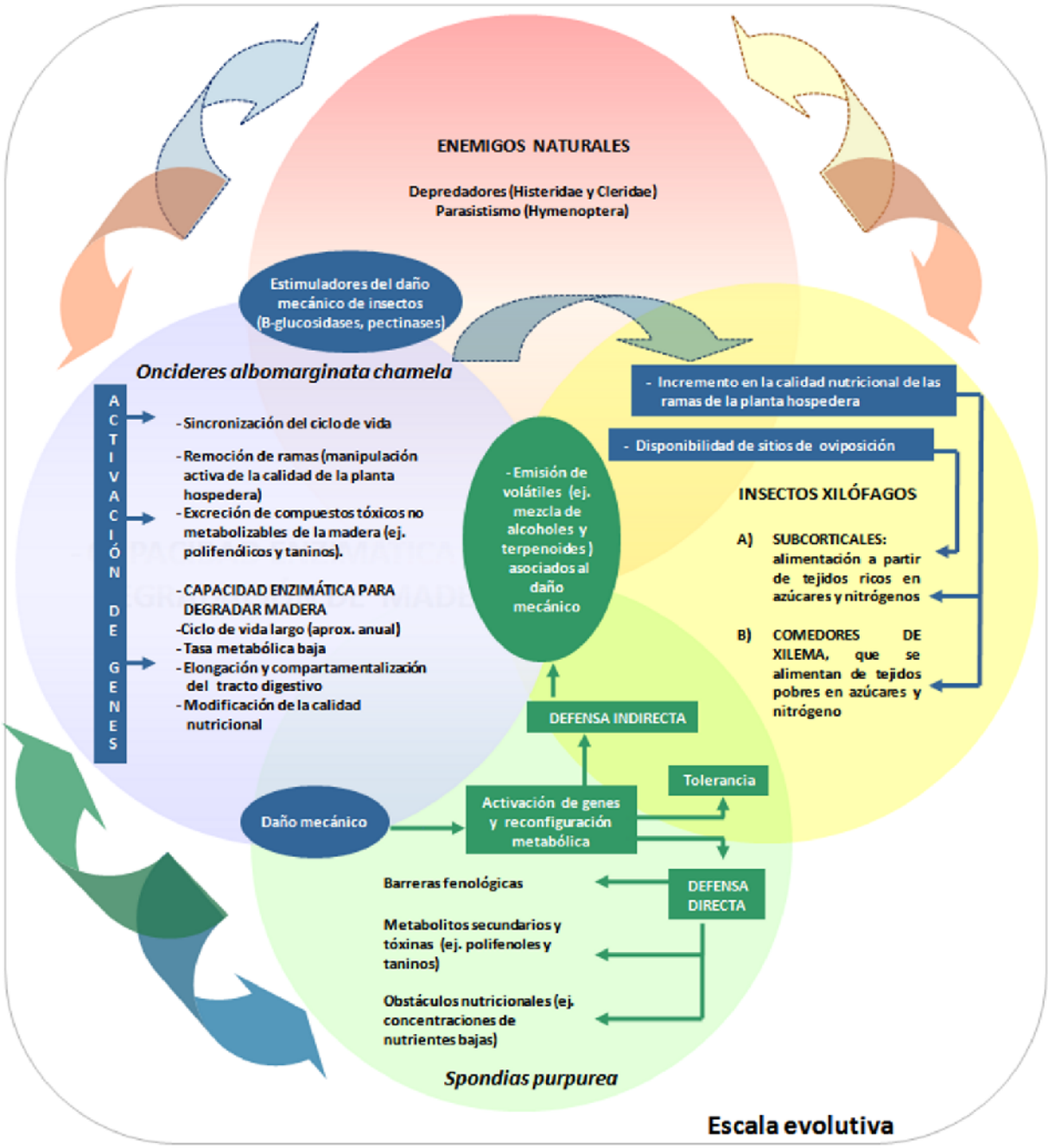

Figura 1. Interacciones bióticas mediadas por el proceso de ingeniería natural del ecosistema realizado por el escarabajo barrenador *Oncideres albomarginata chamela.* Los óvalos muestran las señales involucradas en el establecimiento de la interacción entre los grupos; las flechas sólidas indican interacciones directas; las flechas punteadas indican las interacciones indirectas.

S. purpurea comúnmente conocido como ciruelo, es un árbol tropical que pertenece a la familia Anacardiaceae que incluye un gran número de especies tóxicas (Mitchell 1990). *S. purpurea* produce taninos, fenoles y resinas, compuestos tóxicos que han sido asociados a las respuestas de defensa de la planta contra el ataque de insectos (Maldonado 2007). *S. purpurea* es además un árbol caducifolio que presenta un período de producción de hojas de Junio-Diciembre, un período de floración de Enero-Marzo y un período de producción de frutos de Febrero a Junio (Bullock y Solís-Magallanes 1990, Bullock 1994). Esta marcada variación en la fenología puede representar a su vez barreras fenológicas para la actividad de los insectos herbívoros asociados a *S. purpurea,* entre los cuales se encuentra el barrenador de ramas *O. albomarginata chamela* (Uribe-Mú y Quesada 2006).

O. albomarginata chamela es un herbívoro especialista que remueve ramas de *S. purpurea* en las cuales oviposita y desarrolla su progenie (Uribe-Mú y Quesada 2006). Una consecuencia importante de remover las ramas del árbol es que con ello evita las respuestas de defensa de la planta al interrumpir el flujo de savia (Forcella 1982). La remoción de ramas que representa un mecanismo de manipulación activa de la planta hospedera, es considerado uno de los mecanismos más especializados que exhiben los insectos herbívoros para confrontar las respuestas de defensa de las plantas (Karban y Agrawal 2002). Por lo tanto, este comportamiento puede representar una adaptación importante de *O. albomarginata chamela* para evadir las respuestas de defensa de *S. purpurea.* Sin embargo, al interrumpir el flujo de savia de la planta, las larvas de *O. albomarginata chamela* deben enfrentarse a la falta de suministro de azúcares solubles y proteínas en el tejido que se desarrollan, lo cual impone obstáculos nutricionales para su desarrollo. En este sentido, *O. albomarginata chamela* al remover las ramas fértiles de *S. purpurea* durante la época seca (una vez que el árbol ha tirado las hojas y reasignado los recursos) cuando las ramas han acumulado la máxima concentración de carbohidratos no-estructurales (Bullock 1992) y nitrógeno (Uribe-Mú and Quesada 2006), incrementa la calidad nutricional de las ramas (Forcella 1982). Aunado a esto, *O. albomarginata chamela* presenta otras adaptaciones morfológicas y fisiológicas como la

capacidad enzimática apropiada para la digestión de madera (Cap. 1 y 3), la elongación y compartamentalización del tracto digestivo y tasas metabólicas bajas, que le permiten vencer los obstáculos nutricionales de la planta y completar su desarrollo en las ramas removidas (Haack y Slansky 1987). Otra manipulación importante, está representada por la capacidad de las hembras de *O. albomarginata chamela* para realizar numerosas incisiones a lo largo de la rama donde depositan los huevos, debido que estas incisiones constituyen sitios potenciales de oviposición para otras especies de insectos barrenadores que ovipositan de manera oportunista en las grietas y huecos naturales presentes en la corteza de las plantas (Hanks 1999, Lieutier et al. 2004). En conjunto, todas las adaptaciones mencionadas tienen consecuencias importantes tanto en el establecimiento de la interacción *O. albomarginata chamela-S. purpurea* como en el establecimiento de las interacciones insecto-insecto (mediadas por el proceso de ingeniería natural del ecosistema; cap. 2 y 3) entre más de 25 especies de 8 familias de insectos, que incluyen especies de escarabajos xilófagos secundarios (subcorticales y del xilema), escarabajos depredadores y avispas parasitoides (cap. 2). Estos insectos (xilófagos secundarios y enemigos naturales) podrían ser atraídos por compuestos volátiles (ej. mezcla de alcoholes y terpenos) emitidos por *S. purpurea* durante el ataque de *O. albomarginata chamela* (Allison et al. 2004), dado que los colonizadores secundarios arriban a los árboles de *S. purpurea* cuando *O. albomarginata chamela* está realizando el trabajo de remoción de ramas y oviposición.

El principal factor involucrado en el establecimiento de estas interacciones insecto-insecto es la ingeniería natural del ecosistema llevada a cabo por *O. albomarginata chamela,* debido a que el establecimiento de la comunidad entera depende de este proceso de creación de hábitats con una mayor disponibilidad de sitios de oviposición y nutrientes (Cap. 2). Este proceso fue responsable del 95% de la abundancia de los insectos y del 82% de la riqueza de especies de la comunidad. En general, estos resultados son consistentes con los efectos positivos sobre la diversidad de artrópodos reportados para otros insectos que funcionan como ingenieros naturales del

ecosistema (Martinsen et al. 2000, Bailey y Whitham 2003, Lill y Marquis 2003, Kagata y Ohgushi 2004).

Como se muestra en la figura 1 la ingeniería del ecosistema llevada a cabo por *O. albomarginata chamela* desencadena un gran número de interacciones (efecto "bottom-up") con otros miembros de la comunidad que incluyen interacciones directas e indirectas (Price et al. 1986, Damman 1993). Esto se debe a que los cambios causados por el ingeniero del ecosistema en una parte de la planta pueden incrementar la heterogeneidad de los recursos como el alimento, sitios de oviposición y hábitats, y con ello la riqueza y abundancia de especies (presas potenciales), que finalmente repercuten la abundancia de depredadores que pueden agregar nuevas interacciones al sistema (Ohgushi 2008). Los efectos "bottom-up" iniciados por herbívoros son comunes en los sistemas terrestres (Dickson y Whitham 1996, Fernandes et al. 1999, Sipura 1999, Kagata et al. 2005, Rodríguez-Saona et al. 2005, Nakamura et al. 2006, Kaplan y Denno 2007, Bukovinszky et al. 2008, Evans 2008). Sin embargo, pocos estudios han demostrado efectos "bottom-up" iniciados por insectos herbívoros ingenieros del ecosistema (Martinsen et al. 2000, Bailey y Whitham 2003).

Entre las interacciones directas (competencia, depredación y parasitismo) asociadas a la ingeniería del ecosistema por *O. albomarginata chamela*, es probable que la competencia directa (por explotación *sensu* Damman 1993) de recursos se presente con una menor intensidad, debido a que las larvas de los escarabajos xilófagos secundarios ocupan diferentes partes de las ramas (ej. tejidos subcorticales y/o xilema) (cap. 2 y 3), y emergen en diferentes momentos a lo largo del año (cap. 2), lo cual sugiere la existencia de un reparto tanto temporal como espacial de los recursos que puede reducir la intensidad de la competencia (Tilman, 1982, Morin 1999). Esto concuerda con la hipótesis general de que la competencia interespecífica directa no es un factor principal que estructura las comunidades de insectos herbívoros (Fritz 1995, Ohgushi 2005, pero ver Damman 1993). No obstante, la competencia indirecta (por interferencia *sensu* Damman 1993) puede tener un efecto importante en la comunidad de

insectos secundarios en ramas de *S. purpurea,* debido a que las primeras especies que emergen (y que son las más abundantes) pueden agotar los recursos para las especies que emergen después.

Otras interacciones que pueden tener efectos importantes en la comunidad de insectos asociada a la ingeniería del ecosistema por *O. albomarginata chamela*, son las interacciones mediadas por enemigos naturales (tanto directas como indirectas). Los escarabajos *Teretriosoma nigrescens* (Histeridae) y *Enoclerus quadrisignatus* (Cleridae) que emergen de las ramas de *S. purpurea,* son depredadores que controlan las poblaciones naturales de sus presas (Bostrichida, Buprestidae y Scolytidae), por lo que potencialmente pueden controlar la abundancia y regular de manera indirecta la competencia entre las especies de escarabajos que coexisten en las ramas de *S. purpurea.* Estas interacciones en complejos multi-especies, han sido reportadas como uno de los principales factores que estructuran las comunidades de insectos herbívoros (Price et al. 1986, Damman 1993, Fritz 1995, Stamp 2001, Ohgushi 2005, 2008, Denno y Kaplan 2007, Bukovinszky et al. 2008). Por lo tanto, un reto importante para estudios futuros es estudiar los efectos de las interacciones mediadas por enemigos naturales, así como distinguir los efectos de las interacciones indirectas de los efectos puramente tróficos.

Desde esta perspectiva, las comunidades representan redes de interacciones complejas que resultan tanto de procesos ecológicos, como de procesos evolutivos (Strauss e Irwin 2004). Actualmente existen dos propuestas con enfoques diferentes (pero ambas relacionadas con el concepto de fenotipo extendido) para integrar estos procesos en una escala de comunidad: la genética de comunidades y la herencia ecológica. La genética de comunidades vincula la variación genética de una especie (generalmente plantas), llamada fundadora, en la composición de la comunidad asociada a esta, partiendo de la hipótesis que el genotipo de una especie fundadora tiene un efecto sobre la adecuación del resto de las especies en la comunidad (Antonovics 2003, Cavender-Bares y Wilczek 2003, Neuhauser et al. 2003, Whitham et al. 2003, 2008). Por su parte, la herencia ecológica, estudia el efecto de las modificaciones del ambiente

realizadas por los ingenieros del ecosistema en la evolución de los organismos que usan los hábitats modificados (Odling-Smee et al. 1996, pero ver Dawkins 2004). Este proceso evolutivo sólo se presenta en aquellos casos en los que la ingeniería de ecosistemas tiene un efecto en las generaciones subsecuentes de los organismos que usan los hábitats modificados (Odling-Smee et al. 1996). En estudios previos (Uribe-Mú 2006, Uribe-Mú y Quesada 2006) se encontró que la variación individual en el contenido de nitrógeno tuvo un efecto significativo sobre la proporción de ramas de *S. purpurea* que remueve *O. albomarginata chamela.* Por lo tanto, es posible que la variación genética de las poblaciones de *S. purpurea* tenga un efecto sobre los niveles de herbivoría de *O. albomarginata chamela*, y por ende en el éxito reproductivo de esta especie, lo cual afectaría a su vez a la comunidad de insectos asociada a las ramas que remueve *O. albomarginata chamela*. En cuanto a la herencia ecológica, varias líneas de evidencia sugieren que la ingeniería natural del ecosistema tiene un efecto sobre la evolución de la comunidad de insectos que usan el hábitat modificado por *O. albomarginata chamela*: estas interacciones son estables a lo largo del tiempo (cap. 2); los insectos asociados también usan las plantas hospederas alternas de *O. albomarginata chamela* (Equihua y Atkinson 1986, Chemsak y Noguera 1993); y estas interacciones pueden ser estables en una escala espacial, puesto que algunas de las especies asociadas a la ingeniería del ecosistema por *O. albomarginata chamela* usan ramas removidas por otras especies de cerambícidos cercanamente relacionadas a *O. albomarginata chamela* en diferentes regiones tropicales y subtropicales del mundo (Polk y Ueckert 1973, Hovore y Penrose 1982, Ramírez-Martínez et al. 1994, Feller y Mathis 1997). Estudios futuros que confirmen estás hipótesis son necesarios.

Por lo anterior, este trabajo tiene un aporte importante en el estudio de interacciones de complejos multi-especies incorporando tanto las interacciones directas (tróficas) como las interacciones indirectas. Esta propuesta de estudios ofrece una nueva perspectiva para el estudio de la evolución de las interacciones en las comunidades, y de cómo se origina y mantiene la biodiversidad en los ecosistemas terrestres (Thompson 1996, Bruno et al. 2003, Ohgushi 2005, 2008, Ohgushi et al. 2007).

Otro aporte importante del presente trabajo está relacionado con la evolución de la capacidad de los insectos para degradar tejidos vegetales. Durante más de un siglo, se ha creído que los animales no pueden digerir la lignocelulosa que constituye los tejidos vegetales sin el establecimiento de relaciones simbióticas con microorganismos como bacterias, hongos y protozoarios, debido a que los animales no son capaces de producir sus propias enzimas líticas (Cleveland 1924). Sin embargo, en la última década se ha comprobado a nivel genético que algunas de estas enzimas están presentes en el genoma de diversos animales invertebrados, como nematodos, anélidos, moluscos y artrópodos, incluidos los insectos (revisado en Cap. 4), tal como se demostró en el capítulo 1 del presente trabajo. Esta nueva información ha generado un debate interesante en la literatura científica sobre si estos genes han sido adquiridos recientemente a través de procesos como la transferencia horizontal de genes (Yan et al. 1998, Davis et al. 2000, Scholl et al. 2003, Kikuchi et al. 2004, Kyndt et al. 2008, Sakamoto y Toyohara 2009), o si fueron transmitidos verticalmente a partir de un ancestro común de los animales invertebrados (Bachman y McClay 1996, Watanabe y Tokuda 2001, 2010; Lo et al. 2003, Davison y Blaxter 2005). Este debate hizo evidente una pregunta fundamental ¿cómo han evolucionado los diferentes mecanismos que los animales utilizan para la degradación de lignocelulosa? Actualmente existe considerable literatura sobre la digestión de tejidos vegetales en insectos que permite empezar a explorar esta pregunta desde una perspectiva filogenética. Por lo tanto, el segundo objetivo general del presente trabajo, fue analizar la evolución de los genes de celulasas en los animales (Cap.1) y de los mecanismos involucrados en la degradación de pared celular vegetal en insectos (Cap. 4). Para analizar la evolución de los genes de los insectos, se realizaron análisis filogenéticos de las proteínas codificadas por los genes de celulasas de *O. albomarginata chamela* y otros genes de origen animal como los nemátodos, moluscos y otros escarabajos, con los genes reportados para bacterias, hongos, protozoarios. Los resultados obtenidos (Cap. 1) indican que las familias de genes a las que pertenecen los genes de celulasas de *O. albomarginata chamela* (GH5 y GH45) incluyen genes ancestrales para los organismos metazoarios. En el caso de la familia GH5 los análisis

filogenéticos sugieren que al menos un gen estuvo presente en el ancestro común de los animales invertebrados (Cap. 1), mientras que para la familia GH45, el número limitado de secuencias de genes disponibles no permite realizar conclusiones rigurosas sobre el origen y evolución de estos genes (ver discusión Cap. 1). Estos resultados enfatizan la necesidad de realizar estudios que analicen sistemáticamente (por lo menos una especie representante de cada orden) la presencia de genes y enzimas lignocelulolíticas en los animales invertebrados.

Por otra parte, para analizar la evolución de mecanismos involucrados en la degradación de pared celular vegetal en insectos, se realizó una revisión de la literatura sobre la digestión de tejidos vegetales en insectos (Cap. 4). Está revisión reveló que la capacidad endógena o mecanismo libre de simbiontes está presente en 16 órdenes de insectos incluidos los insectos vivos más ancestrales (Zygentoma), así como los parientes más cercanos de los insectos (colémbolos y crustáceos). Con base en esta información, se realizó un análisis de reconstrucción de caracteres ancestrales, que indicó que el mecanismo independiente de simbiontes es el carácter ancestral para los insectos, y probablemente uno de los factores relacionados con la evolución y diversificación de los insectos. Los resultados obtenidos en estos capítulos representan un aporte relevante para la ciencia, porque aportan nueva información en el campo altamente debatido del estudio de la digestión de tejidos vegetales por los insectos y animales en general. Además, la revisión del capítulo 4 representa el primer análisis de la evolución de la capacidad de degradación de pared de celular vegetal en insectos con una base filogenética, donde se discuten las implicaciones de esta capacidad endógena en la evolución y diversificación de los insectos. Sin embargo, aún se requiere mucha información para confirmar este escenario evolutivo, particularmente para aquellos grupos de insectos que no se han estudiado.

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Apéndice 1

DESCRIPCIÓN DE TÉCNICAS USADAS EN EL CAPÍTULO 1

A1.1 Extracción de DNA por el método de CTAB-polivinilpirrolidona (Chen y Ronald 1999)

Moler 0.5 g de tejido en un mortero con nitrógeno líquido hasta obtener un polvo muy fino. Colocar el tejido pulverizado en un microtubo 1.5 ml estéril con 700 µl buffer de extracción (CTAB 2%, NaCl 1.42M, EDTA pH 8.0 0.02M, Tris.HCl pH 8 0.2M, PVP-40 2-10%, β-mercaptoetanol 1%) precalentado a 65°C, mezclar suavemente por inversión, e incubar 10 min a 65°C con inversiones ocasionales. Agregar 570 µl cloroformo-isoamílico (24:1), mezclar nuevamente por inversión y centrífugar a13 000 rpm durante 10 min para separar las fases. Después de la centrifugación, el homogeneizado se separa en tres fases, una inferior que corresponde al cloroformo-isoamílico, una intermedia con los restos celulares y por último una fase superficial acuosa (en la cual se encuentran los ácidos nucleicos) que se remueve a un microtubo estéril cuidando no llevarse fase orgánica. Agregar un volumen igual de isopropanol frío a cada tubo y se mezclar por inversión. Precipitar el DNA a –20°C durante 1 hora, y posteriormente centrifugar a 13,000 rpm durante 5-8 min para formar una pastilla que contiene el DNA. Decantar el sobrenadante y lavar la pastilla formada con etanol al 70% (frío), secar hasta evaporación del etanol y resuspender la pastilla en 100 µl de agua destilada desionizada estéril (dde).

A1.2 Extracción de DNA a partir de patas mediante técnica de Fet et al. (2000).

Moler las patas de un individuo en un mortero con Nitrógeno líquido hasta obetener un polvo muy fino, y cuidando que el tejido no se descongele. Colocar el tejido pulverizado en un microtubo 1.5 ml estéril que contenga 900 µl de Buffer Lifton [Tris.HCl 0.1M, SDS 1%, EDTA 0.05M, aforado con sacarosa 0.2M hasta alcanzar el volumen final], mezclar por inversión e incubar en hielo por 20 min. Agregar 100 µl de

acetato de potasio 8M, mezclar por inversión y colocar en hielo 15 min. Centrifugar a 13 000rpm 15 min y transferir la fase superior (DNA en solución) a un microtubo estéril. Agregar 100 µl de cloroformo:isoamílico (24:1) y aforar a 1.5 ml con fenol, incubar 5 min en hielo y centrifugar 5 min a 13 000 rpm. Recuperar la fase acuosa superior a un microtubo 1.5 ml estéril y aforar a 1.5 ml con cloroforo:isoamilico (24:1), centrifugar 5 min a 13 000 rpm y transferir nuevamente la fase superior a un microtubo estéril. Precipitar el DNA adicionando 100 µl de acetato de amonio 10M y un volumen de etanol absoluto frío (-20°C) e incubar a -20°C 1 hora o toda la noche. Empastillar el DNA centrifugando a 13 000 rpm durante 30 min, decantar el sobrenandante cuidadosamente para que no se desprenda la pastilla de DNA, y lavar la pastilla con etanol al 70% frío, secar la pastilla y resuspender en agua dde.

A1.3 Eliminación de RNA en preparaciones de DNA

Agregar 3 µl de RNAasa libre de DNAasas (10mg/ml) a la muestra que contiene DNA. Incubar a 37°C durante 1 hora. Remover la proteína de la muestra mediante una limpieza con fenol-cloroformo (Sambrook y Russell 2001): agregar un volumen de fenol:cloroformo (25:25), mezclar por inversión y centrifugar a 12 000 rpm por 5 min; recuperar la fase superior en microtubo 1.5 ml estéril y precipitar con 0.2 volúmenes de acetato de amonio 10M y un volumen de etanol absoluto frío (-20°C); mezclar por inversión e incubar a -85°C 30 min (o toda la noche a -20°C); centrifugar a 13 000 rpm 15 min; secar pastilla de DNA y resuspender en agua dde.

A1.4 Diálisis de DNA

Agregar agua dd estéril en un recipiente estéril de 10ml (por ejemplo, caja de petri). Colocar una membrana de fibra de vidrio de 0.025µm (Milipore Type US) sobre el

agua, cuidando que no se sumerja. Pipetear gotas (10 µl) de DNA sobre la membrana de vidrio y esperar 15-30 min hasta que las moléculas pequeñas de sales y otros contaminantes se hayan eliminado de la muestra de DNA (por difusión).

A1.5 Electroforesis y determinación de concentración de DNA

Para revisar la integridad de las muestras de DNA obtenidas aplicando cada una de las técnicas, separar las muestras por electroforesis en una cámara de electroforesis (BIO-RAD) utilizando buffer de carga tipo III 6X (Bromofenol 0.25%, xilen cianol FF 0.25%, glicerol 30%) en gel de agarosa 1.0 % teñido con bromuro de etidio (10mg/ml), en buffer de corrida TAE (Tris-acetato 0.04 M, EDTA 0.001M pH 8) (Sambrook y Russell 2001).

Diluir 1-5 µl de DNA en un 1 ml de agua dd. estéril. Calcular la concentración de DNA en un espectrofotómetro con luz ultravioleta a partir de la lectura de absorbancia a una longitud de onda de 260 nm (Spectrophotometer Perkin-Elmer), en base a la siguiente fórmula:

$$[\text{ADN}] = \frac{(\text{DO } 260\text{nm})\ (50)\ (\text{FD})}{1\ 000}$$

Donde: DO 260 nm = absorbancia o densidad óptica a 260 nm; FD = factor de dilución

A1.6 Extracción de mRNA por el sistema comercial QuickPrep Micro mRNA Purification Kit (Amersham Bioscience)

A) Preparación de tejido

Incubar en un microtubo de 1.5 ml 500 µl de Buffer de Elución y mantenerlo a 65°C hasta usarse. Agregar 400-600 µl de Buffer de extracción en microtubo 1.5 estéril. Colocar el tejido (0.1 g) y homogenizarlo en el buffer de extracción. Centrifugar a 15 000 rpm por 5 min a 4°C para empastillar los restos celulares. Recuperar el sobrenandante y diluirlo en 2 volúmenes del Buffer de Elución (temperatura ambiente).

B) Preparación de columna de oligo(dT)-celulosa

Colocar 1 ml de oligo(dT)-celulosa en un microtubo de 1.5 ml estéril, y centrifugar a 13000 rpm por 1 min. Aspirar el sobrenadante y descartarlo.

C) Purificación de mRNA

Agregar el tejido homogeneizado en el Buffer de Extracción y Buffer de Elución (1.5 ml aprox.) a la pastilla de oligo(dT)-celulosa. Mezclar por inversión durante 3 min, centrifugar 10 seg a 10 000 rpm y descartar sobrenandante. Lavar 5 veces con 1 ml de Buffer High Salt: mezclar por inversión 1 min, centrifugar 1 seg y aspirar sobrenadante. Posteriormente, lavar 2 veces con 1 ml de Buffer Low Salt: mezclar por inversión 1 min, centrifugar 1 seg y aspirar sobrenadante. Resuspender la pastilla de oligo(dT)-celulosa en 300 µl de Buffer Low Salt y trasferir la solución a la columna "microspin". Centrifugar la columna a 10 000 rm por 10 seg y descartar lo que fluye. Lavar 3 veces con 500 µl de Buffer Low Salt: mezclar por inversión 1 min, centrifugar 1 seg y aspirar sobrenadante. Finalmente, recuperar el mRNA en un microtubo 2ml nuevo agregando 200 µl de Buffer de Elusión precalentado a 65°C. Mezclar con la punta de la pipeta, centrifugar 10 seg a 10 000 rpm. Una segunda elución puede realizarse para recuperar el 100% del mRNA a partir de la columna.

D) Precipitación del mRNA

Agregar 10 µl de glicógeno (5-10 mg/ml), 40 µl de acetato de potasio (2.5M pH 5) y 1 ml de etanol al 95% frío (-20°C). Mezclar por inversión e incubar 20 min a -85°C. Centrifugar a 15 000 rpm durante 15-20 min a 4°C. Lavar la partilla de mRNA con etanol al 70% y centrifugar 2min a 15 000 rpm. Aspirar

sobrenadante y secar hasta evaporación del etanol. Resuspender pastilla en 3 µl de agua dde con tratada con DEPC.

A1.7 Preparación de medio Luria Bertani o LBA (Ausbel et al. 1992)

Disolver por agitación 10 g de triptona, 5 g de extracto de levadura y 5 g de NaCl en 800 ml de agua destilada, 166.7 µl de NaOH 10N y 14 g de agar bacteriológico previamente disuelto en 100 ml de agua destilada. Aforar a 1 litro con agua destilada. Esterilizar a 15 libras de presión durante 20 minutos y vaciar en cajas de petri en una campana de flujo laminar (Purifier Class II Biosafety Cabinet). Posteriormente, incubar a 37°C (SHEL LAB Incubator) durante toda la noche y se almacenar a 4°C.

A1.8 Cultivo de bacterias

En el caso de los cultivos bacterianos en medio líquido para aislamiento de plásmidos, preparar medio Luria Bertani (LB) carente de agar como se describió previamente. Incular 3ml de medio con una asada de la cepa bacteriana e incubar a 37°C en una incubadora orbital (LAB LINE Incubator-Shaker) en agitación continua a 150-200 rpm toda la noche.

A1.9 Clonación y Transformación

Para la clonación en el vector pCR 2.1 (3.9 Kb) y transformación en la cepa bacteriana Top10 de *Escherichia coli* quimio-competente, ambos del kit comercial TOPO TA Cloning (Invitrogene), preparar una mezcla de reacción de clonación como sigue: en un microtubo de 0.2 ml agregar 50 ng de cDNA purificado a partir de banda, 1 µl de

solución salina (NaCl 1.2M, $MgCl_2$ 0.06M), 1 µl de vector pCR 2.1 (10ng/µl) y agua dde en un volumen final de 6 µl. Mezclar gentilmente e incubar a temperatura ambiente durante 30 min. Posteriormente, incubar en hielo durante 10 min y proceder con la transformación de la cepa bacteriana *E. coli* Top10. Descongelar un volumen de 50 µl de bacterias en hielo y agregar 3 µl del producto de la ligación. Incubar en hielo durante 10-30 min, y posteriormente incubar a 42 °C durante 30 s. Incubar en hielo durante 2 min y agregar 250 µl de medio de cultivo nutritivo SOC (triptona 2%, extracto de levadura 0.5%, NaCl 10mM, KCl 2.5 mM, $MgCl_2$ 10mM, $MgCl_4$ 10mM y glucosa 20mM) a temperatura ambiente, y crecer las bacterias a 37 °C por 1 h en agitación a 200 rpm en una incubadora orbital (LAB LINE Incubator-Shaker). Posteriormente, en una campana de flujo laminar (Purifier Class II Biosafety Cabinet), inocular 50-100 µl de las células transformadas en cajas de petri con LBA-Amp (50µg/µl) conteniendo X-gal (5-bromo-4-cloro-3-indolil-B-D-galactosido) (40mg/ml). Incubar las cajas de petri toda la noche a 37°C (SEP LAB Incubator).

Elegir las clonas bacterianas positivas (con inserto). La selección de clonas bacterianas transformadas se basa en lo siguiente:

- Resistencia a antibiótico. La eliminación de las bacterias no transformadas se debe a que al carecer del plásmido, el cual porta un gen de resistencia a ampicilina, estas células no pueden crecer en un medio con ampicilina.
- Gen reportero. En el medio de LBA-ampicilina crecen bacterias azules y blancas. Las clonas azules corresponden a las clonas transformantes con plásmido sin inserto que presentan el gen de la β-galactosidasa sin interrumpir, el cual codifica la enzima que degrada el X-gal presente en el medio, y produce una coloración azul. Las clonas blancas corresponden a transformantes con plásmido con inserto, debido a que el sitio de inserción se encuentra en medio del gen que codifica la β -galactosidasa, que al estar

interrumpido no se expresa e impide la metabolización del X-gal (Sambrock y Russell 2001).

A1.10 PCR sobre colonias

Para la selección de clonas con inserto de tamaño adecuado que se secuenciaran, con un palillo estéril picar una colonia positiva (blanca) e inocular una mezcla de reacción para PCR [2 µl Buffer para PCR 10X, 0.4 µl $MgCl_2$ 50 mM, 0.4 µl dNTPs 2.5 mM, 0.4 µl primer M13 directo 10pmol, 0.4 µl primer M13 reverso 10pmol, 2 U de enzima Taq polimerasa y H_2O dd hasta un volumen final de 20 µl]. Amplificar mediante PCR con las siguientes condiciones: desnaturalización inicial a 94°C, 4min; 35 ciclos con desnaturalización a 94°C, 30 s; alineamiento a 55°C, 30s; y extensión a 72°C 1 min/Kb del inserto. Revisar las muestras por electroforesis tomando una alícuota de 5 µl TOPO (TA Cloning User Manual, Invitrogene).

A1.11 Minipreparaciones de plásmido (Zhou et al. 1990).

Tomar una muestra de 10-20 clonas blancas crecidas en las cajas de petri con medio LBA-amp con palillos estériles. Con un palillo para cada colonia, primero inocular cada colonia en una caja de petri con medio LBA-Amp (placa madre), y posteriormente con el mismo palillo, inocular 3 ml de medio LB-Amp (50 ng/µl) en un tubo de ensaye. Incubar ambos cultivos a 37°C toda la noche; los cultivos en placa, en una incubadora (SEP LAB Incubator) y los cultivos en medio líquido, en una incubadora orbital con agitación a 200 rpm (LAB LINE Incubator Shaker). Almacenar las placas madre con las colonias transformantes a 4°C.

A partir de los cultivos líquidos, recuperar 1.5 ml de células en 2 microtubos de 1.5 ml estériles; centrifugar las células bacterianas a 13,000 rpm por 8 min (Centrifuge 5415D, Eppendorf) y decantar el sobrenadante de cada tubo. Añadir 300 µl de TEN [Tris/HCl 10 mM pH 8, EDTA 1mM pH 8.0, NaCl 100mM] para resuspender la pastilla de las bacterias. Centrifugar la mezcla a 13,000 rpm por 10 min y descartar el sobrenandante. Resuspender la pastilla nuevamente en 200 µl de solución Birnboim I fría [Glucosa 50mM, EDTA 10mM, Tris-HCl 25mM] por vortéx. Agregar 20 µl de solución de lisozima (Tris-HCl 10mM, lisozima 10mg/ml] recién preparada, y 400 µl de solución Birnboim II [NaOH 0.2M, SDS 1%] a temperatura ambiente; mezclar brevemente por inversión hasta obtener una consistencia viscosa e incubar a temperatura ambiente durante 10 min. Agregar 150 µl de solución Birnboim III fría [1 V de ácido acético glacial, 2.5 V de acetato de potasio 3.8M pH 5.2] que arrastra los residuos de la lisis de proteínas, lípidos y carbohidratos; mezclar por inversión e incubar en hielo 10 min. Centrifugar a 13 000 rpm por 10 min. Posteriormente, transferir el sobrenadante a un microtubo de 1.5 ml estéril, agregar 0.6 V de isopropanol frío (-20°C) y mezclar por inversión. Incubar a -20°C durante 30-60 min y centrifugar a 13 000 rpm por 15 min para empastillar el DNA del plásmido. Decantar el sobrenadante y lavar la pastilla con etanol al 70% frío (-20°C). Secar la pastilla hasta evaporación del etanol, y resuspender en 20 µl de agua dde. Finalmente, eliminar el RNA como se describió anteriormente.

A1.12 Limpieza de plásmidos con bromuro de etidio (Stemmer 1991)

Llevar la preparación de plásmidos a un volumen de 200 µl con H_20 dde. Agregar 140 µl de acetato de amonio 7.5 M y 5 µl de bromuro de etidio (10mg/ml); mezclar por inversión. Agregar 350 µl de fenol:cloroformo:isoamílico (25:24:1) y mezclar nuevamente por inversión. Centrifugar a 13,000 rpm durante 5 min. Transferir la fase acuosa a un microtubo estéril y agregar 700 µl de etanol absoluto. Incubar la mezcla a

-20°C durante 10-15 min, y centrifugar a 13, 000 rpm durante 10 min. Lavar la pastilla de DNA con etanol al 70% frío (-20°C) y secar hasta evaporar el etanol. Resuspender el DNA en 20 µl de H_2O dd estéril.

A1.13 Purificación de DNA en gel mediante el kit comercial NucleoTrap Gel Extraction (Clontech)

Cortar la región del gel de agaraosa que contiene el DNA de interés. Pesar el fragmento de gel. Por cada 100 mg de agarosa, agregar 300 µl de buffer NT1 (600 µl para geles >2% de agarosa). Mezclar por vórtex hasta que el gel se resuspenda completamente (se puede incubar a 50°C para facilitar la disolución del gel). Agregar 4 µl de suspensión NucleoTrap por cada µg de DNA a purificar (mínimo 10 µl para asegurar alta eficiencia de unión). Incubar a 50°C durante 5-15 min, mezclando por vórtex cada 2-3 min. Centrifugar a 13,000 rpm por 30 s a temperatura ambiente y descartar sobrenadante. Agregar 500 µl de buffer NT2 y mezclar por vórtex. Centrifugar a 13,000 rpm por 30 s a temperatura ambiente y remover sobrenadante. Agregar 500 µl de buffer NT3 y mezclar por vórtex. Centrifugar nuevamente a 13,000 rpm por 30 s a temperatura ambiente y descartar sobrenadante. Repetir el paso de lavado con buffer NT3. Eluir el DNA, agregando 50 µl de buffer NE. Resuspender la pastilla por vórtex e incubar 10-15 min a 50°C, mezclándo por vórtex 2-3 veces. Centrifugar a 13,000 rpm por 30 s. Con este paso se recupera aproximadamente el 80% del DNA, se puede repetir este paso de elución para recuperar el resto del DNA.

Si se requiere concentrar el DNA en un menor volumen, precipitar el DNA con 0.1V de acetato de sodio 3M, 1 µl de glicógeno (5-10 mg/ml) y 2.5V de etanol absoluto frío. Incubar a -80°C durante 15-30 min y centrifugar a 13,000 rpm por 15 min. Lavar pastilla con etanol al 70% y secar hasta evaporación del etanol. Resuspender con el volumen necesario de H_2O dde.

A1.14 Síntesis de cadena sencilla de cDNA (Transcripción reversa) con el kit comercial SMART RACE (Clontech).

Para la síntesis de cDNA para el extremo 5´ (5´RACE), preparar la siguiente mezcla de reacción: 200-500 ng de RNAm en un volumen de 3 µl, 1 µl de primer 5´-CDS, 1 µl de SMART II oligo. Mezclar y centrifugar (un pulso) para bajar el contenido. Incubar la mezcla a 70°C durante 2 min y transcurrido este tiempo transferir a hielo 2 min. Centrifugar (un pulso) y agregar 2 µl de buffer first-strand 5X, 1 µl de DTT 20mM, 1 µl de dNTPs 10mM, 1 µl Super Script II RNAase H Retro-Transcriptasa (Invitrogene). Mezclar y centrifugar (un pulso). Incubar la mezcla a 42°C durante 90 min. Posteriomente agregar 10 µl de H_2O dde estéril, mezclar e incubar a 71°C 8 min.

La síntesis de cDNA para el extremo 3´ (3´RACE), se hace de la misma manera que para el extremo 5´, sustituyendo el primer 5´-CDS por el primer 3´-CDS y el SMART II oligo por H_2O dde.

A1.15 Amplificaciones rápidas de los extremos de cDNA (Amplificaciones RACE) con el kit comercial Advantage 2 PCR (Clontech).

Preparar la siguiente mezcla de reacción: 34.5 µl H_2O dde, 5 µl buffer advantage 2 PCR 10X, 1µl dNTPs 10mM, 1µl oligonucleótido específico (10pmol ó 100pmol en caso de que se trate de olinucleótido degenerado), 5 µl de UPM primer 10X, 2.5 µl de cDNA (5´RACE o 3´RACE, según sea el caso), 1 µl advantage 2 Polymerase 50X.

Las condiciones de generales de PCR difieren según se trate de un oligonucleótido degenerado o específico. Para PCR con oligonucleótido degenerado

utilizar un programa Touchdown con descenso de temperatura de alineamiento (ramping down), con las siguientes condiciones: 1 ciclo inicial de desnaturalización a 95°C 2min; 3 ciclos de 95°C 5 s, 57°C 10 s, 72°C 3 min; 3 ciclos de 95°C 5 s, 54°C 10 s, 72°C 3 min; 3 ciclos de 95°C 5s, 51°C 10 s, 72°C 3 min; 3 ciclos de 95°C 5 s, 48°C 10 s, 72°C 3 min; 3 ciclos de 95°C 5 s, 45°C 10 s; 72°C 3 min; 18 ciclos de 95°C 5s, 45°C 10 s, 72°C 3 min; y un ciclo para extensión final a 72°C 7 min. Para PCR con oligonucleótido específico, las condiciones generales son: desnaturalización inicial a 95°C 4 min, 25 ciclos con una desnaturalización a 94°C 30 s, alineamiento a la temperatura de alineamiento del oligonucleótido específico durante 20 s, y extensión a 72°C 1 min/Kb. Tomar una alícuota de 5µl de la reacción del PCR y revisarla por electroforesis, si el producto de amplificación no es visible, regresar la mezcla de reacción al termociclador, y correr 5-10 ciclos más en las condiciones antes descritas, pero agregando un ciclo de extensión final a 72°C, 7 min.

A1.16 Construcción de la librería de cDNAs o de expresión con el kit comercial Creator SMART (Clontech)

Purificar RNAm a partir del tejido, células o condiciones de interés. Sintetizar cadena sencilla de cDNA preparando la siguiente reacción: 25-50 ng de RNAm en un volumen máximo de 3µl, 1µl de SMART IV oligonucleotide, 1µl de CDS III/3´PCR primer. Mezclar y centrifugar (un pulso) para bajar contenido. Incubar a 72°C 2 min, y después en hielo durante 2 min. Centrifugar (un pulso), y agregar: 2 µl de buffer First-stand 5X, 1µl de DTT 20mM, 1µl de dNTPs 10mM, 1 µl Super Script II RNAase H Retro-Transcriptasa (Invitrogene). Mezclar y centrifugar (un pulso). Incubar la mezcla a 42°C durante 60 min y mantener el tubo en hielo si se continuará inmediatamente, o almacenar a -20°C.

Amplificar el cDNA por PCR de larga distancia (LD PCR), para ello preparar la siguiente mezcla de reacción: 80 µl de H_2O dde, 2 µl de cDNA, 10 µl de buffer advantage 2 PCR 10X, 2 µl dNTPs 10mM, 2 µl 5´PCR primer, 2 µl CDS III/3´PCR primer, 2 µl advantage 2 polymerase mix 50X. Mezclar y centrifugar (un pulso). Precalentar un termociclador a 95°C. Amplificar bajo las siguientes condiciones: desnaturalización inicial a 95°C 1min, 22-26 ciclos de 95°C 10s, 68°C 6 min. Revisar por electroforesis y elegir el número de ciclos que genere la mayor cantidad de fragmentos entre 500-3000 pb. Cortar la región del gel desde la región que corresponde a fragmentos de (i) 500-3000pb y (ii) >3000pb. Recuperar el DNA a partir de gel y limpiarlo. Precipitar el DNA con 250 µl de etanol absoluto frío, 1 µl de glicógeno (5-10 mg/ml) y 10 µl de acetato de sodio 3M. Incubar a -80°C 10 min, centrifugar a 15 000 rpm 15min a 4°C. Lavar pastilla con etanol al 70%, secar hasta evaporación de etanol, y resuspender en 10 µl de H_2O dde.

Para clonar los cDNAs, preparar 3 reacciones de ligación al vector como se muestra a continuación:

	Ligación A (µl)	**Ligación B(µl)**	**Ligación C(µl)**
Cdna	0.5	1.0	1.5
vector pDNR-LIB	1.0	1.0	1.0
buffer de ligación	0.5	0.5	0.5
ATP (10mM)	0.5	0.5	0.5
T4 DNA ligasa	0.5	0.5	0.5
H_2O dde	2.0	1.5	1.0

Incubar a 16°C toda la noche. Agregar 95 µl de H_2O dde y 1.5 µl de glicógeno (5-10 mg/ml). Mezclar y agregar 280 µl de etanol al 95% frío. Incubar a -80°C 10min y

centrifugar a 15,000 rpm 15 min. Secar y resuspender en 1 µl de H_2O dde. Descongelar en hielo 50 µl de células electrocompetentes. Agregar el cDNA preparado anteriormente (1 µl) a las células y mezclar con movimientos suaves de la punta de la micropipeta. Transferir células a una celda de electroporación. Transformar las células bajo las siguientes condiciones: 200 Ω, 25 *F*, 1.5 KV/cm, 1 s. Transferir las células a microtubo estéril y agregar 1 ml de medio SOC (triptona 2%, extracto de levadura 0.5%, NaCl 10mM, KCl 2.5 mM, $MgCl_2$ 10mM, $MgCl_4$ 10mM y glucosa 20mM) a temperatura ambiente, y crecer las bacterias a 37 °C por 1 h en agitación a 200 rpm en una incubadora orbital (LAB LINE Incubator-Shaker). Inocular 50, 100, 150, 200, y 250 µl de las células transformadas en cajas de petri con LBA-Amp (50µg/µl) conteniendo X-gal (40mg/ml) e IPTG (O.1 M). Incubar las cajas de petri toda la noche a 37°C. Seleccionar y recuperar colonias positivas en placa madre y revisar el tamaño de los insertos para elegir colonias para secuenciación como se describió anteriormente.

A1.17 Ensayo tipo Southern Blot con Digoxigenina mediante el sistema comercial DIG Nonradioactive nucelic acid labeling and detection (Roche)

1. Preparación de sondas marcadas con DIG por PCR

Preparar las siguientes mezclas de reacción (control sin marcar y para sonda que se marcará) sobre hielo: (i) 50 ng de plásmido que contenga el fragmento que se usará como sonda, 1 µl de oligonucleótido específico directo (10pmol), 1 µl de oligonucleótido específico reverso (10pmol), 2.5 µl Buffer para PCR 10X, 1 µl de $MgCl_2$ 50mM, 2 µl dNTPs 10mM, 2 U de enzima taq polimerasa y H_2O dde hasta un volumen final de 25 µl; (ii) 50 ng de plásmido que contenga el fragmento que se usará como sonda, 1 µl de oligonucleótido específico directo (10pmol), 1 µl de oligonucleótido específico reverso (10pmol), 5 µl Buffer para PCR 10X, 1 µl de

$MgCl_2$ 50mM, 5 µl mezcla de dNTPs marcados con UTP-DIG 10mM, 2-3 U de enzima taq polimerasa y H_2O dde hasta un volumen final de 50 µl. Revisar el marcado de las sondas por electroforesis; la sonda marcada deberá ser ligeramente más grande que la no marcada (entre 100-200 pb más).

2. Reparación de membranas

Preparar las siguientes reacciones de restricción (una por cada gen):

A) 10 µg de DNA del organismo de interés, 4 µl de Buffer NE 2 10X, 20 U de la enzima Mse I y H_2O dde hasta un volumen final de 40 µl e incubar a 37°C toda la noche.

B) 10 µg de DNA del organismo de interés, 2 µl de Buffer NE 3 10X, 20 U de la enzima Not I y H_2O dde hasta un volumen final de 40 µl e incubar a 37°C toda la noche.

C) 10 µg de DNA del organismo de interés, 2 µl de Buffer NE 2 10X, 20 U de la enzima Xho I y H_2O dde hasta un volumen final de 40 µl e incubar a 37°C toda la noche.

D) 10 µg de DNA control negativo, 2 µl de Buffer NE 3 10X, 20 U (1.5 µl) de la enzima Eco RI y H_2O dde hasta un volumen final de 40 µl e incubar a 37°C toda la noche.

E) 10 µg de DNA del organismo de interés, 2 µl de Buffer NE 2 10 X, 20 U (1 µl) de la enzima Xho I y H_2O dde hasta un volumen final de 40 µl. Incubar a 37°C durante 1 hora. Posteriormente, agregar 20 U (1 µl) de la enzima Hind III e incubar la reacción toda la noche a 37°C.

Separar los productos de las reacciones de restricción por electroforesis en geles de agarosa 2% en TAE 1X teñido con bromuro de etidio. Una vez separadas las muestras, depurinar el DNA colocándo el gel en HCl 0.25M hasta que cambie de color azul a amarillo-verde. Lavar el gel con agua destilada y colocarlo en 100ml de buffer de desnaturalización (NaCl 1.5M, NaOH 0.5M) durante 30 minutos a temperatura

ambiente y en agitación suave hasta que el color amarillo-verde cambie nuevamente a azul. Lavar con agua destilada y colocar en 100ml de buffer de neutralización (NaCl 1.5M, Tris-HCl 0.5M pH7.2, EDTA 0.001M) dos veces durante 15 minutos a temperatura ambiente y en agitación.

Equilibrar membrana de nylon Hybond –N+ (Amersham) en SSC 10X durante 10 min. Montar el blot de capilaridad para transferir el DNA: colocar un puente de papel filtro Whatman #4 sobre un soporte sólido; colocar el gel sobre el papel filtro; colocar la membrana sobre el gel, y sobre la membrana, dos piezas de papel filtro; colocar una capa de aprox. 5 cm de papel absorbente, una placa de plástico y un contrapeso. Transferir el DNA con SSC 10X toda la noche y una vez transferido, secar la membrana a temperatura ambiente y fijar el DNA con irradiación de luz UV a 1320 1 μjoules X 100 (UV Stratalinker Stratagene).

3. Hibridación

Prehumeder la membranas en H_2O dde, y lavarla con SSC 2X (NaCl, Citrato de Sodio) durante 15 minutos. Prehibridar la membrana durante 2-4 horas a 37°C en horno de hibridación en 1 ml buffer de hibridación/10 cm^2 de membrana [SSC 5X, N-lauril-sarcosine 0.1% w/v, SDS 0.02%, agente bloqueador para una dilución 1:20, formamida 50%]. Preparar el un volumen necesario (3.5 ml/100 cm^2) de buffer de hibridación [SSC 5X, N-lauril-sarcosine 0.1% w/v, SDS 0.02%, agente bloqueador para una dilución 1:20, formamida 50%]. Tomar de 2-4 μl de sonda marcada con DIG/ml de buffer de hibridación y llevar a un volumen final de 50 μl con H_2O dde; desnaturalizar la sonda a 95°C durante 5 min y colocar inmediatamente en hielo; centrifugar (un pulso) para bajar el contenido. Concluido el tiempo de prehibridación, descartar el buffer de prehibridación y agregar el buffer de hibridación, así como la sonda marcada con DIG previamente desnaturalizada. Hibridar toda la noche a 37 °C.

Colocar la membrana en un recipiente de plástico y lavar dos veces con buffer de baja astringencia (SSc 2X, SDS 0.1%) a temperatura ambiente durante 5 min en agitación. Después lavar dos veces con buffer de alta astringencia (SSC 0.5%, SDS 0.1%) a 65°C durante 15 minutos cada uno.

4. Detección

Una vez terminados los lavados de astringencia, incubar la membrana en una dilución 1:10 de la solución de bloqueo en 1 ml/cm^2 de buffer A estéril [Tris-HCl 100mM, NaCl pH 9.5 300mM] durante 1 h con agitación suave a temperatura ambiente. Centrifugar el anticuerpo anti-digoxigenin-AD 5 min a 10,000 rpm y preparar una dilución 1:10,000 (75mU/ml) en una solución w/v de BSA [BSA 0.5% en buffer A]. Agregar 0.3 ml de esta solución/cm^2 de membrana. Incubar 1 h a temperatura ambiente.

Remover el conjugado no unido, al lavar la membrana tres veces con 5 ml un solución v/v de tween 20 [Tween 20 0.3% en buffer A] por cada cm^2 de membrana, durante 10 min a temperatura ambiente con agitación suave. Drenar el exceso de buffer y equilibrar la membrana en 20 ml de buffer de detección [Tris-HCl 0.1M; NaCl pH 9.5 0.1M] durante 3 min.

Colocar la membrana sobre una película fotográfica usada, evitando la formación de burbujas y la desecación de la membrana. Aplicar 1 ml de una dilución1:500 de CSPD en buffer de detección por cada 100 cm^2 de membrana. Incubar 5 min a temperatura ambiente, y posteriormente 10 min a 37°C para potenciar quimioluminiscencia. Cubrir la película con plástico cuidando no formar burbujas, y colocar la película cubierta dentro de un cassette de exposición. En condiciones de total oscuridad, poner sobre la membrana de 1-3 películas fotográficas Kodak X-OMAT para obtener registro de las señales de hibridación, e incubar 1 h a 37°C (para revelados rápidos), de 24-48 h a temperatura ambiente para genes unicopia. Revelar la placa colocándola 3 min en solución reveladora (dektrol), 2 min en agua y finalmente 3 min en solución fijadora. Lavar con agua corriente y dejar secar.

A1.18 Determinación de actividad enzimática con azul de tetrazolium como agente cromogénico mediante la técnica propuesta por Jue y Lipke (1985).

En este ensayo se determina la cantidad de azúcares reductores producidos después de la incubación de carboximetilcelulosa (CMC) como substrato con extractos enzimáticos. Para la preparación del agente cromogénico: pesar 1 g de azul de tetrazolium (Sigma-Aldrich) y agregarlo a 500 ml de NaOH 0.1M. Calentar la mezcla a 60°C con agitación vigorosa hasta obtener una solución de color amarillo-claro (sobrecalentar resulta en la precipitación de material negro, presumiblemente diformazano). Preparar una solución 1M de tartrato de sodio-potasio en agua. Mezclar en volúmenes iguales y filtrar la solución final con un filtro Durapore.

Para los ensayos enzimáticos: preparar la siguiente mezcla de reacción: 25 µl de extracto crudo y 75 µl de substrato. Incubar a 37 °C durante 5-60 min. Agregar 800 µl de la solución del agente cromogénico, e incubar a 95°C 5min. Incubar en hielo 5 min. Cuantificar azúcares reductores con un espectrofotómetro a una longitud de onda de 650 nm.

Para cualquier ensayo enzimático se debe generar una curva de calibración con el azúcar adecuado para el substrato, que se llamará estándar (i.e. glucosa para celulasas, xilosa para xilanasas, ácido galacturónico para poligalacturonasas). Para ello, preparar soluciones con 5, 10, 15, 20, 25, 30 µg del estándar en 100 µl de H_2O dde. Realizar el ensayo enzimático con cada una de las soluciones como se describió anteriormente. Graficar absorbancia a 650 nm (eje y), y µg del estándar (eje x) y comprobar la linearidad del ensayo (valores r^2 cercanos a 1).

Todos los ensayos enzimáticos deben incluir un control negativo, un control positivo y el estándar. El control negativo consiste en 100 µl de H_2O dde tratada como

anteriormente se describió. Para el control positivo agregar 75 µl de substrato e incubar a 37 °C durante 5-60 min; después, agregar 800 µl de la solución del agente cromogénico y 25 µl del extracto crudo e incubar a 95°C 5min y en hielo 5 min más; finalmente cuantificar azúcares reductores como se describió antes. El estándar consiste de una solución que contenga 10 µg disueltos en 100 µl de H_2O dde.

Una unidad (U) de actividad enzimática se define como la cantidad de enzima que produce 1 µmol de azúcar reductor/min.

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Apéndice 2

ASSOCIATION OF LIFE STAGES OF LONGHORN BEETLES (CERAMBYCIDAE) USING THE UNIVERSAL DNA BARCODING REGION

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ABSTRACT

DNA barcoding has increased the attention on the use of specific regions of mitochondrial cytochrome oxidase I gene (COI) to diagnose and delimit species. In the Neotropics, congeneric cerambycids often colonize the same branches, and morphological characters that would distinguish larval species are not known. Using DNA sequences of the universal DNA barcoding region of the COI gene, we identified three larval species of the family Cerambycidae (Coleoptera): *Ataxia alpha, Estoloides chamelae* and *Lissonotus flavocinctus*. These results suggest that DNA-assisted taxonomy using the COI universal barcoding region might be a useful tool to identify insect larvae to the species level.

Keywords: mitochondrial cytochrome oxidase I gene, larval identification, insect systematics, Cerambycidae

INTRODUCTION

Cerambycidae (Polyphaga: Chrysomeloidea) constitutes one of the most diverse insect groups, with more than 35,000 species in about 4,000 genera (Hanks 1999). These longhorn beetles are generally characterized by their pseudopentamerous tarsi, the presence of antennal tubercles, and long antennae that range from one quarter to three times the length of the body (Martínez 2000). Cerambycids are phytophagous: adults feed on a wide spectrum of plant resources including leaves, seeds, sap, roots, flowers, bark and fungi (Duffy 1960, Linsley 1959, 1961, Hanks 1999, Allison et al. 2004); most larvae bore living, recently dead or decaying tissues of woody plants, but some are confined to the roots or to the pith of herbaceous plants (Hanks 1999, Martínez 2000).

Many cerambycid species play important roles in ecosystem processes such as: alteration of tree architecture (Whitman and Mooper 1985, Feller 2002, Martínez et al. 2009); effects on growth, reproduction and gender expression (Whitman and Mooper 1985, Martínez et al. 2009, Uribe-Mú and Quesada 2006), nutrient cycling (Amman 1976, Schowalter 1981, Feller 2002); resource regulation (Duval and Whitford 2008); ecosystem engineering (Calderón-Cortés et al. *submitted manuscript*); and alteration of the composition and hydrology of forests (Bethlahmy 1975, Feller and McKee 1999, Lundquist and Negron 2000, Duval and Whitford 2008). However, some cerambycids can also act as biological vectors for the transmission of tree-killing nematodes and fungal pathogens of trees, and others can be serious pests of wood products (Allison et al. 2004). Therefore, they can cause substantial economic losses to commercial forests, agricultural and wood products, and landscape ornamentals (Schowalter and Flip 1993, Hanks 1999, Allison et al. 2004). The cryptic wood-boring habits of many cerambycids make them ideally suited for introduction as exotics in wood products, dunnage and nursery stocks (Allison et al. 2004). These introductions have caused severe damages worldwide (e.g. Haack and Poland 200, Nowak et al. 2001). Due to their ecological and economic importance, cerambycids have stimulated a great number of studies about

their: taxonomy, host plant interactions, ecological roles, chemical ecology and physiology, among others. However, most of the taxonomic studies have focused on adults (e.g. Linsley 1961, Arnett 1963, Lawrence and Newton 1995, Linsley and Chemsak 1995), and there is little information about larval taxonomy (Craighead 1923, Duffy 1953, 1957, 1960, 1963, 1968).

In the Neotropics, congeneric cerambycids often colonize the same host plants (Feller and Mathis 1997, Travakalian et al. 1997, Berkov et al. et al. 2007), and the morphological characters that would distinguish their larvae are not known. In many insects, the mitochondrial cytochrome oxidase I (COI) gene is considered sufficiently variable for species level identification (Hebert et al. 2003a, b, 2004, Hajibabaei et al. 2005, Monaghan et al. 2005, Roe and Sperling 2007, Smith et al. 2007, Burns et al. 2008). This gene is amongst the most widely used in species level systematics of insects. and has been advocated as an ideal marker to underline a DNA-based taxonomy or DNA barcode (Hebert et al. 2003a, b). DNA sequences can be used to identify larval stages for which diagnostic morphological characters are not known. This identification can be possible if larvae and adults are collected and analyzed together (e.g. Caterino and Tishechkin 2006, Berkov et al. 2007). In this study we used the sequence of the "DNA barcoding region" of the COI gene to assign larval genotypes to adult genotypes of three cerambycid species previously identified.

MATERIALS AND METHODS

Study site

The collection of cerambycids (both adults and larvae) was conducted in the Chamela-Cuixmala Biosphere Reserve at Chamela Biological Station, UNAM (19°30'N, 105°03'W) located on the Pacific coast of Jalisco, Mexico. The vegetation is tropical dry

forest with a mean annual rainfall of 707 mm and a dry season that extends from November to June (Bullock 1988).

Study system

Reproductive branches from 2-3 cm in diameter of the tropical tree *Spondias purpurea* L. (Anacardiaceae) are girdled and completely detached by the Cerambycidae (Lamiinae) beetle *Oncideres albomarginata chamela* Chemsak and Gisbert to oviposit and develop its offspring within them (Uribe-Mú and Quesada 2006). Most detached branches remain hanging from the trees and over the shrubs, and others can fall to the ground. The reproductive period of this species begins in October and finishes in February; eggs pupate and develop inside detached branches until the adults emerge after 6-8 months later in low densities (Uribe-Mú and Quesada 2006). Adult females of *O. albomarginata chamela* are the only that detach and immediately oviposit the branches of *S. purpurea* in the study site (Uribe-Mú and Quesada 2006), but after a certain time period other cerambycid species take advantage of the branches detached by *O. albomarginata chamela* and oviposit on them as well (Calderon-Cortés et al. *submitted manuscript;* chapter 2 of this thesis*)*.

For rearing the adult cerambycids that colonize *S. purpurea* branches, we collected 150 (50 branches per year of sampling) detached branches of *S. purpurea* colonized by *O. albomarginata chamela,* during December 2006-January 2007, December 2007- January 2008 and December 2008-January 2009. These branches (that were sampled as part of an ecological study of *O. albomarginata chamela*) were marked. These branches were left hanging on the source tree during 30-45 days to allow the colonization of secondary cerambycid species. After this period, all branches were enclosed in mesh bags (< 0.5 mm of aperture) to prevent any further escape. Branches collected in mesh bags were placed in a exclosure at the study site, and maintained at local environmental conditions. Emerging cerambycids from each branch enclosed in a

mesh bag were collected monthly. Taxonomic identification of species that emerged was carried out by the cerambycid specialist Dr. Felipe A. Noguera.

For larval samples, we collected detached branches of the tree *S. purpurea* (two-three months after cerambycid colonization) during April 2007, 2008 and 2009. The collected branches were transported to the laboratory. Branches were dissected and larvae inside them were harvested and classified at subfamily level using reported literature (Böving & Craighead, 1931; Duffy, 1960) and separated according to morphological characters.

Molecular methods

Adult and larvae collected were sterilized in 70% ethanol and frozen at -80°C. DNA was purified from 100 mg of tissue of one individual (3-5 individuals per species/morphotypes), following a modified DNA isolation method (Calderón-Cortés et al., *unpublished data;* Appendix 3 of this thesis). The remaining body fragments were mounted as vouchers. Purified DNA was used as template for the amplification of the 5′end of COI gene (a fragment of ~ 650 base pairs corresponding to the DNA universal barcoding region) with the forward 5′-GGTCAACAAATCATAAAGATATTGG-3′ (LCO) and reverse 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ (HCO) using Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. Polymerase chain reactions (PCRs) were carried out using a ramping-down of the annealing temperature ('touchdown'; -2°C for every five cycles) program. PCR amplification was started using the following settings: 94°C/30 s (denaturing), 50°C/30 s (base annealing temperature of first three cycles) and 72°C/1 min (extension), and was continued until the base annealing temperature reached the final condition of 44°C. Under the final conditions, the amplification was continued for 20 cycles. PCR-products were sent to Macrogen Sequencing Service (Macrogen Inc., Seoul, Korea) for sequencing using the forward LCO primer and the reverse HCO primer.

Data analysis

We assembled a library of COI sequences for adult cerambycids: *Ataxia alpha, Estoloides chamelae, Eutrichillus comus, Lagocheirus araeniformis, Lissonotus flavocinctus;* and a sympatric Bostrichidae species *Melalgus* for using as outgroup. To identify the larval species, we aligned larval COI sequence data from our unidentified specimens with adult COI sequences from our adult library using Clustal W software (Thompson et al. 1994). Phylogenetic analyses were performed under maximum parsimony and Bayesian criteria, using the programs PAUP*v 4b10 (Swofford, 2000) and MrBayes vs. 3.1.2 (Huelsenbeck & Ronquist, 2001). For maximum parsimony analyses, the most parsimonious trees were estimated using the heuristic search option [TBR branch swapping, saving only a single tree in each case] with random sequence addition (five random replicates). Support for the different clusters was evaluated by bootstrap analysis using the full heuristic search option with 1000 replicates. For Bayesian analyses, an appropriate model of nucleotide substitution for each GHF analysis was determined using the program Modeltest 3.7 (Posada et al. 1998) based on the Akaike Information Criterion (AIC). The best fit model of evolution was a GRT+I+ G model. For Bayesian criteria, the analyses were performed using the selected model of substitution. A total of 10 000 trees were obtained based on the settings ngen = 1000 000, samplefreq = 100. Prior to estimating the support of the topologies found, we checked the convergence overall chains (4) when the log likelihood values reached the stationary distribution. The first 2000 trees were 'burn in' and discarded, and a 50% majority rule consensus tree of the remaining trees was generated. We used the *Periplaneta americana* (AM114927) and *Melalgus* sp. (sympatric Bostrichidae species sequenced in this study) as outgroups. Uncorrected pairwise distances among all specimens were estimated using Mega 4 software (Tamura et al. 2007). The number of haplotypes in each species was estimated using the DnaSP v5 software (Librado and Rozas 2009).

RESULTS

The trees obtained by both methods were similar in overall topology (Fig. 1). We were able to unambiguously identified three larvae species: *Ataxia alpha* (L2-1 to L2-5: Lamiinae)*, Estoloides chamelae* (L3-2, L3-3, L3-4, L3-5, L3-8 and L3-9: Lamiinae) and *Lissonotus flavocinctus* (L1-2, L1-3 and L1-5: Cerambycinae) (Fig. 1, Table 1 and 2). Uncorrected pairwise distances between larval sequences and closely related adult sequences (intraspecific distances) range from 0.4 to 0.7% (Table 1), whereas uncorrected pairwise distances among cerambycid species range from 18.5 to 28.7% (Table 2). The number of haplotypes per species ranges from 2 to 6 (Table 3).

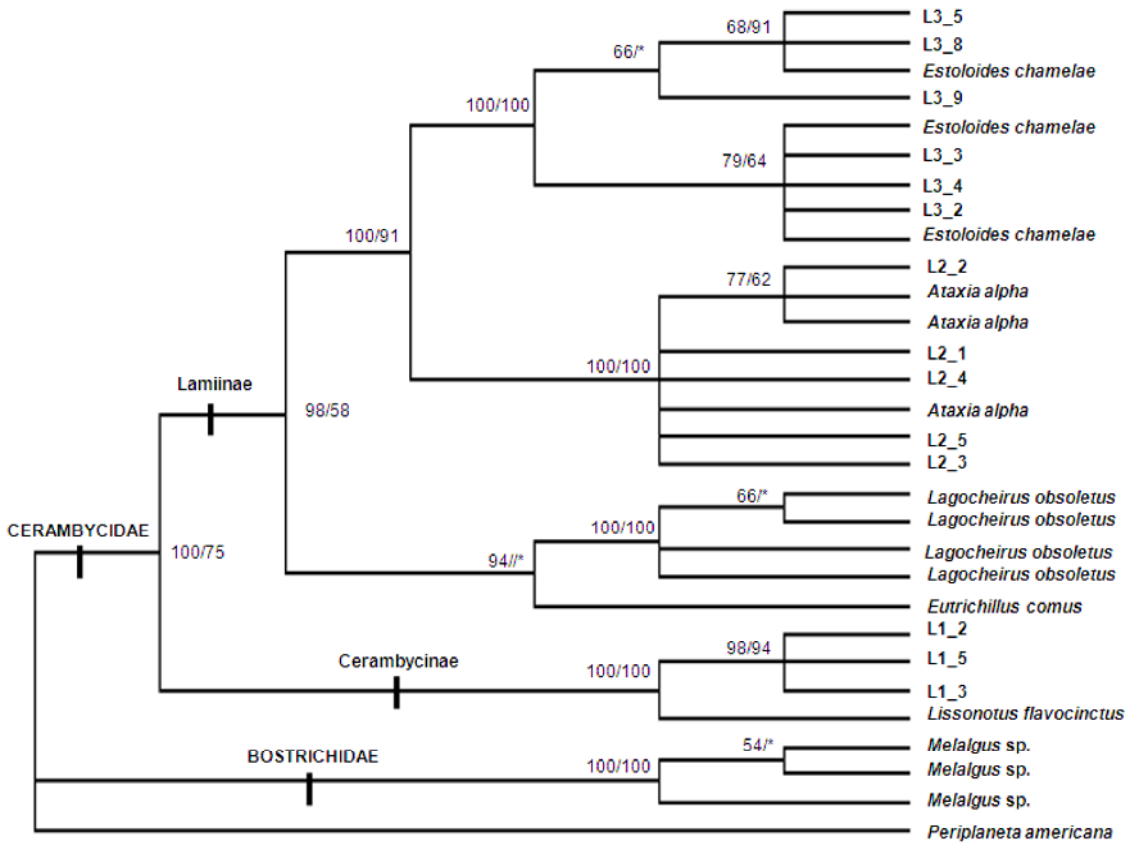

Figure 1. Analysis of the COI sequences (DNA universal barcoding region) from unidentified cerambycid larval specimens, and sequences from adult cerambycids reared from *Spondias purpurea* branches. The shown topology and branch lengths were inferred using MrBayes vs. 3.1.2 with the GRT+I+G model. COI sequences of the cockroach *Periplaneta americana* and the Bostrichidae beetle *Melalgus* sp. were included as outgroups. Numbers above diagonal indicate posterior probability values from Bayesian analysis. Numbers below the diagonal indicate bootstrap percentage values from a bootstrap analysis inferred using the same alignment with PAUP*4.0. The asterisks represent branches that were not supported in 50% or more of bootstraps. Phylogenetic tree was edited using Dendroscope software (Huson et al. 2007).

Table 1. Intraspecific uncorrected pairwise distances.

Species	**% Distance (SD)**
Ataxia alpha	0.4% (±0.2)
Estoloides chamelae	0.7% (±0.2)
Lagocheirus araeniformis	0.4% (±0.2)
Lissonotus flavocinctus	0.4% (±0.2)
Melalgus sp.	0.4% (±0.2)

Table 2. Interspecific uncorrected pairwise distances of Cerambycidae species.

Species	*A. alpha*	*E. chamelae*	*E. comus*	*L. araeniformis*	*L. flavocinctus*
A. alpha	-	5	8.0	6.5	8.3
E. chamelae	18.5	-	7.7	6.6	8.4
E. comus	26.1	25.9	-	5.7	9.4
L. araeniformis	23.1	23.2	20.9	-	6.4
L. flavocinctus	27.1	27.2	28.7	22.5	-

Distances (%) are shown below diagonal, and standard deviations (%) are shown above diagonal.

Table 3. Analysis of DNA polymorphisms

Species	Sequences	Polymorphic sites	Haplotypes	Haplotype diversity (SD)
Ataxia alpha	8	9	6	0.893 (±0.11)
Estoloides chamelae	9	15	5	0.833 (±0.10)
Lagocheirus araeniformis	4	5	3	0.833 (±0.22)
Lissonotus flavocinctus	4	6	3	0.833 (±0.22)
Melalgus sp.	3	4	2	0.667 (±0.31)

DISCUSSION

In this study we used DNA sequence data to facilitate the identification of cerambycid larvae from an assembled library of COI sequences of previously identified adult cerambycids. There is a considerable controversy regarding the use of short segments of DNA, or "DNA barcodes", to aid in species-level identification (Monaghan et al. 2005, Berkov et al. 2007, Roe and Sperling 2007). However, DNA barcoding studies focused on the identification of pre-identified species have been successful in the delimitation of species (Hebert et al. 2003a, 2004, Smith et al. 2007, Burns et al. 2008), because the sequence divergences are much lower among individuals of a species [1-2%, usually much less], than between closely related species [> 6.5%] (Moore 1995, Hebert et al. 2003b, 2004; Monaghan et al. 2005). Berkov et al. (2007) reported 28% of pairwise divergence between distantly related congeneric beetles. Our results showed contrasting sequence divergences: 0.4-0.7% for intraspecific divergences *vs.* 18.5 to 28.7% for congeneric divergences, indicating that each cluster of adult cerambycids and closely related larval sequences correspond to one species. These results suggest that DNA-assisted taxonomy using the COI universal barcoding region might be a useful tool to identify and associate unknown insect larvae to the species level. However, few studies have utilized DNA sequences to achieve this (e.g. Alaire and Bilton 2005, Miller et al. 2005, Caterino and Tisheckin 2006). Larval identification has important applications for pest management, but it requires a more representative database of identified sequences to be broadly applicable (Caterino and Tisheckin 2006).

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Apéndice 3

A SIMPLE AND RAPID METHOD FOR DNA ISOLATION FROM XYLOPHAGOUS INSECTS*

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ABSTRACT

Published methods to isolate DNA from insects are not always effective in xylophagous insects because they have high concentrations of phenolics and other secondary plant compounds in their digestive tracts. A simple, reliable and labor-effective cetyltrimethylammonium bromide- polyvinylpyrrolidone (CTAB-PVP) method for isolation of high quality DNA from xylophagous insects is described. This method was successfully applied to PCR and restriction analysis, indicating freedom from common contaminating compounds. DNA isolated by CTAB-PVP method could be used in most molecular and genetic analysis.

Keywords: xylophagous insects; phenolics; PCR; restriction digestion; *Oncideres albomarginata chamela*

INTRODUCTION

Insect species that directly feed on wood play important functional roles in forest ecosystems, as they contribute to nutrient cycling (Amman 1976, Schowalter 1981, Feller 2002). Moreover, most species can be serious pests of forests and wood products, having economically important consequences for the forest and timber industries (Allison et al. 2004, Knizek and Beaver 2004). Due to their ecological and/or economical importance, research on systematics, phylogenetics, ecological genetics and molecular methods for detection and control of insect pests are needed (Caterino et al. 2000, Brockerhoff et al. 2006). For most molecular studies, the isolation of high quality DNA is an essential prerequisite. Nevertheless, the isolation of high quality DNA from xylophagous insects is usually cumbersome, because xylophagous insects tend to concentrate high amounts of plant phenolics and tannins in their digestive tracts (Strauss and Zangerl 2002, Chown and Nicolson 2004). Most of the published methods for insect DNA isolation are SDS/proteinase K based protocols (Henry et al. 1990, Aljanabi and Martínez 1997, Reineke et al. 1998, Hill and Gutierrez 2003, Juen and Traugott 2005) and commercially available kits (Stone et al. 2007, Ball and Armstrong 2008). These methods usually use adult specimens or specific tissues from thorax, head, wings or leg muscles to avoid contaminants. However, due to the presence of phenolics and other plant contaminants in the digestive tract of xylophagous insects, these methods are not useful to isolate high quality DNA when is necessary to include the digestive tract in the tissue for DNA isolation (Serrano et al. 1999, Juen and Traugott 2006, Stone et al. 2007, Ball and Armstrong 2008), or when insects are too small to dissect them. Additionally, SDS based methods and commercially available kits tend to produce low DNA yields with short storage life from tissues rich in phenolics (Lodhi et al. 1994, Zidani et al. 2005), which make them unsuitable for some molecular applications (e.g. Southern blot analysis, construction of genomic libraries, DNA fingerprinting, etc.).

Phenolics are recognized as the major contaminants in DNA preparations from plants (Couch and Fritz 1990, Lodhi et al. 1994, Kim et al. 2004). Phenolics are powerful oxidizing agents that can reduce the yield and purity of DNA by binding covalently with the extracted DNA, thereby, inhibiting further enzymatic modifications of the DNA such as restriction endonuclease digestion and polymerase chain reaction (PCR) (Lodhi et al. 1994, Horne et al. 2004, Friar 2005, Padmalatha and Prasad 2006, Arif et al. 2010). Higher concentrations of cetyltrimethylammonium bromide (CTAB) and the addition of antioxidants such as polyvinyl-pyrrolidone (PVP) and β-mercaptoethanol to the extraction buffer can help remove phenolics in DNA preparations of plants (Lodhi et al. 1994, Kim et al. 1997, Chen and Ronald 1999, Horne et al. 2004, Li et al. 2007). However, PVP usually is not used in the methods reported for DNA isolation from insects (Henry et al. 1990, Aljanabi and Martínez 1997, Reineke et al. 1998, Serrano et al. 1999, Feeley et al. 2001, Hill and Gutierrez 2003).

In this study we evaluated the traditionally used CTAB method for isolation of DNA from the xylophagous insect, *Oncideres albomarginata chamela* (Coleoptera: Cerambycidae), and developed an inexpensive and rapid method for xylophagous insects modifying several existing PVP-based methods developed for plant DNA isolation (Doyle and Doyle 1990, Lodhi et al. 1994, Kim et al. 1997, Chen and Ronald 1999, Horne et al. 2004, Li et al. 2007) . We evaluated the quality of the DNA isolated using this modified method by restriction endonuclease digestions and PCR. The isolated DNA was suitable for these molecular applications. The CTAB-PVP method was also used for DNA isolation in three additional xylophagous beetles: *Ataxia alpha, Estoloides chamelae* and *Lissonotus flavocinctus* (Cerambycidae), confirming that this modified method can be applicable to other xylophagous insects.

MATERIALS AND METHODS

DNA isolation

For DNA isolation, we used larvae at the last instar of the borer beetle *Oncideres albomarginata chamela*, because this life stage presents the highest concentration of phenolics and other plant contaminants (*pers. obs.*). DNA was isolated using the CTAB "traditional" isolation method proposed by Doyle and Doyle (1990), and a CTAB-PVP modified method. 100 mg of tissue were ground into a fine powder using a mortar and pestle and adding liquid nitrogen. The ground tissue was transferred to a 1.5 ml tube and homogenized in 1 ml of prewarmed at 60°C extraction buffer [20 mM ethylene diamide tetraacetic acid (EDTA) pH 8.0, 100 mM Tris-HCl pH 7.5, 1.4 M NaCl, 2% w/v CTAB, 4% w/v PVP-40]. 2% v/v β-mercaptoethanol was added to the extraction buffer just prior use. Samples were incubated at 60°C for 30 min with occasional mixing, and cooled to room temperature. Two microliters of RNAase (1 mg/ml) were added to the solution and incubated at 37°C 15 min. One volume of chloroform:isoamyl alcohol (24:1) was added, and the sample was emulsified by gently inverting shaking and finally centrifuged for 15 min at 13000 rpm. The top aqueous phase was transferred to a clean tube. A second chloroform:isoamyl extraction was performed when aqueous phase was cloudy due to the presence of PVP. Two volumes of cold (-20°C) 95% ethanol were added to the sample, mixed well and incubated at -20°C until DNA strands were visible. DNA strands were recovered using a sterile Pasteur pipette and washed with 70% ethanol, centrifuged for 5 min at 13000 rpm, dried and finally eluted in sterile analytic grade H_2O.

Comparison of the efficacy of the DNA isolation methods

DNA was visualized by electrophoresis on a 1% TAE agarose gel, stained with ethidium bromide, and visualized under UV light. The quality of DNA isolated by the CTAB traditional method and the CTAB-PVP modified method was estimated by measuring the A260/280 absorbance ratio using a spectrophotometer (Perkin-Elmer Corp., Norwalk, CT, USA). To evaluate the quality of the isolated DNA, 100 ng of fresh DNA and 100 ng of DNA stored during three months were used as template for the amplification of the 5′end of the mitochondrial cytochrome oxidase I (COI) gene (a fragment of ~ 650 base pairs corresponding to the DNA universal barcoding region) with the forward 5′-GGTCAACAAATCATAAAGATATTGG-3′ (LCO) and reverse 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ (HCO) primers, using Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. PCR amplifications were carried out using a ramping-down of the annealing temperature ('touchdown'; -2°C for every five cycles) program. PCR amplification was started using the following settings: 94°C/30 s (denaturing), 50°C/30 s (base annealing temperature of first three cycles) and 72°C/1 min (extension), and was continued until the base annealing temperature reached the final condition of 44°C. Under the final conditions, the amplification was continued for 20 cycles. Additionally, the quality of the DNA isolated by both methods was evaluated by a restriction analysis, for which 10 µg of DNA isolated by each method were incubated overnight with 10 U Xba I, Not I and Eco RI, and analyzed on 1% agarose gels.

Evaluation of the CTAB-PVP modified method in other xylophagous species

To test if the modified method is applicable to other xylophagous insects, we isolated DNA from larvae of three additional cerambycid xylophagous beetles, *Ataxia alpha, Estoloides chamelae* and *Lissonotus flavocinctus* as previously described. Isolated DNA

from each species was stored during three months. After the storage period, DNA was digested with Eco RI, and used as template for PCR amplifications as previously described.

RESULTS AND DISCUSSION

Biological material for DNA isolation varies in contaminants content according its origin (e.g. organism, tissue, life stage) (Aljanabi and Martínez 1997, Hill and Gutierrez 2003, Friar 2005). Therefore, the type and condition of specimens and tissues are key factors in selecting a DNA isolation method. A DNA isolation method from tissues rich in phenolics and tannins (such as those present in the digestive tracts of xylophagous insects), must remove these secondary compounds from DNA preparations. Phenolics and other secondary compounds cause damage to DNA and/or inhibit restriction endonucleases and Taq polymerases (Lodhi et al. 1994, Friar 2005, Padmalatha and Prasad 2006, Arif et al. 2005, Li et al. 2007). The CTAB based methods which are widely used, occasionally could not remove all phenolics from DNA preparations (Lodhi et al. 1994). Antioxidants are commonly used to address problems related to phenolics; examples include β-mercaptoethanol, PVP, bovine serum albumin (BSA), among others (Zidani et al. 2005, Puchooa and Venkatasamy 2005). PVP forms complex hydrogen bonds with phenolics and co-precipitates with cell debris upon cell lysis (Lodhi et al. 1994, Kim et al. 1997, Michiels et al. 2003). Hence, when extract is centrifuged in the presence of chloroform, the PVP-phenolics complexes accumulate at the interface between the organic and aqueous phases and can be eliminated. On the other hand, high concentrations of β-mercaptoethanol, helps reduce the browning in DNA preparations produced by the oxidation of phenolics (Horne et al. 2004, Li et al. 2007). To test the effect of the inclusion of PVP and an increased concentration of β-mercaptoethanol in our DNA isolation method, we compared this method with traditionally used CTAB method (Doyle and Doyle 1990). The results indicated similar

yields (~50 µg/100mg fresh tissue) of high molecular weight DNA using both methods (Fig.1). Nevertheless, the ratio A260/280 for CTAB method (1.21-1.32) and for CTAB-PVP modified method (1.69-1.76) indicated a higher contamination level in the DNA isolated by the traditional CTAB method.

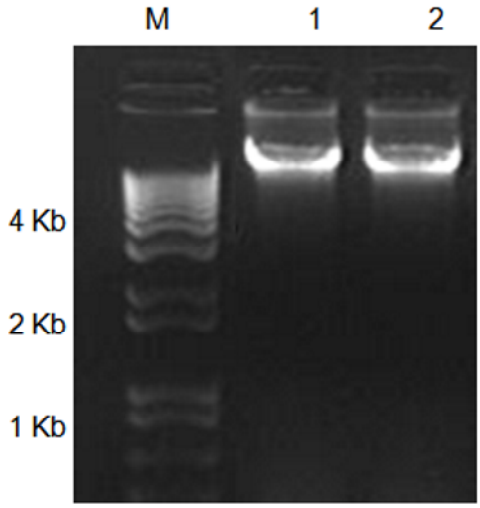

Figure 1. Agarose gel analysis of DNA prepared from *Oncideres albomarginata chamela* larvae with two DNA isolation methods. M, DNA size marker (1kb plus DNA ladder, Invitrogen); Lane 1, genomic DNA isolated with the CTAB method; Lane 2, genomic DNA isolated with the CTAB-PVP method.

The isolated DNA using both methods was tested for PCR amplification reactions. Amplifications of a COI gene fragment using fresh DNA obtained with both methods were successfully achieved. However, when DNA purified by both methods was stored during three months, and used as template for PCR, the amplification product was observed only for the CTAB-PVP isolated DNA (Fig. 2). These results indicate that DNA isolated by the traditional CTAB method is not suitable for longer storage periods. Similar results have been previously reported [18, 22]. DNA preparations containing phenolics have a shorter storage lifespan [18]. This confirms that DNA isolated by the

CTAB-PVP method had lower concentrations of polyphenolics than the traditionally used CTAB method.

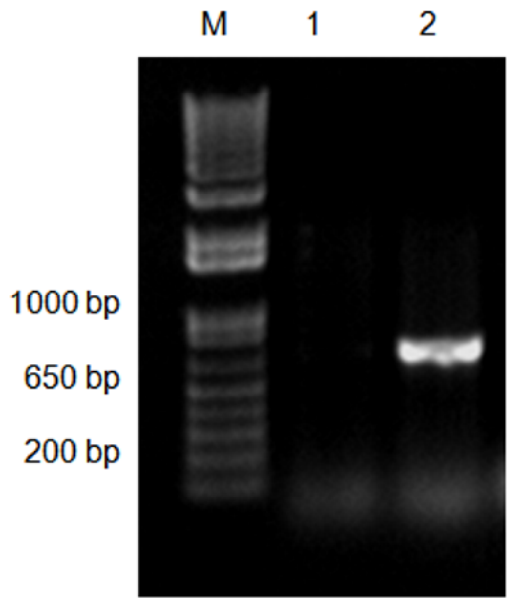

Figure 2. Amplification of a COI gene fragment using DNA isolated from *Oncideres albomarginata chamela* larvae and stored during three months. M, DNA size marker (1kb plus DNA ladder, Invitrogen); Lane 1, genomic DNA isolated with the CTAB method; Lane 2, genomic DNA isolated with the CTAB-PVP method.

The purity and quality of isolated DNA also were validated by digestion with different restriction endonucleases. The results showed a complete digestion of CTAB-PVP isolated-DNA (Fig 3B), while CTAB isolated-DNA was only partially restricted (Fig. 3A), indicating the presence of contaminants in this DNA preparation. The CTAB-PVP method demonstrated to be applicable to other xylophagous insects, since isolated DNA from three additional species of xylophagous beetles proved amenable for PCR amplification (Fig. 4) and restriction digestion (Fig. 5).

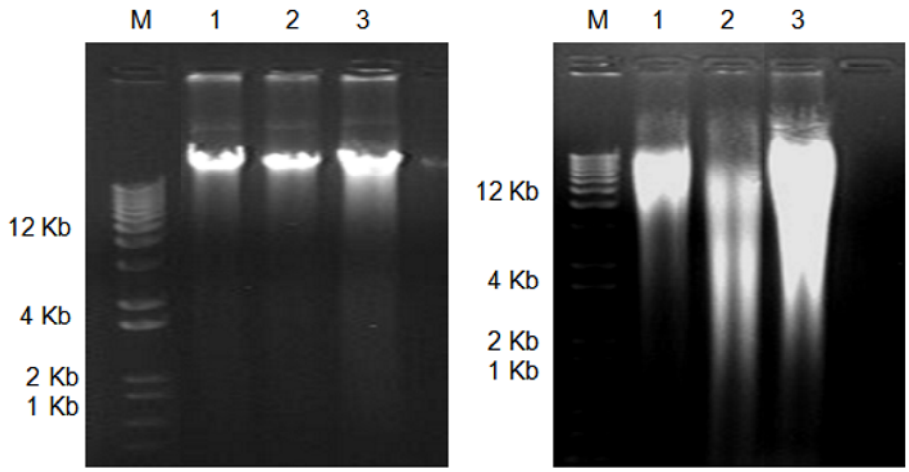

Figure 3. Analysis of *Oncideres albomarginata chamela*-DNA digested with different restriction enzymes and separated by electrophoresis in a 1% agarose gel. A) Genomic DNA isolated with the CTAB method: M, DNA size marker (1kb plus DNA ladder, Invitrogen); Lane 1, restriction digestion with Xba I; Lane 2, digestion with Not I; Lane 3, restriction digestion with Eco RI. B) Genomic DNA isolated with the CTAB-PVP method: M, DNA size marker (1kb plus DNA ladder; Invitrogen, Carlsbad, CA, USA); Lane 1, restriction digestion with Xba I; Lane 2, restriction digestion with Not I; Lane 3, restriction digestion with Eco RI.

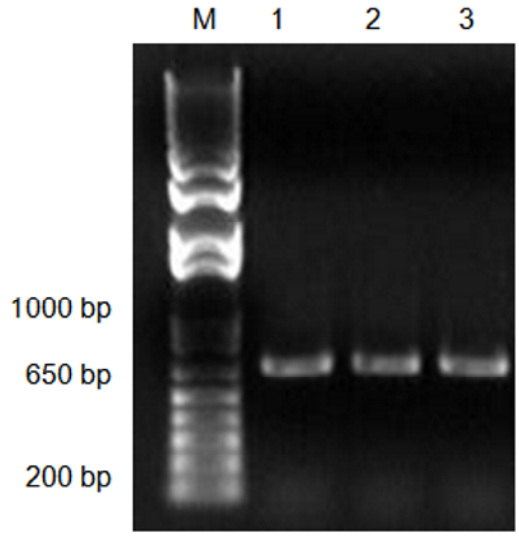

Figure 4. Amplification of a COI gene fragment using DNA isolated from larvae of three xylophagous beetles and stored during three months. M, DNA size marker (1kb plus DNA ladder; Invitrogen, Carlsbad, CA, USA); Lane 1, *Ataxia alpha*; Lane 2, *Estoloides chamelae;* Lane 3, *Lissonotus flavocinctus.*

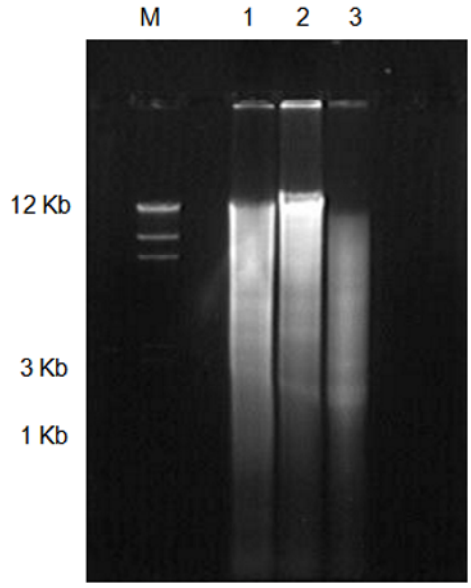

Figure 5. Analysis of three xylophagous beetles-DNA digested with Eco RI and separated by electrophoresis in a 1% agarose gel. Lane 1, *Ataxia alpha*; Lane 2, *Estoloides chamelae;* Lane 3, *Lissonotus flavocinctus.*

In conclusion, the proposed CTAB-PVP method for DNA isolation seems to be suitable for most of the genetic and molecular analysis. This method is rapid, simple and efficient for the isolation of DNA from xylophagous insects which possess high concentrations of plant compounds that can interfere with DNA extraction and analysis.

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