NIDIFICACIÓN Y RECONOCIMIENTO EN HEMBRAS DE *MANUELIA POSTICA* (XYLOCOPINAE: APIDAE) Y ANÁLISIS FILOGENÉTICO DE XYLOCOPINAE; IMPLICANCIAS EN LA EVOLUCIÓN DE LA SOCIABILIDAD EN APIDAE

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A Jeanette, quien ha sido apoyo permanente y por quien sigo adelante.

A Flor y Antonio, quienes son ejemplo y de quienes más aprendo.

A Marisol y Sergio, a quienes por distintas razones admiro.

A Emilia y Valentina, quienes sólo me regalan alegría.

RESÚMEN BIOGRÁFICO

Mi interés por la ciencia, particularmente por la entomología, comenzó a muy temprana edad, incentivado por algunos profesores y apoyado permanentemente por mis padres. Formé parte de las Juventudes Científicas de Chile, asociadas al Museo Nacional de Historia Natural, participando en trabajo de terreno, laboratorio, y en congresos estudiantiles, desde el año 1983. De gran estímulo fue haber obtenido en 1985 el premio anual que la Academia Chilena de Ciencias entregaba a un trabajo científico desarrollado por escolares. Es el único diploma que conservo enmarcado en mi hogar, y que tal vez con el transcurso de los años se transforme en el más importante. Ingresé a la Universidad Metropolitanta de Ciencias de la Educación, en gran parte motivado por la presencia del Instituto de Entomología que funciona en dicha institución, donde efectué mi especialización profesional. En 1995 obtuve el grado de Licenciado en Ciencias de la Educación con mención en Entomología, y luego el título de Profesor de Biología y Ciencias Naturales, en 1998, del cual siempre me he sentido orgulloso. El año 2002 egresé del Magíster en Ciencias, mención en Entomología, impartido en el Instituto de Entomología, y el año 2003 ingresé al programa de Doctorado en Ciencias, mención en Ecología y Biología Evolutiva del cual ahora egreso. Durante todos estos años he conocido, en diverso grado de profundidad, científicos nacionales y extranjeros, de los cuales he aprendido enfoques metodológicos y formas de trabajo, más que solo conocimientos. Esto ha conducido inexorablemente a originar y desarrollar, desde hace algunos años, una línea de investigación propia, en el campo de la evolución de la sociabilidad en insectos, cuyos resultados han comenzado a materializarse, parte de los que se reflejan en esta tesis doctoral, así como en las actuales y venideras publicaciones.

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RESUMEN

En especies eusociales de Hymenoptera, las hembras son más tolerantes hacia compañeras de nido que hacia no compañeras de nido, en tanto que en especies solitarias las hembras son agresivas hacia cualquier hembra conespecífica, con excepción de algunas especies en las cuales se ha sugerido que el comportamiento solitario deriva de especies eusociales. La evolución del estado eusocial desde el estado solitario ha sido hipotetizado en diversos taxones de Hymenoptera, y ha ido acompañado de una modificación del patrón agresivo no discriminativo exhibido por las hembras solitarias, a un patrón de discriminación conductual característico de las hembras eusociales. La evolución de los rasgos sociales debe ser entendida en un marco filogenético, ya que es posible que la eusociabilidad se haya originado por diferentes rutas y por distintas razones en diferentes grupos. Grupos monofiléticos que contienen especies que presentan un amplio rango de sociabilidad han emergido como un valioso modelo para estudiar las transiciones en la evolución de sociabilidad. La subfamilia Xylocopinae es uno de tales grupos y ha sido reconocida como el linaje más basal dentro de la familia Apidae. La tribu Manueliini ha sido propuesta como el grupo hermano de todos los demás Xylocopinae, aunque tal hipótesis ha sido cuestionada. En el contexto de la evolución de la sociabilidad en Xylocopinae y de los rasgos conductuales relacionados, los principales objetivos de esta investigación son: i) presentar una nueva hipótesis filogénetica para Xylocopinae, basada en datos nucleotídicos, incluyendo todas las especies del género *Manuelia*, ii) describir la biología de la nidificación de *Manuelia postica*, una especie solitaria de la

tribu Manueliini, para identificar potenciales rasgos precursores de la sociabilidad en una especie que no tiene especies eusociales cercanamente relacionadas, iii) desarrollar experimentos para demostrar la ocurrencia del reconocimiento entre hembras compañeras de nido; lo cual ha sido sugerido sobre la base de observaciones efectuadas en un contexto natural; y evaluar el papel que los compuestos cuticulares juegan en este fenómeno, y iv) explorar la posibilidad de igualación de fenotipos por autoreferencia en una especie en que cada individuo permanece aislado de los otros durante todo el período de desarrollo. Los análisis filogenéticos indicaron que la tribu Manueliini es el linaje más basal de Xylocopinae. Los principales resultados demostraron que *M. postica* presenta algunos rasgos típicos de la vida solitaria y otros inusuales para abejas solitarias, y que han sido descritos en especies sociales filogenéticamente más apicales. Además, el reconocimiento entre hembras compañeras de nido mediado por compuestos cuticulares, y el reconocimiento de parentesco basado en igualación de fenotipos por autoreferencia, fueron demostrados por primera vez en una especie solitaria de la familia Apidae. La posición filogenética de Manueliini sugiere que algunos rasgos conductuales propuestos como precursores de la sociabilidad, tales como el reconocimiento entre compañeras de nido y el reconocimiento de parientes, exhibidos por especies eusociales más apicales que *Manuelia,* representan la retención de un estado ancestral en Xylocopinae. Los resultados aquí presentados permiten preguntarse si existe un patrón conductual de mayor tolerancia entre hembras compañeras de nido que entre no compañeras de nido en otras especies solitarias de Apidae. De ser así, la modificación de un patrón conductual

agresivo no discriminativo exhibido por las especies solitarias habría sido necesario en la evolución de la sociabilidad de otros grupos taxonómicos.

ABSTRACT

In eusocial Hymenoptera, females are more tolerant towards nestmate than towards non-nestmate females; instead, in solitary Hymenoptera females are aggressive towards any conspecific female, with the exception of some species in which solitary behavior has been suggested to derive from eusocial species. The evolution of the eusocial state from the solitary state has been hypothesized in several taxa of Hymenoptera, and requires a modification of the aggressive pattern exhibited by solitary females to a discrimination pattern characteristic of eusocial ones. Evolution of social traits is best considered in the context of phylogeny, since it is possible that eusociality has arisen by different routes and for different reasons in different taxonomic groups. Groups containing species ranging from solitary to social have emerged as a valuable model to study the transitions in social evolution. The Xylocopinae subfamily is one such monophyletic group, which has been recognized as the most basal lineage within the family Apidae, and the tribe Manueliini has been proposed as sister group of all other Xylocopinae, although such hypothesis has been questioned. In the context of the evolution of sociality and related behavioural traits in the Xylocopinae, the main purposes of this research are: i) to present a new phylogenetic hypothesis, based on nucleotide data, for Xylocopinae, including all species of the genus *Manuelia*, ii) to describe the nesting biology of *Manuelia postica*, a solitary species in tribe Manueliini, to identify potential precursor traits of sociality in a solitary species which does not have a closely related eusocial species, iii) to develop experiments to demonstrate the occurrence of nestmate recognition, as suggested by observations in a natural

context, and the role that cuticular compounds play in this phenomenon, and iv) to explore the possibility of self referent phenotype matching in a species in which each individual remains isolated from the others during all the developing period. Phylogenetic analyses indicated that the tribe Manueliini is the most basal lineage of Xylocopinae. The main results demonstrated that *M. postica* presents some features typical of solitary life, and others which are unusual in solitary bees and which have been reported in phylogenetically more apical social species. Moreover, nestmate recognition mediated by cuticular compounds and kin recognition based on self referent phenotype matching in a solitary species of Apidae were demonstrated for the first time. The position of Manuellini within Xylocopinae suggests that some behavioral traits proposed as precursors of sociality, such as nestmate and kin recognition exhibited by eusocial species more apical than *Manuelia*, represent the retention of an ancestral state in the Xylocopinae. Moreover, if and when other solitary species of Apidae are examined, higher tolerance between nestmate than between non-nestmate females might prove to be a more frequent phenomenon than previously thought, and modification of an agonistic non-discriminative pattern in solitary species has probably been necessary for social evolution in other taxonomic groups.

INTRODUCCION

En especies del orden Hymenoptera, la vida solitaria se caracteriza porque habitualmente sólo una hembra construye un nido (o habita uno vacío) sin la presencia de otra hembra, y es responsable del cuidado de su progenie en desarrollo, aún cuando nunca está en contacto directo con ella. Los individuos inmaduros permanecen aislados de cualquier conespecífico durante todo su desarrollo hasta alcanzar el estado adulto. Las especies en que ocurre contacto entre la hembra y su progenie inmadura, hasta antes que la generación filial alcance el estado adulto, son categorizadas como subsociales. En contraposición, el comportamiento social requiere de un grupo de hembras adultas dentro de un nido, que manifiestan cooperación con diferente grado de complejidad. Si las hembras son de la misma generación y efectúan las mismas tareas, el nivel de organización es comunal, pero si hay división de la labor reproductiva (habitualmente una reina y más de una obrera), o al menos una aproximación a esas castas, la organización es semisocial. El nivel de organización es eusocial si en la agrupación existen dos o más generaciones de hembras adultas, con división de la labor reproductiva, en que la reina es la madre de las obreras (Batra, 1966; Michener, 1969; 1974; Wilson, 1971).

Un rasgo distintivo de las hembras de especies eusociales de Hymenoptera es la discriminación conductual, que puede verse reflejada en un patrón de comportamiento agresivo diferencial de acuerdo con la procedencia de las hembras; aquellas que han nacido y se han desarrollado en el mismo nido (compañeras de nido), son más tolerantes y/o menos agresivas entre sí que

aquellas provenientes de nidos diferentes (no compañeras de nido) (Buckle & Greenberg, 1981; Breed, 1998; Inoue et al., 1999; Boulay & Lenoir, 2001; Beekman et al., 2002). Por el contrario, las hembras de especies solitarias no presentan un patrón de discriminación conductual, ya que habitualmente son intolerantes hacia cualquier hembra (Hölldobler & Michener, 1980; Kukuk, 1992; Field, 1992, Packer, 2000). Aunque investigaciones recientes efectuadas en especies solitarias de la familia Halictidae (Hymenoptera) sugieren tolerancia entre hembras compañeras de nido, dicha capacidad en tales especies puede representar una rasgo derivado de ancestros eusociales (Wcislo, 1997a; Jeanson et al., 2005).

En Hymenoptera, tradicionalmente se ha hipotetizado que el comportamiento eusocial habría evolucionado a partir de especies solitarias de manera directa, o de manera indirecta, desde un nivel solitario a uno subsocial o semisocial (Wheeler, 1928; Sakagami & Michener, 1962; Lin & Michener, 1972; Michener, 1985; Carpenter, 1989; Gadagkar, 1990; Danforth, 2002). Se ha sugerido que en tal ruta evolutiva el patrón de comportamiento agresivo sin discriminación entre conespecíficos, característico de especies solitarias, evolucionó a un patrón de discriminaión conductual en el que las hembras son agresivas sólo hacia no compañeras de nido, rasgo característico de las especies eusociales (Hölldobler & Michener, 1980; Moynihan, 1998; Wcislo, 2000). La modificación de tal patrón de comportamiento agresivo entre hembras conespecíficas es más probable si éstas se han desarrollado juntas y establecen posteriores agrupaciones dentro del nido exhibiendo mutua tolerancia, o cuando los individuos inmaduros tienen la posibilidad de interactuar entre sí y/o con su

progenitora (Lin & Michener, 1972; Michener, 1969). Otros rasgos conductuales que han sido propuestos como prerrequisitos en la evolución del comportamiento social son i) la existencia de un extenso período de vida reproductiva de las hembras que fundan los nidos, de modo que la sobreposición de generaciones de hembras adultas sea posible dentro del nido, y ii) el comportamiento de defensa del nido en cuyo interior se encuentra la progenie en desarrollo. Todos estos rasgos conductuales, por lo tanto, están relacionados con la biología de la nidificación (Michener, 1969; 1974).

Por otra parte, la modificación en el comportamiento agresivo desde especies solitarias que no discriminan hacia especies sociales que sí lo hacen, requiere de la capacidad de reconocimiento entre hembras conespecíficas (Michener & Smith, 1987). Tal capacidad es mediada en insectos sociales principalmente por señales químicas (Howard & Blomquist, 2005) que pueden tener un origen endógeno y/o exógeno (Smith & Breed, 1995). Las señales endógenas son producidas por los individuos miembros de un nido o colonia y corresponden a compuestos químicos glandulares o secretados por la cutícula (Hölldobler & Michener, 1980). Los hidrocarburos cuticulares han sido frecuentemente incluidos en esta última categoría (Breed, 1998; Singer, 1998). Las señales exógenas corresponden a sustancias químicas absorbidas por la cutícula de los insectos, que determinarían el olor específico de la colonia (Hölldobler & Michener, 1980). Estos olores provienen del material del nido (Gamboa et al., 1986; Breed et al., 1988; Singer & Spelie, 1992) y/o de los alimentos (Smith & Breed, 1995). Si la capacidad de reconocimiento entre hembras compañeras de nido está mediada por estímulos de origen endógeno, entonces tal

capacidad corresponde al fenómeno de reconocimiento por parentesco (Getz & Smith, 1983; 1986). Se ha planteado que en los insectos sociales, fundamentalmente del orden Hymenoptera, el reconocimiento entre parientes se basa principalmente en el mecanismo de igualación de fenotipos (Wyatt, 2005), en que un individuo aprende las señales provenientes de parientes que participan en su crianza, habitualmente sus padres o hermanos (referencia de un pariente), y/o provenientes de él mismo (auto-referencia), los almacena en su memoria (patrón de reconocimiento) y luego compara el fenotipo de individuos encontrados por primera vez con el patrón de reconocimiento almacenado (Sherman et al., 1997). Sin embargo, sólo el mecanismo de igualación de fenotipos basado en referencia de parientes ha sido demostrado inequívocamente en especies eusociales (Buckle & Greenberg, 1981; Stuart, 1988; 1992; Soroker, 1995).

La gran mayoría de los estudios enfocados en el proceso de reconocimiento entre hembras compañeras de nido, así como respecto del origen (endógeno y/o exógeno) y procesamiento (igualación de fenotipos) de los estímulos involucrados, han sido efectuados en especies eusociales, principalmente del orden Hymenoptera (Formicidae, Vespidae, Halictidae y Apidae) (veáse revisiones de Hölldobler & Michener, 1980; Howard & Blomquist, 1982; Getz, 1991; Jaisson, 1991; Smith & Breed, 1995; Singer, 1998, Gamboa, 2004; Howard & Blomquist, 2005), debido a la importancia que tal fenómeno tiene para esas especies eusociales, pues permite el mantenimiento de la estructura social de una colonia (Hölldobler & Michener, 1980; Hölldobler & Wislon, 1990; Crozier & Pamilo, 1996), el nepotismo o conductas favorables hacia parientes

cercanos (Keller, 1997) y es una condicion necesaria en la evolución de la eusociabilidad, de acuerdo con la teoría de selección por parentesco (Hamilton, 1964a,b). Hasta la fecha no se han realizado estudios de tales aspectos en aquellas especies solitarias donde se ha propuesto tolerancia entre hembras de un mismo nido y posibilidad de reconocimiento entre éstas (Wcislo, 1997a; Jeanson et al., 2005).

Muchos grupos taxonómicos de insectos, tales como el orden Isoptera, la familia Formicidae, y las tribus Euglossini, Bombini, Meliponini y Apini, dentro de la subfamilia Apinae (Apidae), están conformados sólo por especies altamente sociales (Wilson, 1971; Crozier & Pamilo, 1996), en las que la sociabilidad es compleja y obligada (no facultativa), cuyos grupos hermanos o cercanamente emparentados no contienen ni especies solitarias ni especies con un nivel de organización social básico (Schwarz et al., 2007). En tales taxones es difícil reconstruir posibles rutas evolutivas desde la vida solitaria hacia la vida eusocial debido a que no existen especies actuales solitarias ni especies representativas de estados sociales intermedios (Danforth, 2002; Danforth et al., 2003; Schwarz et al., 2007). Sin embargo, existen otros grupos en los cuales los niveles de organización social varían entre las especies, los cuales permiten entender cómo han evolucionado las formas más avanzadas de sociabilidad (Wilson, 1971; Lin & Michener, 1972; Michener, 1974; 1985). Entre éstos, la subfamilia Xylocopinae, grupo basal de la familia Apidae (Michener, 2000) (Fig.1a), ha emergido como un importante modelo para estudiar la evolución de la sociabilidad (Michener, 1990; Schwarz et al., 1997; 1998; 2007; Tierney et al., 2002), ya que contiene especies que presentan gran

diversidad de niveles sociales, y también especies basales solitarias (Michener, 1969; 1974; 2000).

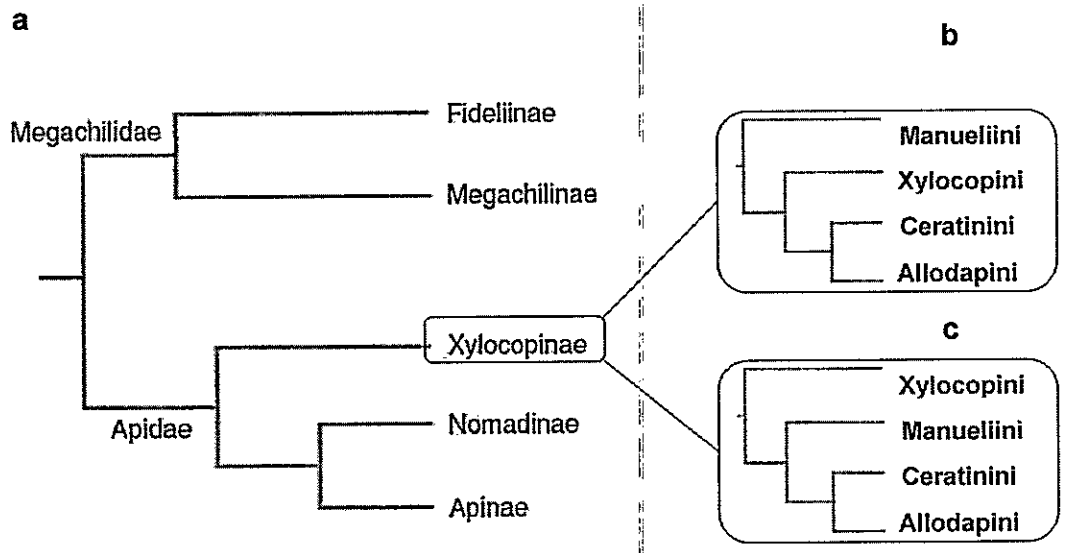

Figura 1. Relaciones filogenéticas propuestas para la subfamilia Xylocopinae dentro de la familia Apidae (a) (Michener, 2000), y de las tribus de Xylocopinae con Manueliini en la posición más basal (b) (Sakagami & Michener, 1987), y con Xylocopini en tal posición (c) (Roig-Alsina & Michener, 1993; Engel, 2001).

La subfamilia Xylocopinae está constituída por las tribus Manueliini, Xylocopini, Ceratinini y Allodapini (Michener, 2000) y, bajo el esquema de niveles de organización social (Batra, 1966; Michener, 1969; 1974; Wilson, 1971), la sociabilidad en Allodapini es más avanzada que en Ceratinini (Sakagami & Maeta, 1977; 1995) y en Ceratinini es más avanzada que en Xylocopini (Sakagami & Maeta, 1995; Maeta et al., 1996). Manueliini es la única tribu constituída sólo por especies solitarias (Daly et al., 1987; Michener, 2000), en algunas de las cuales se ha sugerido la existencia de rasgos conductuales que han sido hipotetizados como precursores en la evolución de la sociabilidad en otras especies solitarias de Xylocopinae (Sakagami & Maeta, 1977). Estos rasgos están relacionados con la biología de la nidificación (Claude-Joseph, 1926; Michener, 1985; Daly et al., 1987). La tribu Manueliini está representada sólo por el género relicto *Manuelia* (Michener, 1979; Daly et al., 1987; Michener, 2000), y ha sido hipotetizada como el grupo hermano de las otras tribus en Xylocopinae (Sakagami & Michener, 1987; Michener, 2000) (Fig.1b), aunque dos reconstrucciones filogenéticas, efectuadas sobre la base de caracteres morfológicos y análisis de parsimonia, han cambiado la posición de *Manuelia* por *Xylocopa* (tribu Xylocopini) (Roig-Alsina & Michener, 1993; Engel, 2001) (Fig.1c). Además, el género *Manuelia* no presenta especies hermanas eusociales, ni en Xylocopini (tribu filogenéticamente más cercana) y tampoco en Megachilidae, familia hermana de Apidae (Michener, 2000) (Fig.1a), por lo tanto, su comportamiento solitario no corresponde a una reversión evolutiva a partir de un estado ancestral eusocial, como ha sido planteado para especies solitarias de Halictidae (Wcislo, 1997a; Wcislo & Danforth, 1997; Danforth, 2002; Danforth et al., 2003; Jeanson et al., 2005).

PROBLEMA

El estudio de la evolución de la sociabilidad debe estar acotado al marco filogenético de un grupo, más que a la búsqueda de explicaciones generalizadas en grupos muy diversos (Gadagkar, 1990; Costa & Fitzgerald, 1996). Idealmente, estas entidades filogenéticas (Wcislo, 1997b) deben ser aquellas en que existen especies con diversidad de niveles sociales y especies no sociales (Schwarz, et al., 2007), como la subfamilia Xylocopinae (Michener, 2000). En este contexto cabe preguntarse si en especies solitarias de la subfamilia Xylocopinae, en las que la ausencia de sociabilidad no represente una reversión evolutiva, existen rasgos conductuales relacionados con la biología de la nidificación, considerados precursores de la vida social. En este sentido, también es relevante conocer si la capacidad de reconocimiento y discriminación entre hembras compañeras de nido está presente en dichas especies, lo cual permitiría re-considerar en la familia Apidae la hipótesis de la modificación del patrón agresivo no discriminativo caracterísitico de especies solitarias, a un patrón de discriminación conductual, característico de las especies eusociales. Además, debido a que en las especies solitarias de Xylocopinae los individuos permanecen aislados de cualquier conespecífico durante todo su desarrollo hasta alcanzar el estado adulto, estos taxones emergen como modelos para poner a prueba la existencia del mecanismo de igualación de fenotipos basado en auto-referencia, hasta ahora no demostrado en especies solitarias de Apidae. *Manuelia postica* será la especie escogida para responder tales interrogantes, debido a que pertenece a uno de los géneros solitarios y basales dentro de Xylocopinae, que no presenta grupos

hermanos, ni filogenéticamente cercanos que exhiban eusociabilidad, y en cuyas especies congenéricas ha sido sugerida la existencia de rasgos conductuales hipotetizados como precursores de la vida social (Sakagami & Maeta, 1977). Por último, debido a que la posición filogenética de las tribus Xylocopini y Manueliini permanece no resuelta, un aspecto fundamental para el posterior análisis de los resultados obtenidos es la reconstrucción filogenética de la subfamilia Xylocopinae, sobre la base de caracteres moleculares que por primera vez incluirá a las cuatro tribus de tal subfamilia, así como también a las tres especies de *Manuelia*: *M. gayi*, *M. gayatina* y *M. postica*.

HIPÓTESIS

La tribu Manueliini representaría el grupo hermano de los demás Xylocopinae, de modo que rasgos conductuales que han sido hipotetizados como precursores de sociabilidad en las otras tribus de esta subfamilia, tendrían un origen previo al origen de tales taxones.

Manuelia postica es una especie solitaria que presentaría rasgos conductuales vinculados con la biología de la nidificación, hipotetizados como precursores de sociabilidad en otras especies de la subfamilia Xylocopinae, que posibilitarían la tolerancia entre las hembras compañeras de nido. Dicha tolerancia permitiría en *M. postica* la capacidad de reconocimiento y discriminación entre las hembras, rasgo característico de las especies eusociales y filogenéticamente más apicales en la familia Apidae.

Debido a que en otras especies de *Manuelia*, y de Xylocopinae, los individuos inmaduros se desarrollan aislados de cualquier conespecífico hasta después de su emergencia, es esperable en *M. postica* la existencia de reconocimiento entre hembras basado en igualación de fenotipos por autoreferencia.

OBJETIVOS

Esta investigación tiene cuatro objetivos generales, cada uno de los cuales surge a partir de las hipótesis propuestas y son desarrollados en cada uno de los anexos:

- Presentar una nueva hipótesis filogenética basada en datos moleculares para la subfamilia Xylocopinae, incluyendo la posición de las especies del género *Manuelia*. En el capítulo 1 se presentan los resultados de reconstrucciones filogenéticas efectuadas con métodos de máxima parsimonia, máxima verosimilitud e inferencia bayesiana, utilizando dos genes mitocondriales y uno nuclear, que sitúan al género *Manuelia* en la posición más basal de la subfamilia Xylocopinae.

- Conocer aspectos relacionados con la biología de la nidificación en *Manuelia postica*, que entreguen antecedentes respecto de rasgos que han sido propuestos como precursores de sociabilidad en especies filogenéticamente relacionadas, y que permitan evaluar la potencialidad de reconocimiento entre hembras, en un contexto natural. En el capítulo 2 se describe el ciclo biológico de *M. postica*, con énfasis en el período de construcción de nidos y de crianza de la progenie, y la arquitectura de los nidos. También se describen y discuten interacciones conductuales relacionadas con la biología de la nidificación, en condiciones naturales.

- Establecer si las hembras de *M. postica* presentan la capacidad de reconocimiento de conespecíficas, evaluada a través de la respuesta agonística en presencia de hembras que i) han nacido y se han desarrollado en el mismo nido (compañeras de nido), y ii) provienen de nidos diferentes (no compañeras de nido). En el capítulo 3 se presentan y discuten los resultados de experimentos efectuados en el campo, que demuestran la capacidad de reconocimiento y discriminación conductual de las hembras, sobre la base de su procedencia (compañeras de nido y no compañeras de nido). Además, se presentan y discuten los resultados de bioensayos realizados en el laboratorio, en los cuales se manipularon los compuestos cuticulares de las hembras, que demuestran que tales compuestos están involucrados en el reconocimiento. Por último, se presentan los compuestos cuticulares identificados y se comparan entre hembras compañeras de nido, y entre hembras no compañeras de nido.

- Determinar si en las hembras de *M. postica* el reconocimiento entre conespecíficas está basado en el mecanismo de igualación de fenotipos por autoreferencia. En el capítulo 4 se presentan y discuten los resultados de un experimento de "cross-fostering" montado en el campo y un posterior bioensayo realizado en el laboratorio, que demuestra el reconocimiento de parentesco en hembras de *M. postica* basado en la igualación de fenotipos por autoreferencia.

CAPÍTULO 1

Molecular phylogeny and hypothesis testing of the basal lineage in the subfamily Xylocopinae (Apidae)

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ABSTRACT

The subfamily Xylocopinae has been recognized as the most basal lineage within the family Apidae. This subfamily comprises four tribes: Manueliini, Allodapini, Ceratinini, and Xylocopini. Diverse phylogenetic analyses using morphological data have been performed by several authors attempting to reveal the relationships among those tribes. Nevertheless, the relative position of Manueliini and Xylocopini remains unclear, since both tribes have been placed as the most basal lineage within this subfamily. Excepting Manuelliini, several studies of molecular phylogenetics have been achieved in Xylocopinae, focused in relationships within each tribe. Therefore, currently there is a lack of evidence, derived from molecular data, to evaluate the alternate hypotheses concerning the phylogenetic relationships among tribes of Xylocopinae. Here

we present results of a molecular phylogeny using sequences of *Col*, *Cytb* and EF-1αF1 from members of the four tribes of Xylocopinae. We used data available from other studies and included new data generated for the three species belonging to the tribe Manueliini. The competing phylogenetic hypotheses of Xylocopinae, derived from morphological data, were here evaluated through phylogenetic hypothesis testing. In addition, we analyzed the phylogenetic signal provided by EF-1αF1 and EF-1αF2 in a large dataset, in order to determine the cause of the observed incompatibility between mitochondrial genes and EF-1αF1 detected in this study. Our results indicate that Manueliini is the most basal lineage of Xylocopinae. We also discuss the implication of our findings in the context of the evolution of sociality in this group of bees.

INTRODUCTION

The subfamily Xylocopinae is currently conceived as the most basal clade in the Apidae (bees) phylogeny (Michener, 2000). Xylocopinae was formerly included within the family Anthophoridae but, after a cladistic analysis using morphological data it was finally included within Apidae (Roig-Alsina and Michener, 1993). This subfamily includes four tribes: 1) Manueliini, a monogeneric taxon which occurs predominantly in south-central Chile (Daly et al., 1987); 2) Xylocopini, a monogeneric worldwide distributed group; 3) Ceratinini, including four genera and several subgenera very abundant on all continents excepting Australia, where only one species is found; 4) Allodapini,

comprising eleven genera predominantly distributed in Africa and Australia (See Michener, 1979 and 2000 for biogeographical and systematical issues, respectively).

The subfamily Xylocopinae has emerged as a valuable model to study early stages and evolution of sociality (e.g. Michener 1985, 1990; Schwarz et al., 1997, 1998, 2007; Tierney et al., 2002), since it includes species ranging from solitary to social levels of nesting behavior and social organization (Michener, 1969, 1974, 2000). Manueliini contains only solitary species, i.e., each female makes her own nest (Daly et al., 1987; Michener, 1985, 2000; Flores-Prado et al., 2007). In Xylocopini, most species are solitary, some have communal life (Michener, 1985) and others show an incipient reproductive division of labor among adult females of the same generation, referred to as semisocial level of organization (Gerling et al., 1989). Ceratinini has long been considered to consist of solitary species (Sakagami and Maeta, 1977, 1987; Maeta et al., 1997a) but others can be regarded as subsocial species (Sakagami and Maeta, 1977). Interestingly, some species belonging to Ceratinini exhibit a rudimentary caste system, being considered as incipiently eusocial (Okazaki, 1987; Sakagami and Maeta, 1995). Finally, all Allodapini species display traits of subsocial modes of life (Michener, 1974) and some of them have reached different degrees of sociality (reviewed in Schwarz et al., 2007). Moreover, some species belonging to Allodapini have been indicated as highly eusocial, since their organization is based on morphological castes (Houston, 1977). In conclusion, sociality in species of Allodapini appears to be more advanced than in Ceratinini (Sakagami and Maeta, 1977; Sakagami and Maeta, 1995),

remaining more incipient in Xylocopini (Maeta et al, 1996), and being absent in Manueliini (Claude-Joseph, 1926; Michener, 1985; Daly et al., 1987; Flores-Prado et al., 2007). Therefore, diverse evolutionary histories about the sociality in Xylocopinae can be depicted, depending on the phylogenetic hypotheses under consideration.

Manueliini, constituted by the genus *Manuelia* Vachal, includes only three species: *M. gayi*, *M. postica* and *M. gayatina* (Sakagami and Michener, 1987). *Manuelia* have been proposed as a relict lineage (Daly et al., 1987; Michener, 1979, 2000) exhibiting high morphological differentiation among its members: "*This genus contains three species, so different that they could well be placed on different genera or at least subgenera*" (Michener, 2000). Despite the relationships among the tribes belonging to Xylocopinae were analyzed by Sakagami and Michener (1987), Roig-Alsina and Michener (1993), and Engel (2001), the phylogenetic placement of *Manuelia* remains controversial. Roig-Alsina and Michener (1993) obtained two minimum-length trees differing in the positions of the tribes Manueliini and Xylocopini, by using morphological data in the subfamily Xylocopinae. One tree showed *Manuelia* as the most basal and *Xylocopa* in the next branch, while the other reversed these positions. Engel (2001) explored the relationships among the tribes of Xylocopinae on the basis of morphological characters, including a Baltic amber fossil. The results of that analysis were in agreement with one of the topologies presented by Roig-Alsina and Michener (1993), in which Xylocopini was the most basal lineage in Xylocopinae followed by Manueliini: (Xylocopini (Manueliini (Ceratinini, Allodapini))). Phylogenetic relationships, inferred on the basis of morphological

and molecular characters of extant species of Apidae, have included species belonging to different tribes of Xylocopinae (Cameron, 1993; Schwarz et al., 1998; Mardulyn and Cameron, 1999; Ascher et al., 2001; Cameron and Mardulyn, 2001; Leys et al., 2002; Bull, et al., 2003). However, species of Manueliini have not been included as a part of the ingroup for molecular phylogenetic inference.

The aim of this study is a) to contribute with a phylogenetic hypothesis for relationships among tribes of Xylocopinae, based on nucleotide data; b) to test the competing hypotheses on the basal lineages of Apidae (Manueliini versus Xylocopini). The results of this work could contribute with new insights about the phylogeny of xylocopine bees, as well as with the necessary phylogenetic framework for studying the evolution of sociality at this basal lineage of Apidae.

MATERIAL AND METHODS

Samples and Sequencing

Samples of *Manuelia postica*, *Manuelia gayi*, and *Manuelia gayatina* were collected in Central Chile and used to obtain nucleotide sequences from fragments of mtDNA *Col* (cytochrome oxidase I), mtDNA *Cytb* (Cytochrome b) and EF-1αF1 (elongation factor copy F1). Those genes have been previously used in studies of molecular phylogenetics within other tribes of Xylocopinae: Xylocopini (Leys et al., 2000), Allodapini (Bull et al., 2003; Schwarz et al., 2004,

2006), and Ceratininii (Bull et al., 2003; Cronin, 2004; Fuller et al., 2005; Schwarz et al., 2006).

Single bees were homogenized and DNA was extracted using the DNeasy Tissue Kit (Qiagen). PCR amplification was performed by mixing 1.0 µl of the bee DNA with 49 µl of PCR master mix. Reaction conditions for the PCR were 1× reaction buffer, 0.1 mM each dNTP, 0.2 µM each primer, and 2.5 units of *Taq* Polymerase (New England Biolabs). Forward and reverse primers (source between parenthesis), size fragment, and annealing temperature used for PCR were as follows: a) *Col* = FORCOI (atgtgctacacatcgctagc) and REVCOI (atgatcgctcgatcgatcgcg) (Former et al., 1986), 602 bp, 55^{O} C ; b) *Cytb* = CYTbF (atgtgtgcgagcgatagcta) and CYTbR (gtgcgctatacggctagcg) (this study), 651 bp, 60^{O} C; c) EF-1αF1 = FOREF1 (agtgcggtatgtacatga) and REVEF1 (gtgatagacacatcgttaga) (this study), 452 bp , 62^{O} C.

An MJ Research thermocycler was used to incubate the reactions for 2 min at 95 °C, and cycle 35 times at 95°C for 0.5 min, 0.5 min at the annealing temperature, and 72 °C for 1 min followed by 10 min at 72°C. PCR products was purified with the MinElute PCR Purification Kit (Qiagen). Purified PCR products were sequenced with Big Dye Terminator Chemistry V3 (ABI). 5-10 ng of amplified target was added to 4.5 µl of Big Dye reaction mix with 0.4 µM of primer to a total volume of 10 µl. Reactions were ramped to 96°C at 2.5°C/sec and cycled 30 times for 10 sec at 96°C, 5 sec at 50°C, and 2 min at 60°C. Sequences were cleaned using Wizard $^{(r)}$ Magnesil Green Sequencing Reaction Clean-Up System (Promega) and analyzed with an ABI 3730.

Sequences from species representing the tribes Xylocopini, Allodapini, and Ceratinini, and also from *Apis mellifera* and other insects were retrieved from Genbank (See list of accessions in Table I and Appendix 1).

Table 1. Accession of nucleotide sequences used in this study.

Tribe	Species	Accession *Coi*	Accession *Cytb*	Accession EF-F1
-	*Apis mellifera*	AY114482.1	L06178.1	X52884.1
Allodapini	*Braunsapis unicolor*	DQ149658.1	AF072666.1	AJ416776.1
Allodapini	*Brevineura ploratula*	DQ149674.1	AJ416824.1	AJ416769.1
Allodapini	*Compsomelissa borneri*	DQ149675.1	AJ416840.1	AJ416784.1
Allodapini	*Exoneura robusta*	DQ149661.1	AJ416815.1	AJ416760.1
Allodapini	*Exoneurella tridentata*	DQ149665.1	AF072670.1	AJ416766.1
Allodapini	*Macrogalea infernalis*	EF103592.1	EF103597.1	EF103602.1
Ceratinini	*Ceratina flavipes*	AY250190.1	AY250200.1	AY250210.1
Ceratinini	*Ceratina iwatai*	AY250191.1	AY250201.1	AY250211.1
Ceratinini	*Ceratina japonica*	AY250192.1	AY250202.1	AJ416849.1
Ceratinini	*Ceratina okinawana okinawana*	AY250194.1	AY250204.1	AY250214.1
Xylocopini	*Xylocopa bombylans*	AY005227.1	AY005254.1	AY005281.1
Xylocopini	*Xylocopa frontalis*	AY005248.1	AY005275.1	AY005302.1
Xylocopini	*Xylocopa pubescens*	AY005236.1	AY005263.1	AY005290.1
Xylocopini	*Xylocopa virginica virginica*	AY005231.1	AY005258.1	AY005285.1
Manueliini	*Manuelia gayatina*			
Manueliini	*Manuelia gayi*			
Manueliini	*Manuelia postica*			

Aligning and saturation analysis

Alignment was performed by using ClustalX (Thompson et al., 1997) and checked using Bioedit (Hall, 1999). Saturation of phylogenetic signal was measured by plotting transitional and transvertional uncorrected distances for each codon position against maximum likelihood distances for the complete gene fragment estimated under the GTR+G model of nucleotide substitution. Pairwise matrices for corrected and uncorrected distances were obtained using Paup4.0b10 (Swofford, 2002).

Homogeneity test

In order to test for congruence among data partitions, 10^4 replicates of the partition homogeneity test (IDL, Farris 1994, 1995) were run in PAUP, comparing the phylogenetic signal between both mitochondrial genes and between mtDNA and EF-1αF1. Other set of partitions were additionally used in this analysis by including and excluding thirds codon positions (see below).

Phylogenetic signal of EF-1αF1

The phylogeny derived from EF-1αF1 was incompatible with that obtained from the mitocondrial genes (see Results) and disagreed with the previously proposed phylogenetic relationships among tribes of Xylocopinae, in particular with the relationship Ceratinini + Allodapini originally proposed by Sakagami and Michener (1987) and strongly supported by other surveys of phylogenetic

inference using morphological data (reviewed in Engel, 2001). Therefore, the phylogenetic signal of EF-1αF1 was analyzed.

To determine the origin of error in the phylogeny derived from EF-1αF1, a phylogenetic analysis was performed using the complete dataset of both copies of EF-1α (F1 and F2) available in Genbank within Xylocopinae (Taxid: 78170). Additionally, sequences of *Apis mellifera* (basal to Xylocopinae), *Drosophila melanogaster* (outgroup, basal to Apoidea) *Bombyx mori*, *Tribolum castaneum*, and three species of *Nassonia* were included for this purpose (See the list of accession numbers in Appendix 1). The objectives of this analysis were: 1) to verify the orthology of EF-1αF1 in Xylocopinae; 2) to evaluate the phylogenetic signal of EF-1αF1 within the tribe Xylocopini, since this tribe was paraphyletic in our preliminary analysis (see figure 1); and 3) to identify potential pseudogenes of EF-1αF1 affecting the species tree inference. This phylogeny was inferred from both nucleotide and aminoacid sequences using 4 million generations in MrBayes. The optimal model of nucleotide substitution used was GTR + I + G, as derived from the AIC test in MrModeltest. WAG + Γ + I was the optimal model of aminoacid substitution, as derived from ProTest (Cuff et al., 2000). Other options and parameters for MrBayes were set by default. Computation of consensus trees and posterior probabilities were as described in the phylogenetic analysis section (see below).

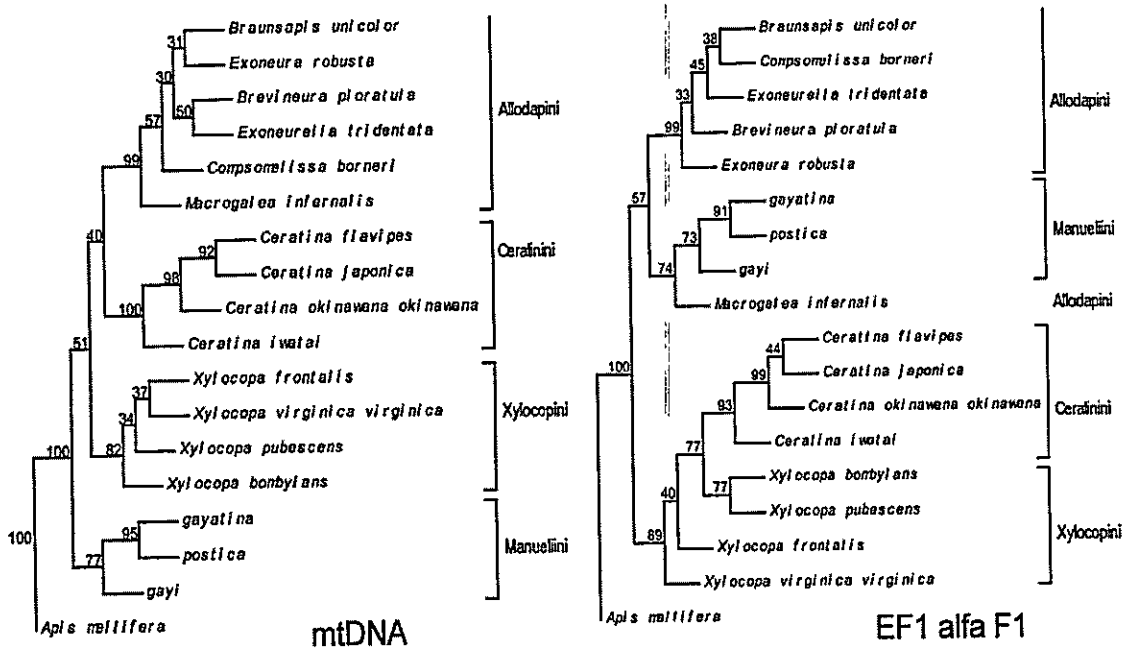

Figure 1. Phylogeny derived from a) mitochondrial and b) EF1 genes. Conflict between both sources of phylogenetic signal is clear, affecting the relative positions of each tribe. The topology obtained from mitochondrial genes is strongly supported by morphological evidence.

Phylogeny of Xylocopinae

Phylogenetic analyses were executed in PAUP 4.0b10 for maximum parsimony (MP) and maximum likelihood (ML), and in MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001) for Bayesian analysis (BA). Optimal models of nucleotide substitution, supported by the Akaike Information Criterion test (AIC; Akaike, 1974), were evaluated using ModelTest (Posada and Crandall, 1998, 2001) for ML and MrModeltest 2.0 (Nylander, 2004) for BA. Parameters for the priors of topology inference, used in BA, were also tested and selected using MrModeltest 2.0. The confidence values for each clade in MP and ML were assessed by bootstrap (Felsenstein, 1985) with 10^3 pseudoreplicates, heuristic searching, and random-addition of sequences. In the BA analysis, two runs were conducted using 4 million generations in four independent chains. The number of generations needed to reach the stationary state were evaluated by plotting the likelihood values (-lnL). Only generations above the stationary were included in the computation of the consensus tree, applying the 50% majority rule.

Hypothesis testing

The two alternate hypotheses on the basal lineage of Xylocopinae ("Xylocopini-basal" versus "Manueliini-basal") were compared by using the approximately unbiased test (AU test; Shimodaira, 2001, 2002) as implemented in the package Consel, version 0.1i. First, maximum likelihood trees enforced either to "Xylocopini-basal" or the "Manueliini-basal" constrains were obtained in PAUP,

using heuristic search under maximum likelihood optimization criterion. GTR+I+G was used as a model for nucleotide substitutions, since it was the best fitted model according to the AIC test. The respective parameters were allowed to be estimated for each tree during the heuristic search to ensure accurate estimations of likelihoods especially for suboptimal trees. Site-wise likelihoods were then estimated for each tree and used as input in the AU test. The scaled bootstrapping and estimation of probabilities and interval confidences were then performed by using the default options in the respective programs included in Consel.

RESULTS AND DISCUSSION

This study represents the first attempt to elucidate the phylogenetic relationships among the four extant tribes of Xylocopinae using molecular data. That purpose was achieved using nucleotide sequences from the mitochondrial genes *Col* and *Cytb* and from the nuclear gene EF-1α F1. The three species belonging to Manueliini were included in the analyses, but sampling within Xylocopini, Ceratinini and Allodapini was limited to a small number of species, representing as distant lineages as possible. Therefore, this study is not suitable to contribute with new insight on relationships within tribes. Instead, this work was especially conceived to contrast between the two hypotheses on the basal lineage within this subfamily.

Substitution saturation on third codon positions

Saturation on third codon positions was detected in the two mitochondrial genes used in this work. Percentage of observed transitions were lower than transversions starting from distances, corrected by the general time reversible model plus the gamma parameter, as small as 0.05 substitutions/site (Figure 2). The average corrected distances (substitutions/site) was 0.17 for *Col* and 0.23 for *Cytb*. Distances below 0.05 were detected only among three species belonging to the genus *Ceratina*: *C. japonica*, *C. flavipes* and *C. okinawana* (data not shown). Thus, these results indicate that phylogenetic analysis should be performed either including or excluding third codon positions, in order to explore the consequences of including random signal or excluding a fraction of the phylogenetic signal, respectively. Third codon positions of EF-1α F1 did not display substitution saturation detectable using this approach. However, contrary to the expected under normal patterns of molecular divergence among species, GTR + G did not correct distances from EF-1α F1, since corrected distances were always lower than uncorrected distance: the average of transitions/GTR+G distances was 4.49 and for transversions it was 2.41.

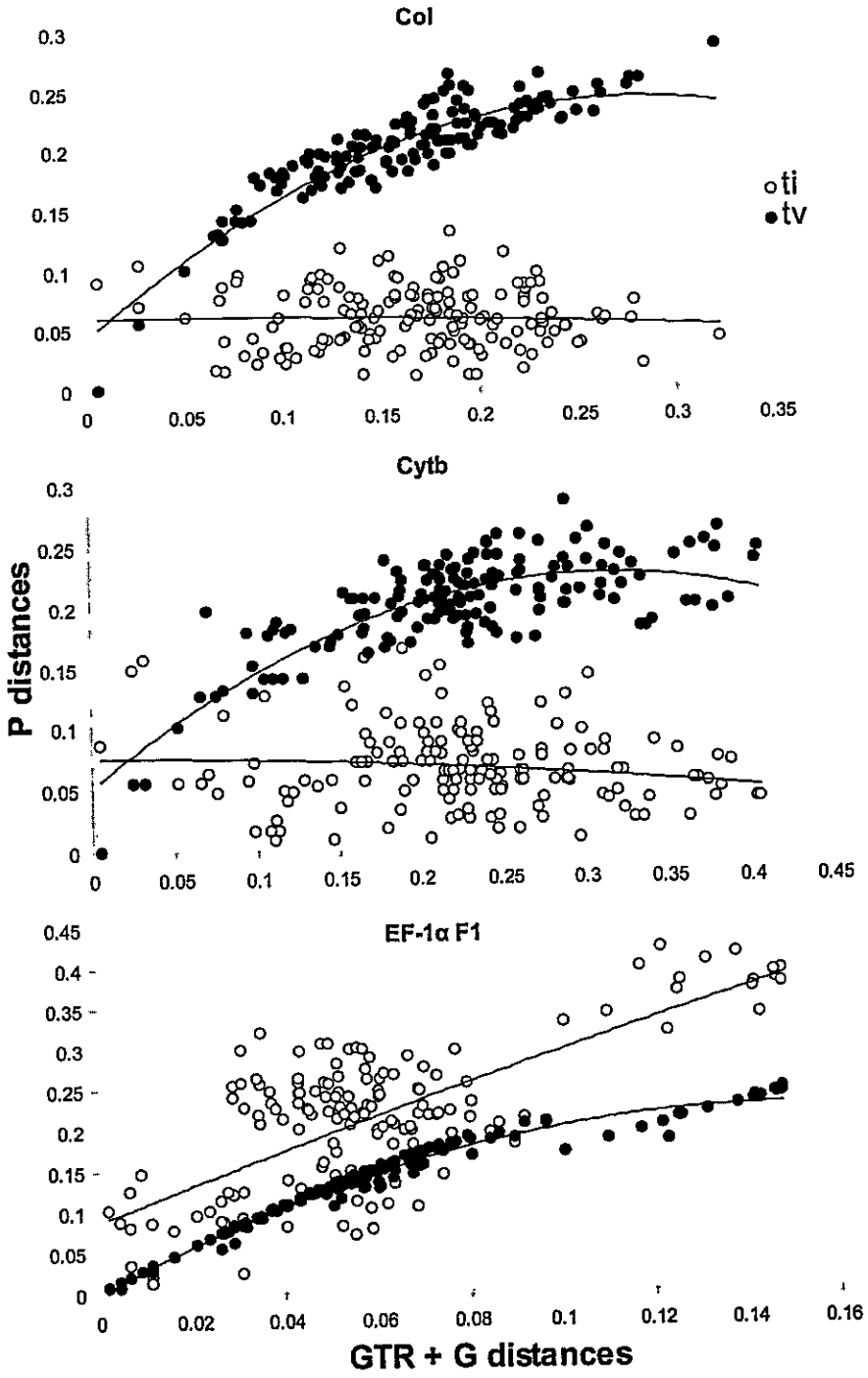

FIGURE 2. Saturation on third codon positions. Comparison between GTR+G distance (X axis) and uncorrected distances for third codon positions (Y axis) estimated from *Col*, *Cytb*, and EF-1α F1 sequences. Lines correspond to regression lines (order 2) fitted to transitions and transversions.

Congruence between partitions

Phylogenetic signal was concordant between both mitochondrial genes either including or excluding third codon positions ($P_{\text{homogeneity test}}$ = 0.602 and 0.621 respectively). However, conflict between phylogenetic signal derived from mitochondrial genes and EF-1α F1 was detected when third codon positions were included ($P_{\text{homogeneity test}}$ = 0.032), but that discordance vanished when third codon positions were excluded from the dataset ($P_{\text{homogeneity test}}$ = 0.991). Conflict was also observed when exclusion of third codon positions was restricted to mitochondrial genes ($P_{\text{homogeneity test}}$ = 0.011) but absent when that exclusion was limited to EF-1α F1 ($P_{\text{homogeneity test}}$ = 0.997). Overall those results indicate that the discordant phylogenetic signal between mitochondrial genes and EF-1αF1 resides on third codon positions. Maximum parsimony trees derived from mitochondrial and EF-1αF1 genes (Figure 1) showed generalized incongruence between both topologies. The phylogeny derived from EF-1αF1 is strongly incompatible with previous studies on the phylogenetic relationships among the tribes of Xylocopinae using morphological data, which strongly support the derived clade Ceratinini + Allodapini and differ on the basal placements of Manueliini and Xylocopini (Sakagami and Michener, 1987; Roig-Alsina and Michener, 1993; Engel, 2001). Therefore, the phylogenetic signal of EF-1αF1 and the paralogous EF-1αF2 was further analyzed in order to determine the cause of this phylogenetic incongruence.

Phylogenetic noise from EF-1α F1

The phylogenetic analysis using a large dataset of EF-1αF1 and F2 revealed that both copies are well differentiated and inaccuracies of gene annotation were not observed (Figure 3, Appendix 1). Presence of pseudogenes was also discarded since the topology obtained from aminoacid sequences did not show evidence of substantial change in open reading frames and every tribe and genus remained monophyletic (Appendix 2). Finally, lack of phylogenetic signal in this gene was detected specifically within the genus *Xylocopa* (tribe Xylocopini), represented here by 26 species, since it was paraphyletic displaying several independent branches basal to *Apis mellifera* + Xylocopini + Allodapini (Figure 3). In another phylogeny of the Subfamily Xylocopinae, excluding third positions, the tribe Xylocopini recovered the monophyly (Appendix 3) indicating lack of phylogenetic signal on third codon positions, in agreement with the results of the homogeneity test. In this work, substitution saturation was preliminary evaluated by plotting transitional and transversional distances from third positions against ML distances estimated from each gene. Thus, estimation of corrected distances was based on the assumption that first and second codon positions contained enough phylogenetic signal. However, false negative results can be obtained from that approach if poor phylogenetic signal is contained on first and second codon positions in addition with saturated third codon position. Estimations of the proportion of invariant sites (Pi) showed that third codon position covers 83% of the variant sites in EF-1αF1, while the first and second codon positions accounted for only 13.2% and 3.8% respectively. For comparison, in the concatenated mitochondrial genes

those values were 28.2% (first position), 30.77% (second position) and 41.03% (third position). In addition, values of the gamma shape parameter, directly proportional to rate of molecular changes, were 0.35, 0.39 and 2.48 for third codon positions from *Coi*, *Cytb* and EF-1αF1 respectively, indicating very high rate of change on the third codon position of EF-1αF1.

Therefore, in subsequent phylogenetic analyses we excluded third codon positions in the EF-1αF1 data set. To determine the precise cause for lack of phylogenetic signal in the EF-1αF1 third codon positions in the tribe Xylocopini is out of the scope of this work, but extreme saturation of substitutions in that lineage is a plausible explanation.

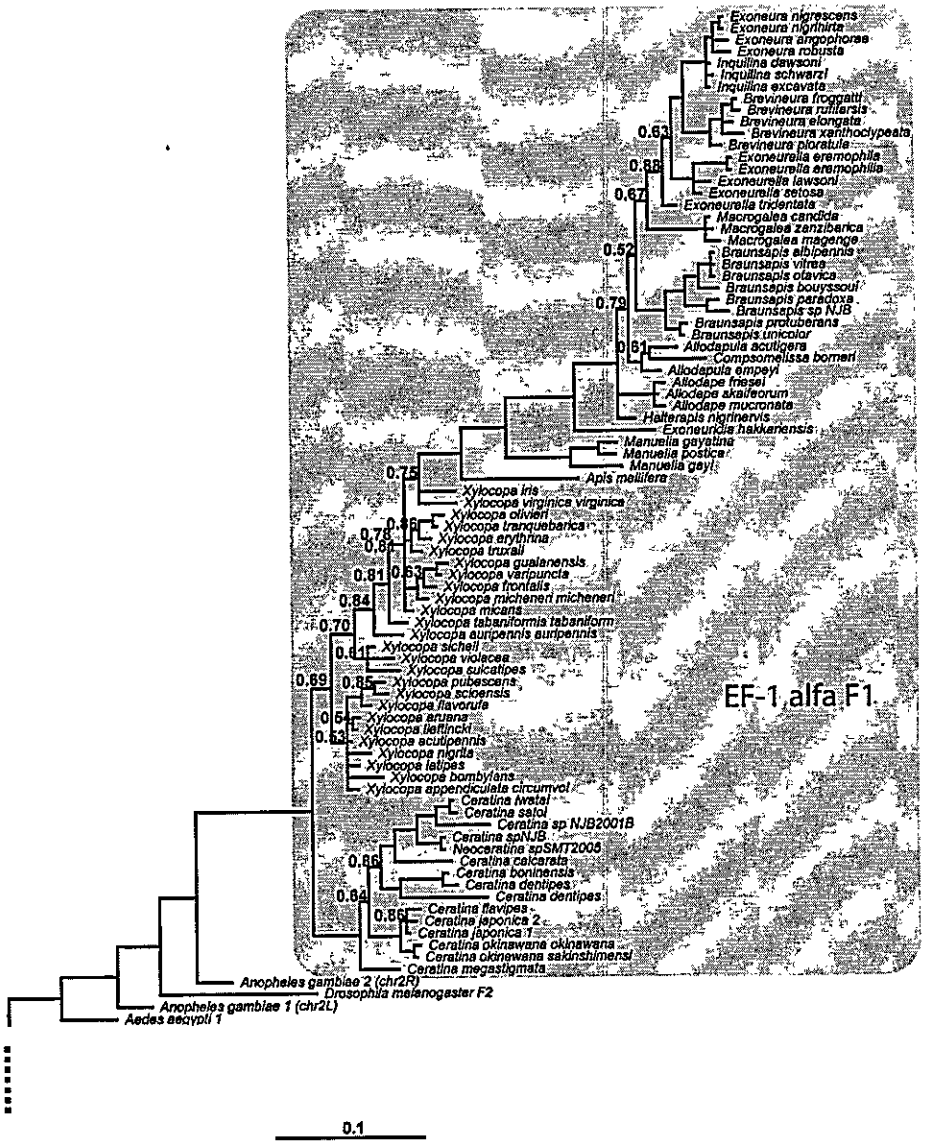

Figure 3. Bayesian phylogeny using EF-1αF1 and EF-1α F2 nucleotide sequences from species of Xylocopinae and including *Apis mellifera*, *Bombyx mori*, *Drosophila melanogaster*, *Tribolum castaneum*, and three species of *Nassonia*. Numbers at the nodes indicate posterior probabilities. Only values under 0.90 are shown.

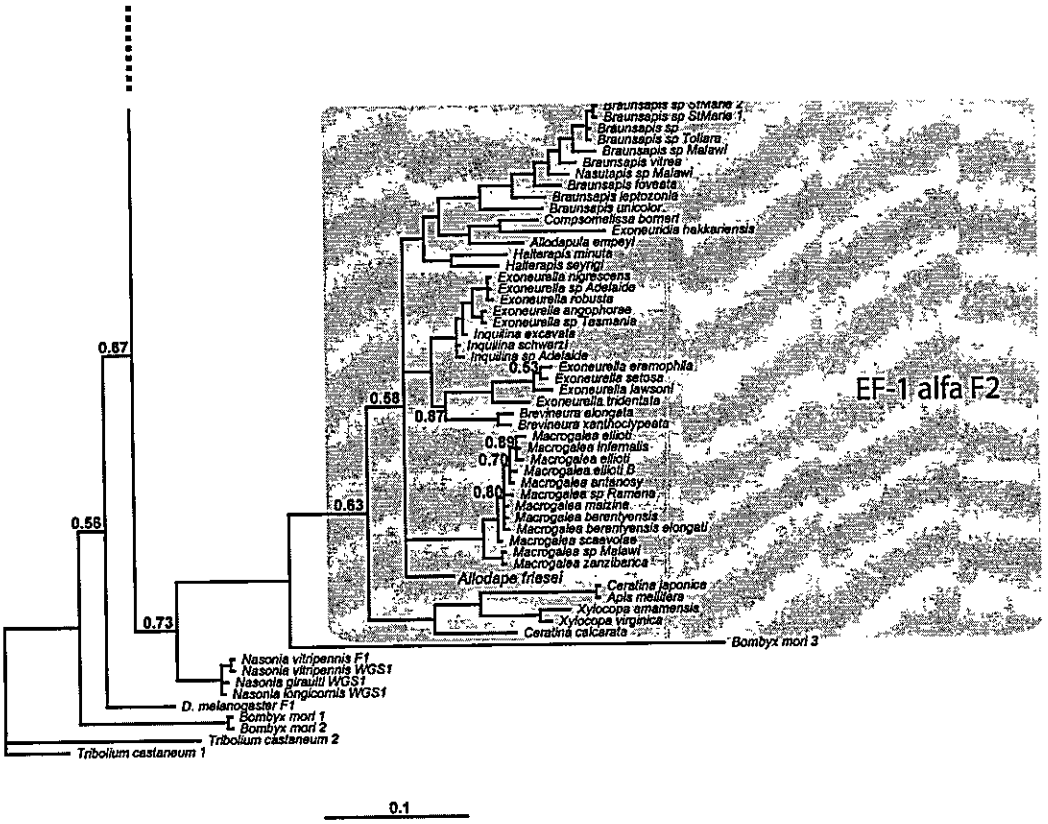

Figure 3, continuation.

Phylogeny of Xylocopinae

Trees obtained either including or excluding third codon positions from mtDNA concatenated with first and second codon positions of EF-1αF1 resulted in the same topology, which placed Manueliini as the basal tribe followed by Xylocopini and the sister tribes Ceratinini and Allodapini, in agreement with the phylogenetic relationships proposed by Sakagami and Michener (1987).

Statistical supports for among-tribes nodes were higher when third codon positions were excluded from mtDNA genes than those obtained including those positions (Figure 4). Thus, substitution saturation observed for mtDNA genes reduced the statistical power, and removal of noisy data improved the confidence on the inferred trees. This is predictable when the distant relationships among the tribes here studied is taking into account.

Low support was obtained for the node connecting Allodapini and Ceratinini (Figure 4). However, that clade is strongly supported for a set of plesiomorphic morphological characters (Roig-Alsina and Michener, 2003). Low support for that node, derived from our dataset, is likely a consequence of the short branch connecting with the last common ancestor shared with the tribe Xylocopini. A short interval of time occurred between the split of Xylocopini and the bifurcation Allodapini/Ceratinini, as shown in the trees. That period of time is probably insufficient to gain phylogenetic signal from first and second codon positions, since only eight characters were plesiomorphic for the Allodapini + Ceratinini clade (data not shown).

The basal placement of Manueliini was supported by the relatively high bootstrap values obtained for the monophyly of Xylocopini + Ceratinini + Allodapini in addition to the moderate support for the monophyly of Manuelini (Figure 4). Taking into account branch lengths, the node connecting the three species of Manueliini predated the origin of any other tribe within the Xylocopinae, being near to the origin of the subfamily. The basal placement of Manueliini and its relatively early origin, in addition to retention of morphological plesiomorphic characters, support Michener's viewpoint on this tribe as a "relict taxon" within Xylocopinae (Michener 1979, 2000; Daly et al., 1987).

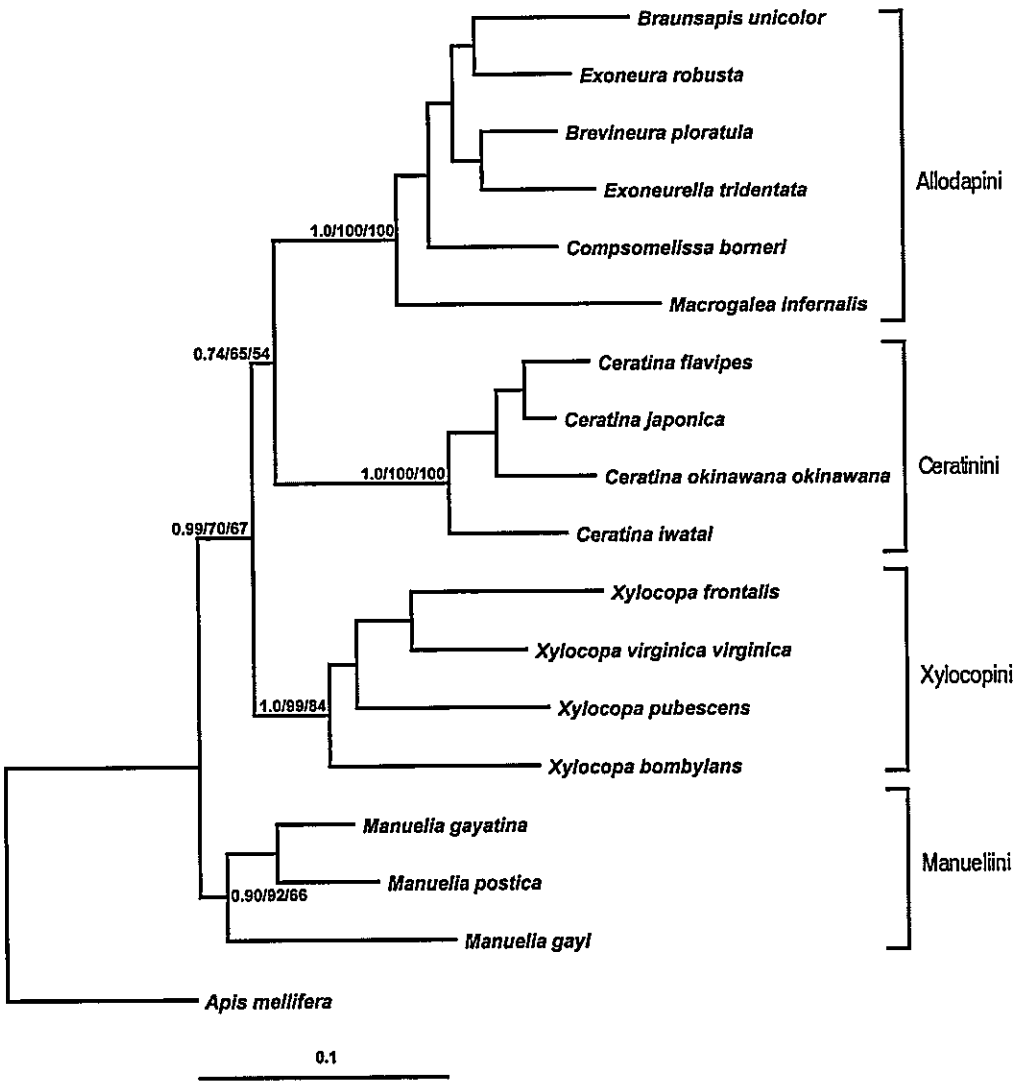

Figure 4. Bayesian phylogeny among tribes of Xylocopinae inferred from the concatenated nucleotide sequences using the *Col*, *Cytb* and EF-1αF1 datasets. The numbers at the nodes indicate Bayesian posterior probability percentages (2 X 106 generations), and bootstrap values from maximum likelihood (103 replicates) and maximum parsimony (104 replicates), respectively. Only supports for among tribes nodes are shown.

Hypothesis testing

The -log likelihoods for the alternate topologies enforcing the basal placement of Manueliini and Xylocopini were 7016.07 and 7024.61 respectively. The AU test resulted in a significant difference in the likelihoods between both constrains (Table 2). In addition, the posterior probability for the basal placement of Xylocopini was zero. In other words, the hypothesis "Xylocopini basal" was never retained as a credible tree during the Markov chain - Montecarlo coupled generations (MCMC) in the Bayesian phylogenetic analysis. In concordance with those results, the basal placement of Manueliini was observed as a result from the different methods, datasets and strategies of phylogenetic reconstruction used in this study. Overall, this hypothesis concerning the basal lineage within Xylocopinae is strongly supported by the data analyzed in this study.

Table 2. Contrast among topologies representing the two alternative hypotheses on the basal lineage of the subfamily Xylocopinae.

Topology	Partition			
	mtDNA		mtDNA + EF1	
	-lnL	P (AU test)	-lnL	P (AU test)
Manueliini basal	6268.55	0.821	7036.48	0.754
Xylocopini basal	6276.00	0.036	7045.40	0.042

Implications of Manueliini basal position in the Phylogeny of the Xylocopinae

According to our results, Manueliini tribe is the sister group to all other Xylocopinae tribes. Therefore, it is likely that some components of social behavior in the xylocopine species with some degree of sociality (from subsociality to advanced eusociality) had an origin prior to the origin of the tribes Xylocopini, Ceratinini and Allodapini. Topology of Xylocopinae with Manueliini at the base, followed by Xylocopini and Ceratinini as sister group of Allodapini is in agreement with general levels of sociality proposed for the subfamily, according to Batra (1966), Michener (1969, 1974), and Wilson (1971). Manueliini contain only solitary species (Michener, 1985, 2000; Daly et al., 1987; Flores-Prado et al., 2007); in Xylocopini sociality has emerged but remains more incipient than that of Ceratinini species (Sakagami and Maeta, 1995; Maeta et al, 1996), and sociality in Allodapini is more advanced than that of Ceratinini species (Sakagami and Maeta, 1977; Sakagami and Maeta, 1995). Nesting behavior typical of solitary species, i.e. cell construction and mass provisioning, is plesiomorphic for Xylocopini and Ceratinini species. In tribes Ceratinini and Allodapini subsociality has evolved, although in a different mode. In some species of Ceratinini there is a direct and continuous care of brood because females open cells, remove feces, and reconstruct partitions between cells (Sakagami and Maeta, 1977; Maeta et al, 1997b). In Allodapini brood do not develop in cells, and are reared progressively by adult females (Michener, 1990; Schwarz et al., 1998). Removal of the partitions observed in Ceratinini species has been regarded as a preadaptation to the total abandonment of the

unit cell system (Michener, 1974; Maeta et al., 1997b), as occurs in all species of the tribe Allodapini (Schwarz et al., 2007). Therefore it is likely that removal of partitions has arisen only once, in the ancestor of both tribes. Semisociality and incipient eusociality have emerged in Xylocopini, Ceratinini and Allodapini species, therefore the basic system of functional castes (absent in Manueliini) is synapomorphic for such xylocopine species. Existence of morphological castes is an apomorphy for some eusocial Allodapini species.

Manuelia is a solitary and relict genus (Michener, 1979; Daly et al., 1987) exhibiting several morphological ancestral features (Michener 2000) and containing species that posses typical behavioral traits of solitary bees (Michener, 1969; 1974), such as cell construction pattern, food provisioning strategy (Michener, 1985; Daly et al., 1987), and no contact between immature siblings, and between them and their mother (Flores-Prado, et al., 2007). *Manuelia* exhibits unusual traits for solitary species, such as mutual tolerance between nestmate females, guarding behavior during breeding time, and existence of hibernating assemblages (Daly et al., 1987; Flores-Prado et al., 2007). To evaluate if these (and other) behavioral characters are precursors of sociality in social species within Xylocopinae, as has been proposed by Michener (1969, 1974) and Sakagami and Maeta (1977), a phylogenetic comparative approach is required, which includes as many taxa as possible, in addition to information on nesting biology from social and non-social species, in order to reconstruct ancestral states of social life in the subfamily Xylocopinae. That approach could contribute with new insights about the transitions from solitary to social modes of life.

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CAPÍTULO 2

Nesting biology, life cycle, and interactions between females of *Manuelia postica*, a solitary species of Xylocopinae (Hymenoptera: Apidae)

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Abstract The Xylocopinae contains four tribes with species which show a range of nesting habits, from solitary to social. The Manueliini is the sister group to all other Xylocopine tribes, with one genus, *Manuelia*, of three species found mainly in Chile. This is a solitary genus, whose biology is scarcely known for

two species, *M. gayatina* and *M. gayi*, and so far completely unknown for *M. postica*. This paper reports on nesting substrates, nest architecture, nesting behaviours, life cycle, and interactions between females at nesting sites, for *M. postica*. The results indicate that *M. postica* presents some features which are typical of solitary life, and also some features which are unusual in solitary bees but have been reported in phylogenetically more apical social species. Our findings open interesting questions on the ecological scenarios involved in the evolution of sociality within the Xylocopinae.

Key words solitary bee, nesting behaviour, nest architecture, *Manuelia postica*

INTRODUCTION

The Xylocopinae (Hymenoptera: Apidae) is currently hypothesised as the sister group to other Apidae subfamilies (Michener, 2000). It has emerged as a valuable model to study transitions in social evolution (e.g. Schwarz et al. 1997; 1998; 2007, Tierney et al. 2002) because it contains species ranging from solitary to social in nesting behaviour and social organisation (Michener 2000). In the Xylocopinae, some solitary species exhibit features unusual in non-social life, which have been proposed as prerequisites for evolution to social life (Michener 1974; 2000). Several of such features are related to nesting biology: a) protection of immature offspring through guarding behaviour by the mother, b) physical contact between the mother and her developing offspring while she cleans their cells, c) existence of hibernating assemblages enabling contact between siblings, and sometimes between siblings and their mother, and d)

tolerance between these nestmate individuals inside the nest (Michener 1969; 1974; 1985). Although such features are probably precursors to social life, none of them is sufficient for the development of sociality (Sakagami and Maeta 1977). To evaluate whether these behavioural characters are precursors of sociality in the Xylocopinae, information is needed on the nesting biology of non-social sister groups of eusocial taxa, which may enable phylogenetic comparative analyses aimed at reconstructing ancestral states of social life.

The Xylocopinae contains four tribes: Manueliini, Xylocopini, Allodapini, and Ceratinini (Daly et al. 1987; Sakagami & Michener 1987). Nesting and social behaviour have been studied in several species of Xylocopini, Allodapini, and Ceratinini (reviews on Xylocopini: Gerling et al. 1989; Michener 1985; 1990; on Allodapini: Schwarz et al. 1997; 1998; on Ceratinini: Michener 1985; Sakagami & Maeta 1995). Manueliini is a monogenerical tribe constituted by the genus *Manuelia* Vachal, which includes only three species: *M. postica, M. gayi,* and *M. gayatina* (Sakagami & Michener 1987) occuring predominantly in Chile (Daly et al. 1987). *Manuelia* has been proposed as a relict genus (Michener 1979; Daly et al. 1987), constituting a sister group to all other Xylocopinae (Sakagami & Michener, 1987), and retaining several ancestral morphological features (Michener 2000). It thus represents an interesting taxon for the study of potential ancestral states of nesting biology. The only data published on the biology of the genus *Manuelia* refer to limited field observations and nest dissections of *M. gayi* and *M. gayatina* (Claude-Joseph 1926; Daly et al. 1987). In this paper we report on nesting substrates, nest architecture, nesting behaviours, life cycle, and interactions between females at nesting sites, for *M. postica.*

MATERIALS AND METHODS

Nesting and life cycle observations

All observations were made at Altos de Lircay National Park, east of Talca, Chile (35°29' S; 70°58'W), during yearly field campaigns starting in October (early spring) of one year and ending in April (mid autumn) of the next year, from 2001 until 2006. Eighteen observation periods were included in the most intensive campaign, from the third week of October, 2005 to the last week of April, 2006 (for exact dates, see Table 1). Nests were collected between 8:00 and 11:00 h. Prior to collection, nest entrances were blocked with Teflon tape affixed with masking tape. Twenty-five nests were collected in each period, and dissected in the field to permit observations on nesting behaviour, nest architecture, and life cycle.

Behaviors observed in nesting sites

During December 2005, presence or absence, and position (head or abdomen showing through the nest entrance) of *M. postica* females at the nest entrance were determined in the field in fifty-four nests, between 16:00 and 19:00 h. In a random sample of those nests (N = 28), the number and sex of adults inside the nests were recorded. Additionally, during January 2006 the behaviours of females at or near the nest entrance were recorded in each of ten nesting sites (with a mean of *ca.* 5 nests per site) observed on separate days from 9:00 to 12:00 and from 16:00 to 19:00 h.

RESULTS

Nesting substrates

Most nests of *M. postica* were found in dead stems (N = 810) or in dry internodes of live stems (N = 17) of *Chusquea quila* (Poaceae: Bambuseae). Occasionally, nests were found in dead stems of *Aristotelia chilensis* (Elaeocarpaceae) (N = 12), and *Rubus ulmifolius* (Rosaceae) (N = 15). The nests in these three plant species were similar in terms of linearity of their structure, cell disposition, location and number of nest entrances, and position of cells with respect to the nest entrance. The detailed descriptions which follow refer to nests in *C. quila*.

Nest construction, architecture, and occupancy

Nest construction by females of *M. postica* began in early spring, and continued until late spring (Table 1). The nests consisted of a tunnel with cells arranged linearly along the plant stem, and separated by biconcave partitions made of wood particles (Figure 1). Nests had one entrance in the middle portion of the tunnel. In nests dissected at the first and last observation periods (N = 50), distinct cells were not observed. Most nests during the other observation (breeding) periods had cells only at one side of the nest entrance (327 out of 400 dissected nests).

In the first observation period in early spring, most nests contained hibernating assemblages of F_0 individuals. Thereafter, no hibernating F_0

assemblages were seen. During the last observation period, nests contained hibernating F_1 assemblages. Adult females found inside nests in the intemediate period corresponded to the parental generation, F_0. When females were present (N = 221 out of 400), the most frequent number of such females was one (N = 196), followed by two (N = 23), and three (N = 2). Larvae appeared in late spring. By mid summer most nests contained newly emerged adults, and hibernating F_1 assemblages were first detected. The proportion of nests containing F_1 hibernating assemblages increased steadily until the last observation period, and so did the number of adults present in them (Table 1).

Fig. 1 Characteristics of nests of *M. postica*: diagram of a nest containing an entrance hole, an entrance chamber, and four cells separated by partitions, two at each side of the chamber.

Table 1 Observations of *M. postica* nests and their number of food masses and individuals. During each observation period, 25 nests were examined. F_0 = parental generation.

		2005												2006					
Main stage	**Observation**	Oct 15-16	Nov 16-17	Nov 26-27	Dec 1-3	Dec 6-8	Dec 13-15	Dec 20-22	Dec 27-30	Jan 3-6	Jan 10-13	Jan 17-20	Jan 24-27	Feb 6-10	Feb 14-17	Mar 1-3	Mar 27-29	Apr 7-10	Apr 28-30
Nests under construction	Opening of nest entrance	6	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Tunnel lengthening	3	6	2	2	3	2	1	0	0	0	0	0	0	0	0	0	0	0
	Construction of food mass	0	1	6	6	13	8	4	2	3	1	0	0	0	0	0	0	0	0
Nests with the given number of F_0 adults present	One female	0	10	14	14	17	17	22	13	19	15	21	20	14	0	0	0	0	0
	Two females	0	4	5	3	1	1	2	1	1	1	1	1	2	0	0	0	0	0
	Three females	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
	One male	0	0	1	0	0	1	1	0	2	0	1	0	0	0	0	0	0	0
	Two males	0	0	0	0	0	2	0	1	0	1	1	0	0	0	0	0	0	0
	Three males	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Nests without F_0 individuals		0	10	5	8	6	3	0	9	3	8	1	4	9	0	0	0	0	0
Nests with hibernating F_0 assemblages		16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of adults found in nests with F_0 assemblages	Females	35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Males	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nests with hibernating F_1 assemblages		0	0	0	0	0	0	0	0	0	0	0	0	0	3	7	11	15	25
Nests with F_1 individuals developing inside cells		0	16	23	23	22	23	24	25	25	25	25	25	25	22	18	14	10	0
Number of adults found in nests with F_1 hibernating assemblages	Females	0	0	0	0	0	0	0	0	0	0	0	0	0	7	17	29	49	63
	Males	0	0	0	0	0	0	0	0	0	0	0	0	0	5	12	8	6	38

Life cycle and brief description of immature stages

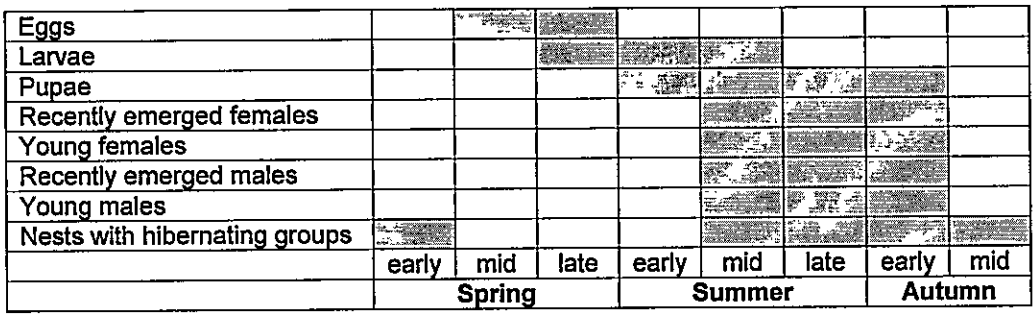
The life cycle of *M. postica* is presented in Table 2 and summarised in Fig. 2. Food masses were semisolid to solid, nearly rectangular, with the main axis along the tunnel, side in contact with cell surface convex, and opposite side flat. Eggs were found on the flat side of food masses, each with its main axis parallel to the main axis of the food mass. The shape of eggs was that of a slightly curved cylinder with convex ends, roughly 3 mm in length and 1 mm in diameter, whitish in colour but nearly transparent, and with a smooth and shiny chorion. First instar larvae were translucent, and they rested on top of nearly intact food masses. Pre-defaecating larvae were readily recognised because food masses had been modified but there were no faeces visible. Post-defaecating larvae were identified because the food masses had been consumed and faeces were evident inside the cell. Larvae were considered prepupae when they showed a marked increase in sclerotisation of the head capsule, the colour of the body cuticle had changed from bright white to opaque white, and a body constriction had developed between what would become the thorax and the abdomen. In general, larvae did not have tubercles, hairs or spicules, their bidentate mandibles had a concavity (as in *Ceratina* spp.), and they showed a basal tapering in the more slender apex (as in *Xylocopa* spp.), antennal papillae, and a small salivary opening (as in *Ceratina* spp. and *Xylocopa* spp.). Five consecutive types of pupae were easily identified on the basis of the colour of the eyes, the body, and the last abdominal segments. In general, pupae showed a protuberance in the femur, and spines on the coxa, trochanter, and also on the second to the last abdominal tergum; this latter

exhibits a strong medium acute projection (as in *Xylocopa* spp.). The newly emerged adults from the first generation were recognised by their whitish incompletely developed wings. Finally, young females and young males from the first generation were easily identified because they had fully developed wings and they were found inside their breeding cells.

Table 2 Stages of *M. postica* individuals inside nests dissected. At each date, 25 nests were examined and the number of individuals at each stage noted. These numbers were transformed into percentages within each stage. Maximum percentage values for each stage have been highlighted.

Stage	2005												2006					
	oct 15-16	nov 16-17	nov 26-27	dec 1-3	dec 6-8	dec 13-15	dec 20-22	dec 27-30	jan 3-6	jan 10-13	jan 17-20	jan 24-27	feb 6-10	feb 14-17	mar 1-3	mar 27-29	april 7-10	april 28-30
Egg	0	25.2	27.7	29.4	17.6	0	0	0	0	0	0	0	0	0	0	0	0	0
Larva, translucent	0	0	16.5	16.5	16.5	26.2	10.7	7.77	2.91	2.91	0	0	0	0	0	0	0	0
Larva, pre-defaecating	0	0	0	1.57	8.66	20.5	38.6	14.2	2.36	3.94	7.09	3.15	0	0	0	0	0	0
Larva, post-defaecating	0	0	0	0	0	0	7.34	37.9	25.4	7.34	13	7.91	1.13	0	0	0	0	0
Larva, pre-pupa	0	0	0	0	0	0	0	14.3	28.6	14.3	14.3	14.3	14.3	0	0	0	0	0
Pupa, white eyes, unpigmented body	0	0	0	0	0	0	0	2	42	16	14	18	6	2	0	0	0	0
Pupa, brown eyes, unpigmented body	0	0	0	0	0	0	0	0	7.81	35.9	20.3	9.38	25	1,56	0	0	0	0
Pupa, black eyes, unpigmented body	0	0	0	0	0	0	0	0	0	20.3	25.3	19	11.4	12.7	7.59	3.8	0	0
Pupa, black body, last three segments of abdomen unpigmented	0	0	0	0	0	0	0	0	0	13.6	27.3	31.8	9.09	4.55	4.55	4.55	4.55	0
Pupa, black body, last three segments of abdomen red	0	0	0	0	0	0	0	0	0	0	0	10.5	28.9	26,3	21.1	5.26	7.89	0
Females, newly emerged	0	0	0	0	0	0	0	0	0	0	0	5.56	16.7	44.4	16.7	11.1	5.56	0
Males, newly emerged	0	0	0	0	0	0	0	0	0	0	0	0	14.3	57.1	9.52	14.3	4.76	0
Females, young	0	0	0	0	0	0	0	0	0	0	0	0	0	22.2	44.4	24.4	8.89	0
Males, young	0	0	0	0	0	0	0	0	0	0	0	0	0	25.9	66.7	7.41	0	0

Fig. 2 Summary of the life cycle of *M. postica*, based on data in Tables 2 and 3.



Measurements and comparisons related to nest biology

Table 3 reports data obtained from nests analysed during the breeding period, some of which is used to compare *M. postica* with other bee species (see Discussion). Cells containing females were longer than cells containing males (ANOVA: $F_{1,69} = 83.77$; $p < 0.001$), but their diameters did not differ significantly (ANOVA: $F_{1,69} = 2.39$; $p > 0.1$). Wings were longer and intertegular distances greater in females than in males (ANOVA: $F_{1,88} = 189.65$, $p < 0.001$; $F_{1,88} = 161.35$; $p < 0.001$, respectively).

In a sample of 20 nests, differences in the number of cells in nests with one (N = 10) or two (N = 10) females were not significant (Mann-Whitney *U*-test: Z = -1.22, P = 0.22). Differences in the mean dry weight of food masses, between nests with one (N = 10) or two (N = 10) females were not significant either (Mann-Whitney *U*-test: Z = -0.48, P = 0.62).

The number of adult individuals per nest (in hibernating nests found in the last observation period) was negatively correlated with mean wing length of individuals in each nest (Pearson product-moment correlation: N = 25; r = -0.50; p = 0.01), and with mean intertegular distances of individuals in each nest

(Pearson product-moment correlation: N = 25; r = -0.62; p = 0.0008). This data were used to assess a potential trade-off between the size and number of progeny.

Table 3 Data obtained from dissection of *Manuelia postica* nests in the field, during all observation periods between October 2005 and April 2006. Lengths (mm) and weights (mg) were determined in the lab.

Period	Parameter	N	Mean	Min	Max	Std. dev.
Breeding	Cell length (with brood)	95	9.03	7.2	10.2	0.62
	Cell diameter (with brood)	95	4.59	3.9	5.8	0.28
	Partition thickness	50	2.25	1.1	3.1	0.40
	Nest entrance diameter	30	2.88	2.8	3.0	0.04
Food mass provisioning	Cells /nest	20	3.05	2.0	5.0	0.83
	Food mass dry weight	61	4.23	4.0	4.44	0.11
Late breeding	Cell length (with female)	44	9.97	8.1	11.0	0.63
	Cell diameter (with female)	44	4.71	4.0	5.2	0.28
	Cell length (with male)	27	8.46	7.0	9.7	0.75
	Cell diameter (with male)	27	4.61	4.0	4.9	0.19
Aggregation of individuals, partitions destroyed	Tunnel length	25	66.17	40.8	141.7	24.22
	Tunnel diameter	25	4.39	4.1	4.8	0.16
	Individuals/nest	25	4.04	2.0	8.0	1.93
	Wing length, female	57	7.90	7.4	8.4	0.27
	Intertegular distance, female	57	2.11	1.9	2.4	0.13
	Wing length, male	33	7.05	6.2	7.6	0.30
	Intertegular distance, male	33	1.78	1.5	2.1	0.11

Guarding behaviour

Observations of fifty-four nests monitored during part of the breeding period (December, 2005) are summarised in Table 4. When females were present at the nest entrance, either their head (inspecting behaviour) or their abdomen (guarding behaviour) could be seen from the outside. When the nest was disturbed, such as by a movement of the stem, inspecting females turned

around and blocked the nest entrance with the apical metasomal tergum, thus adopting a guarding posture. While most nests had a female at the entrance, no males were ever found in such position. Nests with only one female were more frequent than nests with two females (Table 4).

Table 4 Data from observation and dissection of *Manuelia postica* nests in the field. In parenthesis, number of nests observed (total = 54). Number of nests dissected: a = 3, b = 15, c= 10.

	Type of nest		
	With inspecting female (3)	With guarding female (36)	Without female at entrance (15)
Nests with one female	100% a	86.6% b	70% c
Nests with two females		13.3% b	10% c
Nests without females			20% c

Interaction between females in nesting sites

During part of the breeding period (January, 2006), ten nesting sites (with a mean of *ca.* 5 nests per site) were selected and each one of them observed during one day from 9:00 to 12:00, and from 16:00 to 19:00 h. A total of 92 behavioural events were registered at the nest entrance, near the nest entrance, or towards the nest entrance (Table 5). The most frequent behaviour was a type of agonistic interaction at the nest entrance, consisting of the

approach by an intruder female flying or walking with hovering movements, while the nest entrance was blocked by a guarding female, and the intruder subsequently touching and pushing the resident female.

Table 5 Behaviour of *M. postica* females at or in the vicinity of the entrance of nests. The total number of behavioural events recorded was 92.

Site where behaviour was observed	Behaviour observed	% times observed
Towards nest entrance	Female walks straight towards nest without guarding female	1.4
	Female performs hovering walk towards nest with guarding female	4.1
	Female performs straight flight towards nest without guarding female	1.4
	Female performs hovering flight towards nest with guarding female	17.8
Near nest entrance	Intruder female displaces guarding female	6.9
	Frontal approach beween intruder female and guarding female	2.7
	Pursuit of one female by another	1.4
	Aggression in flight between two females	2.7
At nest entrance	Female enters nest without guarding female	5.5
	Inspecting female extends her head out and displaces intruder female	4.1
	Intruder female antennates towards inspecting female, and moves away	2.7
	Intruder female pushes guarding female	21.9
	Intruder female touches guarding female with its legs	27.4

DISCUSSION

Nesting substrates and nest architecture

Xylocopinae species nest in dead wood, or branches of plants and trees, digging into stems, occupying hollow stems, or using pre-existing cavities (Gerling et al. 1989; Maeta et al. 1992; Steen & Schwarz 2000). Nests of *M. postica*, as for other species of Xylocopinae including *M. gayi* and *M. gayatina* (Claude-Joseph 1926), were found in dry stems.

Xylocopine bees construct two main types of nests: branched and linear (unbranched) (Gerling et al. 1989; Michener 1990). Linear nests with the entrance at one end of the tunnel have one cell construction zone, thus making communal life, *sensu* Michener (1974), virtually impossible (Sakagami & Maeta 1995). Linear nests with the entrance in the middle of the tunnel have two cell construction zones; in this case, both solitary and social nesting has been documented (Sakagami & Maeta 1995). Nests of *M. postica* were linear, and some contained cells at both sides of the nest entrance, suggesting incipient social nesting. Moreover, nests of *M. postica* have been observed with more than one female of the parental generation during the breeding period. Although these facts may be taken as indications of a certain degree of intrinsic sociality, nests with cohabitating females have been documented in other solitary species (Michener 1974). The cohabitation in a nest of two females of certain bee species can represent a temporary event or a long lasting situation (Sakagami and Maeta, 1977), and be determined by ecological restrictions such as availability of pollen or nest sites, and by genetic factors such as degree of

genetic relatedness between females (Hogendoorn & Leys 1993; Schwarz et al. 1998). Work in progress in our laboratory is oriented towards distinguishing between these two possibilities.

Cells containing young females were longer than those containing young males, consistent with the greater dimensions of females (wing length and intertegular distance) compared with males. Studies on other bee species (*Apis mellifera, Diadasina distincta*) have also demonstrated that individuals of the larger sex occupy cells of greater dimensions (Taber & Owens 1970; Martins et al. 1999), whereas in *Xylocopa abbreviata*, in which sexes do not differ in size there is no difference in size between male and female cells (Ramalho et al. 2004). These facts suggest that females of *M. postica* can build cells to a given length depending on the sex of individual which will develop inside that cell. This behavioural pattern requires: i) that *M. postica* females can control the size of cells, as has been proposed for the queen and workers of *Apis mellifera* (Koeniger 1970; Pratt 1998), and ii) an haplo-diploid sex determination system allowing control of the sex of the egg oviposited, as has been demonstrated broadly in Hymenoptera (Page et al. 2002; Normark 2003).

Finally, there was a negative correlation between the number of individuals found inside a nest and their mean size (estimated by wing length and intertegular distance). If females have a fixed amount of resources available for reproduction, then the occurrence of a trade-off between the number and size of the progeny is likely, as has been described for most studies of semelparous arthropods that exhibit no parental care (related to continuous food provisioning), as is the case of *M. postica* (review: Fox & Czesak 2000).

Life cycle and nesting behaviour

The data in tables 1 and 2 suggest there is one generation of *M. postica* per year, as in some Xylocopine species of temperate regions (Gerling & Hermann 1978). The breeding period begins in late spring and ends in mid autumn, as in other Xylocopine species (Michener 1985). Hibernating assemblages of adults of both sexes may be found both in late summer and autumn and also in early spring, at the beginning of the breeding season.

The genus *Manuelia* is considered solitary at the time of nest construction and food provisioning (Michener 1985; 2000). This proposal was based on descriptions of nest biology of *M. gayi* and *M. gayatina* (Claude-Joseph 1926; Daly et al. 1987). According to our observations, *M. postica* exhibits many characteristics of solitary behaviour (*sensu* Michener 1969) during nest construction and provisioning. Immature individuals were always observed inside cells with intact partitions. This observation strongly suggests a lack of contact between the immature siblings, and also between them and their mother, as has been reported for many, but not all, Xylocopine species (Sakagami & Michener 1987; Maeta et al. 1992). Contact between siblings, as in some Xylocopine species in which the first adults to emerge destroy cell partitions and pass over the younger sibs in order to reach the entrance of the nest (Michener 1985; Sakagami & Maeta 1995), was not registered in *M. postica*. On the other hand, the pattern of cell construction, food mass provisioning, egg laying, cell closure, and sequential repetition of these activities, is typical of solitary bees (Michener 1974). Finally, when pupae reach the adult stage they remain inside their cells for some days before destroying

the cell partitions. When all partitions are destroyed, adults constitute a hibernating assemblage, as in other Xylocopine species (Michener 1985).

Guarding behaviour

Females of *M. postica* at the nest entrance exhibit either guarding (92.3% of cases observed) or inspecting (7.7% of cases) postures. When the nest is disturbed by an intruder, the guarding females remain in their posture, thereby preventing the entry of the intruder. These behaviours are similar to those of *Braunsapis hewitti*, an Allodapini species which is seen inspecting in 4.5% of cases and guarding in 93.5% of cases (Maeta et al. 1992), but different from *Ceratina*, whose females often turn around, face the intruder, and secrete an odoriferous substance from the mouth (Maeta et al. 1992). This type of defensive behaviour has not been observed in *Braunsapis hewitti* (Maeta et al. 1992) nor in *M. postica*.

Guarding behaviour as described in *M. postica* is widespread in the Xylocopinae, i.e., Ceratinini (Sakagami & Maeta 1977; Michener 1985; Maeta et al. 1992), Allodapini (Mason 1988; Maeta et al. 1992; Hogendoorn & Schwarz 1998; Steen & Schwarz 1998), and Xylocopini (Ramalho et al. 2004), and may reflect the retention of a primitive defence mechanism closely related to nest architecture, i.e., a single, narrow nest entrance allowing an effective blockading with the metasoma (Ramalho et al. 2004).

On the other hand, guarding behaviour has two important advantages in social nesting bees: i) more extended foraging trips by a female while another one remains at the nest entrance and hence, higher accumulation of pollen

loads, and ii) defence against conspecific or heterospecific invaders (Hogendorn & Velthuis 1993). In relation to the first advantage, a higher foraging time when nests are occupied by two or more females has been correlated with an increase in the number of cells constructed in some Xylocopine species (Sakagami & Maeta 1977; Michener 1985). In *M. postica*, neither the number of cells per nest nor the mean dry weight of food masses per cell differed between nests with one or two females. Therefore, it is likely that in those nests of *M. postica* with two females inside during the rearing period, there is no coordinated foraging and guarding as described in social nesting Xylocopine bees.

In relation to the second advantage, guarding behaviour suggests strong pressure from conspecific individuals, i.e., usurpers and robbers, and heterospecific ones, i.e., predators and parasites (Ramalho et al. 2004). In *M. postica*, only attempts by conspecific individuals to enter the nests were observed. On the other hand, we found progeny of the parasitoid, *Macrogotea gayi* (Hymenoptera: Ichneumonidae), inside nests of *M. postica* containing larvae and/or pupae, and have observed its oviposition behaviour: when a female of *M. gayi* lands on *C. quila* stems, she walks around feeling the stem surface with her antennae before curving her abdomen, inserting her ovipositor through the nest wall, and laying an egg directly inside a cell. Therefore, the guarding female cannot prevent parasitism, so this interaction does not constitute a pressure leading to guarding behaviour, further suggesting that guarding behaviour in *M. postica* is mainly a defence against conspecifics.

Interaction between females

In other Xylocopine species, robber conspecific females approach cautiously, and turn away as soon as they find a resident female, and nest usurper conspecific females make direct approaches, are not deterred by the presence of a resident female, and normally push her aside when present (Hogendoorn & Velthuis 1993). Some behavioural interactions observed between *M. postica* females at the nest entrance were similar to these. Thus, intruders showed cautious approaches or aggressive behaviours near or at the nest entrance. On the other hand, the presence of two (or three) females inside a nest suggests that a resident female allowed the entry of an intruder female, or that these females remained together after hibernation (Stark et al. 1990; Hogendoorn & Velthuis 1993). Some of these cohabiting females displayed tolerant interactions (i.e., permitting passing; one or both female passing venter-to-venter in opposite directions). Taken together, these facts suggest nestmate discrimination ability in *M. postica*.

CONCLUSIONS

This paper describes field observations on the nest biology of *M. postica* and on interactions between females, and compares the data with those from phylogenetically related species. The main characteristics of *M. postica* are: i) it is a univoltine species, ii) it shows cell construction and food provisioning strategies typical of solitary bees, iii) some nests allow two zones of cell

construction, iv) females can regulate cell size depending on the sex of the individual which will develop inside, v) nests with two females are not more productive than nests with one, vi) the number of individuals per nest correlated negatively with the size of those individuals, vii) females do not have contact with their developing offspring, viii) there is no contact between immature siblings, ix) females defend their nest through guarding behaviour, and x) hibernating assemblages occurr after the breeding period. The observation that a female of a solitary bee shares her nest with one and occasionally two females opens some interesting questions, for instance: can such females recognise nestmate conspecifics and discriminate non-nestmates? Do females found inside a nest during the breeding period show a high degree of relatedness? Under what ecological circumstances do two or more females of a solitary bee species share a nest? We are currently engaged in finding answers to these questions, which will contribute to our understanding of social evolution in the Apidae.

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CAPÍTULO 3

Nestmate recognition in *Manuelia postica* (Apidae, Xylocopinae): an eusocial trait is present in a solitary bee

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ABSTRACT

In eusocial Hymenoptera, females are more tolerant towards nestmate than towards non-nestmate females. In solitary Hymenoptera, females are generally aggressive towards any conspecific female. Field observations of the nest biology of *M. postica* suggested nestmate recognition. Laboratory experiments were performed involving two live interacting females, or one live female interacting with a dead female. Live females from different nests were more intolerant to each other than females from the same nest. Females were more intolerant towards non-nestmate than towards nestmate dead females. When dead females were washed with pentane, no differences in tolerant and intolerant behaviors were detected between non-nestmates and nestmates females. Females were more intolerant towards nestmate female carcasses

coated with the cuticular extract from a non-nestmate, than towards non-nestmate female carcasses coated with the cuticular extract from a nestmate. Compositions of cuticular extracts were more similar between females from the same nest than between females from different nests. The results demonstrate for the first time nestmate recognition mediated by cuticular chemicals in a solitary species of Apidae. The position of *Manuelia* at the base of the Apidae phylogeny suggest that nestmate recognition in eusocial species apical to *Manuelia* represents the retention of a primitive capacity in the Apidae.

Keywords: nestmate recognition, Hymenoptera, Apidae, solitary bee, cuticular compounds

1. INTRODUCTION

Conspecific recognition plays a key role in the evolution of cooperative behaviour between relative (e.g. Hamilton 1964a) and non relative animals (e.g. Trivers, 1971). It is based on traits related to the identity of individuals, kinship, or membership to categories such as nestmate or non nestmate (review: Wilson, 1975). Nestmate recognition is widespread among eusocial insects (Smith & Breed 1995, Breed 1998; Breed *et al.* 2004a), and can be inferred from the outcome of the interaction between conspecifics from the same or different nests (or colonies) (Gamboa 2004). For instance, females of social species of Hymenoptera attack non-nestmates more often than nestmates, and nestmates are mutually tolerant, i.e. they exhibit little mutual aggression (Buckle & Greenberg 1981; Michener & Smith 1987; Inoue *et al.* 1999). In contrast, high levels of aggressive behaviours towards conspecifics have been observed in

solitary species (Hölldobler & Michener 1980; Field 1992; Kukuk 1992). From an evolutionary point of view, the transition from solitary to social life requires a regulation of such aggressiveness and therefore intraspecific recognition, because individuals within a group must develop reciprocal tolerance to maintain cohesion (Lin & Michener 1972; Michener & Smith 1987; Jeanson *et al.* 2005).

Most research on discrimination between hymenopteran conspecifics from the same and different nests or colonies, and the associated recognition cues have been performed in social species (reviews: Gadagkar 1985; Smith & Breed 1995; Singer 1998; Gamboa 2004; Howard & Blomquist 2005), probably due to the importance of this process for such species, i.e., to support the social structure of a colony (Hölldobler & Wilson 1990), to maintain high cooperation and low aggression necessary for communal life (Paxton *et al.* 1999), and from an evolutionary viewpoint, to maximise inclusive fitness (Hamilton 1964a,b). Nevertheless, some evidence has been presented which suggests that solitary species may also exhibit mutual tolerance and nestmate recognition abilities. Thus, Wcislo (1997) reported that reproductively inactive females of *Lasioglossum (D.) figueresi*, a largely solitary species belonging to a basal branch in the phylogeny of the Halictidae (Danforth *et al.* 2003), recognise familiar females and consequently modify their agonistic behaviour.

The family Apidae includes numerous solitary species, particularly among the Xylocopinae, its most basal subfamily (Michener 2000). However, no studies in this subfamily have demonstrated nestmate recognition through behavioural assays comparing conspecifics from the same or different nests. A particularly interesting solitary taxon in the Xylocopinae is the genus *Manuelia*,

which has been proposed as the most basal taxon in the phylogeny of Xylocopinae (Sakagami & Michener 1987), which in turn is the most basal subfamily of the Apidae (Michener 2000). Moreover, none of the known species of the family Megachilidae, the sister group of Apidae, exhibit eusocial behaviour (Michener 1974), suggesting that the solitary behaviour in *Manuelia* derives from a common ancestor to Apidae and Megachilidae without eusocial behaviour. Recently, Flores-Prado, Chiappa & Niemeyer (unpublished results) described the occasional presence of two females inside nests of *M. postica*, suggesting a certain degree of tolerance between nestmate conspecifics, and the agonistic interactions between a guarding female and an intruder, suggesting rejection between non-nestmate conspecifics. These observations support the idea that nestmate recognition is a capacity present in a natural context in *M. postica*. In this paper we describe experiments with paired females from the same or different nests which show the occurrence of nestmate discrimination. Finally, we demonstrate the role that cuticular compounds play in nestmate recognition.

2. MATERIALS AND METHODS

(a) *Females of* M. postica

Females of *M. postica* were extracted from fifty-four nests constructed inside stems of *Chusquea quila* (Poaceae: Bambuseae) at the Altos de Lircay National Park, east of Talca, Chile (35°29' S; 70°58' W). These females were used to perform the experiments with live females in the field. Another group of seventy-three nests which were judged to contain young adults before they destroy the

partitions separating individual cells (Flores-Prado, Chiappa & Niemeyer, unpublished results), was collected and transported to the laboratory in Santiago. These nests were kept at low temperature (10 to 15 C) and females were withdrawn from them as needed to conduct the experiments.

(b) *Recognition between two live females*

The following experiments were performed in the field. Two live females (from the same generation) were placed at the extremes of a 20-cm-long Tygon® tubing whose 5-mm-internal diameter was similar to that of the galleries where the bees live in nature; thereafter, the two extremes of the tubing were joined together forming a "circular tube" arena (Breed *et al.* 1978). The agonistic and tolerant behaviours of both females were recorded using an "instantaneous sampling" method (Altmann 1974), in which the activity of the bees was observed for 5 seconds at 1-min intervals during 20 minutes. The behaviours were defined on the basis of those reported for other species of Apidae (e.g., Michener 1969; 1974), and were classified as tolerant or intolerant according to patterns described by several authors for species of Apoidea (e.g., Smith & Weller 1989; Breed & Julian 1992; Kukuk 1992; Wcislo 1997; Paxton *et al.* 1999; Pabalan *et al.* 2000; Packer 2000). Thus, the behaviours were scored as tolerant if: i) females were near each other (less than one body length) (tolerant approach), ii) females were in contact with each other with no signs of mutual aggression (tolerant contact), or iii) one female passed by the other venter to venter (pass). If a female exhibited a C-posture (she curls her abdomen while her mandibles and sting are pointed towards the other female) or if she was observed pushing, biting, stinging, or touching the other female with her legs,

the behaviour was classified as intolerant. If females remained far away (more than one body length) from each other (intolerant spacing), or if one female facing the other moved back, the behaviours were also classified as intolerant. An agonistic index for the interaction between two females was modified from Barki *et al.* (1992) and Lehner (1996) as:

$$AI_{A+B} = 1/20\ [\sum B_A + \sum B_B + \sum B_{A+B}]$$

where B_A and B_B are the number of intolerant behaviours performed only by female A or B, respectively, while the other one is at rest, B_{A+B} is the number of intolerant behaviours performed simultaneously by both females, and 20 is the maximum potential number of behaviours observed during the 20 min period.

Two treatments were performed: a) an intra-nest treatment, consisting of two females from the same generation taken from the same nest, and b) an inter-nest treatment with two females from different nests. Each treatment was replicated 31 times, using a new circular tube in each trial. Since results from similar experiments have indicated a relationship between aggressive behaviour and body size (Smith & Weller 1989; Hogendoorn & Veltuis 1999), the length of the forewing from its base to the tip of each female was measured as an estimation of body size (Breed *et al.* 1978; Smith & Weller 1989).

(c) *Recognition between a live and a dead female*

The following experiments were performed in the laboratory. The recognition bioassay (adapted from Ruther *et al.* 1998; 2002) involved two females (from the same generation): a "treated" dead female, and a "test" living female. The

treated female was placed at one end of a 7-cm-long glass tube whose 5-mm-internal diameter was similar to that of the galleries where the bees live in nature, and the test female was placed at the opposite end. After placing the two bees inside the tube, the ends were sealed with teflon stoppers.

The behaviour of the test female was video-recorded during 20 min. Behavioural events and states were determined during tape playback and were analysed using the software The Observer 3.0 (Noldus). The behavioural events were the same as described for the experiment performed in the field, i.e. passing, pushing, biting, stinging, touching with the legs, C-posture, and moving backwards. In addition, the duration of the following behavioural states was determined: i) a tolerant approach, if the test female remained near the dead female (less than one body length), ii) a tolerant contact, if the test female was in contact with the dead female but did not exhibit aggressive behaviours, iii) an intolerant spacing, if the test female remained far away from the dead female (more than one body length), iv) an intolerant contact, if the test female was in contact with the dead female and exhibited aggressive behaviours, and v) an intolerant escape, if the live female moved away from the dead female, and bit or pushed the stopper at the end of the glass tube. The total number of tolerant and intolerant events was counted, and the duration of tolerant and intolerant states was assessed.

Three bioassays were performed with two treatments in which the treated female was a nestmate or a non-nestmate of the test female. The treated female was: a) a dead female, killed by exposure to pentane vapors; b) a pentane-washed air dried carcass, or c) a pentane-washed carcass which had been previously coated with the extract made from another individual female. In

the latter case, if the dead female was a nestmate of the test female, it was coated with the extract of a non-nestmate of the test female; if the dead female was a non-nestmate of the test female, it was coated with the extract of a nestmate of the test female. Each treatment in each bioassay was replicated 15 times. Each replicate was performed with a different pair of females and a different glass tube. Temperature was maintained between 23 and 25 C during the experiments.

The use of dead females in these bioassays not only allows the manipulation of cuticular compounds but also avoids the potential problem that females can adjust or modify their agonistic behaviour in response to the behaviour of the opponent (Smith & Weller 1989; Schneider *et al.* 2001; Breed *et al.* 2004b).

(d) *Extraction of cuticular compounds*

Cuticular compounds were extracted by individually immersing females in glass vials containing 250 µL pentane, enough to cover their body completely, during 45 min; extracts were maintained at -18 C until they were analysed (Salvy *et al.* 2001, Saul-Gershenz & Millar 2006).

(e) *Chemical analysis*

Cuticular extracts were concentrated to 30 µL with a flow of pure nitrogen. Ten µL of the concentrated extract were injected in splitless mode into a gas chromatograph (GC model HP-5890, Hewlett-Packard, Palo Alto, CA) fitted with a capillary column (SPB5, 30m x 0.25 mm ID, Supelco, Deerfield IL, USA) and directly coupled to a mass detector (MD model HP-5972, Hewlett-Packard, Palo

Alto, CA) with an integrated data system. Helium was used as carrier gas at 2 ml/min. The GC oven was programmed to remain at 150 C for 5 min, then to increase to 260 C at a rate of 5 C/min, and finally to remain at 260 C for 20 min. Ionisation by electron impact (70 eV) was carried out at 280 C. The presence or absence of a given compound in the chromatographic profile of each individual extract was determined by comparison of mass spectra with a library database, and comparison of retention indexes with those of authentic standards - when available - or with data from the literature. Identifications were considered positive if coincidence between experimental and library mass spectra was higher than 90% (values were typically higher than 95%), and if retention indexes did not differ by more than 5 units (differences were typically less than 3 units).

The extracts from 17 females from 8 different nests with 2 or 3 females each, were analysed. This allowed the setting up of 10 intra-nest comparisons and 126 inter-nest comparisons. The composition of cuticular extracts was compared between females from the same nest and from different nests using the simple matching coefficient (Krebs 1989), calculated as the number of positive and negative matches (in terms of presence or absence of a given compound) between female pairs divided by 28, the total number of compounds identified. Thus, the simple matching coefficient for each comparison varied from 0 to 1.

(f) *Statistical analyses*

The agonistic index for the interaction between two live females obtained in intra and inter-nest treatments were compared using one-way ANOVA (Sokal &

Rohlf 1995). The agonistic index for the interaction between two live females from either the intra-nest or the inter-nest treatments was correlated with differences in forewing length using Pearson product-moment correlation (Sokal & Rohlf 1995). The number of tolerant and intolerant behavioural events and the duration of tolerant and intolerant behavioural states in bioassays involving dead females were compared between treatments (intra and inter-nest) using the Mann-Whitney *U* test because data did not show a normal distribution (Siegel & Castellan 1988). Simple matching coefficients for females from the same or different nests were compared using the Mann-Whitney *U* test because data did not show a normal distribution (Siegel & Castellan 1988).

3. RESULTS

(a) *Recognition between two live females*

Experiments in the field involving two live females demonstrated that females were more aggressive towards non-nestmates than towards nestmates. Thus, the agonistic index in the inter-nest treatment (two females from different nests) was higher than in the intra-nest treatment (two females from different nests) (mean ± S.D.= 1.39 ± 0.49 and 0.99 ± 0.75, for inter-nest and intra-nest treatments, respectively; one-way ANOVA: $F_{1,60} = 6.05$, $p < 0.05$). Furthermore, the agonistic index was not correlated with differences in body size (forewing length) of females used either in the intra-nest treatment ($n = 31$; $r = 0.24$; $p = 0.19$) or in the inter-nest treatment ($n = 31$; $r = -0.26$; $p = 0.14$).

(b) *Recognition between a live and a dead female*

Results of the laboratory bioassays involving a dead and a living (test) female showed that test females were more intolerant towards non-nestmate dead females than towards nestmate dead females, as demonstrated by an increased number of intolerant behavioural events (Fig. 1A), and the increased duration of behavioural states (Fig. 1D). When the treated dead female was washed with solvent, no differences were apparent between non-nestmates and nestmates for any of the parameters analysed (Figs. 1B and 1E). Nevertheless, when the pentane-washed carcasses were coated with cuticular extracts the test females were more intolerant towards nestmate female carcasses coated with the cuticular extract from a non-nestmate, than towards non-nestmate female carcasses coated with the cuticular extract from a nestmate (Fig. 1C). This reversal of the behavioural pattern by application of cuticular extracts also occurred in the duration of behavioural states (Fig. 1F). Opposite patterns were found when duration of tolerant behavioural states were considered (Figs. 1D and 1F), but not when the mean number of tolerant behavioural events was considered (Figs. 1A and 1C). On the other hand, test females were more tolerant towards nestmate dead females than towards non-nestmate dead females in terms of duration of behavioural states (Fig. 1D) and, when the solvent-washed carcasses were coated with cuticular extracts the test females were more tolerant towards non-nestmate female carcasses coated with the cuticular extract from a nestmate, than towards nestmate female carcasses coated with the cuticular extract from a non-nestmate (Fig. 1F).

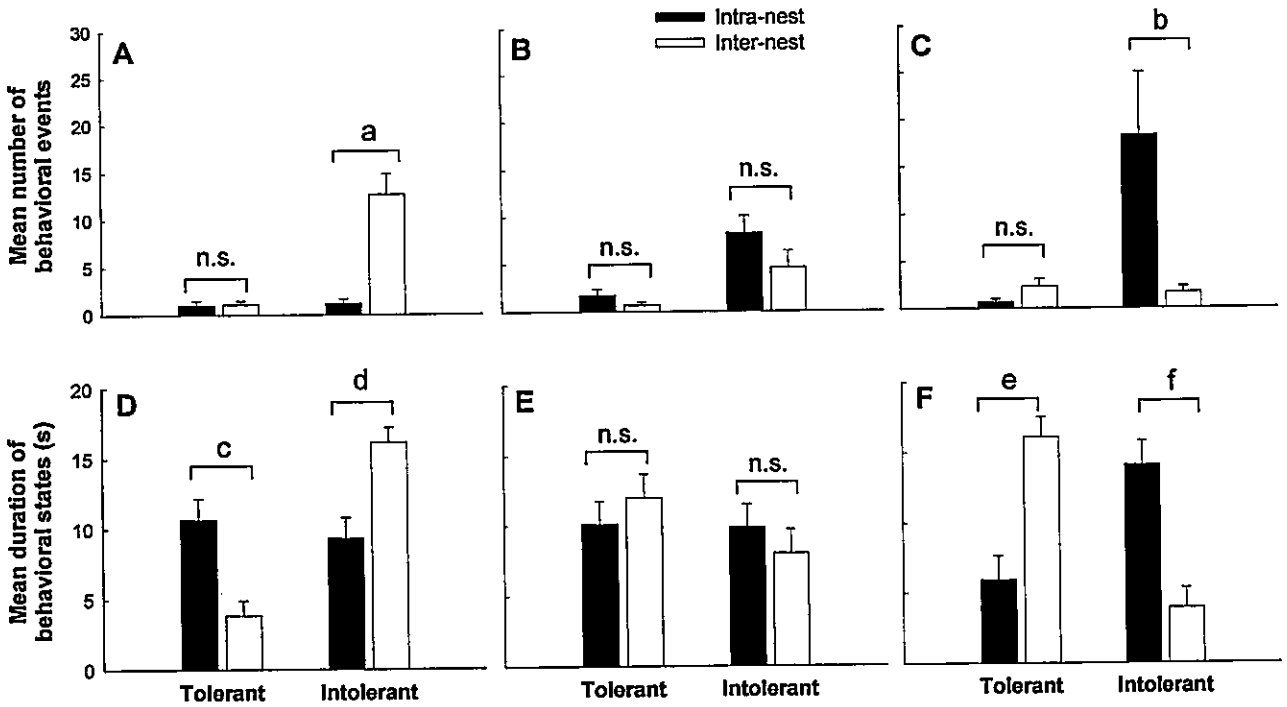

Figure 1. Mean number of tolerant and intolerant behavioural events and mean duration of behavioural states of *Manuelia postica* females in the presence of a dead female from the same nest (intra-nest treatment) or from a different nest (inter-nest treatment). Behaviours were recorded from bioassays involving: A, D = dead females; B, E = washed dead females; and C, F = washed and coated dead females (see Materials and Methods). The observation period was 20 minutes. Statistics for significant comparisons (Mann-Whitney *U*-test): a ($Z = -4.33$, $p < 0.001$), b ($Z = 2.34$, $p = 0.019$), c ($Z = 2.92$, $p < 0.01$), d ($Z = -2.92$, $p < 0.01$), e ($Z = -3.75$, $p < 0.001$), and f ($Z = 3.75$, $p < 0.001$).

(c) *Cuticular compounds*

Cuticular compounds were extracted from 17 females of *M. postica* from 8 different nests. Table 1 shows the compounds identified in the extracts and their abundance. They were carboxylic acids and esters, and hydrocarbons (alkanes and alkenes). The most abundant compounds were hydrocarbons, particularly C23 and C25 saturated hydrocarbons, as found in the solitary bee, *Habropoda pallida* (Apidae, Apinae) (Saul-Gershenz & Millar 2006). With the exception of 10-heneicosane, all compounds have been previously described as constituents of cuticular extracts of Hymenoptera (El-Sayed 2007). The number of compounds detected in any given female ranged from 10 to 24 (mean ± SD = 20.1 ± 4.18). The number of females in which a given compound occurred ranged from 3 to 17 (mean ± SD = 12.1 ± 4.07). The mean area of chromatographic peaks which could not be identified corresponded to 2.0 ± 2.6 % (mean ± SD) of the total chromatographic area.

Females from the same nest were more similar between each other than females from different nests (simple matching coefficient, mean ± SE: 0.764 ± 0.026 and 0.683 ± 0.011, for comparisons of females from the same or different nests, respectively; Mann-Whitney *U*-test: $Z = 2.25$, $p = 0.024$).

Table 1. Cuticular compounds identified in extracts of females of *Manuelia postica*.

Compound	Retention index	N° of females [a]	Abundance (% ± SD) [b]	Identification method [c]
Hexadecanoic acid, ethyl ester	1991	7	0.21 ± 0.41	MS,RI,ST
Hexadecanoic acid, methyl ester	2010	3	0.05 ± 0.12	MS,RI,ST
10-Heneicosene[d]	2076	13	1.44 ± 2.32	MS,RI
Heneicosane	2095	17	3.91 ± 4.77	MS,RI,ST
Octadecanoic acid	2164	6	0.65 ± 1.27	MS,RI,ST
(Z)-9-Octadecenoic acid, ethyl ester	2175	9	0.88 ± 1.61	MS,RI,ST
Octadecanoic acid, ethyl ester	2190	6	0.41 ± 1.01	MS,RI,ST
Docosane	2201	15	0.67 ± 0.45	MS,RI,ST
Acetic acid, octadecyl ester	2212	10	0.35 ± 0.57	MS,RI,ST
(Z)-9-Tricosene	2278	16	15.97 ± 13.70	MS,RI,ST
Tricosane	2299	17	26.86 ± 10.92	MS,RI,ST
Tetracosene[d]	2370	12	0.45 ± 0.51	MS,RI
1-Tetracosene	2392	7	1.07 ± 2.53	MS,RI
Tetracosane	2400	13	2.58 ± 4.14	MS,RI,ST
Pentacosene[d]	2465	9	1.29 ± 1.77	MS,RI
4-Pentacosene	2469	13	3.92 ± 3.22	MS,RI
Pentacosane	2498	17	14.98 ± 10.71	MS,RI,ST
Hexacosane	2590	16	0.69 ± 0.43	MS,RI,ST
Heptacosene[d]	2663	10	1.19 ± 1.29	MS,RI
13-Heptacosene[d]	2670	15	3.63 ± 3.51	MS,RI
Heptacosane	2699	17	4.50 ± 2.42	MS,RI,ST
Octacosane	2795	15	0.43 ± 0.35	MS,RI,ST
Nonacosadiene[d]	2848	16	4.30 ± 2.61	MS,RI
Nonacosadiene[d]	2855	15	1.45 ± 1.38	MS,RI
Nonacosene[d]	2866	9	1.20 ± 1.97	MS,RI
Nonacosene[d]	2872	12	2.13 ± 1.94	MS,RI
Nonacosene[d]	2877	9	1.66 ± 1.82	MS,RI
Nonacosane	2898	17	1.17 ± 0.39	MS,RI,ST

[a] number of females in which the compound was found.
[b] percentage of chromatographic area of compound in relation to total area of the chromatogram.
[c] MS = mass spectrum, RI = retention index in relation to *n*-alkanes, ST = standard compound.
[d] isomer undetermined.

4. DISCUSSION

(a) *Recognition between two live females*

Flores-Prado, Chiappa & Niemeyer (unpublished results) described the occasional presence of two females inside nests of *M. postica*, and the agonistic interactions between a guarding female and an intruder, suggesting nestmate recognition capacity. Moreover, these observations support the idea that some components of kin discrimination are already present in *M. postica* in a natural context, as shown earlier for other solitary species (Wcislo 2000). Nestmate recognition ability was tested in experiments which demonstrated that females were more aggressive towards non-nestmates than towards nestmates, as in eusocial species of bees (Buckle & Greenberg 1981; Michener & Smith 1987; Inoue *et al.* 1999).

Body size has been shown to affect the outcome of intraspecific interactions between hymenopterans (Gamboa & Dropkin 1979; Sullivan & Strassmann 1984). For example, laboratory observations of the interaction between nestmates and non-nestmates of the same generation have demonstrated that larger female bees are more aggressive than smaller ones (Smith & Weller 1989; Hogendoorn & Velthuis 1999; Arneson & Wcislo 2003). The differential agonistic responses exhibited by females of *M. postica* were not correlated with differences in body size, suggesting that in this experimental context visual stimuli are not particularly important in conspecific interactions and that other stimuli, such as chemical cues, affect the outcome of encounters between nestmates and non-nestmate females.

(b) *Recognition between a live and a dead female*

The perception of chemical cues as signals for nestmate recognition has been demonstrated in laboratory bioassays measuring agonistic responses in social Hymenoptera (Roulston *et al.* 2003). Bioassays developed in eusocial Hymenopterans in which cuticular compounds have been removed and reapplied, or have been modified by addition of compounds from external sources, have pointed to cuticular hydrocarbons as nestmate recognition pheromones (Ruther *et al.* 2002; Dani *et al.* 2001; 2005). Our results are unequivocal about the central role that cuticular compounds play in nestmate recognition in *M. postica*. Firstly, test females of *M. postica* discriminated dead females depending on whether they were nest or non-nestmates. Secondly, extraction of the potential recognition signal led to the disappearance of the associated discrimination patterns. Thirdly, the pattern of discrimination observed towards normal dead females was changed by coating the solvent-washed carcasses with cuticular extracts. Finally, the composition of cuticular extracts was more similar between females from the same nest than between females from different nests. Hence, this series of bioassays unequivocally demonstrate that cuticular compounds are the cues employed by *M. postica* females in nestmate recognition.

The importance of cuticular hydrocarbons in intraspecific communication has been demonstrated in a wide diversity of social Hymenoptera (Singer 1998; Tsutsui 2004; Howard & Blomquist 2005). The present report is the first to demonstrate nestmate recognition and to show the role of cuticular chemicals in nestmate recognition in a largely solitary species of Apidae, *M. postica*. Nestmate recognition has been suggested as one of the characteristic attributes

of eusocial species (Breed *et al.* 2004a); the present results show that it is not an exclusive ability of eusocial insects. Furthermore, since *Manuelia* is at the base of the Apidae phylogeny and possesses several ancestral features (Michener 2000), nestmate recognition exhibited by more apical eusocial species may represent the retention of a primitive capacity in the Apidae.

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CAPÍTULO 4

Kin Recognition by Self Referent Phenotype Matching in a Solitary Bee

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Abstract

The recognition of conspecifics is a central issue to social behavior. In eusocial hymenopterans, recognition through kin referent phenotype matching has been clearly demonstrated, while self referent phenotype matching has been a subject of controversy. In this paper, we demonstrate kin recognition based on self referent phenotype matching in the solitary bee, *Manuelia postica*, using cross-fostering field experiments involving the single transfer of larvae and subsequent laboratory recognition bioassays with females. This is the first time that such phenomenon has unequivocally been demonstrated in an hymenopteran. Given the basal position of *Manuelia* in the phylogeny of the Apidae, this capacity may represent an ancestral recognition mechanism in this family.

Brief report

Throughout the animal kingdom recognition of fellow group members or kin conspecifics is a central issue to social behavior. This ability has usually been attributed to phenotype matching, whereby an individual learns some phenotypic trait of its familiar conspecifics and/or some aspect of its own phenotype, stores its representation in memory as a template, and later matches this template to the phenotype of an unfamiliar conspecific; thus, individuals can acquire cues from kin or from itself (*1*).

Eusocial hymenopterans are a valuable model for studying kin recognition and its underlying mechanisms (*2*). They recognize conspecifics through kin referent phenotype matching; the template is learned during contact between workers or contact of the workers with the secretions used in comb construction (*3*).

Manuelia postica is a solitary bee species which constructs a tunnel in stems of *Chusquea quila* (Poaceae), deposits a food mass and an egg at its end, builds a partition with wood particles, and repeats these last two processes up to seven times before the nest is finished. The individuals which develop inside the cells have no contact between themselves or with their mother during the breeding period (*4*). However, females do show nestmate recognition (*5*). Thus, *M. postica* represents an ideal system for testing the occurrence of self referent phenotype matching.

Cross-fostering experiments were set up. One larva was withdrawn from a cell *a* of nest *A*, and another from a cell *b* of a different nest *B*. The larva withdrawn from cell *b* was placed in cell *a*, and viceversa. In this way, each

manipulated nest contained one foster larva and one or more non-transferred larvae. When the larvae were judged to have reached adulthood (*6*), the nests were brought to the laboratory. Females were withdrawn from the nests to conduct recognition experiments between two non-kin females from the same nest (N = 14), and between two kin females from different nests (N = 13). The females were placed at the ends of a glass tube which was then sealed with teflon stoppers. Video recording and playback allowed the determination of occurrence of behavioral events and duration of behavioral states. A behavioral event was scored as tolerant if one female passes by the other venter to venter, and as intolerant if a female exhibits a C-posture, is observed pushing, biting, stinging, or touching with the legs the other female, or if one female facing the other moves back. A behavioral state was scored as tolerant if females remain near each other or if they are in contact with each other with no signs of mutual aggression, and as intolerant if they are in contact and exhibit aggressive behaviors, remain far away from each other, or one of them attempts to move away from the other.

The number of intolerant behaviors and the duration of intolerant behavioral states was higher between non-kin females from the same nest than between kin females from different nests ($F_{1,25}$ = 10.3, $P < 0.01$; and $F_{1,25}$ = 7.82; $P < 0.01$, respectively) (Fig. 1A). Furthermore, the duration of tolerant behavioral states was higher between kin females from different nests than between non-kin females from the same nest ($F_{1,25}$ = 7.82; $P < 0.01$) (Fig. 1B).

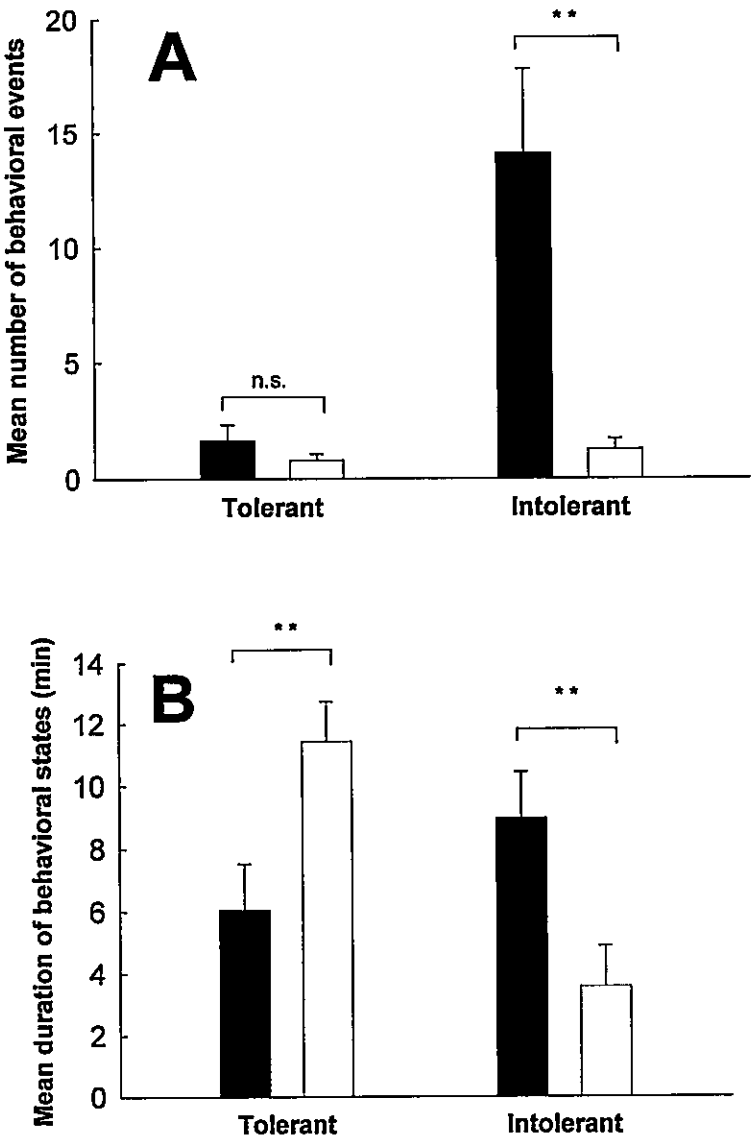

Fig. 1. Mean number of tolerant and intolerant behavioral events (A) and behavioral states (B) of *Manuelia postica* females in the presence of non-relative females reared in the same nest and of relative females developed in different nests. The observation period was 15 minutes. n.s. = non significant; **: $p<0.01$. □ = relative females; ■ = non-relative females.

These results show that *M. postica* uses self referent phenotype matching for conspecific recognition. This is the first time that such phenomenon has unequivocally been demonstrated in an hymenopteran. Given the basal position of *Manuelia* in the phylogeny of Apidae (*7*), this capacity may represent an ancestral mechanism of recognition in this family. Although this feature may still be present in eusocial bees, its demonstration in such species remains controversial due to the difficulty of entirely eliminating pre-imaginal (*8*) and social (*9*) learning.

Supporting Online Material

The cross fostering experiment was set up in Altos de Lircay National Park, Chile (35°29' S; 70°58' W); female exchanges were started during the last week of november 2005 and continued until the first week of january 2006. This is the period when nests are most often found with pre-defecating larvae inside (see Figure 2). Ninety-four nests were sagitally opened, but 30 of them could not be used because they contained less than two pre-defecating larvae inside. Exchanges of larvae were performed in 64 nests; thereafter, nests were closed and left in the field for *ca.* 80 days, a period sufficient for the larvae to develop into adults (see Figure 2). Ten nests were damaged in the field; the rest was brought to the laboratory and kept at 9 °C until they were opened to withdraw females to be used in recognition experiments. Only forty nests could finally be used in the bioassays, since in the remainder nests: i) the partitions between cells had been destroyed by the growing individuals, *i.e.*, hibernating

assemblages had formed (N= 3), only dead females were found (N = 3), or foster larvae turned out to be males (N = 8).

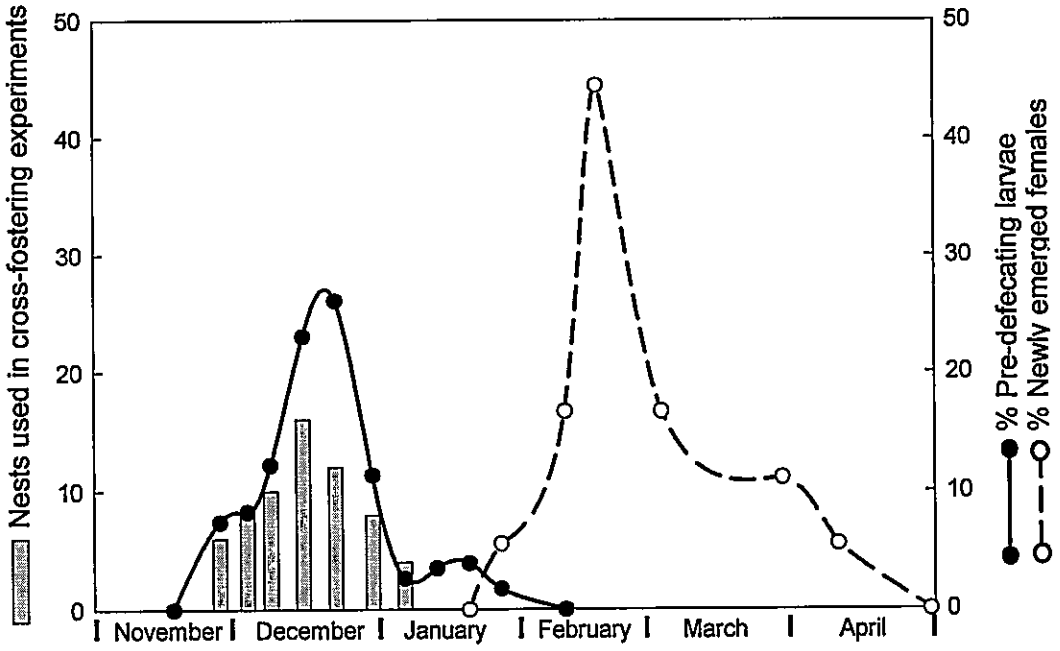

Figure 2. Abundance of pre-defecating larvae and newly emerged females of *Manuelia postica* between november 2005 and april 2006, and instances when cross fostering experiments were set up.

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RESULTADOS GENERALES

Los principales resultados obtenidos en esta investigación son los siguientes:

La tribu Manueliini, representada sólo por el género *Manuelia*, es el grupo más basal en la filogenia de la subfamilia Xylocopinae, de modo que un taxón compuesto sólo por especies solitarias constituye el grupo hermano de las demás tribus en Xylocopinae.

Manuelia postica presenta rasgos conductuales, relacionados con la biología de la nidificación, típicos de especies solitarias, y posee otros rasgos que han sido propuestos como precursores de la sociabilidad en Xylocopinae.

Las hembras de *M. postica* tienen la capacidad de reconocimiento de conespecíficas, rasgo característico de especies eusociales y filogenéticamente apicales en Apidae. Dicha capacidad se refleja en el comportamiento agonístico diferencial exhibido por las hembras; éstas son más intolerantes hacia no compañeras de nido que hacia compañeras de nido. La capacidad de reconocimiento de hembras conespecíficas está mediada por compuestos cuticulares.

Las hembras de *M. postica* presentan la capacidad de reconocimiento de parentesco basada en el mecanismo de igualación de fenotipos por autoreferencia, es decir, en el aprendizaje de señales producidas por sí mismas. Por consiguiente, las señales que mediarían el reconocimiento entre

parientes, son de tipo endógeno al igual que en otras especies eusociales y filogenéticamente apicales en Apidae.

DISCUSION GENERAL

Aspectos generales de la sociabilidad en las tribus de Xylocopinae

La subfamila Xylocopinae ha emergido como un valioso modelo para el estudio de estados sociales tempranos y de la evolución de la sociabilidad (e.g. Michener, 1985; Michener, 1990; Schwarz et al. 1997; 1998; 2007, Tierney et al. 2002), ya que contiene especies cuyo nivel de organización social se extiende desde la subsociabilidad hasta la eusociabilidad, y también posee especies solitarias (Michener, 1969; 1974; 2000). Cabe destacar que en Xylocopinae la sociabilidad es un rasgo complejo que presenta diferentes estados, por lo cual en muchas especies ha resultado difícil adscribir algún nivel social en particular (Michener, 1985; 1974), bajo el esquema de organización social categorizado por Batra (1966), Michener (1969; 1974) y Wilson (1971). Se ha planteado, en términos generales, que la sociabilidad de Allodapini es más avanzada que en Ceratinini (Sakagami & Maeta, 1977; Sakagami & Maeta, 1995) y más avanzada en Ceratinini que en Xylocopini (Sakagami & Maeta, 1995; Maeta et al, 1996). La tribu Manueliini contiene sólo especies solitarias, en las que cada hembra construye su propio nido siguiendo un patrón de construcción de celda, aprovisionamiento de alimento, postura de huevos, cierre de la celda, y repetición de tal secuencia (Daly et al., 1987; Michener, 1985; 2000, Flores-Prado et al., 2008a).

En la tribu Xylocopini no existen especies subsociales (en las que la hembra progenitora cuida a su progenie en desarrollo, de manera directa y contínua).

La mayoría de las especies son solitarias y algunas presentan vida comunal (con diferentes hembras nidificando en diferentes túneles de un nido ramificado) (Michener, 1985). En unas pocas especies existe una incipiente división de la labor reproductiva (Gerling et al., 1989).

La tribu Ceratinini contiene muchas especies solitarias (Sakagami & Maeta, 1977; 1987; Maeta et al., 1997a), algunas de las cuales han sido propuestas como subsociales desde que se registró en ellas un cuidado directo de la progenie, debido a que la hembra progenitora destruye los tabiques que separan las celdas en un nido, remueve las fecas de las larvas, y reconstruye los tabiques (Sakagami & Maeta, 1977). El nivel comunal de organización es imposible debido a que los nidos son construídos en tallos muy delgados que no tienen el espacio suficiente para nidos ramificados (Michener, 1985). Algunas especies exhiben un sistema de castas rudimentario, por lo cual se ha propuesto en ellas un nivel de organización eusocial incipiente (Okazaki, 1987; Sakagami & Maeta, 1995).

Todas las especies de Allodapini pueden exhibir un estado subsocial dentro de su ciclo de vida (Michener, 1974). Algunas especies muestran diversos grados de sociabilidad, con variabilidad inter e intraespecífica, hasta el desarrollo de castas funcionales (Schwarz et al., 2007). Algunas especies son altamente eusociales pues presentan castas diferentes tanto funcional como morfológicamente (Houston, 1977; Schwarz et al., 2005).

Rasgos conductuales precursores de la sociabilidad en Xylocopinae

Algunas especies solitarias de la subfamilia Xylocopinae presentan rasgos inusuales para especies solitarias de abejas, los cuales han sido propuestos como prerrequisitos para la evolución de la vida social (Michener, 1969; 1974). Varios de estos rasgos están vinculados con la biología de la nidificación; i) cohabitación de más de una hembra dentro de un nido, exhibiendo mutua tolerancia, ii) contacto físico entre la hembra progenitora y su progenie en desarrollo, iii) protección de los individuos inmaduros por parte de la madre, por medio de conductas de defensa en la entrada del nido, iv) extenso período de vida de la hembra progenitora, en etapa reproductiva, de modo que pueda existir sobreposición de generaciones adultas dentro del nido, y v) existencia de agrupaciones hibernantes de hembras en el interior de los nidos (Michener 1969; 1974; 1985). Aunque tales rasgos son probablemente precursores de la vida social, ninguno de ellos por sí mismo garantiza el origen de la sociabilidad (Sakagami & Maeta, 1977; Michener, 1990). A continuación se describen cada uno de estos rasgos conductuales y se comparan entre las especies de Xylocopinae.

Tolerancia mutua entre las hembras. Habitualmente las hembras de especies solitarias de himenópteros son agresivas hacia cualquier hembra conespecífica y el grado de tolerancia entre hembras, necesario en la vida grupal (Michener, 1969; 1974), aumenta con el nivel de sociabilidad de las especies (Batra, 1968). Sin embargo, en especies solitarias o con un nivel básico de sociabilidad, se ha registrado con baja frecuencia cohabitación de

dos hembras dentro del nido, y en ocasiones más de dos, durante el período de fundación del nido, lo cual es una evidencia de tolerancia entre las hembras. La cohabitación puede representar un evento temporal o un estado permanente (Sakagami & Maeta, 1977), dependiendo de restricciones ecológicas, como por ejemplo, la disponibilidad de recursos para nidificación y/o alimentación, y el grado de parentesco entre las hembras (Hoogendoorn & Leys, 1993; Dunn & Richards, 2002 Schwarz et al., 2007). Cohabitación durante el período de construcción del nido ha sido reportado en las tribus Manueliini (Flores-Prado et al., 2008a), Xylocopini (Gerling & Hermann, 1978; Hogendoorn & Leys, 1993; Hogendoorn & Velthuis, 1999), Ceratinini (Sakagami & Maeta, 1977; Katayama & Maeta, 1979) y Allodapini (Schwarz et al., 1998).

Contacto físico entre la hembra y su progenie en desarrollo. Las especies de Xylocopinae, excepto el subgénero *Proxylocopa* (Xylocopini), nidifican en ramas, tallos o troncos secos (Gerling et al., 1989; Maeta et al., 1992; Steen & Schwarz, 2000). Las hembras construyen dos tipos de nido, lineales o ramificados, que consisten en uno o más túneles, respectivamente, con celdas dispuestas en serie y separadas por tabiques hechos con partículas del sustrato de nidificación (Gerling et al., 1989; Michener, 1990). En algunas especies de la tribu Ceratinini las hembras abren las celdas que contienen larvas, remueven las fecas y reconstruyen los tabiques (Sakagami & Maeta, 1977; Maeta et al., 1997b). Tal conducta ha sido considerada como una preadaptación al abandono total de las celdas (Michener, 1974; Maeta et al., 1997b), como ocurre en todas las especies de Allodapini, tribu hermana de Ceratinini (Schwarz et al, 2007). Adicionalmente, la evolución de la sociabilidad

en Allodapini ha sido relacionada con la transición de una estrategia de aprovisionamiento del alimento dentro de las celdas, sin contacto entre la hembra progenitora y su descendencia (rasgo característico de Manueliini, Xylocopini y la mayoría de las especies de Ceratinini), a una estrategia de alimentación progresiva de las larvas, por parte de la hembra progenitora, en un nido sin celdas, rasgo característico de casi la totalidad de especies de Allodapini estudiadas (Michener, 1985; 1990). Aunque algunas especies filogenéticamente más apicales de Allodapini presentan una estrategia de aprovisionamiento de alimento, la sociabilidad en esta tribu es considerada un estado ancestral, ya que las especies basales, que tienen una estrategia de alimentación progresiva, exhiben cierto grado de sociabilidad (Schwarz et al., 2007).

Comportamiento de defensa del nido. El comportamiento de guardia descrito en *Manuelia postica*, en que el metasoma de la hembra bloquea la entrada del nido (Flores-Prado et al., 2008a), ha sido registrado en especies de las tribus Xylocopini (Ramalho et al., 2004), Ceratinini (Sakagami & Maeta, 1977; Michener, 1985; Maeta et al., 1992) y Allodapini (Mason, 1988; Maeta et al., 1992; Hogendoorn & Schwarz, 1998; Steen & Schwarz, 1998), y puede representar la retención de un sistema primitivo de defensa en Xylocopinae, el cual es efectivo debido a la existencia de un sólo orificio en la mayoría de los nidos, y a la dimensión de tal orificio que permite el bloqueo completo.

Extenso período de vida en las hembras. La sobreposición de generaciones de hembras adultas puede ocurrir si la madre está presente en el nido cuando

la progenie alcanza su estado adulto, lo cual permitiría el contacto entre la madre y su descendencia. Esta característica ha sido registrada en especies de las tribus Xylocopini, Ceratinini y Allodapini (Hogendoorn & Velthuis, 1999; Schwarz et al., 2003), y ha sido sugerida para especies de la tribu Manueliini (Daly et al., 1987)

Agrupaciones hibernantes. Estas agrupaciones, también llamadas agrupaciones pre-reproductivas, consisten en un grupo de adultos recién emergidos dentro del nido que han destruído los tabiques que separan las celdas (Michener, 1985). Agrupaciones hibernantes, ya sea temporales o permanentes, han sido observadas en las tribus Manueliini (Daly et al., 1987; Flores-Prado et al., 2008a), Xylocopini (Michener, 1990; Martin, 1991), Ceratinini (Michener, 1985) y Allodapini (Michener, 1985; Tierney et al., 2002). En *M. gayi*, cada adulto permanece dentro de sus celdas durante el invierno sin destruir los tabiques que separan las celdas, lo cual ha sido propuesto como un rasgo ancestral en Xylocopinae (Daly et al., 1987) y que concuerda con la posición más basal de *M. gayi* en la filogenia reconstruída en este estudio.

Reconocimiento entre hembras conespecíficas

En Xylocopinae son pocos los estudios que sugieren o demuestran la capacidad de reconocimiento entre hembras conespecíficas. El reconocimiento de compañeras de nido ha sido demostrado en una especie de Xylocopini con nivel básico de sociabilidad, la cual presenta un sistema incipiente de castas funcionales (Hogendoorn, 1996). En algunas especies de Allodapini ha sido

demostrado el reconocimeinto de parentesco entre hembras que fundan un nuevo nido (Schwarz et al., 1998). En especies eusociales de la subfamilia Apinae (Apidae), el reconocimiento entre compañeras de nido, y entre parientes, también ha sido demostrado (Breed, 1998; Inoue et al., 1999; Buchwald & Breed, 2005, Harano & Sasaki, 2006), así como el papel de los compuestos cuticulares como señales de reconocimiento entre las hembras (Breed & Stiller, 1992; Singer, 1998; Fröhlich et al., 2001; Buchwald & Breed, 2005; Dani et al., 2005). Sin embargo, el mecanismo que subyace al reconocimiento no ha sido tan ampliamente estudiado. La igualación de fenotipos por autoreferencia ha sido el único mecanismo demostrado en sólo una especie, *Apis mellifera* (Getz & Smith, 1983; 1986), aunque dicha demostración ha sido cuestionada debido la dificultad de eliminar el aprendizaje pre-imaginal (Alexander, 1991) y social (Hauber & Sherman, 2000) de las hembras sometidas a los experimentos. En *M. postica* el reconocimiento entre compañeras de nido está mediado por compuestos cuticulares, entre los que destacan los hidrocarburos (Flores-Prado, et al., 2008b). Dicho fenómeno también puede corresponder a reconocimiento por parentesco, como fue demostrado a partir de los experimentos de "cross-fostering" y de los posteriores bioensayos en el laboratorio. Debido a que se demostró el mecanismo de igualación de fenotipos basado en autoreferencia, las señales de tipo endógeno son las que mediarían el reconocimiento entre parientes, al igual que en otras especies eusociales (Smith & Breed, 1995). Esto no contradice un posible efecto de señales provenientes del material vegetal del nido, que podrían reforzar las señales que posibilitan el reconocimiento entre hembras compañeras de nido.

Implicancias de la posición basal de Manueliini en la filogenia de Xylocopinae

Manuelia es un género solitario y relicto (Michener, 1979; Daly et al., 1987), que exhibe diversos rasgos morfológicos ancestrales (Michener 2000), y contiene especies que poseen rasgos conductuales típicos de especies solitarias de abejas (Michener, 1969; 1974), tales como i) el patrón de construcción de celdas y la estrategia de aprovisionamiento de alimento dentro de éstas (Michener, 1985; Daly et al., 1987), ii) la ausencia de contacto entre los individuos inmaduros, y iii) la ausencia de contacto entre éstos y su madre (Flores-Prado et al., 2008a). Sin embargo, también exhibe rasgos conductuales inusuales para especies solitarias de abejas, tales como i) la presencia de dos o más hembras cohabitando un nido, aunque en baja frecuencia, durante el período de construcción del nido, lo que permite inferir tolerancia mutua entre dichas hembras, ii) la conducta de defensa en la entrada del nido, realizada por una hembra durante el período de desarrollo de la progenie, y iii) la existencia de agrupaciones hibernantes (Daly et al., 1987; Flores-Prado et al., 2008a). En esta investigación se propone por primera vez una filogenia de Xylocopinae elaborada sobre la base de secuencias nucleotídicas que incluye todas las tribus de dicha subfamilia. Todas las reconstrucciones efectuadas, utilizando genes mitocondriales, arrojaron una topología en cuya base se sitúa Manueliini, ratificando la hipótesis filogenética propuesta por Sakagami & Michener (1987) y Michener (2000), elaborada sobre la base de caracteres morfológicos. Los altos valores de soporte del clado Manueliini permiten proponer efectívamente a *Manuelia* como el grupo hermano de los demás Xylocopinae, lo cual hace

posible interpretar que algunos componentes del comportamiento social presentes en especies de Xylocopinae, que han sido propuestos como precursores de sociabilidad, tengan un origen previo al origen de las tribus Xylocopini, Ceratinini y Allodapini. Además, la topología aquí presentada concuerda con la idea general de sociabilidad más avanzada en Allodapini y Ceratinini que en Xylocopiini (Sakagami & Maeta, 1977; 1995; Maeta et al., 1996), de acuerdo con los niveles de organización social propuestos para Hymenoptera (Batra, 1966; Michener, 1969; 1974; Wilson, 1971). Por otra parte, tanto la capacidad de reconocimiento de hembras compañeras de nido, como el fenómeno de reconocimiento de parentesco, corresponderían a la retención de un estado ancestral en las especies eusociales y filogenéticamente apicales de la familia Apidae, tanto en la subfamilia Xylocopinae, como en la subfamilia Apinae. El mecanismo de igualación de fenotipos por autoreferencia representaría un rasgo ancestral en *M. postica*, probablemente compartida con otros Xylocopinae, pero hasta el momento sólo demostrada en dicha especie.

CONCLUSIONES

Manuelia postica es una especie solitaria que presenta algunos rasgos conductuales, relacionados con la biología de la nidificación, que han sido propuestos como prerrequisitos o precursores de la sociabilidad en Xylocopinae. Para evaluar si dichos rasgos son precursores de la sociabilidad en especies de la subfamilia Xylocopinae, es necesario una nueva hipótesis filogenética robusta, que incluya tantos taxones como sea posible, además de información respecto del estado de tales rasgos en especies solitarias y sociales. Así se podrán efectuar análisis filogenéticos comparativos que permitan reconstruir los estados ancestrales en Xylocopinae. La filogenia aquí presentada, aunque robusta, constituye un marco filogenético básico que permite hacer comparaciones generales entre las tribus de Xylocopinae y entre las especies de *Manuelia*.

Manuelia postica es una especie solitaria que presenta la capacidad de reconocimiento de hembras conespecíficas, rasgo característico de especies eusociales y filogenéticamente apicales en Apidae. Dicha capacidad se refleja en el comportamiento agonístico diferencial exhibido por las hembras; éstas son más intolerantes hacia no compañeras de nido que hacia compañeras de nido. Es poco probable que tal fenómeno sea explicado por la retención de un rasgo proveniente de ancestros eusociales, debido a que en toda la familia Megachilidae, hermana de Apidae, no existen especies eusociales, y *Manuelia* es el linaje más basal en la subfamilia Xylocopinae, que a su vez es basal

respecto de la subfamilia Apinae, grupo caracterizado por contener las tribus altamente eusociales en la familia Apidae.

El patrón de discriminación conductual exhibido por las hembras de *M. postica* obliga a reconsiderar la hipótesis de modificación de un patrón de comportamiento agresivo sin discriminación, característico de la vida solitaria, a un patrón de comportamiento diferencial, característico de la eusociabilidad, necesario en la evolución del comportamiento social. Es probable que si se manipulan experimentalmente otras especies solitarias de la familia Apidae, se descubra que la capacidad de reconocer y discriminar sobre la base de la procedencia de las hembras (compañeras de nido y no compañeras de nido) no es un fenómeno distintivo de las especies eusociales. De ser así, en la evolución del comportamiento eusocial a partir del comportamiento solitario, no fue necesario la modificación del patrón de agresividad no discriminativo, al menos en la familia Apidae.

Manuelia postica presenta la capacidad de reconocimiento de parentesco basada en el mecanismo de igualación de fenotipos por autoreferencia. Este descubrimiento es interesante debido a que i) la capacidad de reconocimiento de parentesco representa una condición necesaria en la evolución de la eusociabilidad, de acuerdo con la teoría de selección por parentesco (Hamilton, 1964a,b), ii) el mecanismo de igualación de fenotipos ha sido demostrado en otras especies de himenópteros eusociales, aunque basado en referencia de parientes, lo cual se ajusta más al modo de vida de tales especies, en las que los individuos inmaduros necesariamente tienen contacto frecuente con

parientes, y iii) el mecanismo de igualación de fenotipos por autoreferencia se ajusta al modo de vida de especies solitarias de Xylocopinae, tales como *M. postica*, en que los individuos inmaduros permanecen aislados en sus celdas de crianza, sin contacto con parientes, hasta después de la emergencia de los adultos.

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APÉNDICES

Appendix 1.

Table 1: Taxa and accession numbers of sequences of EF-1α retrieved from Genbank.

family	subfamily	tribe	species	accessions
Apidae	Xylocopinae	Allodapinii	*Allodape friesei*	DQ149701
Apidae	Xylocopinae	Allodapinii	*Allodape friesei*	DQ149694
Apidae	Xylocopinae	Allodapinii	*Allodape mucronata*	AJ416773
Apidae	Xylocopinae	Allodapinii	*Allodape skaifeorum*	AJ416774
Apidae	Xylocopinae	Allodapinii	*Allodapula acutigera*	AY247264
Apidae	Xylocopinae	Allodapinii	*Allodapula empeyi*	DQ149704
Apidae	Xylocopinae	Allodapinii	*Allodapula empeyi*	DQ149695
Apidae	Xylocopinae	Allodapinii	*Braunsapis albipennis*	AJ416777
Apidae	Xylocopinae	Allodapinii	*Braunsapis bouyssoui*	AJ416778
Apidae	Xylocopinae	Allodapinii	*Braunsapis foveata*	EF190112
Apidae	Xylocopinae	Allodapinii	*Braunsapis leptozonia*	EF190111
Apidae	Xylocopinae	Allodapinii	*Braunsapis otavica*	AJ416780
Apidae	Xylocopinae	Allodapinii	*Braunsapis paradoxa*	AJ416779
Apidae	Xylocopinae	Allodapinii	*Braunsapis protuberans*	AJ416775
Apidae	Xylocopinae	Allodapinii	*Braunsapis sp. Malawi*	DQ160177
Apidae	Xylocopinae	Allodapinii	*Braunsapis sp. NJB-2001*	AJ416782
Apidae	Xylocopinae	Allodapinii	*Braunsapis sp. St-Marie-1*	DQ160175
Apidae	Xylocopinae	Allodapinii	*Braunsapis sp. St-Marie-2*	DQ160176
Apidae	Xylocopinae	Allodapinii	*Braunsapis sp. Taolagnaro*	DQ160174
Apidae	Xylocopinae	Allodapinii	*Braunsapis sp. Toliara*	DQ160173
Apidae	Xylocopinae	Allodapinii	*Braunsapis unicolor*	DQ149703
Apidae	Xylocopinae	Allodapinii	*Braunsapis unicolor*	AJ416776
Apidae	Xylocopinae	Allodapinii	*Braunsapis vitrea*	DQ149702
Apidae	Xylocopinae	Allodapinii	*Braunsapis vitrea*	AJ416781
Apidae	Xylocopinae	Allodapinii	*Brevineura elongata*	DQ149718
Apidae	Xylocopinae	Allodapinii	*Brevineura elongata*	AJ416772
Apidae	Xylocopinae	Allodapinii	*Brevineura froggatti*	AJ416770
Apidae	Xylocopinae	Allodapinii	*Brevineura ploratula*	AJ416769
Apidae	Xylocopinae	Allodapinii	*Brevineura rufitarsis*	AY005276
Apidae	Xylocopinae	Allodapinii	*Brevineura xanthoclypeata*	DQ149717
Apidae	Xylocopinae	Allodapinii	*Brevineura xanthoclypeata*	AJ416771
Apidae	Xylocopinae	Ceratininii	*Ceratina boninensis*	AY250208
Apidae	Xylocopinae	Ceratininii	*Ceratina calcarata*	AY362998
Apidae	Xylocopinae	Ceratininii	*Ceratina calcarata voucher Cedu656*	AY585108
Apidae	Xylocopinae	Ceratininii	*Ceratina dentipes*	AY250209
Apidae	Xylocopinae	Ceratininii	*Ceratina flavipes*	AY250210
Apidae	Xylocopinae	Ceratininii	*Ceratina iwatai*	AY250211
Apidae	Xylocopinae	Ceratininii	*Ceratina japonica*	DQ149700
Apidae	Xylocopinae	Ceratininii	*Ceratina japonica*	AY250212
Apidae	Xylocopinae	Ceratininii	*Ceratina japonica*	AJ416849
Apidae	Xylocopinae	Ceratininii	*Ceratina megastigmata*	AY250213

Apidae	Xylocopinae	Ceratininii	*Ceratina okinawana okinawana*	AY250214
Apidae	Xylocopinae	Ceratininii	*Ceratina okinawana sakinshimensis*	AY250215
Apidae	Xylocopinae	Ceratininii	*Ceratina satoi*	AY250217
Apidae	Xylocopinae	Ceratininii	*Ceratina sp. ALC-2003*	AY250216
Apidae	Xylocopinae	Ceratininii	*Ceratina sp. NJB-2001*	AJ416850
Apidae	Xylocopinae	Ceratininii	*Ceratina sp. NJB-2001*	AJ416848
Apidae	Xylocopinae	Allodapinii	*Compsomelissa borneri*	DQ149719
Apidae	Xylocopinae	Allodapinii	*Compsomelissa borneri*	AJ416784
Apidae	Xylocopinae	Allodapinii	*Exoneura angophorae*	DQ149705
Apidae	Xylocopinae	Allodapinii	*Exoneura angophorae*	AJ416759
Apidae	Xylocopinae	Allodapinii	*Exoneura nigrescens*	DQ149707
Apidae	Xylocopinae	Allodapinii	*Exoneura nigrescens*	AJ416762
Apidae	Xylocopinae	Allodapinii	*Exoneura nigrihirta*	AJ416761
Apidae	Xylocopinae	Allodapinii	*Exoneura robusta*	DQ149706
Apidae	Xylocopinae	Allodapinii	*Exoneura robusta*	AJ416760
Apidae	Xylocopinae	Allodapinii	*Exoneura sp. Adelaide*	DQ149709
Apidae	Xylocopinae	Allodapinii	*Exoneura sp. Tasmania*	DQ149708
Apidae	Xylocopinae	Allodapinii	*Exoneurella eremophila*	DQ149712
Apidae	Xylocopinae	Allodapinii	*Exoneurella eremophila*	DQ149696
Apidae	Xylocopinae	Allodapinii	*Exoneurella eremophilia*	AJ416767
Apidae	Xylocopinae	Allodapinii	*Exoneurella lawsoni*	DQ149713
Apidae	Xylocopinae	Allodapinii	*Exoneurella lawsoni*	AJ416765
Apidae	Xylocopinae	Allodapinii	*Exoneurella setosa*	DQ149711
Apidae	Xylocopinae	Allodapinii	*Exoneurella setosa*	AJ416768
Apidae	Xylocopinae	Allodapinii	*Exoneurella tridentata*	DQ149710
Apidae	Xylocopinae	Allodapinii	*Exoneurella tridentata*	AJ416766
Apidae	Xylocopinae	Allodapinii	*Exoneuridia hakkariensis*	DQ149722
Apidae	Xylocopinae	Allodapinii	*Exoneuridia hakkariensis*	DQ149698
Apidae	Xylocopinae	Allodapinii	*Halterapis minuta*	DQ149720
Apidae	Xylocopinae	Allodapinii	*Halterapis nigrinervis*	AJ416785
Apidae	Xylocopinae	Allodapinii	*Halterapis seyrigi*	DQ149721
Apidae	Xylocopinae	Allodapinii	*Inquilina dawsoni*	AJ416763
Apidae	Xylocopinae	Allodapinii	*Inquilina excavata*	DQ149714
Apidae	Xylocopinae	Allodapinii	*Inquilina excavata*	DQ149697
Apidae	Xylocopinae	Allodapinii	*Inquilina schwarzi*	DQ149715
Apidae	Xylocopinae	Allodapinii	*Inquilina schwarzi*	AJ416764
Apidae	Xylocopinae	Allodapinii	*Inquilina sp. Adelaide*	DQ149716
Apidae	Xylocopinae	Allodapinii	*Macrogalea antanosy*	DQ149724
Apidae	Xylocopinae	Allodapinii	*Macrogalea berentyensis*	EF190109
Apidae	Xylocopinae	Allodapinii	*Macrogalea berentyensis*	EF103601
Apidae	Xylocopinae	Allodapinii	*Macrogalea candida*	AJ416783
Apidae	Xylocopinae	Allodapinii	*Macrogalea ellioti*	EF190107
Apidae	Xylocopinae	Allodapinii	*Macrogalea ellioti isolate 1*	EF103599
Apidae	Xylocopinae	Allodapinii	*Macrogalea ellioti isolate 2*	EF103600
Apidae	Xylocopinae	Allodapinii	*Macrogalea infernalis*	EF103602
Apidae	Xylocopinae	Allodapinii	*Macrogalea magenge*	AY245174
Apidae	Xylocopinae	Allodapinii	*Macrogalea maizina*	EF190108
Apidae	Xylocopinae	Allodapinii	*Macrogalea scaevolae*	EF190110
Apidae	Xylocopinae	Allodapinii	*Macrogalea sp. Malawi*	EF190106
Apidae	Xylocopinae	Allodapinii	*Macrogalea sp. Ramena*	EF103603
Apidae	Xylocopinae	Allodapinii	*Macrogalea zanzibarica*	DQ149723
Apidae	Xylocopinae	Allodapinii	*Macrogalea zanzibarica*	DQ149699
Apidae	Xylocopinae	Allodapinii	*Nasutapis sp. Malawi*	DQ160178
Apidae	Xylocopinae	Allodapinii	*Neoceratina sp. SMT-2005*	DQ149693

Apidae	Xylocopinae	Xylocopinii	*Xylocopa acutipennis*	AY005296
Apidae	Xylocopinae	Xylocopinii	*Xylocopa amamensis*	AY267147
Apidae	Xylocopinae	Xylocopinii	*Xylocopa appendiculata circumvolans*	AY005297
Apidae	Xylocopinae	Xylocopinii	*Xylocopa aruana*	AY005288
Apidae	Xylocopinae	Xylocopinii	*Xylocopa auripennis auripennis*	AY005279
Apidae	Xylocopinae	Xylocopinii	*Xylocopa bombylans*	AY005281
Apidae	Xylocopinae	Xylocopinii	*Xylocopa erythrina*	AY005283
Apidae	Xylocopinae	Xylocopinii	*Xylocopa flavorufa*	AY005293
Apidae	Xylocopinae	Xylocopinii	*Xylocopa frontalis*	AY005302
Apidae	Xylocopinae	Xylocopinii	*Xylocopa gualanensis*	AY005300
Apidae	Xylocopinae	Xylocopinii	*Xylocopa iris*	AY005286
Apidae	Xylocopinae	Xylocopinii	*Xylocopa latipes*	AY005295
Apidae	Xylocopinae	Xylocopinii	*Xylocopa lieftincki*	AY005289
Apidae	Xylocopinae	Xylocopinii	*Xylocopa micans*	AY005277
Apidae	Xylocopinae	Xylocopinii	*Xylocopa micheneri micheneri*	AY005298
Apidae	Xylocopinae	Xylocopinii	*Xylocopa nigrita*	AY005292
Apidae	Xylocopinae	Xylocopinii	*Xylocopa olivieri*	AY005294
Apidae	Xylocopinae	Xylocopinii	*Xylocopa pubescens*	AY005290
Apidae	Xylocopinae	Xylocopinii	*Xylocopa scioensis*	AY005291
Apidae	Xylocopinae	Xylocopinii	*Xylocopa sicheli*	AY005284
Apidae	Xylocopinae	Xylocopinii	*Xylocopa sulcatipes*	AY005287
Apidae	Xylocopinae	Xylocopinii	*Xylocopa tabaniformis tabaniformis*	AY005282
Apidae	Xylocopinae	Xylocopinii	*Xylocopa tranquebarica*	AY005278
Apidae	Xylocopinae	Xylocopinii	*Xylocopa truxali*	AY005301
Apidae	Xylocopinae	Xylocopinii	*Xylocopa varipuncta*	AY005299
Apidae	Xylocopinae	Xylocopinii	*Xylocopa violacea*	AY005280
Apidae	Xylocopinae	Xylocopinii	*Xylocopa virginica*	AY208290
Apidae	Xylocopinae	Xylocopinii	*Xylocopa virginica virginica*	AY005285

Appendix 2.

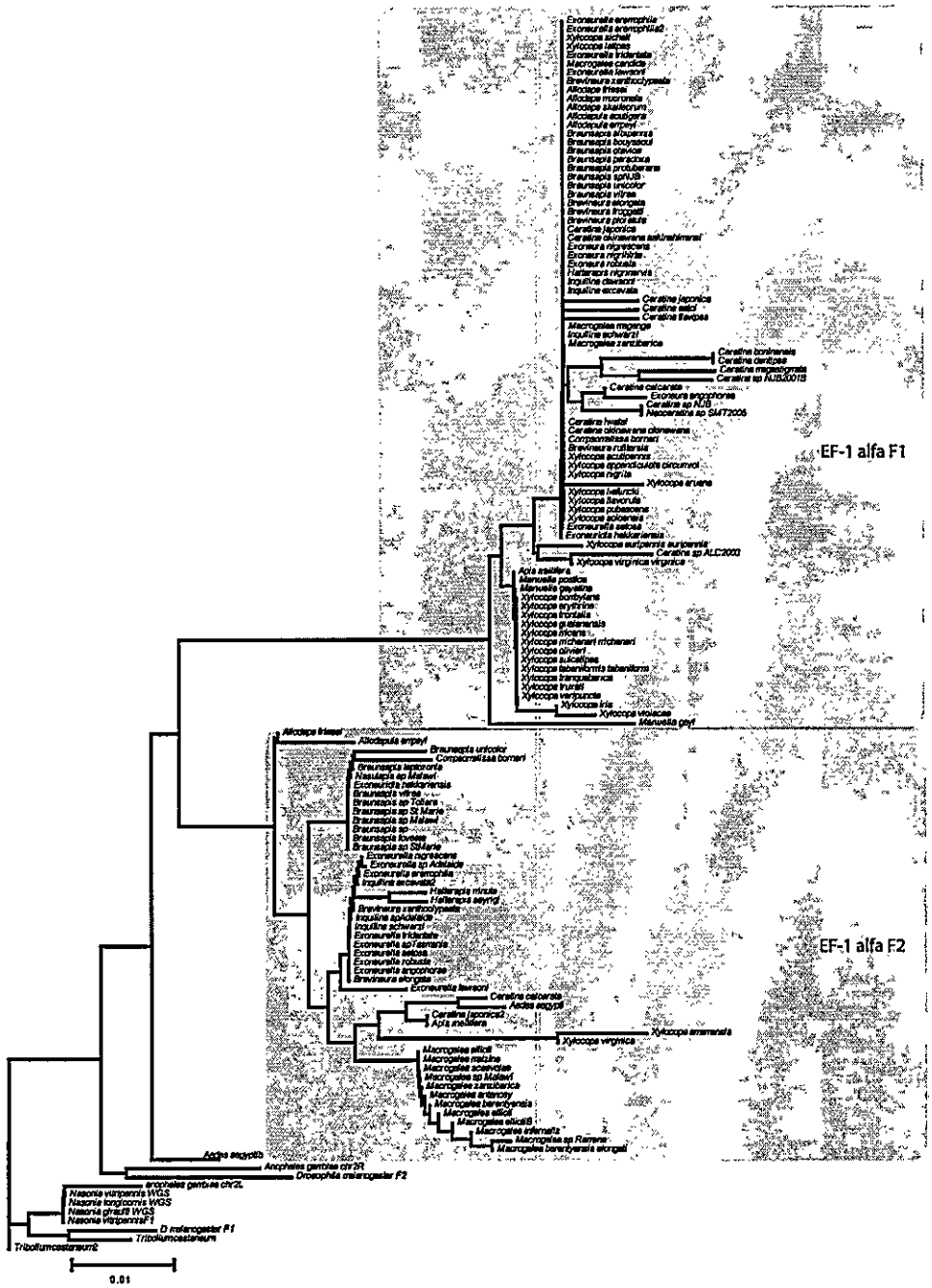

Figure 1. Bayesian phylogeny using EF1-αF1 and EF1-αF2 aminoacid sequences from species of Xylocopinae, and including *Apis mellifera*, *Bombyx mori*, *Drosophila melanogaster*, *Tribolum castaneum* and three species of *Nassonia*.

Appendix 3.

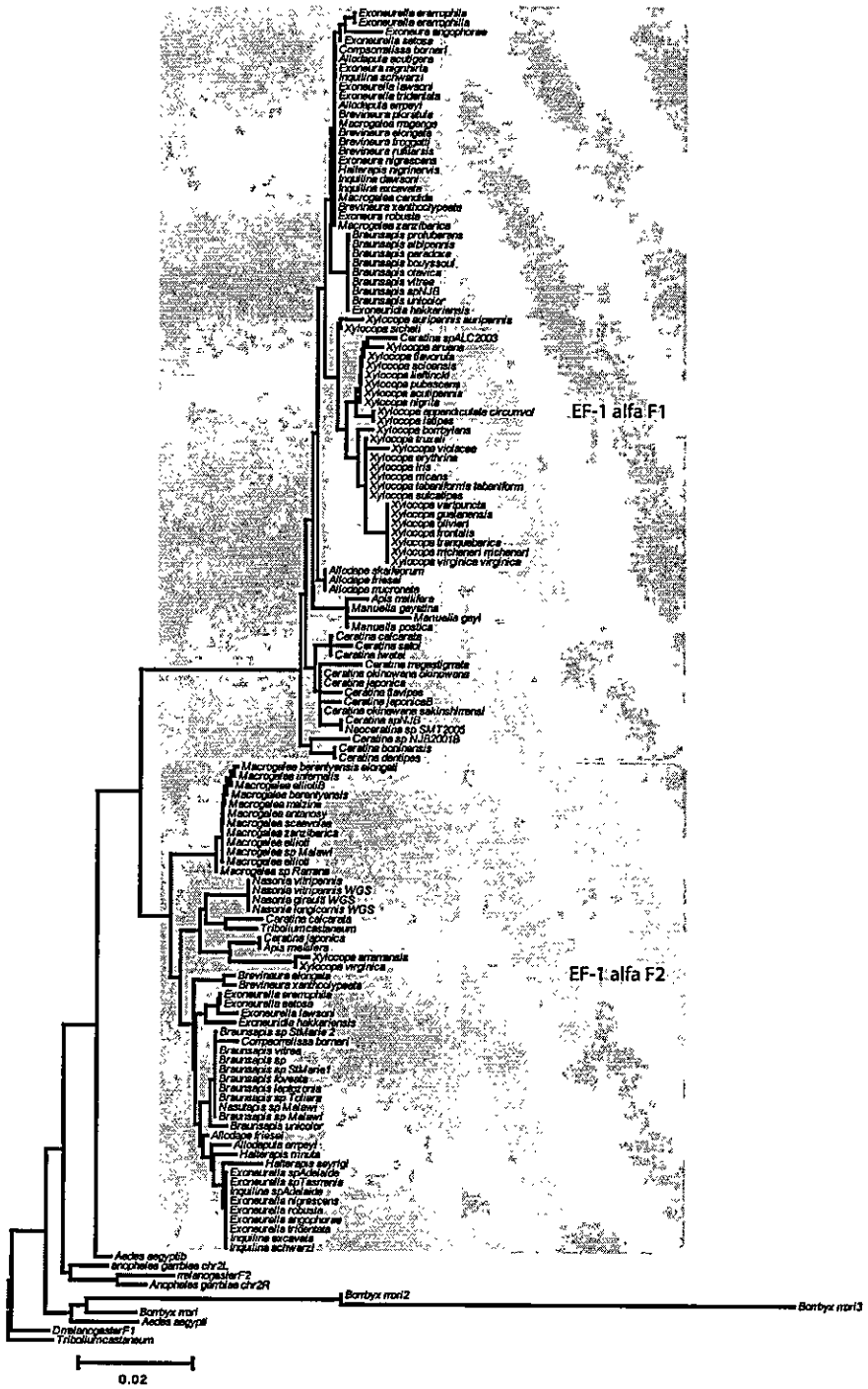

Figure 2. Bayesian phylogeny using EF1-α F1 and EF1-α F2 nucleotide sequences excluding third codon positions from species of Xylocopinae and including *Apis mellifera*, *Bombyx mori*, *Drosophila melanogaster*, *Tribolum castaneum* and three species of *Nassonia*.