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Molecular and morphometric characterization of the genus *Scaptotrigona* (Apidae: Meliponini) in Mesoamerica

Caracterización molecular y morfométrica del género *Scaptotrigona* (Apidae: Meliponini) en Mesoamérica

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That which does not kill us makes us stronger

"*Twilight of the Idols*" by Friedrich Nietzsche

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Resumen

1. ABEJAS SIN AGUIJÓN

Las abejas sin aguijón o meliponinos (Apidae: Meliponini) son un grupo de insectos sociales de la subfamilia Apinae. Esta subfamilia tiene 19 tribus y cuatro de ellas forman el grupo de las abejas corbiculadas, cuya característica común es que tienen una corbícula o cesta de polen en la parte anterior de la tibia de la pata trasera. Estas cuatro tribus son Euglossini (abejas de la orquídeas), Bombini (abejorros), Apini (abejas de la miel) y Meliponini (abejas sin aguijón). Mientras que en la tribu Apini solo hay 11 especies válidas descritas, Meliponini tiene 42 géneros y un elevado número de especies, alrededor de 500 en las áreas tropicales. Dentro de su distribución, el área Neotropical es la que alberga una mayor diversidad y número de especies (alrededor de 400 especies y 33 géneros, Camargo y Pedro, 2007; Michener, 2007).

En comparación con la abeja melífera, la característica diagnóstica de los meliponinos es la ausencia de un aguijón funcional; tanto las obreras como las reinas solo poseen vestigios de él. Además las obreras poseen una estructura pilosa en forma de peine en el extremo más ancho de la tibia, que es conocido como penicillium. La venación de sus alas es mucho más débil que la de otras abejas o incluso inexistente (Wille, 1983). Otra característica importante de estas abejas es la presencia de uñas simples, no bifurcadas y el basitardo carente de aurícula (Quezada-Euán, 2005).

Los meliponinos usan cerumen (una mezcla de cera y resina proveniente de las plantas) como elemento principal en la construcción de sus nidos. Las celdas para el desarrollo de la larva son exclusivas para ese uso (nunca son utilizadas para el almacenamiento de miel o polen a diferencia de lo que ocurre en las colmenas de *Apis mellifera*) y además están en posición vertical. El desarrollo de los meliponinos comprende las mismas etapas que *A. mellifera* aunque es considerablemente más lento (huevo, larva, prepupa, pupa y adulto; Quezada-Euán, 2005).

La formación de las nuevas colonias de las abejas sin aguijón es diferente a la de las abejas melíferas. Mientras que esta última se parte en dos y el nuevo enjambre abandona la colonia vieja, los meliponinos mantienen una gran dependencia de la colonia madre, existiendo un transporte regular de miel, cerumen y cera de la colonia madre a la nueva. El objetivo de esto es crear un depósito de reservas en la colmena recién formada (Kerr, 1950). Este sistema de creación de colonias favorece el establecimiento de nuevas colonias en áreas cercanas a la colonia madre

pero origina endogamia entre las poblaciones (Wille, 1983; Cameron et al., 2004). De hecho, las abejas eusociales son propensas a la endogamia promoviendo de esta forma el llamado "vórtice de extinción" (Zayed y Packer, 2005). Este vórtice es probable que se inicie por la combinación de un tamaño efectivo de la población pequeño (Chapman et al., 2003) y del sistema haplodipoide de determinación sexual. En los himenópteros, el gen de la determinación de sexo complementario (csd) controla el sexo, de forma que las hembras diploides provienen de heterocigotos, los machos haploides de hemicigotos y los machos diploides de homocigotos (Zayed, 2009). La producción de machos diploides tiene un elevado coste genético para las poblaciones ya que estos machos, en su mayoría son estériles (Heimpel y de Boer, 2008) y pueden ocasionar la pérdida de la mitad de los miembros de la colonia, reduciendo la salud de la misma (Carvalho et al., 1995; Green y Oldroyd, 2002). Por lo tanto, cualquier estrategia que incremente las probabilidades de apareamiento con individuos no relacionados genéticamente será beneficiosa para la supervivencia de la colmena. En este sentido, las áreas de congregación de zángano (DCAs por sus siglas en inglés) son un fenómeno común en las abejas sin aguijón (Paxton, 2005). Algunos estudios han mostrado que estas DCAs están formadas por zánganos de entre 20 y 40 colonias en el género *Scaptotrigona* (Paxton, 2000; Kraus et al., 2008) y de hasta 135 en *Trigona collina* (Cameron et al., 2004). Los zánganos evitan las DCAs cercanas, por lo que se incrementa la exogamia, favoreciendo la salud de las poblaciones (Paxton, 2000; Cameron et al., 2004; Kraus et al., 2008; Mueller et al., 2012) y asegurando así el apareamiento de la reina de forma panmíctica (Baudry et al., 1998).

2. LA IMPORTANCIA DE LAS ABEJAS SIN AGUIJÓN

Las abejas sin aguijón son el grupo de abejas más importante en los ecosistemas neotropicales y tienen un papel fundamental como polinizadores generalistas (Roubik, 1989; Martínez-Hernández et al., 1993; Ramalho et al., 1994; Kaminski y Absy, 2006; Michener, 2007). De hecho, su importancia ecológica radica en su especificidad en la polinización de plantas, alrededor del 33 % de las plantas tropicales son exclusivamente polinizadas por estas abejas (Wilms et al., 1996).

Los meliponinos poseen una elevada relevancia económica ya que polinizan eficientemente cultivos tropicales de gran valor económico como el tomate, el chile, el café o el aguacate (Slaa et al., 2006; Quezada-Euán, 2009). Por ello, el uso y manejo de las abejas sin aguijón para la

polinización de cultivos es verdaderamente importante, sobre todo por la gran dependencia que el mundo agrícola tiene de *A. mellifera* (Aizen y Harder, 2009). En este sentido, en muchos lugares y para muchos cultivos la habilidad de la abeja de la miel para polinizar está amenazada o limitada debido a numerosos factores como el síndrome de despoblamiento de las colmenas, la africanización, las enfermedades y parásitos, las limitaciones climáticas y las presiones económicas, (Ormond et al., 1984; vanEngelsdorp et al., 2009), reduciendo su eficacia en algunos cultivos (Ricketts et al., 2004).

Las abejas sin aguijón poseen ventajas en cuanto a su manejo al compararlas con la abeja de la miel: son menos dañinas tanto para el ser humano como para los animales; pueden polinizar eficientemente en los invernaderos (Kakutani et al., 1993). Además la expansión de sus colonias contribuye a la conservación de la diversidad de los meliponinos y la enjambrazón de las colonias es improbable ya que la reina pierde la capacidad de vuelo (Inoue et al., 1984). Pero la meliponicultura (uso y manejo de las abejas sin aguijón) también posee desventajas: las tecnologías para su manejo están, en la mayoría de los casos menos desarrolladas (Jaffé et al., 2015), y tanto la tasa de crecimiento de estas abejas como su producción de miel y cera son menores que en *A. mellifera*.

Además, las abejas sin aguijón tienen gran importancia cultural, ya que fueron las abejas utilizadas en la época precolombina por los nativos en las áreas tropicales de América, especialmente en Mesoamérica, dónde los Mayas hicieron grandes avances para la obtención de su miel y su cera (Crane, 1992; Quezada-Euán et al., 2001). Existe documentación de esa época en numerosas zonas de México como Yucatán, Puebla, Veracruz, o Tabasco (Foster, 1942; Vásquez-Dávila y Solís-Trejo, 1991; González-Acereto, 2008). La meliponicultura siempre ha tenido una gran importancia tanto en la vida social como en la religiosa de los nativos, pero casi desapareció debido a la introducción de la abeja de la miel por parte de los españoles (Schwarz, 1949; Dixon, 1987). Hoy en día, muchas organizaciones están tratando de impulsar y revivir la meliponicultura como parte de la cultura nativa a través de cursos, charlas e innovaciones en las técnicas tradicionales.

3. AMENAZAS

Según numerosos estudios hay evidencias que apuntan a un declive mundial de las abejas (Potts et al., 2010; González-Varo et al., 2013; Goulson et al., 2015). En las últimas cinco décadas, las reservas de *A. mellifera* se ha incrementado en algunas regiones mientras que en otras ha disminuido al igual que las poblaciones de abejas silvestres, como señalan estudios de abejorros (Goulson et al., 2008) y de otras pocas abejas silvestres (Goulson et al., 2015). Por lo tanto, a la vista del declive de las abejas y abejorros en América y en Europa, no es erróneo asumir este declive a nivel mundial (Goulson et al., 2015).

Este declive de la abundancia y riqueza de polinizadores puede afectar drásticamente a las plantas silvestres polinizadas por animales, dando lugar a un descenso de la biodiversidad debido al incremento de la endogamia en las plantas autocompatibles y a la reducción de la producción tanto de semillas como de frutos (Kluser y Peduzzi, 2007). El cambio en la abundancia y composición de los polinizadores también afecta a la estabilidad de los servicios polínicos tanto en cultivos agrícolas como en la flora silvestre (Burkle et al., 2013; González-Varo et al., 2013).

Los principales factores que influyen en este descenso son la pérdida de hábitat (Foley et al., 2005), las prácticas agrícolas y el uso masivo de plaguicidas, las especies invasoras y el cambio climático (Cane, 2001; Cane y Tepedino, 2001; Kremen et al., 2002; NRC, 2007; Dormann et al., 2008; Murray et al., 2009).

La pérdida y fragmentación del hábitat no solo afecta a las abejas, sino a toda la biodiversidad y es también la causa del aislamiento genético de las poblaciones (Ellis et al., 2006; Zayed, 2009). Ricketts et al. (2008) encontraron una relación negativa entre la transformación y la degradación del hábitat y la abundancia de abejas nativas. Esta amenaza es mayor para las abejas sin aguijón ya que necesitan cavidades en troncos de árboles maduros para sus nidos, tienen una reducida capacidad de dispersión y una gran dependencia de la colonia madre (Quezada-Euán et al., 2001; Freitas et al., 2009; Zayed, 2009). La depresión endogámica es un alto riesgo en las poblaciones pequeñas debido al descenso de los heterocigotos que conduce a una reducción de salud de la población (Hedrick, 2000; Frankham, 2005). En estas poblaciones pequeñas, la depresión endogámica es casi inevitable con el paso del tiempo debido a la deriva genética, la pérdida de

diversidad y la reducción de la heterocigosidad. Esto conlleva una disminución en la respuesta evolutiva a cambios en el entorno tales como la aparición de nuevos parásitos, el cambio climático o la introducción de especies (Gaggiotti, 2003). Todo esto afecta a los meliponinos en poblaciones reducidas y se ha observado en distintas especies como *Melipona beecheii* (Quezada-Euán et al., 2007), *M. rufiventris* (Tavares et al., 2007) *M. scutellaris* (Carvalho-Zilse et al., 2009), *Scaptotrigona hellwegeri* (Quezada-Euán et al., 2012) y *S. xanthotricha* (Duarte et al., 2014).

Una de las principales causas de la fragmentación del hábitat es la expansión de la agricultura (Kremen et al., 2002; Silveira, 2004). Algunos estudios han demostrado que las áreas sin cultivos agrícolas poseen una mayor densidad de nidos de abejas sin aguijón (Meléndez et al., 2004; Santos-Leal, 2006). La agricultura conlleva el uso de plaguicidas, y esto se magnifica en Latinoamérica, donde la agricultura está sustentada en gran medida por el uso de estas substancias químicas (Tansey et al., 1995; Pinheiro y Freitas, 2010). Los efectos subletales de los agroquímicos, concretamente de los insecticidas, pueden ser más dañinos que los efectos letales sobre las abejas ya que pueden dañar su capacidades sensitivas y neuromotoras (Pinheiro y Freitas, 2010). Además, si la exposición a estos productos ocurre durante la fase larvaria, puede acarrear problemas en la movilidad de los adultos y en el desarrollo del cerebro (Tomé et al., 2012).

La introducción de especies invasoras puede ser una gran amenaza para las poblaciones nativas de abejas (Stout y Morales, 2009). Uno de los mayores ejemplos es la introducción de *A. mellifera* en América. Es un gran riesgo para las poblaciones nativas debido a la competencia por los recursos, por los lugares de anidación y por la introducción de nuevos patógenos (Roubik, 1989; Freitas et al., 2007).

Así como la pérdida del hábitat, el cambio climático también es una amenaza para la biodiversidad del planeta. Concretamente para las abejas, puede suponer un cambio en la fenología entre las abejas y las plantas polinizadas por estas (Willmer, 2012). El calentamiento global puede afectar también a la composición de las comunidades de polinizadores (Memmott et al., 2007). Otro de los posibles efectos en las abejas ocasionados por el cambio climático, y que ya se ha observado en algunas especies de mariposas (Forister et al., 2010), es un desajuste espacial y temporal entre las flores y las abejas (Schweiger et al., 2008). Esto se espera que suceda sobre

todo en el extremo sur de la distribución de las especies (Goulson et al., 2015). Además de los cambios en la meteorología, los eventos extremos como inundaciones, sequías o las tormentas también puede tener un gran impacto sobre las poblaciones de las abejas, siendo perjudiciales para la mayoría de las colonias (Goulson et al., 2015).

Pero todos estos factores no actúan de manera individual, sino que lo hacen al mismo tiempo, lo que conlleva una sinergia de los efectos sobre las poblaciones de las abejas (Tylianakis et al., 2008). Las diferentes amenazas deben considerarse al mismo tiempo para tratar de entender como las poblaciones de las abejas responden ante esos factores (Potts et al., 2010). Por ejemplo, la exposición a los plaguicidas aumenta la vulnerabilidad de las abejas facilitando la infección de patógenos como *Nosema* (Pettis et al., 2012; Wu et al., 2012). Además, la tasa de infección por parásitos es mayor en las poblaciones que se alimentan de cultivos intensivos debido a que su dieta es más pobre (Alaux et al., 2010). Debido a las complejas interacciones de las distintas amenazas, es de vital importancia ahondar en el conocimiento de la biología y del estado actual de la biodiversidad de las comunidades de abejas nativas (González-Varo et al., 2013).

Esta problemática se acentúa debido a la falta de conocimiento sobre el estado de las especies de abejas sin aguijón. Este grupo de abejas es la tribu más diversa, tanto en comportamiento como en morfología de todas las abejas corbiculadas eusociales (Apini, Bombini y Meliponini) (Michener, 2007). En esta tribu hay cientos de especies y hoy en día, una estimación precisa del número real de especies parece casi imposible debido a la existencia de especies crípticas (Michener, 2007). Aunque varios autores han tratado de realizar un análisis riguroso de algunos de los géneros como *Paratrigona, Aparatrigona* y *Geotrigona* (Camargo y Moure, 1994; 1996) o *Partamona* (Pedro y Camargo, 2003), en la mayoría de los géneros aún existe una importante falta de información. La clasificación filogenética de las abejas sin aguijón se ha realizado con diferentes métodos y por distintos investigadores: Michener (1944) y Schwarz (1948) solo reconocían dos géneros principales, *Melipona* y *Trigona*. Algunos años más tarde Moure (1961), admitía 23 géneros (sin contar subgéneros) del Nuevo Mundo y 10 del Viejo Mundo. El mismo autor reconocía 27 taxones supraespecíficos (géneros y subgéneros) del Nuevo Mundo y posteriormente, en 1971 cambió algunos de los subgéneros al nivel de género (Michener, 2007). Camargo y Pedro (1992) y específicamente Camargo (1989) expusieron un exhaustivo resumen de esas clasificaciones. Sakagami (1982) también presentó un sistema de clasificación diferente. En

este trabajo hemos tratado de arrojar un poco de luz sobre la situación actual de las especies de *Scaptotrigona* Moure 1942 distribuidas en México y el norte de Guatemala.

4. EL GÉNERO *SCAPTOTRIGONA* EN MÉXICO

Este género tiene alrededor de 24 especies distribuidas desde México hasta el norte de Argentina (Michener, 2007). Las colonias de *Scaptotrigona* son de las más abundantes de las abejas sin aguijón en los ecosistemas Neotropicales (Cortopassi-Laurino et al., 2006).

Morfológicamente, es uno de los géneros más robustos dentro de los meliponinos, con un tamaño de cuerpo que varía entre los 4,5 y los 7 mm. Se caracterizan por tener tanto el margen posterior del escutelos como el margen anterior del lóbulo pronotal redondeado (Michener, 2007). Tanto el tórax como la cabeza son altamente punteados. Como la mayoría de las abejas sin aguijón, las del género *Scaptotrigona*, también construyen sus nidos en el interior de troncos, sobre todo en troncos de grandes árboles maduros.

En esta tesis nos hemos centrado en las tres especies del género *Scaptotrigona* presentes en México: *Scaptotrigona hellwegeri* Friese 1900, *S. mexicana* Guérin 1845 y *S. pectoralis* Dalla Torre 1896 cuya distribución se sitúa en México y América Central.

S. hellwegeri es la única de las tres especies que es endémica de México y se ha registrado desde el nivel del mar hasta los 1500 m. Se distribuye a lo largo de la costa del Pacífico y la Sierra Madre Occidental, desde Sinaloa hasta el estado de Oaxaca.

S. mexicana se distribuye a lo largo de la costa Atlántica en el Golfo de México y en la Sierra Madre Oriental, desde Tamaulipas hasta Veracruz. Además también se distribuye a lo largo del eje Neovolcánico, por la Sierra Madre del Sur y por Sierra de Chiapas. Se ha encontrado desde el nivel del mar hasta los 1000 m. Aunque su mayor distribución es en México, esta abeja también se ha encontrado en Belice, Guatemala, Costa Rica y El Salvador.

La tercera *Scaptotrigona* de México, *S. pectoralis* tiene una distribución solapada con *S. mexicana* a lo largo de Centroamérica pero también se ha encontrado en Honduras, Nicaragua y Panamá. En México tiene una distribución parcialmente solapada con *S. mexicana*, pero solo llega hasta el sur de la Sierra Madre Oriental en el estado de Veracruz y está presente en la península de Yucatán (donde *S. mexicana* está ausente). Se ha encontrado desde el nivel del mar hasta los 1200 m. (Ayala, 1999).

S. hellwegeri y *S. mexicana* son morfológicamente más similares, claramente diferenciadas de *S. pectoralis* por la ausencia de una fuerte muesca en la carena occipital. Por otra parte, *S. hellwegeri* y *S. pectoralis* comparten un patrón de coloración naranja y negro, aunque la primera de éstas posee el escuto naranja y el escutelo negro mientras que *S. pectoralis* muestra el patrón de color contrario. *S. mexicana* es la única de las tres que tiene el integumento completamente negro. Estas tres especies tienen un tamaño medio en relación a su género: *S. hellwegeri* (4,7 a 5,1 mm.), *S. mexicana* (5 a 5,3 mm.) y *S. pectoralis* (5,2 a 5,5 mm.). Tanto las obreras como los zánganos tiene un tamaño similar, mientras que la reina es más grande incluso recién emergida de la celda (Ayala, 1999).

4.1. Biología

Las colonias de *S. hellwegeri* no tienen estructuras en la entrada de su nido, sino un gran agujero donde se posicionan varias obreras (González-Acereto, 2008). Por otra parte, *S. mexicana* y *S. pectoralis* construyen sus nidos con una estructura tubular hecha de cerumen donde varias obreras se sitúan como defensa. Esta estructura en forma de trompeta es mayor en *S. mexicana* que en *S. pectoralis*. Estas abejas defienden sus nidos mordiendo a los intrusos y liberando feromonas, siendo *S. mexicana* la menos agresiva de las tres.

S. mexicana es una de las abejas sin aguijón más estudiadas en México debido a su producción de miel y a su fácil manejo. Esta abeja ha sido usada desde tiempos prehispanicos por los nativos en Centro América para curar enfermedades gástricas, respiratorias y de otros tipos mediante el uso de su miel (aunque también de su polen y su propóleo). Varios estudios recientes han probado la efectividad de la miel de *Scaptotrigona* como antimicrobiano natural, mostrando incluso mayor

actividad antibacteriana que las mieles de otras abejas (Enríquez y Dardón, 2006; Catzín Ventura et al., 2009; Rodríguez-Malaver et al., 2009).

4.2. Manejo

La meliponicultura ha sido una práctica habitual en Mesoamérica, y concretamente en México desde la era precolombina. Desde la introducción de la abeja melífera esta práctica ha sufrido un considerable abandono, sin embargo hoy en día existen muchas iniciativas de rescate de la actividad (González-Acereto, 2008). Algunas de las especies de abejas sin aguijón son utilizadas para esta práctica en México, y entre ellas las del género *Scaptotrigona* son consideradas importantes recursos en Puebla, Veracruz y Guerrero, sobre todo por la tribus nativas (González y De Araújo, 2005). *S. mexicana* se usa para la meliponicultura en Chiapas, Veracruz, Puebla, Guerrero y Sinaloa (Manzo, 2009). En lugar de estar en una colmena de madera como *A. mellifera*, *S. mexicana* se explota en unas urnas de barro que hacen la función de colmenas. El manejo tradicional de esta especie, ha situado la localidad de Cuetzalan del Progreso, en el estado de Puebla a la cabeza de la producción de miel de abejas sin aguijón (Guzmán et al., 2011). Además, el uso de *S. hellwegeri* para la meliponicultura está empezando ya que su nivel de producción de miel es aceptable y la calidad de la misma es excelente. Su manejo se produce principalmente en Guerrero, Jalisco y Michoacán (Ayala et al., 2013). *S. pectoralis* también se utiliza en algunas zonas como Yucatán para la producción de miel (González-Acereto et al., 2006; González-Acereto, 2012).

Tanto *S. pectoralis* como *S. mexicana* tienen una gran eficiencia en la polinización de cultivos con elevada importancia económica como el café, el aguacate y el rambután (Ish-Am et al., 1999; Camposeco, 2002; Guzmán, 2002)

4.3. Taxonomía de *Scaptotrigona*

Las especies de *Scaptotrigona* fueron descritas inicialmente por Latraille (1807), considerándolas dentro del género *Trigona.* No fue hasta 1942 cuando Moure definió el género como es en la actualidad (Michener, 2007). Recientemente, varios estudios sobre la filogenia de los meliponinos del Viejo y del Nuevo Mundo han incluido al género *Scaptotrigona*. Rasmussen y Cameron (2007) usaron cuatro marcadores, uno mitocondrial (16S) y tres genes nucleares (opsina, Argk y EF1-α)

para establecer las relaciones filogenéticas de esta tribu. El análisis bayesiano de los cuatro genes resolvió que *Scaptotrigona* era cercana a *Geotrigona, Cephalotrigona* y *Trigona* pero con poco soporte. Por otra parte, en el mismo estudio, la filogenia basada en máxima parsimonia (MP) estableció que *Scaptotrigona* estaba más relacionada con *Paratrigona*, *Partamona* y *Parapartamona*. En un estudio posterior, los mismo autores (Rasmussen y Cameron, 2010) añadieron más muestras y otro marcador nuclear (28S) para esclarecer la filogenia. En esta ocasión, los datos situaron a *Scaptotrigona* como clado hermano de *Oxytrigona* con elevado soporte en el análisis bayesiano. Ramírez et al. (2010) centraron su filogenia en el género *Melipona,* pero analizaron muestras de géneros cercanos. En dicho estudio usando dos marcadores mitocondriales (16S y cox1) y tres nucleares (ArgK, EF1-α y Pol II), *Scaptotrigona* se agrupó con *Trigona* apareciendo *Geotrigona* y *Cephalotrigona* como clados hermanos.

También ha habido problemas taxonómicos en las especies mexicanas de *Scaptotrigona.* Con relación a *S. hellwegeri,* Quezada-Euán et al. (2012) mostraron la existencia de diferencias genéticas y morfométricas significativas entre algunas poblaciones de esta especie revelando la existencia de al menos dos linajes genéticos.

En cuanto a *S. mexicana*, se ha relacionado con *S. luteipennis* ya que ambas son totalmente negras. *S. mexicana* es una especie sinónima con *S. pachysoma* (Ayala, 1999). Ayala (1999) destacó la posible existencia de dos especies diferentes en *S. mexicana*, una de ellas en el centro del país (Ixtapan-México) y la otra en la costa del Pacífico (Zihuatanejo-Guerrero). Las muestras de la costa fueron descritas por Moure como especies distintas. Además Schwarz (1951) también señaló la existencia de dos subespecies en *S. mexicana*, una en México y Guatemala y la otra en Centroamérica (Panamá). Esta última era *S. mexicana suboscuripennis*, que posteriormente se elevó a categoría de especie como *S. suboscuripennis* (Camargo y Pedro, 2013).

S. pectoralis fue dividida en un principio en cuatro subespecies por Schwarz (1951). Dos de ellas estaban localizadas en México, una en el Suroeste y la otra en Yucatán, pero Ayala (1999) consideró ambas subespecies mexicanas como una sola especie diferente de las otras subespecies, *S. pectoralis barrocoloradesis* (Ecuador y Panamá) y *S. pectoralis panamensis* (Costa Rica y Panamá), que fueron posteriormente consideradas como dos especies distintas. Además, esta especie parece que está filogenéticamente más relacionada con *S. luteipennis* que con cualquier

otra especie de México debido a la presencia de la muesca en la carena occipital (Ayala, 1999). *S. luteipennis* se distribuye por América Central (Costa Rica, El Salvador y Panamá y también en el sur de México, Chiapas, Camargo y Pedro, 2013), aunque en la revisión de los meliponinos mexicanos de Ayala (1999) no aparece citada en México.

Debido a todo lo expuesto anteriormente, creemos que una investigación más profunda es necesaria para tratar de esclarecer el estado taxonómico de las especies de *Scaptotrigona*.

5. HERRAMIENTAS Y METODOLOGÍAS PARA EL ESTUDIO DE LA BIODIVERSIDAD

La identificación y el descubrimiento de especies se han basado tradicionalmente en un enfoque morfológico. Sin embargo, el aumento de la pérdida de biodiversidad es un gran desafío para los investigadores y requiere de acciones prontas para la descripción de la diversidad antes de su desaparición (Blaxter, 2004). En las últimas décadas se han desarrollado un gran número de técnicas rápidas y económicas para caracterizar la biodiversidad, destacando el gran auge de las técnicas moleculares.

Los método moleculares, y el código de barras de ADN (DNA barcoding) en particular, se han aplicado mediante dos enfoques distintos que difieren en el tipo y en la cantidad de datos requeridos. El primero es la identificación de especies (o diagnóstico de especies) en el cual los datos de ADN de una muestra sin identificar se comparan con las bases de datos moleculares de individuos identificados (librería de barcoding). El segundo enfoque (delimitación de especies) consiste en el uso de los datos de ADN para descubrir nuevas especies determinando los límites de las mismas.

5.1. Marcadores moleculares y morfológicos

5.1.1. El código de barras genético

El código de barras de ADN utiliza un segmento estandarizado de 658 pares de bases (pb) del gen mitocondrial citocromo oxidasa I (cox1) (Hebert et al., 2003b, 2004a, b) para identificar la biota global de manera satisfactoria (Waugh, 2007). La habilidad del código de barras de ADN para identificar especies radica en las bajas tasas de variación intraespecífica y en las relativamente altas tasas de variación interespecífica (Packer et al., 2008), ya que la secuencia de cox1 se mantiene moderadamente constante entre las poblaciones de una especie pero difiere entre especies próximas, incluso aunque hayan divergido recientemente (Hebert et al., 2003a). Esta técnica se basa en el reconocimiento de especies a través de los valores de distancia genética en relación a los grupos cercanos (Vernooy et al., 2010). El código de barras genético también proporciona la oportunidad de evaluar y entender la diversidad en los grupos en los que las técnicas de taxonomía tradicional presentan algunas dificultades (Köhler, 2007), pudiendo establecer unidades moleculares taxonómicas operativas o MOTUs (Blaxter, 2004). Cabe señalar que varios estudios han discutido que sólo el uso del cox1 no es suficiente para la delimitación de especies de manera precisa debido a factores como la introgresión, la retención de polimorfismos ancestrales, diferencias en flujo génico entre machos y hembras o la elevada variabilidad intraespecífica (Moritz y Cicero, 2004; Will et al., 2005; Dupuis et al., 2012).

En abejas, el código de barras de ADN se ha usado satisfactoriamente para el reconocimiento de taxones no descritos mediante la taxonomía tradicional y para el descubrimiento de especies crípticas (Sheffield et al., 2009; Rehan y Sheffield, 2011), así como para la asociación de castas en especies con dimorfismo entre la reina y las obreras y para aquellas con dimorfismo sexual entre machos y hembras de una especie (Packer et al., 2008). Concretamente para los meliponinos, ha puesto de manifiesto la existencia de un complejo de especies en *Melipona yucatanica* en México permitiendo establecer estrategias de conservación acordes a su historia evolutiva (May-Itzá et al., 2010). De igual forma, ha permitido demostrar la existencia de especies crípticas en *Liotrigona bitika* (Koch, 2010).

5.1.2. Microsatélites

El uso de marcadores moleculares altamente variables como los microsatélites es una forma muy efectiva de estimar parámetros poblacionales en especies amenazadas (Frankham et al., 2002; Hedrick, 2004). Las regiones flanqueantes de los microsatélites se conservan incluso en géneros distintos (Ferreira y Grattapaglia, 1998), por lo que los marcadores diseñados para una especie pueden utilizarse en otra, como los primers diseñados para *Bombus* o *Melipona* pueden usarse en especies de *Scaptotrigona*. Los microsatélites son codominantes y altamente polimórficos (Moritz y Hillis, 1996), por lo que tienen una gran potencia estadística para determinar parámetros poblacionales como la variación inter e intraespecífica, la estructura poblacional o los cambios en el tamaño de la población (Frankham et al., 2008).

En abejas sin aguijón han sido ampliamente utilizados desde el primer análisis para desarrollar marcadores de microsatélite de los genomas de *Melipona bicolor* (Peters et al., 1998); *S. postica* (Paxton et al., 1999) y *Trigona carbonaria* (Green et al., 2001). Estos marcadores se han usado exitosamente en los meliponinos para caracterizar la variabilidad genética (Arias et al., 2006; Francisco et al., 2006; Fernandes et al., 2012) y otros aspectos de la biología de estas abejas (Kraus et al., 2008; Mueller et al., 2012).

5.1.3. Morfometría geométrica

Este es un método rápido y económico basado en la variación de la forma de las alas. Numerosos estudios han demostrado que la morfometría geométrica de las alas es una herramienta fiable y efectiva para resolver incógnitas taxonómicas. Los resultados de la morfometría geométrica normalmente son coherentes con los resultados de los marcadores moleculares.

En la última década, la morfometría geométrica de las alas ha demostrado ser una buena técnica para resolver las diferencias entre las especies y para la identificación de abejas (abejas de la miel: Bouga et al., 2011; da Silva et al., 2015; abejorros: Barkan y Aytekin, 2013; entre otros). En abejas sin aguijón, este método ha mostrado una elevada sensibilidad para el descubrimiento de especies crípticas (Francisco et al., 2008), la identificación de subespecies (Francoy et al., 2008; Tofilsky, 2008) y la variabilidad dentro de las poblaciones (Mendes et al., 2007; Ferreira et al.,

2011; Francoy et al., 2011; Lima Junior et al., 2012; Nunes et al., 2013; Bonatti et al., 2014). Sin embargo para resolver problemas taxonómicos, la información genética debe ser incorporada para incrementar el rigor en la delimitación de especies tal y como se propone en el enfoque de la taxonomía integrativa (Schlick-Steiner et al., 2010).

5.2. Metodologías

5.2.1. Delimitación de especies

Determinar que un individuo pertenece a una especie puede ser una tarea problemática y subjetiva. Esta problemática se agrava cuando sólo se usa un gen ya que pueden existir diferencias entre la historia de la especie (árbol de especies) y los resultados obtenidos de la filogenia de un gen (árbol del gen) (Papadopoulou et al., 2008; Monaghan et al., 2009; Fujisawa y Barraclough, 2013). Estas diferencias son incluso mayores cuando no se comprueba exhaustivamente la eficacia de ese único marcador (Dupuis et al., 2012). Por lo tanto, existe un consenso para el uso de varios genes independiente (mitocondriales y nucleares) para el correcto establecimiento de relaciones filogenética y la delimitación de especies. Los métodos que asignan las muestras objetivamente basándose en un criterio definido pueden ser la solución al problema (Payo et al., 2013). Un ejemplo son los métodos basados en la coalescencia, que permiten testar hipótesis alternativas sobre la divergencia de un linaje reduciendo de esta forma los sesgos introducidos por el investigador que poseen un carácter subjetivo, como son el grado de diferenciación ecológica, morfológica o genética. Por ello, los métodos multilocus basados en coalescencia son replicables y deberían dar el mismo resultado al ser llevados a cabo por distintos investigadores (Fujita et al., 2012).

Estos métodos se han convertido en una herramienta fiable para delimitar y descubrir nuevas especies y ayudar a establecer medidas de conservación correctas que aseguren la supervivencia de dichas especies (Leaché y Fujita, 2010). Pese a que esta técnica está comenzando, ya existen numerosos ejemplos de su aplicación exitosa para resolver complejos de especies crípticas en peces (Niemiller et al., 2012; Bagley et al., 2015), arañas (Satler et al., 2013), ranas (Setiadi et al., 2011), salamanquesas (Blair et al., 2015), lagartos (Barley et al., 2013), pájaros (Zamudio-Beltrán y Hernández-Baños, 2015) o serpientes (Myers et al., 2013).

5.2.2. Taxonomía integrativa

El uso de múltiples disciplinas independientes (como datos moleculares, morfológicos, de comportamiento y/o ecológicos) es una herramienta fiable para resolver problemas taxonómicos (Padial et al., 2010; Andújar et al., 2014). La taxonomía integrativa se basa en la congruencia de diferentes disciplinas las cuales por separado pueden no reflejar de manera adecuada las relaciones y los límites de especies (Schlick-Steiner et al., 2010). Este enfoque necesita al menos dos disciplinas y por consenso se establece que una de ellas debe provenir de datos morfológicos y la otra de datos moleculares (Gibbs, 2009; Padial et al., 2010; Schlick-Steiner et al., 2010; Chesters et al., 2012).

6. OBJETIVOS E HIPÓTESIS

El principal objetivo de esta tesis es caracterizar el estado taxonómico de las poblaciones de Mesoamérica (principalmente de México y del norte de Guatemala) de las especies del género *Scaptotrigona* (*S. hellwegeri, S. mexicana* y *S. pectoralis*) y establecer una premisa fiable para el futuro establecimiento de medidas de manejo y de conservación. Para alcanzar esas metas, hemos establecido unos objetivos concretos en cada capítulo basados en diferentes hipótesis.

Capítulo 1. Código de barras genético en abejas sin aguijón: diversidad genética en el género *Scaptotrigona* de importancia económica en Mesoamérica.

El objetivo principal de este capítulo es establecer una primera aproximación a la diversidad de las tres especies.

Los objetivos específicos son (i) probar la eficacia de la técnica del código de barras genético en la identificación de las especies de *Scaptotrigona* descritas en México, y (ii) su precisión para asignar individuos clasificados morfológicamente a especies definidas por barcoding. (iii) Además queremos evaluar la variación genética intra e interespecífica de cada especie y (iv) descubrir la posible existencia de especies crípticas como se ha señalado en estudios previos (Quezada-Euán et al., 2012).

Las hipótesis de este capítulo sostienen (1) que la técnica del código de barras de ADN diferenciará al menos las especies ya establecidas mediante la taxonomía clásica y (2) que según la taxonomía (Ayala, 1999) *S. mexicana* está más relacionada con *S. hellwegeri* que con *S. pectoralis*.

Capítulo 2. Revelando la biodiversidad de las abejas sin aguijón de Mesoamérica: morfometría geométrica y análisis de microsatélites de *Scaptotrigona mexicana* y *S. pectoralis* (Apidae: Meliponini)

El principal objetivo de este capítulo es confirmar la existencia de diferentes taxones crípticos dentro de la especie *S. mexicana* tanto de la costa atlántica como de la pacífica en México, y detectar si existen diferencias genéticas en *S. pectoralis*, ya que esta especie tiene una distribución casi solapada en México con *S. mexicana*.

Los objetivos específicos son (i) usar la morfometría geométrica y los microsatélites para comprobar la estructura poblacional y la diversidad molecular de *S. mexicana* y *S. pectoralis*, (ii) probar la existencia de al menos dos especies dentro de *S. mexicana*, y (iii) en vista de la diversidad observada en este género, investigar el estado taxonómico de *S. pectoralis.*

Las hipótesis de partida son (1) que *S. mexicana* es un complejo de especies crípticas y (2) que los factores geográficos y ecológicos influyen en las poblaciones de *S. pectoralis* por lo que esta especie presenta una marcada estructura filogeográfica.

Capítulo 3. La delimitación multilocus de especies en abejas sin aguijón mesoamericanas apoya la existencia de especies crípticas en el género *Scaptotrigona* (Apidae: Meliponini)

El principal objetivo de este capítulo es definir de manera precisa el estado taxonómico de las tres especies de *Scaptotrigona* distribuidas en México.

Los objetivos específicos son (i) descubrir cuantas especies hay en *S. mexicana* y *S. hellwegeri* y concretar el estado de *S. pectoralis* y (ii) usar técnicas que reduzcan el criterio subjetivo del investigador para establecer programas de conservación adecuados.

Las hipótesis de partida son (1) que existen especies crípticas en *S. mexicana* y *S. hellwegeri* y (2) que por el contrario, *S. pectoralis* es una sola especie.

7. RESÚMENES

Capítulo 1. Código de barras genético en abejas sin aguijón: diversidad genética en el género *Scaptotrigona* de importancia económica en Mesoamérica.

Las abejas sin aguijón del género *Scaptotrigona* están ampliamente distribuidas a lo largo del área tropical de México e incluyen especies de importancia económica debido a su uso en la meliponicultura. Ya que las colmenas de *Scaptotrigona* están siendo actualmente intercambiadas entre regiones, o al menos existe un elevado potencial de que esto suceda, es importante analizar la extensa diversidad genética de las diferentes poblaciones. La técnica del código de barras genético (DNA barcoding) se ha utilizado con éxito para caracterizar e identificar la biodiversidad de manera rápida y fiable. En este capítulo, todos los individuos de *Scaptotrigona* analizados, fueron correctamente asignados mediante la técnica del código de barras genético a una de las tres especies reconocidas por la taxonomía tradicional (*Scaptotrigona mexicana*, *S. pectoralis* y *S. hellwegeri*). La divergencia intraespecífica mostró un valor medio de 0.70 %, mientras que el valor de la interespecífica fue de 2.79 %. Tal y como establece la taxonomía tradicional, el análisis de las secuencias demostró que *S. mexicana* es genéticamente más cercana a *S. hellwegeri* que a *S. pectoralis*. Los valores de divergencia de las muestras de *S. mexicana* superaron el valor del límite de la variación intraespecífica, lo que sugiere la existencia de especies crípticas en la misma. *S. mexicana* es una de las especies de abejas sin aguijón más explotadas para la producción de miel en Mesoamérica. Estos resultados confirman la hipótesis de que la técnica del código de barras de ADN puede como mínimo diferenciar taxones de abejas sin aguijón aceptados por la taxonomía actual y apuntan a la existencia de diferentes especies dentro de *S. mexicana*.

Capítulo 2. Revelando la biodiversidad de las abejas sin aguijón de Mesoamérica: morfometría geométrica y análisis de microsatélites de *Scaptotrigona mexicana* y S. pectoralis (Apidae: Meliponini)

La morfometría geométrica y los métodos moleculares proporcionan herramientas efectivas para el estudio de la variabilidad de las poblaciones de abejas sin aguijón. Estas poblaciones deben ser protegidas debido al declive mundial de las comunidades de abejas. Basándonos en evidencias previas de la alta diversidad subyacente en las especies de *Scaptotrigona*, en este capítulo, analizamos dos especies, *S. mexicana* y *S. pectoralis,* con ambos métodos para comprobar la existencia de, al menos, dos especies en *S. mexicana*, y para investigar el estado de la especie *S. pectoralis*. Para alcanzar dichos objetivos, se ha medido la variación morfológica de las alas mediante puntos (landmarks) en las intersecciones de las venas alares y se han genotipado siete loci de microsatélite polimórficos en abejas de ambas especies. La morfometría geométrica de la venación alar mostró diferencias entre las poblaciones de *S. mexicana* de la costa del Pacífico (*Sm1*) y las del Atlántico (*Sm2*) y en cambio no se observó diferenciación en las poblaciones de *S. pectoralis*. Los análisis de microsatélites, confirmaron este resultado e incluso mostraron una mayor diferenciación entre las poblaciones al incrementar la distancia geográfica (test de Mantel) en *Sm2* y en *S. pectoralis.* Estos resultados revelan una diferenciación de dos unidades evolutivas en *S. mexicana* y la distribución de la diversidad genética en las especies de *Scaptotrigona*, lo que sugiere la necesidad de una revisión taxonómica, así como el desarrollo de actividades de manejo y conservación para preservar dicha diversidad.

Capítulo 3. La delimitación multilocus de especies en abejas sin aguijón mesoamericanas apoya la existencia de especies crípticas en el género *Scaptotrigona* (Apidae: Meliponini)

Para proteger la biodiversidad de forma adecuada es necesario tener un conocimiento preciso de las especies y de su distribución. Esto es incluso más importante en áreas de elevada diversidad como Mesoamérica. En este contexto, es fundamental la delimitación de especies de una forma objetiva, reduciendo el sesgo causado por los investigadores, para poder establecer las medidas de conservación y manejo adecuadas. Estudios morfológicos y moleculares previos en tres especies de abejas sin aguijón del género *Scaptotrigona* distribuidas en México (*S. mexicana*, *S. pectoralis* y *S. hellwegeri*) sugieren que tanto *S. mexicana* como *S. hellwegeri* son complejos de especies crípticas. En este capítulo se ha testado la delimitación de especies mediante el análisis de secuencias de cinco marcadores moleculares (dos mitocondriales: cox1 y 16S, y tres nucleares: ITS1, EF1-α, ArgK) dentro de un enfoque de coalescencia bayesiano para confirmar el soporte de las especies putativas. Se obtuvieron dos hipótesis diferentes de cuatro (cox1) y seis (16S) especies, usando el modelo mixto generalizado de coalescencia de Yule (GMYC). La validación de dichas hipótesis de especies mediante el análisis bayesiano de delimitación de especies (BPP), respaldó un escenario con cuatro especies donde se confirma que *S. mexicana* es un complejo de dos especies (*Sm1* y *Sm2*) distribuidas a lo largo de las costas del Pacífico y del Atlántico. Por ello, se recomienda el manejo de ambas especies de manera separada evitando el intercambio de colonias para conservar ambos taxones.

8. CONCLUSIONES

Conclusiones generales

Esta tesis ha explorado los patrones de diferenciación dentro de las especies *S. hellwegeri*, *S. mexicana* y *S. pectoralis* distribuidas en México y en el norte de Guatemala. Nuestros resultados de la morfología geométrica y de los análisis moleculares sugieren la existencia de linajes crípticos dentro de estas especies. El género *Scaptotrigona* es más diverso de lo esperado, pero aún existen muchos aspectos sobre su diversidad, distribución y biología por estudiar. Por ello, recomendamos el establecimiento de programas para la conservación de la biodiversidad de las abejas nativas de México y para mejorar la práctica ancestral de la meliponicultura.

Capítulo 1. Código de barras genético en abejas sin aguijón: diversidad genética en el género *Scaptotrigona* de importancia económica en Mesoamérica.

1. El código de barras genético es una técnica rápida y fácil que proporciona información fiable en las especies del género *Scaptotrigona* distribuidas en México.
2. Esta herramienta también es eficiente en la reasignación de individuos erróneamente identificados a la especie a la que pertenecen.
3. Los valores de variación intraespecífica de *S. mexicana* (1.90 %) exceden el límite de la variación intraespecífica (1.58 %) lo que sugiere la presencia de especies crípticas o linajes genéticos dentro de esta especie.
4. La filogenia apoya la hipótesis morfológica de partida en la que *S. mexicana* es más cercana evolutivamente a *S. hellwegeri* que a *S. pectoralis.*
5. Se deben implementar programas de conservación para proteger los hábitats y la biodiversidad del género. Además, específicamente, se deben desarrollar estrategias que eviten el intercambio de colonias de *S. mexicana* entre ambas costas.

Capítulo 2. Revelando la biodiversidad de las abejas sin aguijón de Mesoamérica: morfometría geométrica y análisis de microsatélites de *Scaptotrigona mexicana* y *S. pectoralis* (Apidae: Meliponini)

6. Los análisis moleculares y morfológicos proporcionan una resolución similar en la discriminación entre unidades evolutivas.
7. Ambas técnicas apoyan la hipótesis de la existencia de unidades evolutivas diferentes en *S. mexicana* de las costas pacífica (*Sm1*) y atlántica (*Sm2*), pero no encuentran diferencias en *S. pectoralis*.
8. Pese al gran soporte que tiene la existencia de unidades evolutivas diferentes en *S. mexicana* nuestros resultados no son concluyentes de un evento de especiación. Aun así, sugerimos que se traten como unidades separadas.
9. Se deben desarrollar políticas de manejo para mantener la diversidad en los dos linajes de *S. mexicana*.
10. Recomendamos situar los meliponarios de *S. pectoralis* cerca de áreas silvestres para aumentar la diversidad genética y promover la exogamia.

Capítulo 3. La delimitación multilocus de especies en abejas sin aguijón mesoamericanas apoya la existencia de especies crípticas en el género *Scaptotrigona* (Apidae: Meliponini)

11. El análisis multilocus basado en la coalescencia ha sugerido la existencia de cuatro especies en las *Scaptotrigona* distribuidas en México.
12. El análisis bayesiano de delimitación de especies posee una buena resolución para delimitar especies incluso en bases de datos con una baja señal.
13. Las diferencias en *S. hellwegeri* no son suficientes para considerarla como dos especies, pero existen divergencias genéticas suficientes que apuntan a la existencia de linajes crípticos en *S. mexicana*.
14. Se deben realizar más análisis con muestras de toda la distribución en México y Centro América para confirmar la presencia de dos especies.
15. La población de *S. mexicana* de Chiapas debe ser tratada como una especie diferente para prevenir el flujo génico.

16. Recomendamos la implantación de estrategias de conservación para regular las prácticas apícolas y mejorar los hábitats favorables para los polinizadores.

Introduction

1. STINGLESS BEES

Stingless bees (Apidae: Meliponini) are a group of social insects of the subfamily Apinae. This subfamily has 19 tribes, four of them grouped in the corbiculate bees characterized by the presence of a corbicula or pollen basket on the hind tibiae of the posterior leg (Fig. 1).

Figure 1. Example of a stingless bee (*Trigona*) with a visible pollen basket (picture by Erica Siegel, on http://www.aussiebee.com.au/stingless-bee-feb2013.html at 06/10/15).

The four tribes of corbiculate bees are Euglossini (orchid bees), Bombini (bumble bees), Apini (honey bees), and Meliponini (stingless bees). While within Apini there is one genus and 11 valid species described, Meliponini has 42 genera and a higher number of species, around 500, located at the Tropical areas. In concrete, the Neotropical area holds the highest diversity and number of species (around 400 species and 33 genera, Camargo and Pedro, 2007; Michener, 2007).

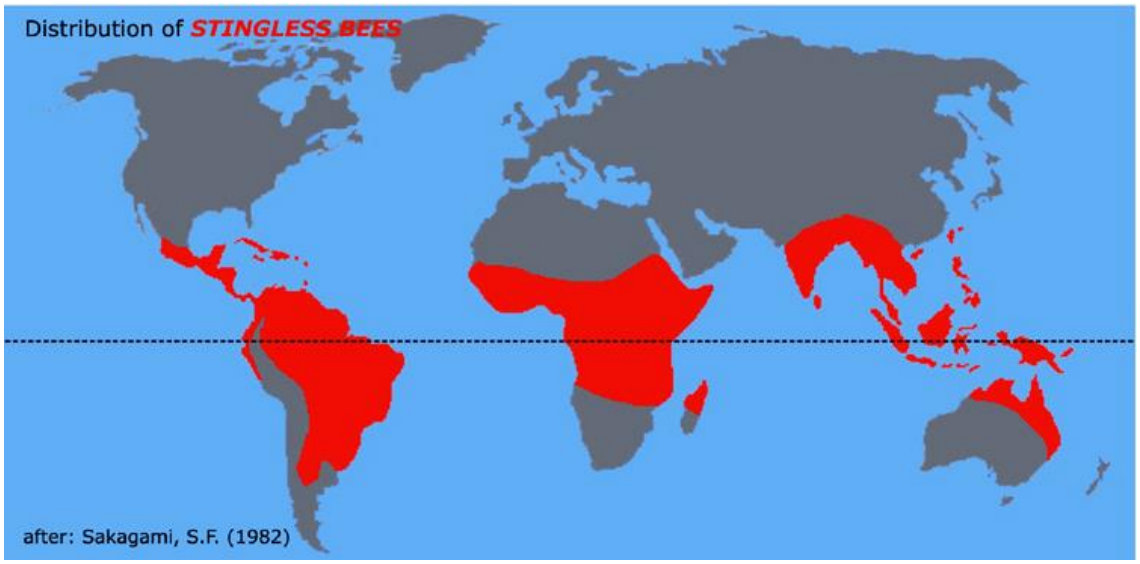

Figure 2. Distribution map of Meliponini (on http://www.b-lab.at/Bilderchens/Distribution-of-stingless-bees.jpg at 06/10/15).

The main anatomic character of Meliponini regarding to honey bees, is the absence of a functional sting; both workers and queens have only traces of it. Moreover, workers have a hairy brush-like structure at the widest end of the tibia, which is known as penicillium. According to other groups of bees the venation of their wings is weaker or even inexistent (Wille, 1983). Other relevant characters are the presence of simple not bifurcated nails and the hind basitarsus without an auricle (Quezada-Euán, 2005).

Stingless bees use cerumen (a mix of wax and resins from the plants) as the main element for building its nests. Cells for larvae development are exclusive for that use (never used for honey or pollen keeping, as does *Apis mellifera*) and have a vertical orientation. The development of stingless bees has the same stages as in *A. mellifera*: egg, larva, prepupa, pupa and adult, but is slower (Quezada-Euán, 2005).

Formation of new colonies in stingless bees is different from honey bees. While *A. mellifera* colonies split in two and the new swarm together with the old queen leaves the old colony, Meliponini new colonies include the new queen and have a great dependence of the mother colony existing a regular transfer of honey, wax and cerumen from the old to the new one. The goal of this movement is to create a deposit of reserves for the daughter colony (Kerr, 1950). This system of colonies creation favors the establishment of new colonies in close areas to the mother

colony, causes inbreeding within populations (Wille, 1983; Cameron et al., 2004). Indeed, Eusocial bees are likely to inbreed thus promoting the so call “extinction vortex” (Zayed and Packer, 2005). This vortex may be initiated by a combination of small effective population size (Chapman et al., 2003) and the effects of the haplodiploidy sex determination system. In Hymenoptera the complementary sex determination gene (csd) controls sex, in the way that diploid females derived from heterozygotes, haploid males from hemizygotes and diploid males from homozygotes (Zayed, 2009). The production of diploids males has a high genetic cost for the populations, since these males are mostly sterile (Heimpel and de Boer, 2008) and can lead to the loss of half of the colony members decreasing the colony fitness (Carvalho et al., 1995; Green and Oldroyd, 2002). Thus, any strategy increasing the probability of mating with unrelated individuals will be beneficial for the survival of the colony. In this sense, drone congregation areas (DCAs) are common phenomena in stingless bees (Paxton, 2005). Some studies have showed that DCAs are composed by drones from about 20 to 40 colonies in the genus *Scaptotrigona* (Paxton, 2000; Kraus et al., 2008) and up to 135 in *Trigona collina* (Cameron et al., 2004). Drones avoid closer DCAs, therefore increasing outbreeding promoting the health of the populations (Paxton, 2000; Cameron et al., 2004; Kraus et al., 2008; Mueller et al., 2012) and assuring this way that the queen can mate in panmixia (Baudry et al., 1998).

2. IMPORTANCE OF STINGLESS BEES

Stingless bees are the most abundant group of bees in the Neotropical ecosystems, playing a key role as generalist pollinators (Roubik, 1989; Martínez-Hernández et al., 1993; Ramalho et al., 1994; Kaminski and Absy, 2006; Michener, 2007). They are ecologically important because of its specificity to pollinate plants: around 33 % of tropical forest plants are exclusively pollinated by these bees (Wilms et al., 1996).

Meliponini has also a great economic relevance since they are efficient pollinators of high economic value tropical crops as coffee, tomato, chili, pepper or avocado (Slaa et al., 2006; Quezada-Euán, 2009). Hence, the use and management of stingless bees for crop pollination is gaining increasing attention due to the dependence of the global agriculture to *A. mellifera* (Aizen and Harder, 2009). In many places and for several crops the ability of honey bees to pollinate is

threaten or limited due to some factors as colony collapse syndrome, africanization, diseases and parasites, climatic limitations and economic pressures (Ormond et al., 1984; vanEngelsdorp et al., 2009) thus reducing the pollination efficiency in some crops (Ricketts et al., 2004).

Stingless bees have advantages over honey bees since they are less aggressive for humans and animals and can pollinate efficiently in greenhouses (Kakutani et al., 1993). Additionally, the dispersion of stingless bees colonies contributes to preserve plant diversity and colony swarming is unlikely since queen lose the ability to fly (Inoue et al., 1984). Beekeeping with stingless bees has also disadvantages: technologies for this kind of beekeeping are less developed in most of the cases (Jaffé et al., 2015), the grow rates in stingless bees are slower than in honey bees and they have lower honey and wax production.

Furthermore, stingless bees has cultural importance, because they were used in pre-Columbian times by natives in tropical America, with special importance in Mesoamerica, where Mayan made great advances in its management to obtain honey and wax (Crane, 1992; Quezada-Euán et al., 2001). There is documentation about management from that time in several areas of Mexico as Yucatan, Puebla, Veracruz, or Tabasco (Foster, 1942; Vásquez-Dávila and Solís-Trejo, 1991; González-Acereto, 2008). Meliponiculture or stingless beekeeping had always been important both in the social and religious life but it almost disappeared by the introduction of *A. mellifera* by Spaniards (Schwarz, 1949; Dixon, 1987). Nowadays many organizations are collaborating to revive and boost meliponiculture as a strong part of the native culture by giving courses, talks and making innovations about traditional techniques.

3. THREATS

According to several studies there are evidences pointing to a worldwide decline of bees (Potts et al., 2010; González-Varo et al., 2013; Goulson et al., 2015). In the last five decades the honey bee stock has increased while feral bee populations have decreased as seen in studies about bumble bees (Goulson et al., 2008) and few other native bees (Goulson et al., 2015). So, according to the observed global decline of bees and bumble bees in America and Europe, is not so unfair to assume this decline worldwide (Goulson et al., 2015).

This decline in the abundance and richness of pollinators can drastically affect to wild animal-pollinated plants, leading to a decrease of biodiversity by increasing the inbreeding in self-compatible plants and reducing the production of seed and fruits (Kluser and Peduzzi, 2007). The change on the abundance and composition of pollinators also affects the stability of the pollinator services on natural and agricultural crops (Burkle et al., 2013; González-Varo et al., 2013).

The main factors affecting this decline are habitat loss (Foley et al., 2005), agricultural activities and extensive use of pesticides, invasive species and climatic change (Cane, 2001; Cane and Tepedino, 2001; Kremen et al., 2002; NRC, 2007; Dormann et al., 2008; Murray et al., 2009).

Habitat loss affects not only bees but also the entire biodiversity and causes habitat fragmentation, which promotes the genetic isolation of populations in small areas where viable populations of bees can not be ensured (Ellis et al., 2006; Zayed, 2009). Ricketts et al. (2008) found a negatively strong relationship between the transformation and degradation of habitats and the abundance of native bees. This threat is enhanced in Meliponini, due to their nesting requirement of cavities in old trunks, their dependence on mother colonies and their low dispersal rate (Quezada-Euán et al., 2001; Freitas et al., 2009; Zayed, 2009). The inbreeding depression is a high risk in small populations because of the decrease of heterozygous could leads to a reduction of the population fitness (Hedrick, 2000; Frankham, 2005). In those small populations, endogamous depression by drift, loss of diversity, and reduction in heterozygosity is almost inevitable over time, and they have less evolutionary response to changes in their environment such as new pathogens, climatic changes or invasive species (Gaggiotti, 2003). These facts affecting stingless bees in small populations, have been detected in different Meliponini species as *Melipona beecheii* (Quezada-Euán et al., 2007), *M. rufiventris* (Tavares et al., 2007) *M. scutellaris* (Carvalho-Zilse et al., 2009), *Scaptotrigona hellwegeri* (Quezada-Euán et al., 2012) and *S. xanthotricha* (Duarte et al., 2014).

The expansion of the agriculture is one of the main causes of the habitat fragmentation (Kremen et al., 2002; Silveira, 2004). Some studies have proven that areas without agriculture have higher density of stingless bees nests (Meléndez et al., 2004; Santos-Leal, 2006). The agriculture entails the use of pesticides, and in Latin America the agriculture is heavily supported

by those chemicals (Tansey et al., 1995; Pinheiro and Freitas, 2010). The sub-lethal effect of the agrochemicals, concretely insecticides on bees can be even worse than the lethal effects on these insects because they can affect to the sensory and neuromotor skills (Pinheiro and Freitas, 2010). Moreover, if exposure occurs on the larva stage it can affect to the brain development and to the mobility of the adults (Tomé et al., 2012).

The introduction of invasive species can be also a threat to the native bees (Stout and Morales, 2009). One of the clearest examples was the introduction of *A. mellifera* in America. This supposed a risk for the native bee populations because of resources competition, competence for nesting sites and the introduction of new pathogens (Roubik, 1989; Freitas et al., 2007).

As the habitat loss, climate change is also an important threat to the biodiversity of the world. More specifically this event could lead to changes in the phenology of the bee-plant pollination relationship (Willmer, 2012). The global warming can also affect the composition of pollinator communities (Memmott et al., 2007). Another change that is likely possible in bees, as has been shown in butterflies (Forister et al., 2010), is the range shifts between bees and flowers, driving to temporal and spatial mismatches (Schweiger et al., 2008). This is expected in the southern edge of the distribution of the species (Goulson et al., 2015). In addition to the weather changes, extreme events as floods, droughts or storms will also have a great impact on bee populations being prejudicial for most of the natural nests (Goulson et al., 2015).

The above-mentioned factors are not acting individually; in fact, they act at the same time generating synergetic effects on bee populations (Tylianakis et al., 2008). So, different threats must be considered together in order to understand how bee populations respond to these factors (Potts et al., 2010). For example, the exposure to pesticides increases the vulnerability of bees that facilitate the infection by invasive pathogens as *Nosema* (Pettis et al., 2012; Wu et al., 2012). Moreover, the infection by parasites is higher in populations settled close to intensive agriculture crops since the diet is poor (Alaux et al., 2010). Due to this complex interaction of threats, is vital to deepen in the knowledge of the biology and present biodiversity status of native bee communities (González-Varo et al., 2013).

This problematic is exacerbated by the lack of knowledge about the status of stingless bee species. This group of bees is the most diverse tribe, both in behavior and in morphology out of the eusocial corbiculates bees (Apini, Bombini and Meliponini) (Michener, 2007). In this tribe, there are hundreds of species and nowadays an accurate approach to the real number of them is hardly impossible due to the existence of cryptic species (Michener, 2007). Even though several authors have tried to make an appropriately analysis of some genera like *Paratrigona, Aparatrigona* and *Geotrigona* (Camargo and Moure, 1994, 1996) or *Partamona* (Pedro and Camargo, 2003), in most of the genera there is still an important lack of information. The phylogenetic classification of stingless bees has been done with different methods by several researchers: Michener (1944) and Schwarz (1948) only recognized two main genera, *Melipona* and *Trigona*, few years later Moure (1961), admitted 23 genera (without taking into account subgenera) from the New World and ten from the Old World. The same author recognized 27 supraspecific taxa (genera and subgenera) from the New World and later, in 1971 changed some of the subgenera to the genus level (Michener, 2007). Camargo and Pedro (1992) and especially Camargo (1989) exposed an exhaustive summary of these classifications. Sakagami (1982) also presented a different classification system. The present work has tried to shed light on the actual situation of *Scaptotrigona* Moure 1942 species distributed in Mexico and northern Guatemala.

4. THE GENUS *SCAPTOTRIGONA* IN MEXICO

The genus *Scaptotrigona* comprehends 24 species distributed from Mexico to northern Argentina (Michener, 2007, Fig. 3). *Scaptotrigona* colonies are within the most abundant of all stingless bees in Neotropical ecosystems (Cortopassi-Laurino et al., 2006).

Figure 3. Distribution map of *Scaptotrigona* species.

Morphologically, this genus is among the most robust of the Meliponini, with a body size ranging from 4.5 to 7 mm (Fig. 4). They are characterize by the posterior margin of scutellum entire and by the anterior margin of pronotal lobe rounded (Michener, 2007). Both the head and the thorax are highly punctuate. As in most of the Meliponini, *Scaptotrigona* nests are usually placed in tree cavities, more often in large old trunks.

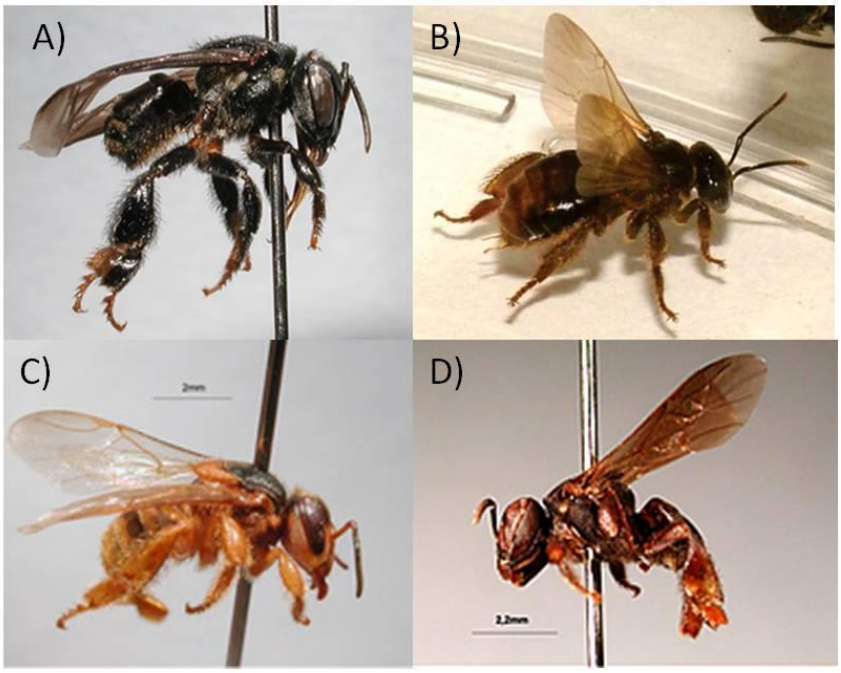

Figure 4. A) *S. bipunctata* (http://www.webbee.org.br/beetaxon/scap.htm on 6/10/15) B) *S. depilis* (http://www.apacame.org.br/mensagemdoce/113/artigo.htm on 6/10/15) C) *S. xanthotricha* (http://www.ib.usp.br/beesp/scaptotrigona_xanthotricha.htm on 6/10/15) D) *S. tubiba* (http://www.ib.usp.br/beesp/scaptotrigona_tubiba.htm on 6/10/15).

In this thesis we have focused on three species of stingless bees from genus *Scaptotrigona*: *Scaptotrigona hellwegeri* Friese 1900, *S. mexicana* Guérin 1845 and *S. pectoralis* Dalla Torre 1896 which are distributed in Mexico and Central America (Fig. 5).

S. hellwegeri is the only endemic Mexican species and has been registered from sea level to 1.500 m. It is distributed across the Pacific coast and Sierra Madre Occidental, from Sinaloa to Oaxaca states.

S. mexicana is distributed across the Atlantic coast in the Mexican Gulf and Sierra Madre Oriental, from Tamaulipas to Veracruz. Moreover, it is distributed across the Neovolcanic axis, Sierra Madre Sur and Sierra of Chiapas. It has been found from the sea level to 1.000 m. This species is also distributed in Belize, Guatemala, Costa Rica and El Salvador.

The third Mexican *Scaptotrigona* species, *S. pectoralis,* has an overlap distribution with *S. mexicana* across most of Central America but it has also been found in Honduras, Nicaragua and Panama. In Mexico, its distribution partially overlaps with *S. mexicana*, but it only reaches the

south of Sierra Madre Oriental in Veracruz state and is present in Yucatan peninsula (where *S. mexicana* is absent). It has been found from sea level to 1200 m (Ayala, 1999).

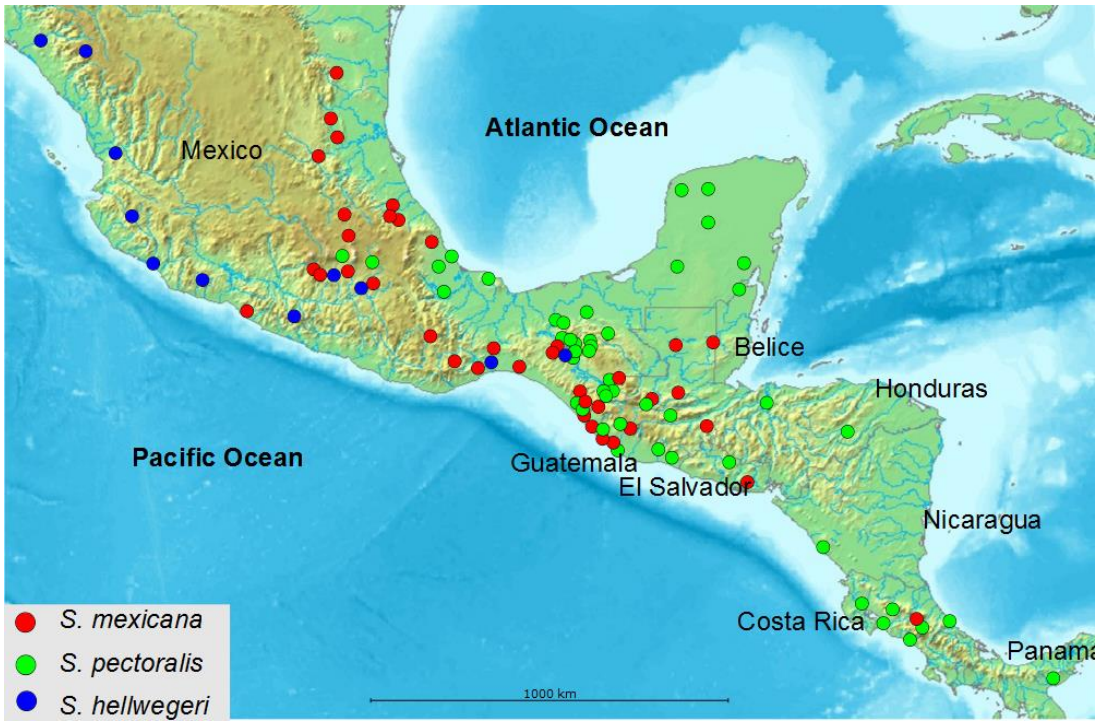

Figure 5. Distribution of localities where *S. hellwegeri* (red), *S. mexicana* (green) and *S. pectoralis* (yellow) have been reported in different studies.

S. hellwegeri and *S. mexicana* are morphologically more similar, clearly differentiated from *S. pectoralis* by the absence of a notch in the occipital carina. On the other hand, *S. hellwegeri* and *S. pectoralis* share an orange and black color pattern, although the former with the scutum orange and the scutellum black while *S. pectoralis* shows the opposite color pattern. *S. mexicana* is the only with a complete black integument (Fig. 6). This three species are medium size according to their group: *S. hellwegeri* (4.7 to 5.1 mm), *S. mexicana* (5 to 5.3 mm) and *S. pectoralis* (5.2 to 5.5 mm). Both workers and drones of these species have similar size, while the queen is larger even just emerged from the cell (Ayala, 1999).

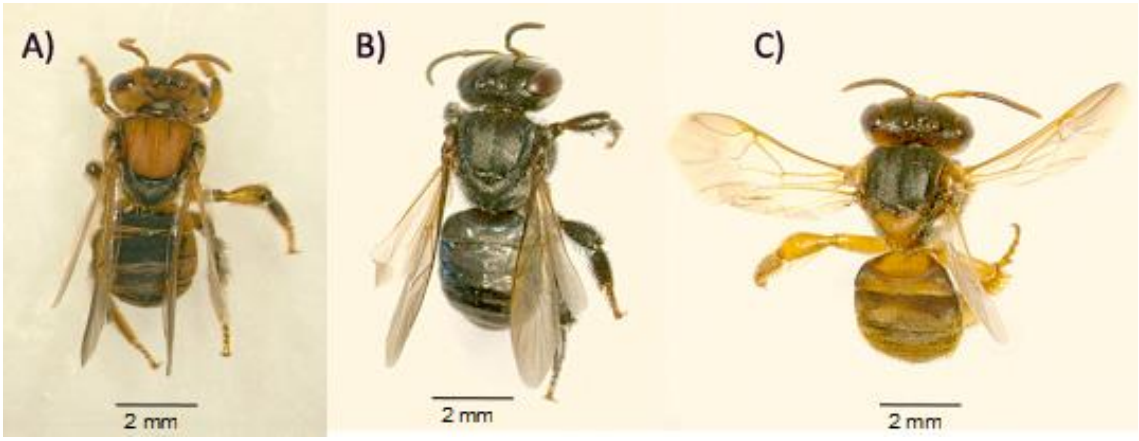

Figure 6. A) *S. hellwegeri*, B) *S. mexicana* and C) *S. pectoralis*.

4.1. Biology

S. hellwegeri colonies do not have an outside structure as entrance but a big hole instead where some workers stand for defense (González-Acereto, 2008). On the other hand, *S. mexicana* and *S. pectoralis* built their nests with a tubular structure made of cerumen at the entrance where workers are placed. *S. mexicana* trumpet shape entrance (Fig. 7) is larger than in *S. pectoralis.* Those bees are aggressive and defend their nest by biting the intruders and releasing a pheromone. *S. mexicana* is the less aggressive.

Figure 7. *S. mexicana* trumpet shape cerumen entrance of a clay pot

S. mexicana is one of the most studied stingless bees in Mexico due to its honey productivity and easy management. This bee has been used since pre-Hispanic times by natives in Central America in order to cure gastric, breathing and other kind of diseases by using its honey (and also

its pollen and propolis). Several recent studies have proven the effectiveness of the *Scaptotrigona* honey as a natural antimicrobial showing even higher antibacterial activity than other honeys (Enríquez and Dardón, 2006; Catzín Ventura et al., 2009; Rodríguez-Malaver et al., 2009).

4.2. Management

Meliponiculture has been a usual practice in Mesoamerica, concretely in Mexico since pre-Columbian times. The introduction of *A. mellifera* caused a considerable decline of this practice, however nowadays it is an ongoing activity (González-Acereto, 2008). Some stingless bees are managed in Mexico and among them *Scaptotrigona* species are valuable resources in Puebla, Veracruz and Guerrero, mostly used by native tribes (González and De Araújo, 2005). *S. mexicana* is widely used for meliponiculture in Chiapas, Veracruz, Puebla, Guerrero and Sinaloa (Manzo, 2009). Instead of a wooden hive as *A. mellifera*, *S. mexicana* is maintained in clay pots (Fig. 8). This traditional management of *S. mexicana* has placed the locality of Cuetzalan in the state of Puebla in the first place of stingless bee honey production (Guzmán et al., 2011). Moreover, the use of *S. hellwegeri* is increasing in Guerrero, Jalisco and Michoacan since the production of its honey is acceptable and its quality excellent (Ayala et al., 2013). *S. pectoralis* is also used in some areas, as Yucatan for honey production (González-Acereto et al., 2006; González-Acereto, 2012).

Figure 8. Meliponary of *S. mexicana* in the locality of Cuetzalan del Progreso (Puebla, Mexico, 2011)

Both *S. pectoralis* and *S. mexicana* have a high efficiency pollinating several crops of high economic importance in Mexico such as coffee, avocado and rambutan (Ish-Am et al., 1999; Camposeco, 2002; Guzmán, 2002).

4.3. Taxonomy of *Scaptotrigona*

Scaptotrigona species were first described by Latraille (1807) as belonging to the genus *Trigona* and was not until 1942 when Moure defined the actual genus (Michener, 2007). Recently, some studies of the Meliponini phylogeny from the Old and the New World have been done including the genus *Scaptotrigona*. Rasmussen and Cameron (2007) used four markers, one mitochondrial

(16S) and three nuclear genes (opsin, Argk and EF1-α) to asset the phylogenetic relationships within this tribe. Bayesian analysis of the four genes solved that *Scaptotrigona* was close to *Geotrigona, Cephalotrigona* and *Trigona* but with low support. On the other hand, in the same study the phylogeny based on the maximum parsimony (MP) established *Scaptotrigona* as more related to *Paratrigona*, *Partamona* and *Parapartamona*. In a posterior study, the same authors (Rasmussen and Cameron, 2010) added more samples and another nuclear marker (28S) to clarify the phylogeny and this time data placed *Scaptotrigona* as a sister clade of *Oxytrigona* with high support by Bayesian analysis. Ramírez et al. (2010) focused on the phylogeny of the genus *Melipona*, but they also analyzed samples from related genera. In this study using two mitochondrial (16S and cox1) and three nuclear markers (ArgK, EF1-α and Pol II) *Scaptotrigona* clustered with *Trigona* being *Geotrigona* and *Cephalotrigona* sister clades.

There have been some taxonomical issues within *Scaptotrigona* Mexican species. In relation to *S. hellwegeri*, Quezada-Euán et al. (2012) showed the existence of significant genetic and morfometric differences among some populations of this species resembling at least two genetic lineages.

Regarding to *S. mexicana*, this species has been related to *S. luteipennis* since both of them are entirely black. *S. mexicana* has been synonymized with *S. pachysoma* (Ayala, 1999). Ayala (1999) reported the possible existence of two different species within *S. mexicana*, one on the center of the country (Ixtapan-Mexico) and the other one in the Pacific coast (Zihuatanejo-Guerrero). Schwarz (1951) also signalized two subspecies in *S. mexicana*, one from Mexico and Guatemala and another one from Central America (Panama). This last named as *S. mexicana suboscuripennis* was later considered as another species *S. suboscuripennis* (Camargo and Pedro, 2013).

S. pectoralis was first divided in four subspecies by Schwarz (1951) two of them distributed in Mexico: one at the Southwest and the other one in Yucatan, but Ayala (1999) considered both of them as a single species distinct of the other two subspecies, *S. p. barrocoloradensis* (Ecuador and Panama) and *S. p. panamensis* (Costa Rica and Panama) that were later considered as two distinct species. Furthermore, *S. pectoralis* seems to be phylogenetically more related to *S. luteipennis* due to the presence of a notch in the occipital carina than to the remaining Mexican *Scaptotrigona*

species (Ayala, 1999). *S. luteipennis* is distributed in Costa Rica, El Salvador and Panama and also in southern Mexico (Chiapas, Camargo and Pedro, 2013), but in the revision of the Mexican Meliponini by Ayala (1999) it was not reported there.

Given these outcomes, a deep research within the genus is needed in order to clarify the taxonomic status of *Scaptotrigona* species.

5. TOOLS AND METHODOLOGIES TO STUDY THE BIODIVERSITY

Species discovery and identification have traditionally relied on a morphological approach. However, the increase of biodiversity loss possess a great challenge for researchers and requires actions for a rapid assessment of its diversity before its disappearance (Blaxter, 2004). In the last decades, different molecular techniques have been developed in order to answer to this problem. Molecular approaches have booming with the goal of providing easy and cheap methods of species identification.

Molecular methods, and DNA barcoding in particular, have been applied by two different approaches that differ in the type and the amount of data required. The first approach is the species identification (or species diagnosis) in which DNA data of an unidentified sample is compared with a molecular database of identified individuals (barcorde library). The second approach (species delimitation) is the use of DNA data to discover new species, determining the species boundaries.

5.1. Molecular and morphological markers

5.1.1. DNA barcoding

Among other approaches, DNA barcoding using a standardized 658 base pairs (bp) segment of the mitochondrial gene cytochrome oxidase I (cox1) (Hebert et al., 2003b, 2004a, b) has been used as an accurate way to identify the global biota (Waugh, 2007). Ability of DNA barcoding to identify species is based in the low rates of intraspecific variation and the relatively high rates of interspecific variation (Packer et al., 2008), since cox1 sequence remains roughly constant within

species but differs between close species, even if they have diverged recently (Hebert et al., 2003a). This technique is built on the recognition of species through the genetic distance values according to similar groups (Vernooy et al., 2010). DNA barcoding also provide the chance to understand and evaluate the diversity within groups where classical taxonomical techniques have showed some difficulties (Köhler, 2007), so it can recognize MOTUs, Molecular Operational Taxonomic Unit (Blaxter, 2004). It should be noted that several studies have argued that cox1 alone is not sufficient to accurately delimit species due to factors such as introgression, retained ancestral polymorphisms, male-biased gene flow or high intraspecific variability (Moritz and Cicero, 2004; Will et al., 2005; Dupuis et al., 2012).

In bees, DNA barcoding has been used for the reconnaissance of taxa not described by morphological characters and for the discovery of cryptic species (Sheffield et al., 2009; Rehan and Sheffield, 2011), for the association of castes in species with dimorphism between queen and workers and for sexual dimorphism in males and females individuals of a given species (Packer et al., 2008). Concretely in Meliponini the existence of a species complex in *Melipona yucatanica* in Mexico allowing to the proposal of conservation strategies according to their evolutionary history (May-Itzá et al., 2010) and the existence of cryptic species in *Liotrigona bitika* (Koch, 2010) have been demonstrated through analysis of the barcode fragment.

5.1.2. Microsatellites

The use of highly variable molecular markers such microsatellites have been a very effective way of estimating population parameters in endangered species (Frankham et al., 2002; Hedrick, 2004). The flanking region of the microsatellites is conserved even among different genera (Ferreira and Grattapaglia, 1998), so markers designed for a specific species can be used in other, as primers designed from *Bombus* o *Melipona* in *Scaptotrigona* species. Microsatellites are codominant and highly polymorphic (Moritz and Hillis, 1996) therefore they have a great statistical power to determinate different parameters within populations as population structure, genetic intra- and interspecific variability and relatedness to population size changes (Frankham et al., 2008).

In stingless bees they have been widely used since the first screening to develop microsatellites markers of the genomes of *Melipona bicolor* (Peters et al., 1998); *S. postica* (Paxton et al., 1999) and *Trigona carbonaria* (Green et al., 2001). These markers have been successfully used within the Meliponini to characterize their genetic variability (Arias et al., 2006; Francisco et al., 2006; Fernandes et al., 2012) and other aspects of the biology of these bees (Kraus et al., 2008; Mueller et al., 2012).

5.1.3. Geometric morphometrics

This fast and cheap technique is based on the variation of wings shape and several studies have shown that geometric morphometrics of wings are a reliable and effective tool to solve taxonomic issues. Results of geometric morphometrics usually are coherent with those obtained with molecular markers.

In the last decade, geometric morphometrics of wings have proven a good tool to resolve variances within species and for the identification of bees (honeybees: Bouga et al., 2011; da Silva et al., 2015; bumblebees: Barkan and Aytekin, 2013; among others). For example this method has shown a great sensitivity to characterize cryptic species (Francisco et al., 2008), subspecies (Francoy et al., 2008; Tofilsky, 2008) and even variability within populations (Mendes et al., 2007, Ferreira et al., 2011; Francoy et al., 2011; Lima Junior et al., 2012; Nunes et al., 2013; Bonatti et al., 2014). However, it is needed to incorporated genetic information to solve taxonomic problems, increasing the rigor in species delimitation, as proposed by the integrative taxonomy approach (Schlick-Steiner et al., 2010).

5.2. Methodologies

5.2.1. Species delimitation

Establishing how each specimen belongs to a species can be a problematic and a subjective task. This is an important issue when only one gene is used, since differences can exist between the history of the species (species tree) and the results obtained in the phylogeny of a gene (gene tree) (Papadopoulou et al., 2008; Monaghan et al., 2009; Fujisawa and Barraclough, 2013). Divergences between both results are even higher when there is not a deep testing of the efficacy

of the single marker (Dupuis et al., 2012). Consequently, there is an accord for using various and independent genes (mitochondrial and nuclear) in order to establish confident phylogenetic relationships and to delimit species. Therefore, methods that objectively assign samples based on a defined criterion may offer a solution to this problem (Payo et al., 2013). Coalescent-based methods let to test alternative hypotheses of lineage divergence reducing investigator-driven biases over methods based on subjectively criteria as the degree of morphological, ecological or genetic differentiation. So that, multilocus coalescent-based methods are replicable and should be reproduced with the same results by different researchers (Fujita et al., 2012). This method has become a reliable tool to delimitate and discover new species and to help establishing right measures of population conservation in order to ensure their survival (Leaché and Fujita, 2010). Although this technique is at the beginning, there are already examples of its success to solve species complex in cases of cryptic species in fishes (Niemiller et al., 2012; Bagley et al., 2015), spiders (Satler et al., 2013), frogs (Setiadi et al., 2011), geckos (Blair et al., 2015), lizards (Barley et al., 2013), birds (Zamudio-Beltrán and Hernández-Baños, 2015) or snakes (Myers et al., 2013).

5.2.2. Integrative taxonomy

The use of multiple independent disciplines (e.g., molecular, morphological, behavioral, and/or ecological data) to solve taxonomic issues in several species has also been proven as a reliable tool (Padial et al., 2010; Andújar et al., 2014). The integrative taxonomy is based on the congruence of different disciplines, as any single dataset may not accurately reflect species limits and relationships (Schlick-Steiner et al., 2010). This approach needs at least two disciplines and the consensus said that one should come from a morphological side and the other from a molecular focus (Gibbs, 2009; Padial et al., 2010; Schlick-Steiner et al., 2010; Chesters et al., 2012).

6. OBJECTIVES AND HYPOTHESIS

The main goal of this thesis is to characterize the taxonomic status of the Mesoamerican (mainly Mexico and northern Guatemala) populations of *Scaptotrigona* species (*S. mexicana*, *S.pectoralis* and *S. hellwegeri*) and to set up a reliable premise for the establishment of further management and conservation strategies. To reach these aims, we have established concrete goals in every chapter based on different hypothesis.

Chapter 1. Barcoding stingless bees: genetic diversity of the economically important genus *Scaptotrigona* in Mesoamerica

The main objective of this chapter is to constitute a preliminary approach to the diversity of the three species.

The specific goals are (i) to test the efficiency of the DNA barcoding technique in the identification of *Scaptotrigona* species described in Mexico, and (ii) its accuracy to asset morphologically classified individuals to barcode-defined species. (iii) Additionally we want to evaluate the intra- and interspecific genetic variation of each species and (iv) to discover the possible existence of cryptic species within them as pointed by previous studies (Quezada-Euán et al., 2012).

The hypotheses of this chapter stands that (1) the DNA barcode technique will differentiate at least the species already determined by classical taxonomy and (2) that according to taxonomy (Ayala, 1999) *S. mexicana* is more related to *S. hellwegeri* than to *S. pectoralis*.

Chapter 2. Shedding light on the biodiversity of Mesoamerican stingless bees: geometric morphometrics and microsatellite analyses of *Scaptotrigona mexicana* and *S. pectoralis* (Apidae: Meliponini)

The main objective of this chapter is to confirm the existence of different taxa within *S. mexicana* species from both Atlantic and Pacific coasts in Mexico, and to detect if there are genetic

differences in *S. pectoralis*, since this species has an almost overlapped distribution in Mexico with *S. mexicana*.

The specific goals are (i) to use geometric morphometry and microsatellites to check the population structure and the molecular diversity of *S. mexicana* and *S. pectoralis*, (ii) to test the existence of at least two species within *S. mexicana*, and (iii) in the light of the diversity observed in this genus to investigate the species status of *S. pectoralis.*

The opening hypothesis are (1) *S. mexicana* is a complex of cryptic species and (2) that geographic and ecological factors influence *S. pectoralis* populations and therefore this species also presents a marked phylogeographic structure.

Chapter 3. Multilocus species delimitation in Mesoamerican stingless bees supports the existence of cryptic species in the genus *Scaptotrigona* (Apidae: Meliponini)

The main objective of this chapter is to define accurately the taxonomical status of the three *Scaptotrigona* species located in Mexico.

The specific goals are (i) to find out how many species are within *S. mexicana* and *S. hellwegeri* and to reveal the status of *S. pectoralis* and (ii) to use techniques that reduce the subjective criteria of the researcher in order to establish the basis of trustful conservation programs.

The starting hypotheses are (1) there are cryptic species in *S. mexicana* and *S. hellwegeri* and (2) in contrast, *S. pectoralis* is a single species.

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Chapter 1

Barcoding stingless bees: genetic diversity of the economically important genus *Scaptotrigona* in Mesoamerica

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Barcoding stingless bees: genetic diversity of the economically important genus *Scaptotrigona* in Mesoamerica

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ABSTRACT

The stingless bee genus *Scaptotrigona* is widely distributed across tropical Mexico and includes economically important species used in stingless beekeeping. As *Scaptotrigona* colonies are currently or potentially translocated across regions, it is important to analyze the extent of genetic diversity from different populations. Herein, every analyzed *Scaptotrigona* individual was correctly assigned through DNA barcoding to the three recognized species (*Scaptotrigona mexicana*, *Scaptotrigona pectoralis*, and *Scaptotrigona hellwegeri*). Intraspecific divergence showed a mean value of 0.70 %, whereas the interspecific value was 2.79 %. As predicted by traditional taxonomy, sequence analyses demonstrated the close affinity of *S. mexicana* with *S. hellwegeri*. However, this also suggested the existence of cryptic species within *S. mexicana*, one of the stingless bees exploited for honey production in Mesoamerica. These results confirm the hypothesis that the DNA barcoding technique may at least differentiate stingless bee taxa accepted by current taxonomy.

Keywords: stingless bees / *Scaptotrigona* / barcoding / cryptic species / Mesoamerica

1. INTRODUCTION

Stingless bees (tribe Meliponini, Michener, 2007) are eusocial insects with an absent functional sting (Wille, 1983) that are distributed in tropical regions with the highest concentration and diversity in the Amazon basin in South America. These bees have great importance as extensive pollinators in Neotropical ecosystems (Roubik, 1989; Michener, 2007; Freitas et al., 2009) and as

an alternative to the domestic honey bee *Apis mellifera* Linnaeus for agricultural pollination purposes (Slaa et al., 2006; Quezada-Euán, 2009). In contrast to *A. mellifera*, stingless bees have several advantages: they are less harmful to humans and domesticated animals, and are also effective pollinators in glasshouses (Kakutani et al., 1993; Heard, 1999; Del Sarto et al., 2005). Despite this, they also have disadvantages, such as a poor level of domestication technologies and the low growth rate compared with *A. mellifera* (Quezada-Euán, 2005).

The genus *Scaptotrigona* is composed of 24 species distributed from Mexico to Argentina (Michener, 2007). Among them, only three *Scaptotrigona* species are currently reported for Mexico (Ayala, 1999): *Scaptotrigona hellwegeri* Friese 1900, *Scaptotrigona mexicana* Guérin 1845, and *Scaptotrigona pectoralis* Dalla Torre 1896 (Ayala, 1999). *S. hellwegeri* is endemic to Mexico across the Pacific coast between Oaxaca and Sinaloa, from the sea level to 1,500 m. *S. mexicana* is distributed from Chiapas to Tamaulipas across the Mexican Gulf coast from the sea level to 1,000 m. *S. pectoralis* is distributed throughout southeast Mexico in Chiapas, the Yucatan peninsula, and in the Gulf coast to Veracruz up to 1,200 m (Ayala, 1999). The distribution of the latter two species continues through Guatemala. Both *S. pectoralis* and *S. mexicana* have a high degree of efficiency in pollinating crops such as avocado (Ish-Am et al., 1999). On the other hand, *S. mexicana* is one of the two stingless bees traditionally exploited for honey production in Mesoamerica and its use in stingless beekeeping is now increasing (Albores-González et al., 2011).

Due to the extensive deforestation of many regions, urban areas have become an alternative microhabitat for these bees. In fact, they have been encountered in wall cavities, although these bees usually nest in hollow trunks. The decline of the stingless bees' habitats is mainly due to anthropogenic factors such as habitat fragmentation and loss, parasites and pathogens, massive pesticide use, or invasive and emergent species like *A. mellifera* (Stout and Morales, 2009). Managed colonies are also subject to translocation among different regions as stingless beekeeping gains interest across the country (Quezada-Euán, 2005). The effect of such practices on the diversity of stingless bee species is unknown but could be potentially detrimental (Quezada-Euán et al., 2012). Given these threats, a rapid and accurate method for the identification of the bee species and their diversity is required to conserve native bee fauna (Gotelli, 2004).

Barcoding is a useful technique for characterizing described and unknown biodiversity (Hebert et al., 2003). In animals, this method is based on the sequence data of a segment of 658 base pairs of the mitochondrial gene cytochrome oxidase I or cox1, and has been used as an accurate way to assess global diversity (Waugh, 2007). In relation to bees, DNA barcoding has proven to be an essential tool in delimiting morphologically non-distinguishable species (Rehan and Sheffield, 2011), to group individuals by sex in dimorphic species (Packer et al., 2008), to associate castes in species with a high sexual dimorphism between queens and workers, and to detect cryptic species (Sheffield et al., 2009). Focusing on stingless bees (tribe Meliponini), DNA barcoding has highlighted the existence of isolated reproductive units in *S. hellwegeri* (Quezada-Euán et al., 2012) as well as in other *Melipona* species such as *Melipona yucatanica* (May-Itzá et al., 2010) and *Melipona beecheii* (May-Itzá et al., 2012).

The aim of this study was to evaluate the DNA barcode technique in order to identify Mexican *Scaptotrigona* species and assign individuals classified by morphology (including non-classified individuals) to the barcode-defined species. The intra- and interspecific genetic variation within *S. mexicana*, *S. hellwegeri*, and *S. pectoralis* were also described to investigate the possible existence of cryptic species (Silveira et al., 2002) as suggested by Quezada-Euán et al. (2012) in *S. hellwegeri*. The opening hypotheses were that (1) DNA barcoding would at least differentiate stingless bee taxa accepted by current taxonomy, and that (2) according to classical taxonomy (Ayala, 1999), *S. mexicana* would be evolutionarily closer to *S. hellwegeri* than to *S. pectoralis*.

2. MATERIALS AND METHODS

2.1. Sampling

As part of an ongoing study of Mesoamerican stingless bee diversity, 88 *Scaptotrigona* colonies (38 of them identified as *S. mexicana*, 33 as *S. pectoralis*, 14 as *S. hellwegeri*, and 3 as *Scaptotrigona sp*) were sampled in different locations throughout their distribution range (Fig. 1), from both managed and feral colonies (Table I). Each sample consisted of three to five worker bees collected from each colony (one to ten colonies per site) and preserved in absolute ethanol at −20 °C. Given the maternal inheritance of the mitochondrial DNA molecule, all of the individuals within each

colony (workers and drones) share the same queen molecule; therefore, just one worker bee per colony was used to characterize the whole colony.

Figure 1. Location of the sampled Mesoamerican *Scaptotrigona* colonies. *S. mexicana* in red; *S. hellwegeri* in blue and *S. pectoralis* in green. Numbers correspond to the locations in Table I.

2.2. DNA extraction and PCR amplification of the barcoding region

Genomic DNA was extracted using a non-destructive protocol, from two right legs dissected from each individual with the DNeasy tissue kit (QIAGEN) following the manufacturer's instructions. Total dilution volume was 100 µl. Vouchers from each colony preserved in ethanol were deposited in the stingless bee collection at the Zoology Laboratory at the Veterinary Faculty (University of Murcia, Spain).

Primers used for the amplification of the cox1 region were LepF (5'-ATTCAACCAATCATAA AGATATTGG-3') and LepR (5'-TAAACTTCTGGATGTCCAAAAAATCA-3') (Sheffield et al., 2009). PCR reactions were carried out in 12.5 µl volume with PureTaq TM Ready-To-Go TM PCR beads (GE Healthcare) in a PTC-200 Thermal Cycler (Biorad). PCR conditions involved an initial denaturation at 96 °C for 2 min, then 35 cycles of 96 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min and a final

extension of 72 °C for 10 min. Amplified PCR products were electrophoresed in 1.5 % agarose gels and then purified with isopropanol and ammonium acetate. Sequencing was performed in both directions using the standard protocol for ABI BigDye(r) Terminator v3.1 Cycle sequencing kit (Applied Biosystems).

2.3. Sequence analysis

DNA sequences of the cox1 region were unambiguously aligned with MEGA 4. Low-resolution ends were eliminated to get a final matrix of 629 bp. Sequences were deposited in GenBank. Nucleotide content was calculated with the MEGA 4 program. DnaSP v5 program (Librado and Rozas, 2009) was used to evaluate genetic variability and calculate the number of haplotypes or identical sequences, their diversity, and the nucleotide diversity.

2.4. Species delimitation

Sequences were compared by the neighbor-joining (NJ) method by applying the correction of the model Kimura 2-parameter (K2P) (Kimura, 1980) as recommended by the Consortium of Barcode of Life (CBOL, http://www.barcoding.si.edu/protocols.html). The analysis of confidence estimates of the relations in the NJ trees was performed with a bootstrap analysis of 2,000 replications with the program MEGA 4.0.2 (Tamura et al., 2007). The strict tree-based method (Ross et al., 2008) was followed for the identification of unclassified and misidentified individuals. This method assumed that query sequences belonged to a specific species if they were incorporated within a cluster (Pettengill and Maile, 2010).

Table I. Sampling data of the analyzed *Scaptotrigona* individuals. Letters in brackets correspond to the locations indicated in Figure 1. N = number of colonies sampled in each location.

Species	Sampling location	N	Colony
S. mexicana	Coyulta, Veracruz, Mexico (1)	7	Managed
S. mexicana	Coatapec, Veracruz, Mexico (2)	6	Managed
S. mexicana[a]	Tlaltetela, Veracruz, Mexico (3)	1	Managed
S. mexicana	Peten, Guatemala (4)	7	Managed
S. mexicana	Tuxtla Chico, Chiapas, Mexico (5)	10	Managed
S. mexicana	Tapachula, Chiapas, Mexico (6)	6	Managed
S. mexicana[b]	San Isidro, Jalisco, Mexico (13)	1	Feral
S. pectoralis	Tapachula, Chiapas, Mexico (7)	8	Feral
S. pectoralis	Tuxtla Chico, Chiapas, Mexico (8)	9	Feral
S. pectoralis	Montes Azules, Chiapas, Mexico (9)	3	Feral
S. pectoralis	Yucatan, Mexico (10)	8	Feral
S. pectoralis[c]	Tlaltetela, Veracruz, Mexico (11)	5	Feral
S. hellwegeri	Guerrero, Mexico (12)	10	Feral
S. hellwegeri	San Isidro, Jalisco, Mexico (13)	2	Feral
S. hellwegeri	Nayarit, Mexico (14)	2	Feral
Scaptotrigona sp[d]	San Isidro, Jalisco, Mexico (13)	3	Feral

a This colony was identified as *S. pectoralis* after barcoding analysis
b This colony was identified as *S. hellwegeri* after barcoding analysis
c One of these five colonies was identified as *S. mexicana* after barcoding analysis
d These colonies were identified as *S. hellwegeri* after barcoding analysis

In order to compare the cluster delimitation of the tree-based method, the Generalized Mixed Yule Coalescent (GMYC) method (Pons et al., 2006; Fontaneto et al., 2007) was applied. This method identified genetic clusters as independently evolving entities by using a maximum likelihood approach to optimize the shift in the branching patterns of the gene tree from

interspecific branches (Yule model) to intraspecific branches (neutral coalescent). Sequences were collapsed to haplotypes with ALTER (Glez-Peña et al., 2010) and an ultrametric tree was generated with BEAST v. 1.5.4 (Drummond and Rambaut, 2007) using a relaxed lognormal clock model, a GTR + I + α substitution model and a coalescence (constant size) tree (Monaghan et al., 2009). The cluster delimitation analysis was carried out using the R package SPLITS (SPecies Lmits by Threshold Statistics) available at http://r-forge.r-project.org/projects/splits/. Both single and multiple threshold optimizations (Monaghan et al., 2009) were analyzed.

TaxonDNA v. 1.5 (Meier et al., 2006) was used to obtain the distribution frequency of intra- and interspecific genetic variability and to evaluate the adequacy of barcoding in identifying stingless bee individuals at the species level. The proportion of correct matches followed three distance-based identification criteria: Best Match (BM), Best Close Match (BCM), and All Species Barcodes (ASB) as described by Meier et al. (2006). The distance below which 95 % of all intraspecific distances are found was used as the cutoff value.

3. RESULTS

3.1. Nucleotide variation analysis

The final analyzed matrix included 629 positions with 592 conserved, 37 variable, and 35 phylogenetic informative positions. Average nucleotide composition showed an A + T bias (T = 46.4 % and A = 32.2 %, C = 11.3 %). No insertions or deletions were observed that could lead to a disruption of the reading frame in the translation, thereby confirming the absence of pseudogenes or NUMTs (López et al., 1994). In total, 15 haplotypes were found (eight in *S. mexicana*, three in *S. hellwegeri*, and four in *S. pectoralis*) with an overall haplotype diversity of 0.907 and nucleotide diversity of 0.019 (Table II). The sequences have been submitted to GenBank under the accession numbers JQ783136-JQ783157.

3.2. Species delimitation

The NJ tree allowed the identification of three undetermined individuals that corresponded to *S. hellwegeri*, and another three individuals previously misidentified by morphometry were

reclassified. In the end, two individuals initially identified as *S. mexicana* were molecularly assigned one to *S. hellwegeri* and a second one to *S. pectoralis*. Likewise, an individual identified as *S. pectoralis* was molecularly assigned to the clade formed by *S. mexicana* individuals (Fig. 2). These results were confirmed by observation of some diagnostic morphological characteristics such as the color of the tergites after sequence analyses. All of the analyzed sequences were properly assigned to their respective clade corresponding to one of the three species following the strict tree-based method (Ross et al., 2008).

Table II. Number and diversity of cox1 haplotypes and nucleotide diversity in *Scaptotrigona* species.

Species	N hap	Hd	Pi
S. mexicana	8	0.862	0.008
S. hellwegeri	3	0.601	0.003
S. pectoralis	4	0.612	0.006
Total	15	0.907	0.019

N hap = number of observed haplotypes, Hd = haplotype diversity, Pi = nucleotide diversity

Genetic K2P distance analyses showed that every species formed a monophyletic group in the NJ tree (Fig. 2). The three species formed well-supported clades with high bootstrap values: 99 (*S. hellwegeri*), 82 (*S. mexicana*), and 100 (*S. pectoralis*). *S. hellwegeri* and *S. mexicana* formed a supported clade (89). Furthermore, at least two clades were observed within each species, each of them corresponding to populations from separated localities within the distribution area of each species.

GMYC analyses showed that both single (LGMYC = 85.3911) and multiple threshold (LGMYC = 85.3911) models had a higher likelihood than the null model (L0 = 83.3653). Both analyses yielded three clusters that agreed with the morphological species. It is noteworthy that the second single threshold model recovered with a higher likelihood two clusters within *S. mexicana*.

The intraspecific divergence values for these three species ranged from 0.00 to 1.90 %, whereas the interspecific divergence values ranged from 1.37 to 3.70 %. There was an overlap of

the intra- and interspecific divergence values in the analyzed individuals between 1.37 and 1.90 %, corresponding to 12.14 % of the values of divergence (Fig. 3). The limit or “cutoff” between intra and interspecific variation was 1.58 % of divergence with 95 % of probability. The mean value of intraspecific divergence in *S. hellwegeri* was 0.30 % (0.00–0.63 %), in *S. pectoralis* was 0.5 % (0.00–1.10 %) and in *S. mexicana* 0.8 % (0.00–1.90 %). *S. mexicana* individuals from Veracruz showed a higher divergence value than the cutoff when compared to the samples from Chiapas.

The proportion of correct matches with Best Match, Best Close Match, and All N hap number of observed haplotypes, Hd haplotype diversity, Pi nucleotide diversity Species Barcodes criteria reached 100 % of the samples.

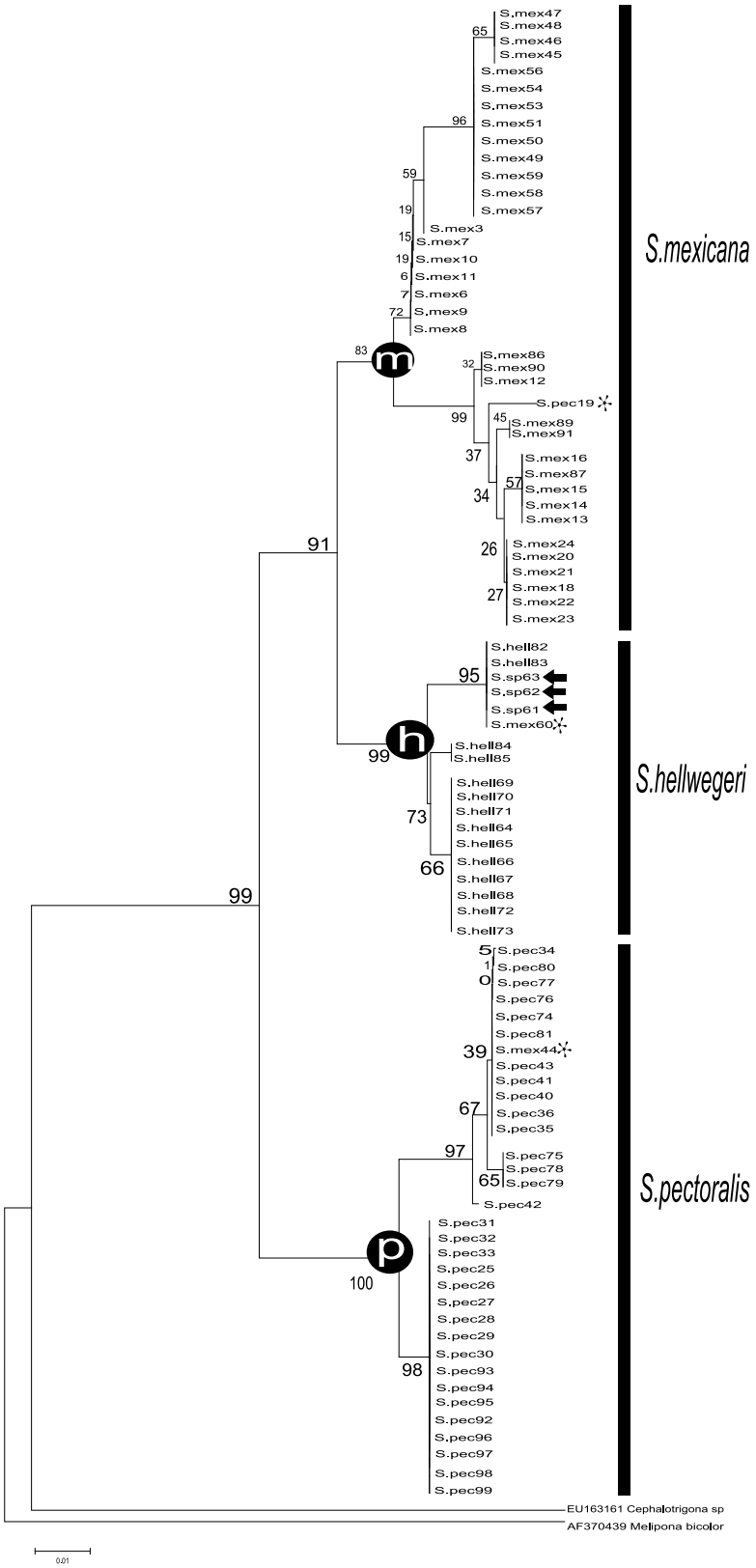

Figure 2. Neighbor-joining tree for Mesoamerican *Scaptotrigona* individuals using Kimura-2-parameters distance. Bootstrap values (2,000 replicates) are shown above each branch. Black circles indicate the clades corresponding to each species, arrows indicate individuals assigned through DNA barcoding to its corresponding species and those with an asterisk correspond to misidentified individuals.

4. DISCUSSION

These findings on the use of DNA barcoding confirm that this technique provided reliable identification of the Mexican species of the stingless bee genus *Scaptotrigona* in congruence with the results obtained for other Hymenoptera taxa (Packer et al., 2008; Gibbs, 2009; Sheffield et al., 2009). It is also an efficient tool for the unequivocal reassignment of individuals previously defined by current taxonomy. In this sense, the DNA obtained from a single leg allows the re-examination of morphological characteristics after molecular analysis. Thanks to the non-destructive DNA extraction method, the vouchers were perfectly conserved, which allowed the re-examination of undetermined or misidentified individuals.

The ability of DNA barcoding to distinguish among species is supported by low values of intraspecific variation and relatively high levels of interspecific variation (Packer et al., 2008). The results for the genus *Scaptotrigona* fulfilled this guideline, as the mean percentage of interspecific variation (2.79 %) was four times higher than the intraspecific value (0.70 %). These values are comparable to those obtained in a comprehensive study of bees (Hymenoptera: Apoidea) from Nova Scotia (Sheffield et al., 2009). In that study of 144 bee species, intraspecific cox1 divergences averaged 0.49 %, which was an expected value due to the elevated mitochondrial evolution rates observed in honey bees (Crozier et al., 1989) and other hymenopterans (Hebert et al., 2003). A ten times higher interspecific divergence than intraspecific divergence has been proposed as a criterion for barcode species identification (Hebert et al., 2004), which was not addressed in the present study. However, the proportion of correctly identified individuals under the Best Match, Best Close Match, and All Species Barcodes criteria reached 100 % of the samples, which shows that barcodes are useful in identifying species within this tribe.

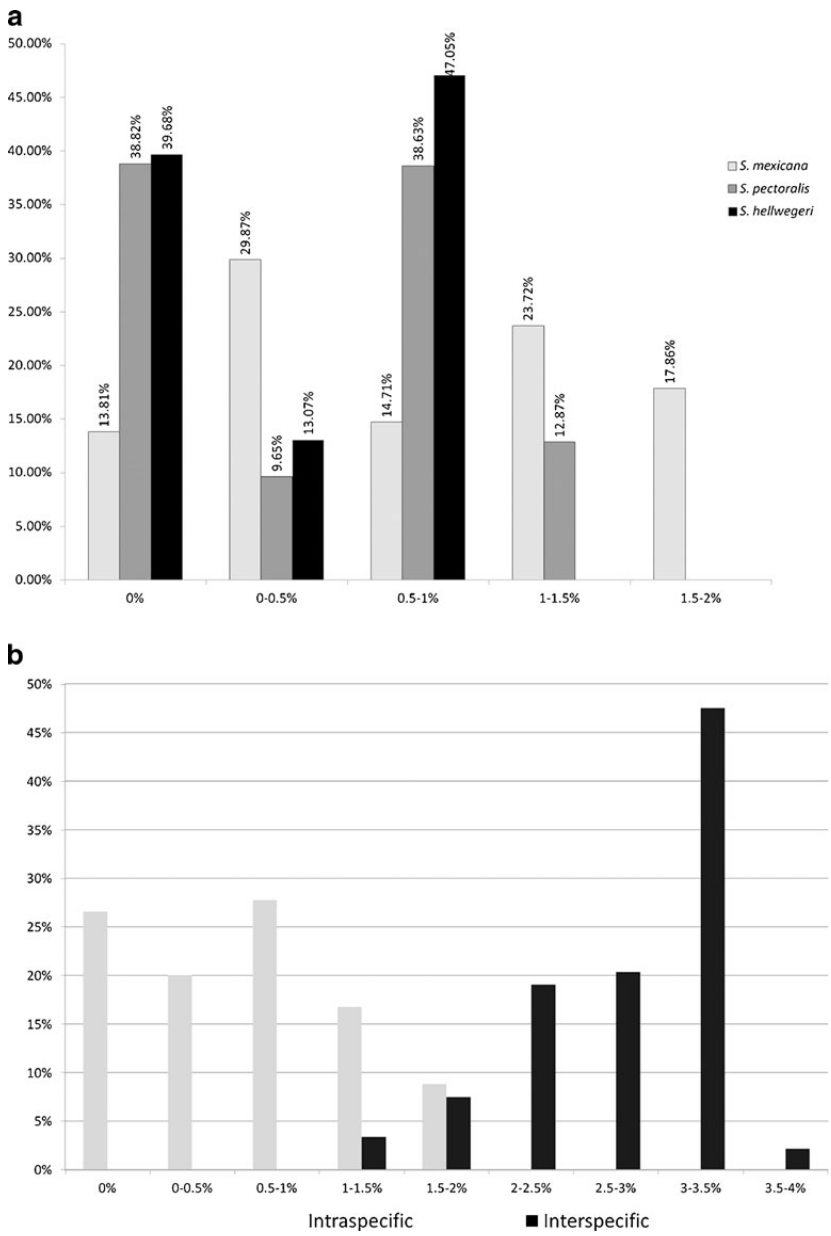

Figure 3. Mean intraspecific (a) and interspecific (b) divergence values for *Scaptotrigona* individuals.

The analysis of the cox1 sequence variation was also helpful in revealing species complexes within taxa with close morphologies, such as *S. mexicana*. In this study, TaxonDNA analysis showed that some intraspecific divergence values within *S. mexicana* (1.58–1.90 %) exceeded the interspecific cutoff (1.58 %), thus suggesting the existence of cryptic species or genetic lineages within this species. These values were found when comparing individuals sampled in Veracruz and Chiapas; these two regions are located in opposite extremes of the species distribution range. This finding is in congruence with genetic distance clustering and the second best solution of the single threshold model. The clustering obtained in the NJ tree also suggested some degree of

differentiation within *S. hellwegeri* and *S. pectoralis* supported by high bootstrap values (95 and 98, respectively), although both species showed intraspecific divergence values that did not exceed the specific cutoff obtained with TaxonDNA. In a previous study performed with samples of the endemic *S. hellwegeri*, significant morphometric differences coupled with complementary analysis of microsatellite loci and the cox1 region resulted in a marked differentiation among populations, which is in accordance with the diversity of habitats occupied by these bees (Quezada-Euán et al., 2012). Given these results, they proposed the existence of genetic lineages (possibly resembling cryptic species) within *S. hellwegeri*. The finding of this hidden diversity requires the implementation of appropriate conservation measures to preserve the populations in their environments and avoid translocations of colonies between distant areas. These criteria should be specifically applied to *S. mexicana*, a species experiencing increasing management for honey production.

The intraspecific divergence found for *S. mexicana* and *S. hellwegeri* might be reflecting unrecognized isolation by distance phenomena within morphological species. In addition, it is expected that the genetic intraspecific divergence would also be reflected in phenotypic differences. In other stingless bee species, morphometric analyses have supported the existence of genetic lineages (*M. yucatanica,* May-Itzá et al., 2010; *M. beecheii* Francoy et al., 2011, May-Itzá et al., unp. data). The morphometric study of *S. mexicana* and *S. hellwegeri* is currently in progress to corroborate this hypothesis. Complementarily, microsatellite analysis of a wider sampling covering the whole distribution range will provide new insights in relation to the extent of the gene flow. This is an important aspect to study given the low dispersal rate in Meliponini due to the reduced dispersion of swarms and short flight distances (Engels and Imperatriz-Fonseca, 1990).

Although the main aim of the barcode analysis was to delineate species boundaries, a phylogenetic signal from the cox1 sequence data could be observed. In this sense, the NJ phylogram and the General Mixed Yule Coalescent (GMYC) results correctly delineated the three species described with traditional morphological analysis and support the hypothesis stated by Ayala (1999) concerning the close evolutionary affinity of *S. mexicana* to *S. hellwegeri*, with *S. pectoralis* as a more distantly related taxon.

In conclusion, Meliponini represents another tribe for which DNA barcoding is a tool for species identification and that provides new insights into the diversity of this group of bees. The main problem in Mesoamerica related to bee conservation is the scarce studies or registers about biodiversity, richness, and the impact of human activities. At present, describing the genetic diversity of organisms spread throughout important biodiversity hotspots like Mesoamerica plays a pivotal role in the scenario of dramatic bee declines and pollination-dependent systems of both wild and managed bees.

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Chapter 2

Shedding light on the biodiversity of Mesoamerican stingless bees: geometric morphometrics and microsatellite analyses of *Scaptotrigona mexicana* and *S. pectoralis* (Apidae: Meliponini)

Shedding light on the biodiversity of Mesoamerican stingless bees: geometric morphometrics and microsatellite analyses of *Scaptotrigona mexicana* and *S. pectoralis* (Apidae: Meliponini)

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ABSTRACT

Geometric morphometrics and molecular methods provide an effective tool for studying the variability of stingless bee populations worth to be protected given its worldwide decline. Based on previous evidence of cryptic lineages within *Scaptotrigona* species, herein we analyze two species *S. mexicana* and *S. pectoralis* with both methods in order to test the existence of at least two species within *S. mexicana*, and investigate the species status of *S. pectoralis*. To achieve these aims we measured wing variation and genotyped bees from both species with seven polymorphic microsatellite loci. We found differences within *S. mexicana* from Pacific (*Sm1*) and Atlantic (*Sm2*) coasts but no differentiation within *S. pectoralis* studying the geometric morphometrics of the wing. Microsatellites confirmed these results, also indicating a tendency toward increased differentiation with increased distance (Mantel test) in *Sm2* and *S. pectoralis*. Our results revealed the pattern of differentiation of two evolutionary units within *S. mexicana* and the distribution of genetic diversity in *Scaptotrigona* species, suggesting the need of future taxonomic revisions and activities for management and conservation.

Keywords: *Scaptotrigona* / stingless bees / geometric morphometrics / microsatellites / cryptic lineages

1. INTRODUCTION

Stingless bees (Hymenoptera: Apidae: Meliponini) are eusocial and haplodiploid insects which are ecologically and economically important pollinators in Neotropical ecosystems (Slaa et al., 2006). This region comprehends most of the diversity of the group with around 400 species (Michener, 2007). The Meliponini tribe has a pantropical distribution (Camargo and Pedro, 1992), with approximately 42 genera endemic to the Neotropical region (Camargo and Pedro, 2013). One of these genera is *Scaptotrigona* that comprehends 24 species distributed from Mexico to Argentina (Michener, 2007). Three of them are reported in Mexico: *S. mexicana* Guérin, 1845 which its distribution range extends to Costa Rica, *S. pectoralis* Dalla Torre, 1896 which distribution continues through Panama, and the endemic *S. hellwegeri* Friese, 1900 (Ayala, 1999). The first two species are efficient pollinators of crops such as avocado (Ish-Am et al., 1999), but the three of them are managed for honey production in greater or lesser degree being *S. mexicana* more widely used for both purposes (Manzo, 2009; González-Acereto, 2012; Ayala et al., 2013). Given the important ecosystem and human services they provide, its commercial use is now increasing (Albores-González et al., 2011) with a potential effect on the gene flow across species (Jaffé et al., submitted). As many other insects, they are also affected by extensive deforestation and the extension of monocrops that occur in these areas (Freitas et al., 2009; Stout and Morales, 2009). These events drive to a potential decline of the stingless bee populations and may lead to an endogamous depression, increase of homozygosity and loss of genetic diversity (Zayed, 2009). In this sense, the lack of information about their richness, diversity, genetic status and distribution is among the most important obstacles to ensure their protection and the establishment of appropriate conservation measures (Brown and Paxton, 2009; Freitas et al., 2009). Furthermore, the existence of cryptic species has been suggested in several stingless bee species (Tavares et al., 2007; Francisco et al., 2008; May-Itzá et al., 2012) and the total number of species could be even higher (Michener, 2007). With the advent of molecular methods and more powerful morphological analyses, the number of cryptic species will probably increase.

In the last decade, geometric morphometrics of wings has proven a good tool to resolve variances within species and for the identification of bees (honeybees: Bouga et al., 2011; da Silva et al., 2015; bumblebees: Barkan and Aytekin, 2013; among others). In stingless bees, this method has shown a great sensitivity to characterize species and even to differentiate populations

(Ferreira et al., 2011; Francoy et al., 2011; Lima Junior et al., 2012; Nunes et al., 2013; Bonatti et al., 2014), but to solve taxonomic problems genetic information should be incorporated to increase rigor in species delimitation, as proposed by the integrative taxonomy approach (Schlick-Steiner et al., 2010).

Species and population identification by genetic methods has made significant progress. Unknown population parameters important for the conservation of endangered species have been depicted from molecular markers (Frankham et al., 2002; Hedrick, 2005). Among them, microsatellites have a great statistical resolution to estimate different parameters and characterize demographic events within species and populations (Luikart and England, 1999). These markers have been successfully used within the Meliponini to characterize their genetic variability (Arias et al., 2006; Francisco et al., 2006; Fernandes et al., 2012) and other aspects of the biology of these bees as the genetic characteristics of the drone congregation areas (Kraus et al., 2008; Mueller et al., 2012).

Previous studies based on morphological and/or molecular markers have suggested the existence of genetically distinct evolutionary lineages (or cryptic species) within the genus *Scaptotrigona* (Quezada-Euán et al., 2012; Hurtado-Burillo et al., 2013; Duarte et al., 2014). In a study on *S. hellwegeri* using a combination of morphometric and molecular methods, Quezada-Euán et al. (2012) found signs of an ongoing speciation process indicating that two populations distributed at different altitude on the Trans-Mexican Volcanic Belt and the Pacific coast should be treated as separate units (Quezada-Euán et al., 2012). Hurtado-Burillo et al. (2013) also suggested the existence of cryptic species within *S. mexicana* colonies in two regions located in opposite extremes of the species distribution range in Mexico (Veracruz and Chiapas) by using the DNA barcoding technique. Recently, up to five distinct clusters with high rates of genetic diversity (microsatellite analysis) were detected in *S. xanthotricha* with a wide distribution in the Brazilian Atlantic Rainforest (Duarte et al., 20014). These authors concluded that they should be considered as units of management and conservation even though they do not have the status of distinct taxonomic units yet.

Because geometric morphometrics and genetic (microsatellites) methods provide an effective tool for studying the variability of stingless bees populations as mentioned before, herein we use

them to resolve taxonomic issues in *S. mexicana* and *S. pectoralis*, two species that share an equivalent distribution area and have similarities in biological and ecological aspects but different management. We aim to: (i) use geometric morphometry and microsatellites markers to check the population structure and the molecular diversity of *S. mexicana* and *S. pectoralis*, (ii) test the existence of at least two species within *S. mexicana*, and (iii) investigate the species status of *S. pectoralis* in the light of the diversity observed in this genus.

2. MATERIALS AND METHODS

2.1. Sampling

This study was carried out across the distribution area of *S. mexicana* and *S. pectoralis* in Mexico and Guatemala. A total of 99 colonies were sampled in 12 localities between 2008 and 2011 (Table I; Fig. 1). Most *S. mexicana* samples were provided by stingless beekeepers that maintain colonies in clay pots or in the original trunks in their own property or in the neighborhood (meliponaries). *S. pectoralis* samples were obtained from non-managed wild colonies located in native forest.

Adult worker bees were collected at the entrance of each colony and preserved in absolute ethanol at -20 ^{o}C. Voucher specimens were deposited in the insect collection at the Zoology Laboratory at the Veterinary Faculty (University of Murcia, Spain).

Table I. Details of *S. mexicana* and *S. pectoralis* sampling in Mexico and Guatemala. N1 = number of colonies used for geometric morphometrics analysis; N2 = number of colonies used for microsatellite analysis. Map code refers to the number labelling each locality in Figure 1.

Species	Locality, state, country	Coordinates		N1	N2	Map Code
		Latitude	Longitude			
S. mexicana	Cacahoatan, Chiapas, Mexico	14.996	-92.167	8	8	10
S. mexicana	Ejido 20 noviembre, Chiapas, Mexico	14.978	-92.265	8	8	11
S. mexicana	Tapachula, Chiapas, Mexico	14.979	-92.266	8	8	12
S. mexicana	Tuxtla Chico, Chiapas, Mexico	14.906	-92.261	21	23	9
S. mexicana	Chilcuahuta, Hidalgo, Mexico	20.331	-99.232	-	1	1
S. mexicana	Melchor de Mencos, Peten, Guatemala	17.066	-89.150	11	11	15
S. mexicana	Cuetzalan del Progreso, Puebla, Mexico	20.017	-97.522	-	1	4
S. mexicana	Tuzamapan de Galeana, Puebla, Mexico	20.065	-97.576	-	1	2
S. mexicana	Coatapec, Veracruz, Mexico	19.451	-96.959	6	6	5
S. mexicana	Coyulta, Veracruz, Mexico	20.248	-97.658	8	9	3
S. pectoralis	Merida, Yucatan, Mexico	20.861	-89.624	8	8	14
S. pectoralis	Tlaltetela, Veracruz, Mexico	19.314	-96.901	4	4	6
S. pectoralis	Palenque, Chiapas, Mexico	17.511	-91.993	3	2	13
S. pectoralis	Tapachula, Chiapas, Mexico	14.966	-92.261	8	8	8
S. pectoralis	Tuxtla Chico, Chiapas, Mexico	14.937	-92.167	9	9	7

Figure 1. Localities in Mexico and Guatemala where specimens were collected: red dots represent *S. mexicana* and green dots *S. pectoralis* colonies. Numbers correspond to the sampling code used in Table I.

2.2. Geometric morphometrics analysis

From one to six specimens from each colony were used in this analysis according to wing availability. Only the right wing of each specimen was used. Wings were photographed with Spot Insight Firewire digital camera (Sterling Heights, USA) adapted to a Zeiss Stemi 2000C Trinocular Zoom Stereomicroscope (Thornwood, USA). We manually plotted twelve homologous landmarks in the wing veins intersections (Fig. 2) using the software tpsDig, version 2.17 (Rohlf, 2013).

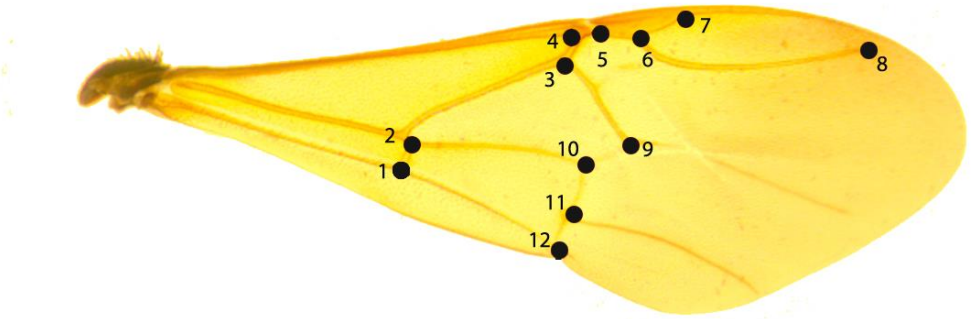

Figure 2. Worker wing with 12 landmarks marked in the vein junctions.

The software MorphoJ, version 1.06c (Klingenberg, 2011) was used to analyze the images that were Procrustes aligned (Bookstein, 1991) in order to identify the points of shape variation. Mean values of the specimens from each colony were used to perform the analyses at the colony level. Principal component (PCA) and canonical variate analyses (CVA) were performed with these data.

Mahalanobis distance values between groups of colonies as defined by PCA analysis were obtained with the software MorphoJ, version 1.06c (Klingenberg, 2011). We also did a Mantel test to investigate whether Procrustes-fitted landmarks coordinates of the wings varied with the geographic distance in each group using Past 3.08 software (Hammer et al., 2001).

2.3. DNA extraction and microsatellites amplification

One worker bee per colony was used for this analysis. DNA was extracted from the leg of each specimen using the DNeasy tissue kit (QIAGEN).

Seven microsatellite loci were amplified in three reactions (two of them multiple). Two of these microsatellites were originally described in *S. postica* (T4–171 and T7–5; Paxton et al., 1999), four in *Melipona bicolor* (Mbi278, Mbi259, Mbi254 and Mbi201; Peters et al., 1998) and one in the bumblebee *Bombus terrestris* (B124; Estoup et al., 1995). Loci T4–171, T7–5 and B124 have been

successfully used in *S. mexicana* (Kraus et al., 2008; Mueller et al., 2012) and *S. hellwegeri* (Quezada-Euán et al., 2012).

PCR reactions were carried out in 12.5 µl volume with PureTaqTM Ready-To-Go TM PCR beads (GE Healthcare) in a PTC-200 Thermal Cycler (Biorad). Amplified fragments (=microsatellite alleles) were detected with an ABI prism 3100 sequencer (Applied Biosystem) and scored with Genemapper version 3.7 (Applied Biosystem).

2.4. Molecular data analyses

The number of clusters (K) present was estimated using a Bayesian model-based clustering method with the software STRUCTURE version 2.2 (Pritchard et al., 2000). Results were based on simulations of 80,000 burn-in steps and 1,000,000 MCMC (Markov Chain Monte Carlo algorithm) iterations. Five runs for each K-value (K = 1−10) were used to estimate the most likely value of K. The number of clusters defined by the value of ΔK as described in Evanno et al. (2005) was used in further analyses.

GenAlex (Peakall and Smouse, 2006) was used to calculate population genetic parameters as allele frequencies, observed (Ho) and expected (He) heterozygosity, number of private alleles (Npa) for each cluster, genetic distance (inferred from pairwise Fst) and principal coordinates (PCoA) analyses based on the first two principal coordinates to find population patterns based on the genetic distance among individual samples. Finally, a Mantel test was performed to check for the correlation of the genetic and geographic distances within each cluster according to a pattern of isolation by distance (IBD). Hardy-Weinberg equilibrium and population differentiation using the Fisher's exact probability test were calculated in Genepop (Raymond and Rousset, 1995a, 1995b).

3. RESULTS

3.1. Geometric morphometrics

The PCA analysis of the 12 landmarks generated 20 relative warp measures. The first 11 factors of these measures had eigenvalues greater than one and explained 95.46 % of the total data variability. The first two PC values explained 64.52 % of the total data variability (PC1 explained 49.31 % and PC2 15.21 %). PCA showed three groups, one with *S. pectoralis* (*Sp*) colonies and two within *S. mexicana* (herein named *Sm1* and *Sm2*). The graphic representation showed that colonies from the Pacific coast (Chiapas, *Sm1*) were placed in the quadrant 4 while the rest of the colonies from the Atlantic coast (Veracruz in Mexico and Peten in Guatemala, *Sm2*) were mainly placed in the quadrant 3. On the other hand, samples of *S. pectoralis* were dispersed in quadrants 1-2 (Fig. 3).

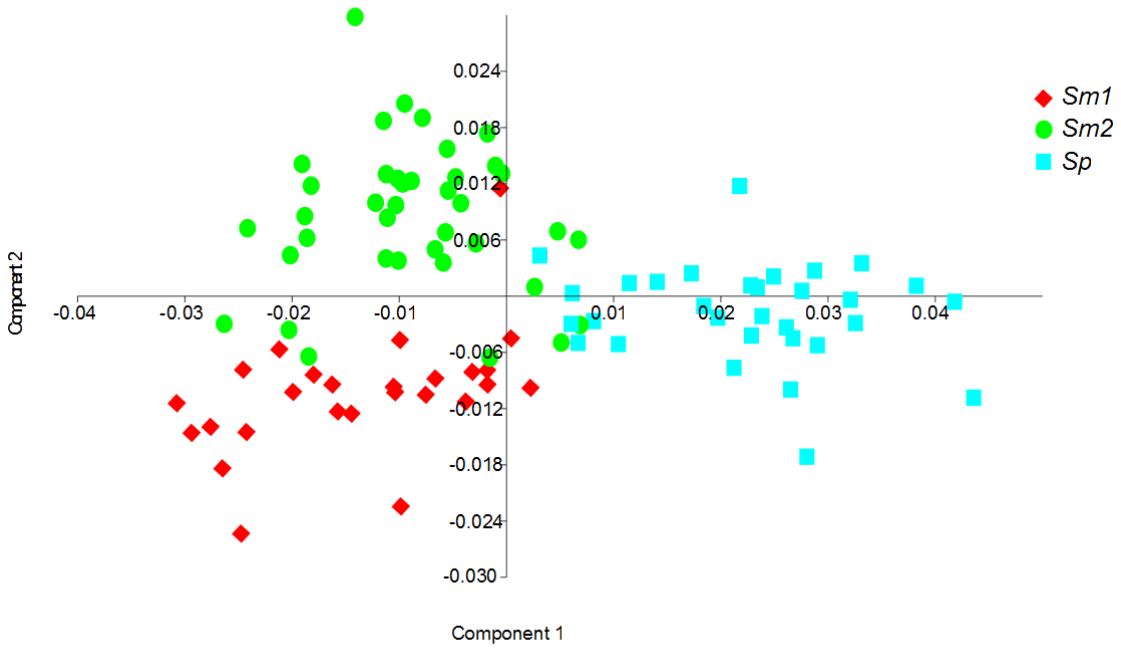

Figure 3. Distribution of the average scores of *Scaptotrigona* colonies against principal components 1 and 2 of the principal component analysis (PCA) based on geometric morphometry data. *Sm* named the two groups observed within *S. mexicana* and *Sp* referred to *S. pectoralis* colonies.

Mahalanobis distances between the groups confirmed the differentiation between *Sm1* and *Sm2* (4.847) although this value was around the half of that observed between *S. pectoralis* and *Sm1* (8.477) and *Sm2* (8.171).

The Mantel test showed no significant correlation between morphological and geographical distances for each putative species (*Sm1*: R= -0.13, p= 0.99; *Sm2*: R=0.012, p= 0.3 and *Sp:* R=0.010, p=0.39).

3.2. Microsatellites

The highest posterior probability of the data set was detected when assuming three clusters (K = 3) in coincidence with the groups detected with morphometric data (Fig. 4). *S. mexicana* was split into two separate clusters that grouped the colonies as previously: *Sm1* (colonies from Chiapas) and *Sm2* (colonies from Veracruz and Peten including in this case two colonies more, one from Puebla and another from Hidalgo). All the colonies corresponding to *S. pectoralis* (*Sp*) grouped together. This information was used in the estimates of the population genetic parameters.

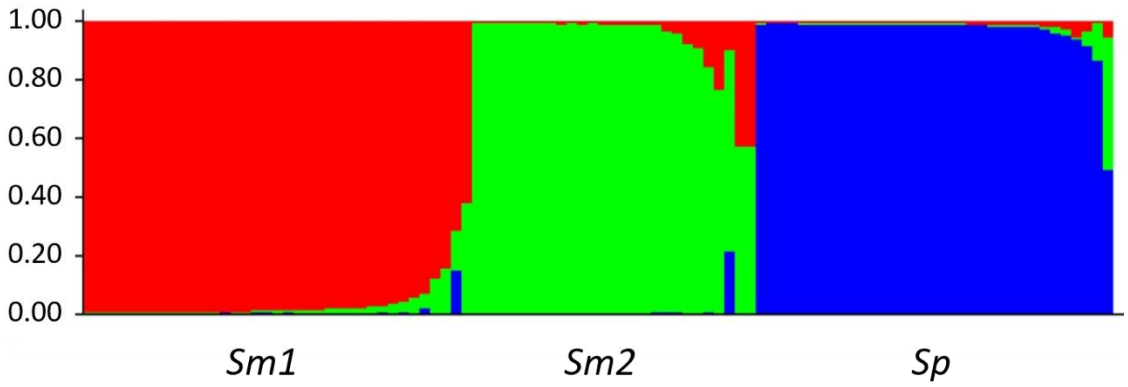

Figure 4. Results of the Bayesian clustering based on STRUCTURE algorithm showing the most probable number of clusters (K = 3). Division of specimens into colored segment represents the assignment probability of that specimen to each of the K clusters (red-*Sm1*; green-*Sm2*; blue-*S. pectoralis*, *Sp*).

Principal coordinates analysis (PCoA) showed samples from the Atlantic coast belonging to *S. mexicana* (*Sm2*) located in quadrant 2 and those from the Pacific coast (*Sm1*) together with two samples from *Sm2* were in the quadrant 1. Samples from *S. pectoralis* were located between the quadrants 3-4 (Fig. 5).

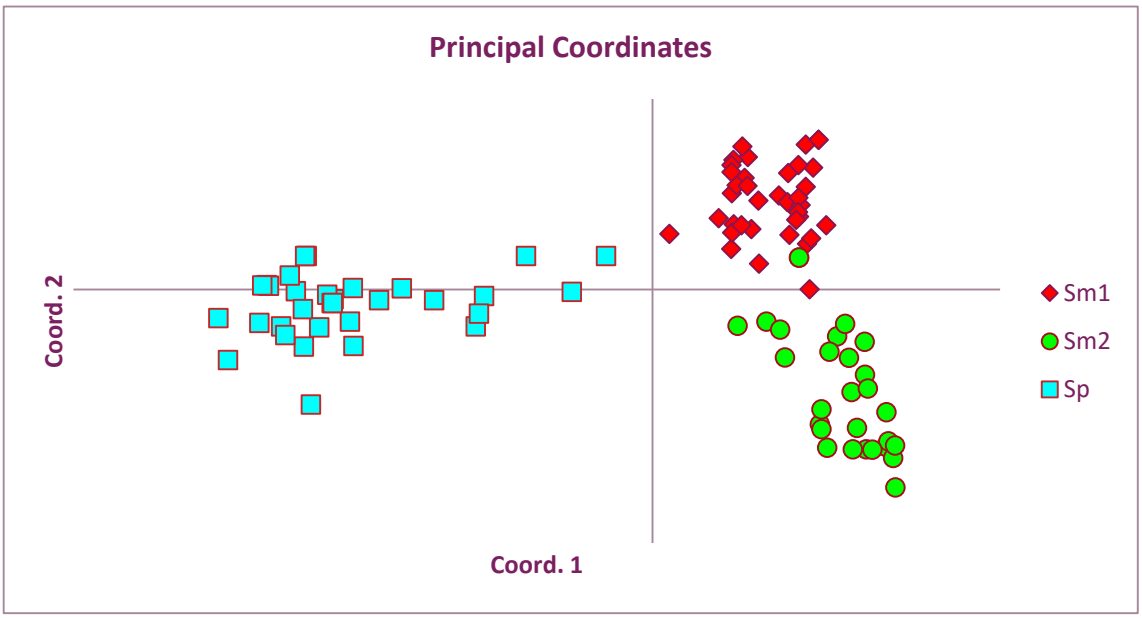

Figure 5. Distribution of *Scaptotrigona* samples based on the genetic distance from microsatellite data analyzed with principal coordinate analysis (PCoA).

For all samples, the number of scored alleles at the seven microsatellite loci varied from one (loci Mbi278 in *Sm1* and Mbi201 in *Sm2*) to 10 (lociB124 in *Sm1* and T4-171 in *S. pectoralis*). The average allele number within clusters varied from 3.86 (*Sm2*) to 4.71 (*Sm1*). Gene diversity measured as expected heterozygosity (He) ranged from 0.40 (*Sm2*) to 0.48 (*S. pectoralis*) on average (Table II). In *S. mexicana* clusters, the number of alleles per locus ranged from one (loci Mbi278 in *Sm1* and Mbi201 in *Sm2*) to eight (loci B124 in *Sm2*) and ten (loci B124 in *Sm1*). Both expected heterozygosity and averaged number of alleles were higher in *Sm1* (He=0.42; Na=4.71) than in *Sm2* (He=0.40; Na=3.86). Total number of private alleles was also higher in *Sm1* (13, average 1.857±0.769) than in *Sm2* (seven, average 1±0.535).

Table II. Microsatellite variation in *S. mexicana* from the Pacific (*Sm1*) and Atlantic (*Sm2*) coasts and *S. pectoralis* (*Sp*). Sample size (N), number of detected alleles (Na), observed (Ho) and expected (He) heterozygosity per locus, number of private alleles (Npa). Mean values ± SE are shown for each cluster.

Pop	Locus	N	Na	Ho	He	Npa
Sm1	Mbi254AAG	32	2	0.250	0.264	0
	Mbi259AAG	37	2	0.270	0.234	0
	Mbi278AAG	37	1	0.000	0.000	0
	Mbi201AAG	37	2	0.162	0.149	1
	T4-171	35	7	0.886	0.798	4
	T7-5	35	9	0.571	0.744	4
	B124	33	10	0.818	0.781	4
	Mean ± SE	35.143	4.714±1.443	0.423±0.129	0.424±0.128	1.857±0.769
Sm2	Mbi254AAG	24	2	0.458	0.499	0
	Mbi259AAG	28	3	0.250	0.223	0
	Mbi278AAG	28	2	0.357	0.375	1
	Mbi201AAG	28	1	0.000	0.000	0
	T4-171	23	4	0.217	0.400	0
	T7-5	25	7	0.920	0.768	3
	B124	28	8	0.429	0.554	3
	Mean ± SE	26.286	3.857±1.01	0.376±0.108	0.403±0.093	1±0.535
Sp	Mbi254AAG	24	5	0.458	0.608	3
	Mbi259AAG	33	6	0.576	0.751	3
	Mbi278AAG	33	3	0.455	0.413	2
	Mbi201AAG	30	3	0.100	0.096	2
	T4-171	28	10	0.929	0.848	8
	T7-5	26	2	0.308	0.311	0
	B124	33	3	0.030	0.340	0
	Mean ± SE	29.571	4.571±1.043	0.408±0.115	0.481±0.101	2.751±1.02

Fisher's exact test of population differentiation ($\chi 2$ = Infinity; df = 14; P = highly significant) showed highly significant genetic differences between all clusters. After applying Bonferroni correction for multiple tests, only *S. pectoralis* deviated significantly from the Hardy-Weinberg equilibrium, while the other two clusters, *Sm1* and *Sm2* were in equilibrium.

Pairwise Fst values between *Sm1* and *Sm2* was 0.233, which is an indicator of high differentiation. The comparison of these two clusters with *S. pectoralis* yielded similar values (0.323 with *Sm1* and 0.375 with *Sm2*).

Isolation by distance was evaluated with the Mantel test for each cluster and showed a significant correlation between genetic and geographic distance in *Sm2* (r = 0.221, P = 0.006) and *S. pectoralis* (r = 0.366, P = 0.0001) but showed no correlation in Sm1 (r = 0.004, P = 0.450).

4. DISCUSSION

Genetic and morphological analyses supported the hypothesis of two lineages within *S. mexicana* (*Sm1* and *Sm2*) and a lack of differentiation within *S. pectoralis.* The clusters found within *S. mexicana* corresponded to two evolutionary units with different distribution, one (*Sm1*) dispersed in the Pacific coast of Mexico and another one (*Sm2*) along the Atlantic Mexican coast to the North of Guatemala. Our outcomes supported previous results pointing to a cryptic species within *S. mexicana* based on a high divergence within *S. mexicana* populations by means of the barcoding method (Hurtado-Burillo et al., 2013). The results obtained here were congruent since both molecular and morphological approaches provided a similar resolution to discriminate between the two evolutionary units. This multidisciplinary approach using both molecular and morphological characteristics has been a useful tool in other studies in stingless bees species (Mendes et al., 2007; Gonçalves, 2010; Francoy et al., 2011; May-Itzá et al., 2012; Bonatti et al., 2014) and also in honey bees (but at the subspecies level Oleksa and Tofilski, 2015).

The differentiation between *Sm1* and *Sm2* has been demonstrated by several population parameters. First, the geometric mophometrics of the wings showed a significant phenetic differentiation resulting in two separate groups.

Secondly, although both lineages within *S. mexicana* shared alleles, a high number of private alleles in both the Pacific *Sm1* and the Atlantic *Sm2*, together with the differences in the allelic frequency of some of the loci indicate the genetic differences between these two evolutionary units. Even though microsatellites markers usually show less allelic diversity when are used in other species than those they have been designed for (Borges et al., 2010), in this study they have been amplified successfully yielding appropriate values for population analysis as in *S. mexicana* (Kraus et al., 2008; Mueller et al., 2012) or in the close species *S. hellwegeri* (Quezada-Euán et al., 2012).

Finally, Fst values (0.23) pointed to a great divergence within *S. mexicana* populations. This value is within the range of those previously reported for other stingless bees from *Scaptotrigona* and *Melipona* genera in studies establishing different units of management and populations with high level of differentiation: 0.113 in *S. xanthotricha* (Duarte et al., 2014) and 0.273 in *S. hellwegeri* (Quezada-Euán et al., 2012), 0.280 in *Melipona beecheii* (Quezada-Euán et al., 2007), 0.492 in *M. yucatanica* (May-Itzá et al., 2010), 0.10 in *M. scutellaris* (Viana et al,. 2013) and 0.210 and 0.250 in *M. rufiventris* populations (Tavares et al., 2007). According to Balloux and Lugon-Moulin (2002), this Fst value (between 0.150 and 0.250) is a sign of great differentiation within populations but is not a conclusive evidence of speciation. This Fst value could be a consequence of low gene flow due to the location of the sampled colonies but it is not conclusive of the total separation in two species. These two evolutionary units are separated by a geographical barrier, the Sierra Madre that may have influenced its distribution as in other Meliponini (May-Itzá et al., 2010, 2012), Hemiptera (Dorn et al., 2009), Coleoptera (Anducho-Reyes et al., 2008) and even terrestrial birds dispersed in the same area (Álvarez and Morrone, 2004; Yáñez-Ordóñez et al. 2008). Historical events may have shaped the distribution of the *Scaptotrigona* species in the Mesoamerican region: while some *S. mexicana* populations probably spread towards the Atlantic coast of Mexico (except Yucatan), other populations moved towards the Central American Pacific region to Costa Rica. In this stingless bee, speciation processes resulting from geographic isolation and different environmental conditions (Kerr 1960; Kerr and Maule 1964) may have contributed to the origin of two separate evolutionary units. The observed divergences between *S. mexicana* populations probably reflect more recent evolutionary processes, among them isolation by distance as detected within *Sm2* with molecular data. The lack of a significant relationship among geographic and phenetic distances suggests wing shape differentiation could be a non-neutral marker

affected by selective pressures (Reed and Frankham, 2001). On the other hand, the lack of IBD on *Sm1* can be explained by the close proximity of the colonies in contrast with the distribution of the *Sm2* colonies in two extremes of the distribution.

Moreover, *S. pectoralis* showed morphological and molecular homogeneity through its populations although they have an equivalent and overlapped distribution with *Sm1* and *Sm2*. This result could be explained by a wider niche breadth of the former species that allows it to colonize a high variety of environmental conditions from tropical dry forest in Yucatan (where *S. mexicana* is absent) to tropical rainforest, resulting in a lower influence of the geography barriers on the gene flow and a wider distribution of this species reaching Panama (Camargo and Pedro, 2013). Recent molecular results even expand its distribution up to Ecuador (Ruiz pers. comm) because in fact the species *S. barrocoloradensis* found there should be considered a subspecies of *S. pectoralis*, following the taxonomic criterion of Schwarz (1951). This homogeneity in molecular and morphological markers within *S. pectoralis* is congruent with previous molecular studies based on mtDNA (Hurtado-Burillo et al., 2013).

However, despite this lack of phylogeographic structure, microsatellites markers show signatures of recent isolation among *S. pectoralis* colonies (as in *Sm2*). Furthermore, *S. pectoralis* is the only species with deviation of H-W equilibrium, a result that can be due to the pooling of populations (Wahlund's effect) or inbreeding. Although this bee has become recently managed in some areas of Mexico (González-Acereto et al., 2006), our samples proceeded from feral colonies and the slight heterozygote deficit could have been probably caused by inbreeding due to habitat loss or fragmentation of areas. The loss of large natural areas in Mesoamerica (Brooks et al., 2002) due to different factors as deforestation has a huge impact in bee populations since it can lead to a forest isolated in small fragments (Brown and Albrecht, 2001), entailing populations decline and extinction with a subsequently loss of genetic diversity (Zayed, 2009). Also the swarming behavior of these bees (Nogueira-Neto, 1997) due to the short rate of dispersion and the dependence of new colonies from the mother colony (Engels and Imperatriz-Fonseca, 1990) influences the differentiation of the populations within species. This short dispersion rates could be an important factor for the existence of morphotypes in stingless bees (Ayala, 1999; Michener, 2007) and for the geographical isolations of populations by preventing the gene flow among them (Van Veen and Sommeijer, 2000; Melo, 2003).

In conclusion, the combined approach of genetic and morphological techniques indicates that *S. mexicana* colonies from both coasts are different evolutionary units. A needed requisite to establish a valid species affirmation is the congruence among several independent lines of evidence (Schlick-Steiner et al., 2010) therefore further studies including more data from more samples covering most of the distribution area of these two putative species and other aspects of their biology are pending to confirm their taxonomic status.

Although the number of colonies analyzed was not high, the results found in this study are enough to establish conservation measures (May-Itzá et al., 2010), so in the light of all the results the two lineages of *S. mexicana* must be treated as separate units in order to avoid the inbreeding and the disappearance of diversity. We also suggest the placement of meliponaries of *S. pectoralis* close to natural areas to promote outbreeding. Programs and strategies focused on maintaining the diversity of these bees must be created in order to preserve the genetic diversity.

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Chapter 3

Multilocus species delimitation in Mesoamerican stingless bees supports the existence of cryptic species in the genus *Scaptotrigona* (Apidae: Meliponini)

Multilocus species delimitation in Mesoamerican stingless bees supports the existence of cryptic species in the genus *Scaptotrigona* (Apidae: Meliponini)

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ABSTRACT

To protect the biodiversity properly is necessary to have an accurate knowledge of the species diversity and distribution. This is even more important in biodiversity hotspots as Mesoamerica. In this context, species delimitation in an objective way reducing investigator-driven bias is basic to establish right management strategies. Previous morphological and molecular studies on three stingless bee species of the genus *Scaptotrigona* distributed in Mexico (*S. mexicana*, *S. pectoralis* and *S. hellwegeri*) suggested that both *S. mexicana* and *S. helwegeri* are cryptic species complexes. Herein we tested species delimitation by analysing sequence information of five markers (two mitochondrial: cox1 and 16S, and three nuclear: ITS1, EF1-α, ArgK) within a Bayesian coalescent framework to confirm the support of the putative species. We obtain two different hypotheses using Generalized Mixed Yule Coalescent (GMYC) model: four (cox1) and six (16S) species. After the validation species step with the Bayesian species delimitation analysis (BPP), we propose a four species scenario confirming that *S. mexicana* is a complex of two species with different distribution (along the Pacific and the Atlantic coasts respectively). We highly recommend to manage them separately avoiding colony exchange in order to conserve both taxa.

Keywords: stingless bees / *Scaptotrigona* / multilocus / cryptic species / BPP / species delimitation

1. INTRODUCTION

To accelerate the taxonomic knowledge and an accurate identification of species are critically important points given the current biodiversity crisis, particularly in taxa that are relatively poorly studied, as well as in areas considered hotspots of biodiversity such as the tropical areas (Myers et al., 2000; Wheeler et al., 2004).

In the last decades, the discovery and delimitation of species are increasing due to the use of DNA-based methods (Knowles and Carstens, 2007). One of the first widely used approaches is to delimit species using a single gene tree what has been applied for a fast and large-scale assessment of species diversity (Lahaye et al., 2008; Papadopoulou et al., 2008; Monaghan et al., 2009; Fujisawa and Barraclough, 2013). However, this approach has been widely criticized because single-locus data represent more precisely the history of a single gene that might not be representative of the organism history, therefore producing misleading results. Consequently, there is a consensus for using different and independent genetic markers (mitochondrial and nuclear) for establishing confident phylogenetic relationships and for delimitating species (Knowles and Carstens, 2007; Galtier et al., 2009; Dupuis et al., 2012).

Several different approaches have been developed for inferring species trees from multilocus markers; however, some of them rely on the reciprocal monophyly criterion, and do not take into account the incongruence between markers. Recently, coalescent-based species delimitation methods have been developed to test alternative hypotheses of lineage divergence (Fujita et al., 2012). These driven-hypotheses methods have the potential to reduce investigator-driven bias in species delimitation.

Stingless bees (Apidae: Meliponini) are one of the key groups of tropical ecosystems (Roubik, 1989). This tribe is the most abundant bee group in the Neotropics, playing an essential role as extensive pollinators in natural and agricultural ecosystems, including high economic value crops as coffee, tomato or avocado (Roubik, 1989; Michener, 2007). In the last decades independent lines of evidence have pointed out to a global decline of pollinators (Biesmeijer et al., 2006; Brosi et al., 2008; Potts et al., 2010) including the tropical areas (Vamosi et al., 2006). Several studies have shown how human disturbance affects to the abundance, diversity and flower visitation rate

of stingless bees (Brown and Albrecht, 2001; Brosi et al., 2007, 2008; Ricketts et al., 2008; Brosi, 2009; Freitas et al., 2009). Despite its importance, there is a rough estimate of the diversity of stingless bees suggesting that they correspond to two thirds of the unknown native bee species in the Neotropics (Brown and Paxton, 2009; Freitas et al., 2009), including many cryptic species yet to be discovered (Michener, 2007). Therefore, attention to this group is necessary in order to get more insights into its biodiversity before it gets lost.

Previous morphological and molecular studies have detected distinct evolutionary lineages within stingless bees species (*Trigona collina*, Theeraapisakkun et al., 2010*; Melipona yucatanica*, May-Itzá et al., 2010; *M. beecheii*, Quezada-Euán et al., 2007; *M. subnitida*, Bonatti et al., 2014; *M. rufiventris,* Tavares et al., 2007; *S. xanthotricha,* Duarte et al., 2014), suggesting the existence of cryptic species (*Heterotrigona itama*, Rasmussen and Cameron, 2007) or revealing them (*M. rufiventris*, Melo, 2003; Tavares et al., 2007; *Liotrigona bitika,* Koch, 2010).

In this study, we have focused on three stingless bees of the genus *Scaptotrigona* distributed in Mexico: *S. mexicana* Guérin 1845, *S. hellwegeri* Friese 1900 and *S. pectoralis* Dalla Torre 1896. *S. mexicana* and *S. pectoralis* are jointly distributed from the Pacific coast of Chiapas state to the south of Veracruz State in the Atlantic coast of the Mexican Gulf. *S. mexicana* has a wider distribution across the neo-volcanic axis at the west and across the Sierra Madre Oriental at the north (Hidalgo, San Luis de Potosi and Tamaulipas States), while *S. pectoralis* reaches Yucatan peninsula, where *S. mexicana* is absent. *S. hellwegeri* is distributed across the Pacific coast, from Sierra Madre Sur to Sierra Madre Occidental mountain ranges reaching Sinaloa State. *S. mexicana* has been managed since pre-Hispanic Mesoamerican cultures, being nowadays of economic and cultural importance. Previous studies based on mitochondrial markers, microsatellites and morphometric analyses have led to hypothesize the existence of cryptic species within *S. mexicana* (Hurtado-Burillo et al., 2013, Chapter 2 of this thesis) and *S. hellwegeri,* (Quezada-Euán et al., 2012).

Herein we further test the existence of cryptic species among the *Scaptotrigona* species distributed in Mexico and northern Guatemala through a multilocus coalescent-based approach. We adopt the biological species concept, recognizing as potential species those groups that have not experienced recent gene flow (although not requiring other evidence of reproductive

isolation). Given that this approach reduces the subjective criteria of the researcher, we expect to clarify objectively the taxonomical status of the three *Scaptotrigona* species. Concretely our aim is to find out how many species are within *S. mexicana* and *S. hellwegeri* and to confirm the specific status of *S. pectoralis*. The results obtained here will provide the foundation for future conservation programs of such important pollinators.

2. MATERIALS AND METHODS

2.1. Sampling

Sampling covered almost the entire geographic range of each species within Mexico and northern Guatemala, including 121 specimens morphologically assigned to three *Scaptotrigona* species (67 *S. mexicana*, 35 *S. pectoralis* and 19 *S. hellwegeri*, Table I). Colonies used in this study were both feral and managed depending on their availability. Specimens were preserved in absolute ethanol at –20 ^{o}C in the laboratory of Zoology of the Veterinary Faculty (University of Murcia, Spain).

Table I. Details of sampling data of the analyzed *Scaptotrigona* species. Between brackets is detailed the number of meliponaries where the specimens were sampled.

Species	Sampling location	Number of colonies/meliponaries	Type of colonies
S. mexicana	Peten, Guatemala	11 (2)	Managed
S. mexicana	Tuxtla Chico, Chiapas, Mexico	23 (3)	Managed
S. mexicana	Tapachula, Chiapas, Mexico	8 (2)	Managed
S. mexicana	Cacahoatan, Chiapas, Mexico	8 (1)	Managed
S. mexicana	Coatapec, Veracruz, Mexico	6 (1)	Managed
S. mexicana	Coyulta, Veracruz, Mexico	8 (1)	Managed
S. mexicana	Cuetzalan, Puebla, Mexico	1 (1)	Managed
S. mexicana	Tuzamapan, Puebla, Mexico	1 (1)	Managed
S. mexicana	Chilcuahuta, Hidalgo, Mexico	1 (1)	Managed
S. pectoralis	Tapachula, Chiapas, Mexico	10 (1)	Feral
S. pectoralis	Tuxtla Chico, Chiapas, Mexico	9 (2)	Feral
S. pectoralis	Montes Azules, Chiapas, Mexico	3 (1)	Feral
S. pectoralis	Tlaltetela, Veracruz, Mexico	5 (1)	Feral
S. pectoralis	Yucatan, Mexico	8 (1)	Feral
S. hellwegeri	San Isidro, Jalisco, Mexico	5 (1)	Feral
S. hellwegeri	Guerrero, Mexico	10 (1)	Feral
S. hellwegeri	Nayarit, Mexico	2 (1)	Feral
S. hellwegeri	La Huerta, Jalisco, Mexico	1 (1)	Feral
S. hellwegeri	Sierra Quila, Jalisco, Mexico	1 (1)	Feral

2.2. Extraction and DNA amplification

Genomic DNA was obtained using two legs of each specimen with the DNeasy tissue kit (QIAGEN) following manufacturer instructions. Four DNA fragments were amplified corresponding to one ribosomal gene (16S, Ramírez et al., 2010) from the mitochondrial genome (mtDNA) and three protein-coding gene fragments (arginine kinase ArgK and elongation factor–1α (EF1-α) F2 copy, Kawakita et al., 2003, and the internal transcribed spacer 1 ITS1, Ji et al., 2003) from the nuclear genome. We also amplified the mitochondrial gene cytochrome oxidase I (cox1, Sheffield et al., 2009) from additional samples not included in the previous study (Hurtado-Burillo et al., 2013). PCR reactions were carried out in 12.5 µl volume in a PTC-200 Thermal Cycler (Biorad). Fragments of the ITS1 and cox1 marker were amplified with PureTaq TM Ready-To-Go TM PCR beads (GE Healthcare) whereas the remaining fragments were amplified using KAPA Biosystem enzyme.

All amplified PCR products were electrophoresed in 1.5 % agarose gels stained with GelRed nucleic acid stain and then purified with isopropanol and ammonium acetate. Sequencing was performed in both directions using standard protocols for ABI BigDye(r) Terminator v3.1 Cycle sequencing kit (Applied Biosystems). All the sequencing reactions were performed with the same primers at the SECUGEN sequencing company (S. L. Madrid, Spain). Sequences were deposited in GenBank (accession numbers in progress).

2.3. Sequence analyses

Available sequence data of the mitochondrial cox1 gene (Hurtado-Burillo et al., 2013) as well as outgroup sequences were included in the analyses. Two *Scaptotrigona* (*S. polysticta* and *S. sp* M102) and two *Trigona* species (*T. chanchamayoensis* and *T. sp* M109) (Genbank accession numbers shown in Table I in Supp. Mat.) were used as outgroups for the phylogenetic analysis.

DNA sequences of all markers were edited with MEGA 6 program (Tamura et al., 2007). Sequences were aligned with MAFFT (Katoh and Toh, 2008) using G-INS-i strategy.

We evaluated genetic variability and calculated the number of haplotypes or identical sequences, their diversity and the nucleotide diversity for each marker using DnaSP v 5 (Librado

and Rozas, 2009). Every analysis was carried out either considering gaps or not. For all markers we estimated the most appropriate substitution model using Akaike information criterion (AIC) in the program jModelTest (Posada, 2008).

To infer haplotype networks of mitochondrial and nuclear markers, we used a median-joining network analysis (Bandelt et al., 1999) implemented in PopART (http://popart.otago.ac.nz).

2.4. Phylogenetic analyses

Two Bayesian independent analyses with 10,000,000 generations were performed with MRBAYES 3.2.2. through the online platform, CIPRES Science Gateway (Miller et al., 2010). Converge between these two analyses was used as a parameter to estimate whether the sampling number of generations was adequate. The commands stoprule=yes and stopval=0.01 were used to stop the analysis when the average value of standard deviation at frequency division hit a value lower or equal to 0.01. Each Bayesian analysis was run either for each marker individually or concatenated (mitochondrial, nuclear and all datasets). Likelihood values were observed with Tracer v1.4 (Rambaut and Drummond, 2007), discarding all trees before stability in likelihood values as a burn-in.

2.5. Species delimitation analysis

Species delimitation was carried out following a two-stage approach (Leaché and Fujita, 2010). The first stage was the species discovery, where the samples were assigned to groups without *a priori* information. Then in a second stage this putative species hypotheses were subsequently validated using Bayesian species delimitation analysis (BPP).

2.5.1. Species discovery

In this stage, we followed three independent approaches: a) morphological identification of the specimens that were identified to currently accepted species based on the external morphological characters using taxonomic keys (Ayala, 1999), b) species hypothesis proposed in previous studies for *S. mexicana* (Hurtado-Burillo et al., 2013, Chapter 2 of this thesis) and *S. hellwegeri,* (Quezada-Euán et al., 2012) and c) species delimitation based on mtDNA data, fitting The Generalized Mixed Yule Coalescent (GMYC) model to the 16S and cox1 sequence data. This method has been proven robust and accurate as a tool for delimiting species when only single-locus information is available (Fujisawa and Barraclough, 2013). Both 16S and cox1 sequences were pruned to haplotypes with ALTER (Glez-Peña et al., 2010) before the analysis to avoid problems for GMYC caused by identical sequences (Monaghan et al., 2009). Ultrametric trees for both markers were obtained separately using BEAST version 1.8.2 (Drummond and Rambaut, 2007). The best fitting model of evolution were TrN+I+G for 16S and TrN+I for cox1. A coalescent (constant population size) tree prior and strict clock were used as both are considered a more adequate and conservative option for generating the tree (Monaghan et al., 2009; Ceccarelli et al., 2012; Powell, 2012). MCMC chains were run for 20 million generations. Resulting trees were pooled removing 25 % of samples as initial burn-in, and consensus trees were obtained in LogCombiner 1.8.2 and TreeAnnotator 1.8.2 (Drummond and Rambaut, 2007).

The GMYC species delimitation analysis (Pons et al., 2006; Monaghan et al., 2009) was done using the SPLITS version 1.0-19 package (Ezard et al., 2009) implemented in R statistical software (R Development Core Team, 2010) for the single threshold option (Fujisawa and Barraclough, 2013; Michonneau, 2015).

2.5.2. Species validation

The Bayesian species delimitation analysis, BPP version 3.1 (Yang, 2015) was used to validate species hypothesis proposed in the species discovery step. This software realized multispecies coalescent-based analyses accounting for incomplete lineage sorting and gene tree conflicts (Yang and Rannala, 2010, 2014; Rannala and Yang, 2013). BPP adopts the biological species concept assuming that gene flow is absent between sister lineages at the time of speciation. The software

uses reversible-jump MCMC to sample different species delimitation models and estimate the posterior probability of each model. It requires a guide tree that was taken from the *BEAST analysis after 30 million generation with a constant model ('Starbeast' Drummond et al., 2012) implemented in BEAST version 1.7.2. As BPP only estimates the posterior probability of all possible ways to collapse the nodes in the guide-tree into fewer species, the highest number of species hypothesis from the discovery step was tested. The new nearest-neighbour interchange algorithm (NNI) reduces the sensitivity of the method to guide-tree (Yang and Rannala, 2014). MtDNA signal can influence the subsequent coalescence species delimitation analyses (Burbrink and Guiher, 2015), therefore, we also tested the effect of inclusion of the mitochondrial phylogenetic signal in the species delimitation analyses.

The priors settings (θ ~ Gamma [1, 10]; τ ~ Gamma [1, 10]) reflect a relatively large ancestral population with deep divergences. These priors are expected to be more conservative generally favouring models with fewer species (Leaché and Fujita, 2010). To investigate the effects of θ and τ, we used alternative priors (θ ~ Gamma [2, 1000]; τ ~ Gamma [2, 2000]) which reflect a relatively small ancestral population and shallow divergences. Each analysis was run at least twice for 250,000 generations, discarding a burn-in of 10,000.

3. RESULTS

Alignment of the mitochondrial 16S fragment (obtained from 117 specimens) revealed two indels (509-511 bp) in *S. hellwegeri* and *S. pectoralis* while cox1 fragment (obtained from 89 specimens) showed no length variation (629 bp) suggesting the absence of nuclear copies (NUMTs) in contrast to other Meliponini species (Cristiano et al., 2012; Ruiz et al., 2013). Both nuclear ArgK (584 bp in 107 specimens) and EF1–α (728 bp in 95 specimens) fragments showed no length variation, whereas the ITS1 fragment sized from 755 to 773 bp (sequenced in 88 specimens) and showed two indels of 3 and 17 bp length in some *S. mexicana* populations. In total 94 out of 121 specimens (78 %) have amplified four or five loci.

The highest nucleotide and haplotype diversity were found in the mtDNA markers, concretely in cox1. In those markers there was almost no difference between haplotype diversity values

obtained with or without considering gaps. On the other hand, nuclear markers showed lower nucleotide and haplotype diversity values. In this case, a decrease of the haplotype diversity was observed when gaps were not considered (Table II).

Table II. Results of the analysis of genetic variability obtained with the different markers used. The values of the number of haplotypes and the haplotype diversity were calculated taken into account or not the gaps present in each marker.

Marker	Nucleotide diversity π	No of haplotypes (with gaps)	Haplotype diversity (with gaps)	No of haplotypes (no gaps)	Haplotype diversity (no gaps)
16S	0.01145	18	0.8748	11	0.8558
cox1	0.02059	20	0.9193	15	0.9104
ArgK	0.00492	18	0.8140	3	0.6041
ITS1	0.00238	7	0.7495	4	0.7351
EF1-α	0.00181	21	0.7451	6	0.3802

Networks obtained from both mtDNA markers showed a clear phylogeographic and more discriminant signal in contrast to nuclear markers. *S. mexicana* was split in two different groups herein named *Sm1* and *Sm2*, including specimens from the Pacific (*Sm1*) and the Atlantic (*Sm2*) coasts, with both mtDNA markers and the nuclear ITS1 (Fig. 1). Moreover, the two mitochondrial networks also showed a geographic structure in *S. hellwegeri* separating southern localities (*Sh1*) from the rest (*Sh2*). The ArgK network grouped the haplotypes per currently accepted species, whereas the EF1-α network clustered the haplotypes together without any clear geographic nor species separation (Fig. 1)

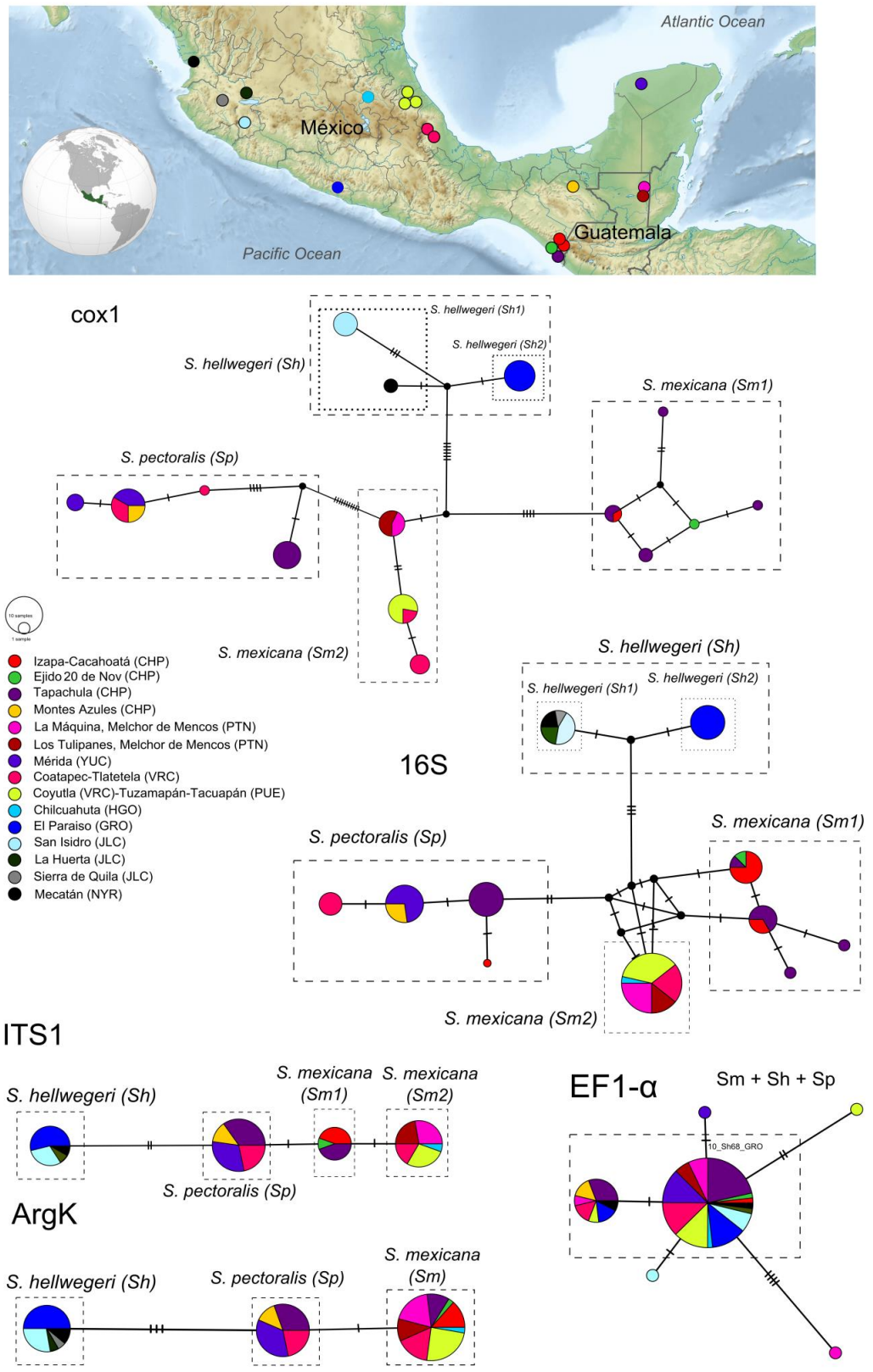


Figure 1. Sampling localities for the three *Scaptotrigona* species and median-joining networks for five markers (16S, cox1, EF1-α, ArgK and ITS1). Each sequenced haplotype is represented by a circle, the size of which is proportional to its overall frequency. Sampling localities are represented by colours on the map and on the networks.

3.1. Phylogenetic trees

Concatenated analyses of mitochondrial fragments showed all *Scaptotrigona* species recovered as monophyletic with high support (pp= 1.0) (Fig. 2a). The species *S. hellwegeri* and *S. pectoralis* were clustered as monophyletic (pp=0.99) in contrast to *S. mexicana* that was recovered in two clades. Individual analyses of each mtDNA marker resulted in a similar topology than concatenated analysis but with lower support (See Fig. 1 in Supp. Mat.).

On the other hand, concatenated nuclear markers resulted in a poorly resolved phylogeny (Fig. 2b). Individual analyses showed also poorly resolved trees, with just one supporting a monophyletic clade for *S. hellwegeri* with the ArgK marker (pp=0.99) (See Fig. 2 in Supp. Mat.). Concatenated analysis using the five markers resulted in a similar topology as the combined analysis of mtDNA markers (See Fig. 3 in Supp. Mat.).

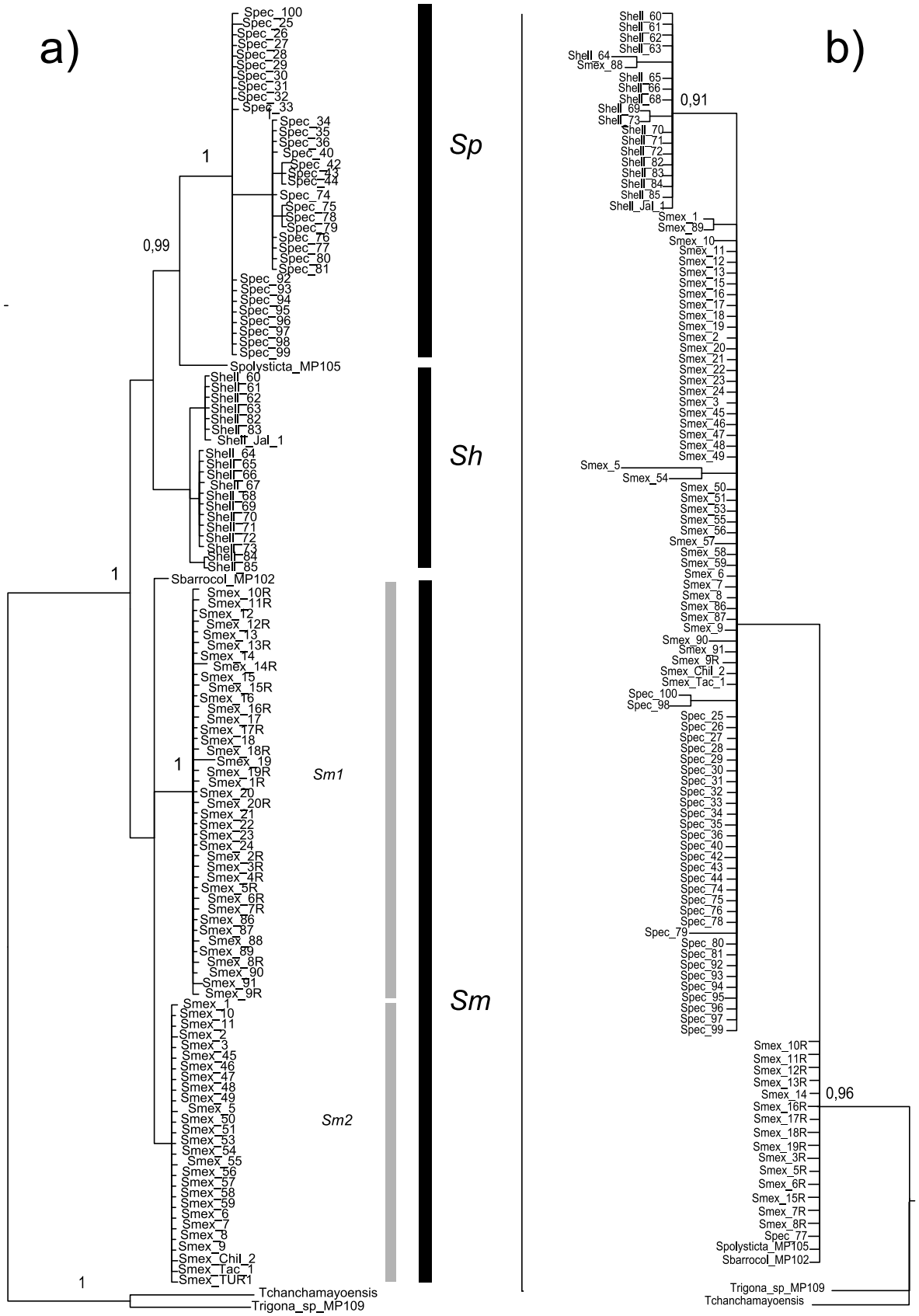


Figure 2. Bayesian phylogeny for the concatenated mtDNA (a) and nuclear (b) markers. Numbers on the nodes are posterior probability (pp) values. Bars and codes represent species hypothesis as explained in the text.

3.2. Species Delimitation

3.2.1. Species discovery step

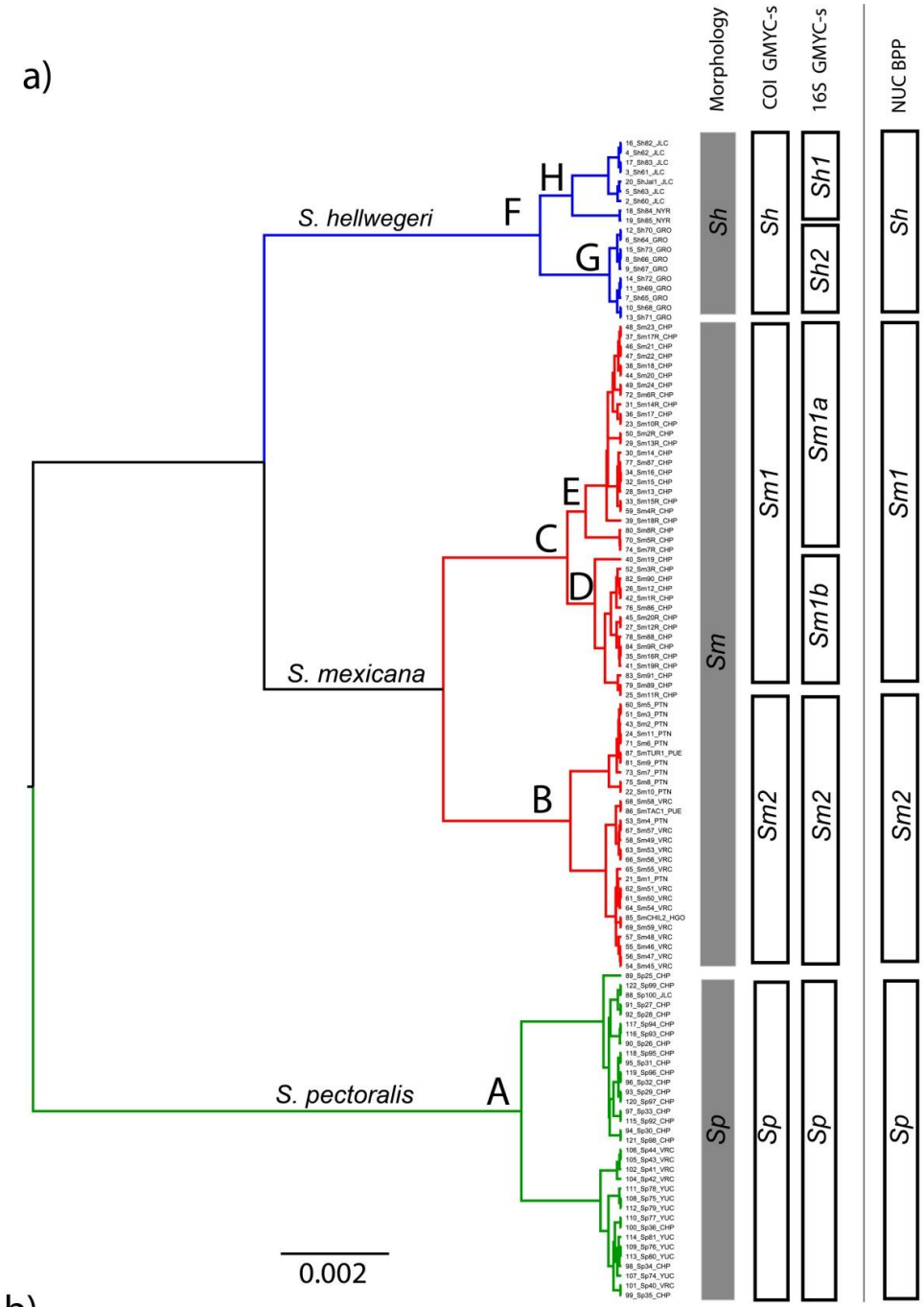
As a first step, we used a three species hypothesis: *S. mexicana* (*Sm*), *S. hellwegeri* (*Sh*) and *S. pectoralis* (*Sp*) following previous taxonomical identification of specimens based on external morphology (Fig. 2a). This contrast with a five species hypothesis (two in *S. mexicana*, two in *S. hellwegeri* and *S. pectoralis*) proposed by previous studies based on morphological and molecular data. However, species discovery based on mtDNA data resulted in two different species hypotheses: (1) GMYC analysis of cox1 sequences resulted in a four species hypothesis (*Sm1*, *Sm2*, *Sh* and *Sp*). *S. mexicana* was divided in two putative species: one including all Chiapas populations (*Sm1*) and another including the remaining eastern and northern populations (*Sm2*); (2) GMYC analysis with 16S data resulted in a six species hypothesis (*Sm1a*, S*m1b*, *Sm2*, *Sh1*, *Sh2* and *Sp*). In this case, *S. mexicana* populations from Chiapas were subdivided in two species (*Sm1a* and *Sm1b*) and *S. hellwegeri* was also subdivided in two putative species: one including northern populations from Jalisco and Nayarit (*Sh1*) and a second one including the population from Guerrero (*Sh2*).

3.2.2. Species validation step

When mtDNA and nuclear markers were combined, the resulting species delimitation was strongly influenced by the phylogenetic signal of the mtDNA markers since nuclear markers (ArgK, EF1-α and ITS1) contained less variable sites (13, 16 and 5 respectively) compared with the mtDNA markers 16S (20) and cox1 (42). Therefore, only the three nuclear markers were considered in the species validation step.

The BPP species delimitation results showed roughly agreement under different settings. Four lineages *Sm1*, *Sm2*, *Sh* and *Sp*; (nodes A, B, C and F, Fig. 3b) were recovered with high support (pp >0.95) for the models with more conservative speciation priors. However, the combination of a small ancestral population and shallow divergences resulted in lower support values (pp= 0.88 and 0.76) for lineages *Sm1* and *Sh* respectively. The species delimitation model with highest posterior probability was the four species model (pp=0.67) under both conservative and alternative priors. This model divided *S. mexicana* in two putative species: southern populations (*Sm1*) and central and northern populations (*Sm2*). The second best model was the five-species model (pp=0.32)

which included the aforementioned *Sm1* and *Sm2* species and two putative *S. hellwegeri* species (*Sh1* and *Sh2*). The six-species model had a marginal probability (pp=0.017).



b)

BPP

	A	B	C	D	E	F	G	H
(θ ~ Γ[1, 10]; τ ~ Γ [1, 10])	1	1	1	0	0	0.99	0.01	0.01
(θ ~ Γ[2, 1000]; τ ~ Γ [2, 2000])	1	1	0.88	0.11	0.11	0.76	0.23	0.23

Figure 3. *Scaptotrigona* species delimitation. a) Concatenated mtDNA tree with the proposed groups in the species discovery and species validation steps. b) Results from BPP under two different priors. Codes represent species hypotheses as explained in the text. Letters correspond to those nodes on the species tree and their pp values.

4. DISCUSSION

Herein the use of a two-stage approach in a multilocus species delimitation through species discovery and validation (Leaché and Fujita, 2010) has increased the knowledge of the taxonomy of the *Scaptotrigona* species distributed in Mesoamerica, although with some considerations. The phylogenetic analysis based on the nuclear markers failed to identify some of the mtDNA clades as monophyletic due to its low phylogenetic signal. Several species delimitation methods also rely upon genetic distances or gene tree monophyly to demark species boundaries. However, a two-stage approach of multilocus coalescence-based species delimitation, do not require reciprocal monophyly of all alleles or fixed differences, allowing to successfully demark species boundaries. Previous studies have shown using simulations that coalescent-based methods have sufficient discrimination power even with small datasets (Yang and Rannala, 2010; Zhang et al., 2011). Our results support that BPP can have enough discriminatory power for reliable species delimitation in Mexican *Scaptotrigona* even using markers that resulted in a lack of monophyly and showed low phylogenetic signal.

In this sense, it must be highlighted the importance of considering the variation of all the markers when they are combined in a multilocus species delimitation. Most of the multilocus species delimitation studies, as this one, combine one or two mitochondrial markers with various nuclear markers (Carstens et al., 2013). When the number of nuclear markers is low, one or few nuclear loci rarely equals the phylogenetic signal of a mitochondrial marker, therefore the species delimitation can be biased by the mitochondrial signal (Burbrink and Guiher, 2015). This must be considered as is known that mitochondrial genome has several issues as introgression (McGuire et al., 2007), sex-biased dispersal (Dávalos and Russell, 2014), NUMTs (Cristiano et al., 2012; Ruiz et al., 2013) or deep divergences without any barrier to gene flow (Irwin, 2002), what can generates misleading results in species delimitation (Funk and Omland, 2003; Petit and Excoffier, 2009).

These results represent a definitive molecular evidence for the taxonomic revision of the *Scaptotrigona* species and pointed to a four-species model, suggesting the existence of a cryptic species within *S. mexicana* and rejecting the split of *S. hellwegeri* populations in two different species. In this regards, our results support previous studies that found significant genetic intraspecific diversification in *S. mexicana* based on barcoding (Hurtado-Burillo et al., 2013) and on

microsatellite and morphometric analyses (Hurtado-Burillo et al., Chapter 2 of this thesis). BPP analysis also divided *S. mexicana* populations in two distinct species, in concrete Chiapas populations (*Sm1*) are significantly differentiated from northern populations (*Sm2*), and they should be considered as a distinct species. In order to describe a new *Scaptotrigona* species further sampling of populations across its entire geographic range, additional markers and independent species delimitation methods in an integrative taxonomy approach (Padial et al., 2010) are needed.

On the other hand, the five-model species that divided *S. hellwegeri* in two taxa was not corroborated by the species delimitation step (nodes H and G, pp=0.01/0.23 in Fig. 3b), although it was proposed as a species hypothesis model in the species discovery step (GMYC of 16S, Fig. 3a). This result did not support the consideration as distinct species of the two genetic lineages observed within *S. hellwegeri* found by Quezada-Euán et al. (2012) using morphometric and microsatellites analyses although we could be witnessing an event of incipient speciation. In relation to *S. pectoralis* all species delimitation analyses have confirmed its specific status in spite of the lack of monophyly for the nuclear markers.

Although previous studies have suggested the existence of cryptic species in Meliponini bees (Melo, 2003, Tavares et al., 2007; Koch, 2010) this is the first study to our knowledge, that objectively and explicitly test the existence of a cryptic species. This is an important approach for a complete and accurate knowledge of the species and population status given the present biodiversity loss in tropical ecosystems, in order to develop right conservation and management measures. The Neotropics harbor one of the largest proportions of biodiversity in the world and are an area with an intense development (Mittermeier et al., 1998). This development entails high anthropogenic impacts that together with extant climate change may pose a high risk of extinction of their taxa (Ricketts et al., 2005; Vamosi and Vamosi, 2008; Bradshaw et al., 2008) Therefore, the identification and delimitation of cryptic species following a highly reproducible and objective method, as the one followed in this study, give support for a proper evaluation of the genetic diversity and for implementing accurate conservation plans (Bickford et al., 2007; Martin et al., 2013; Blair et al., 2015). These programs must be focused on the conservation of natural habitats and on the improvement of the meliponiculture by providing beekeepers with techniques to manage their colonies in an efficient and respectful way.

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SUPPLEMENTARY MATERIAL

Table I. GenBank accession numbers of the sequences obtained in this study. Those corresponding to Meliponini species used as outgroups are also included.

Species	16S	cox1	ArgK	EF	ITS1
Sm1	In progress	JQ783140.1-JQ783146.1 & JQ783150.1	In progress	In progress	In progress
Sm2	In progress	JQ783139.1 & JQ783147.1-JQ783149.1	In progress	In progress	In progress
S. pectoralis	In progress	JQ783151.1-JQ783157.1	In progress	In progress	In progress
S. hellwegeri	In progress	JQ783136.1-JQ783138.1	In progress	In progress	In progress
S. polysticta	EU162934.1	EU163101.1	EU163015.1	EU163185.1	None
S. sp. M102	EU162931.1	None	EU163018.1	EU163182.1	None
T. chanchamayoensis	EU049698.1	KC853315.1	EU049748.1	EU049769.1	None
T. sp. M109	EU162938.1	EU163105.1	EU163022.1	EU163189.1	None

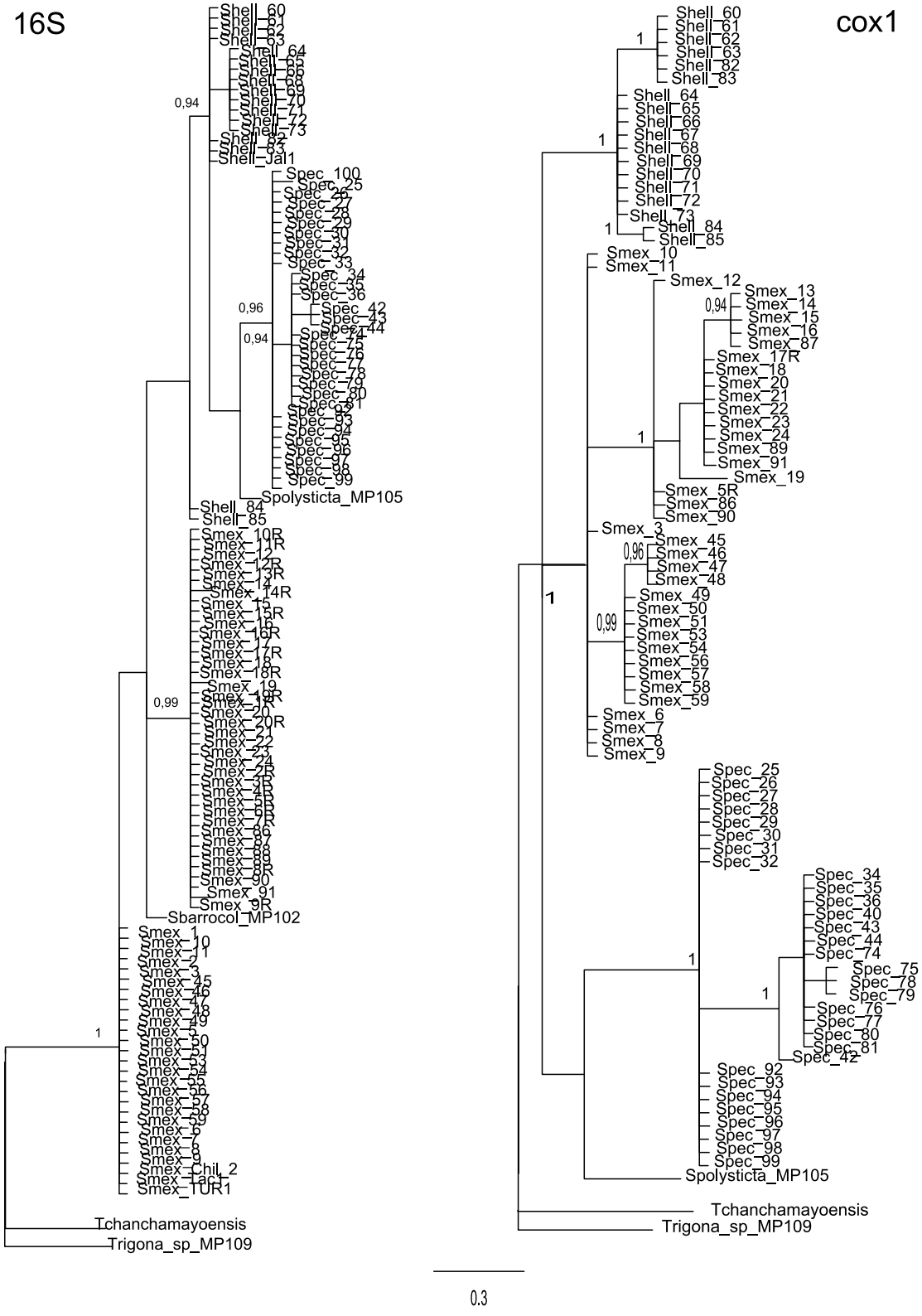


Figure 1. Bayesian phylogeny of single mitochondrial markers (16S and cox1). Any posterior probability value lower than 0.9 was removed.

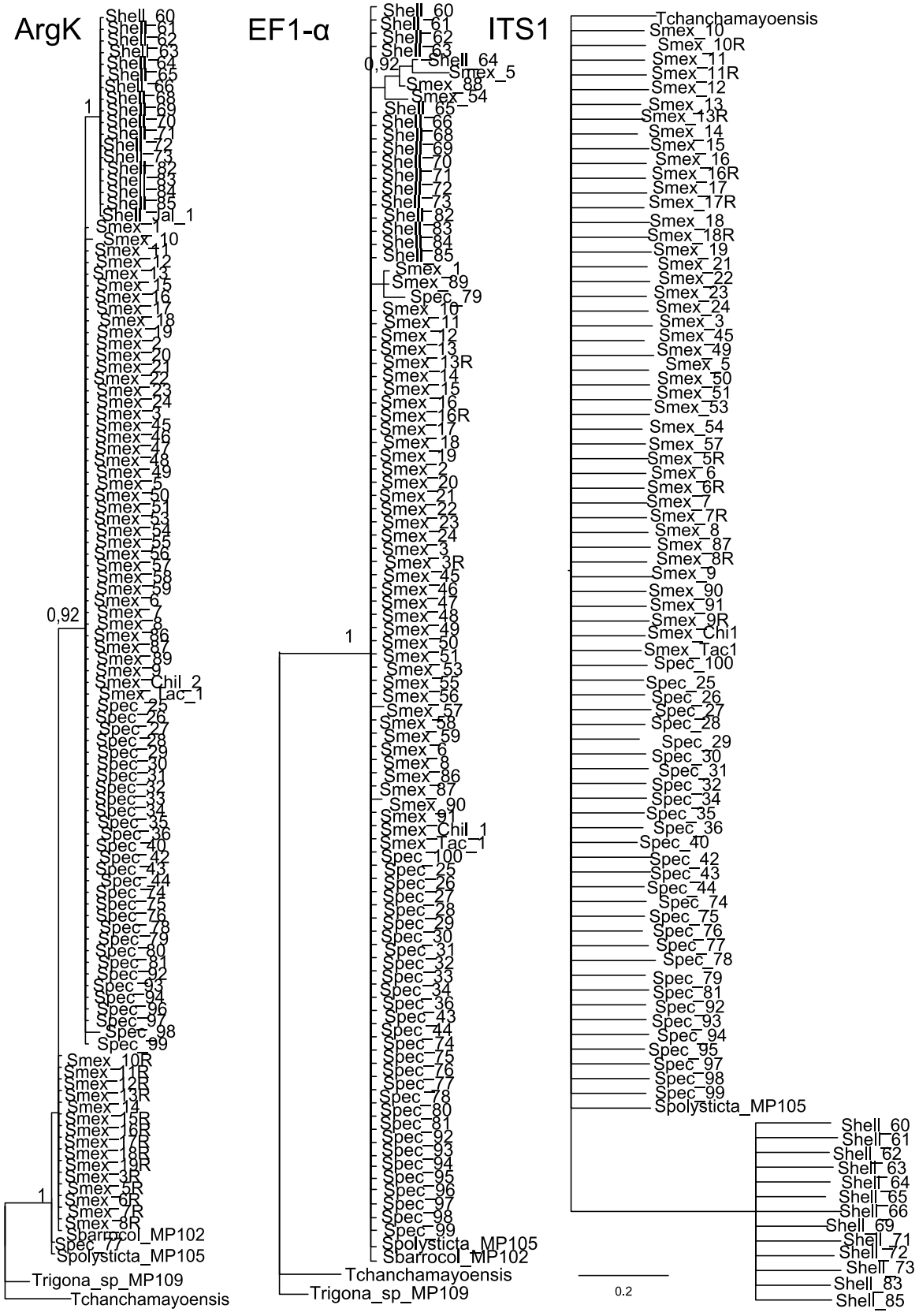


Figure 2. Bayesian phylogeny of single nuclear markers (ArgK, EF1-α and ITS1). Any posterior probability value lower than 0.9 was removed.

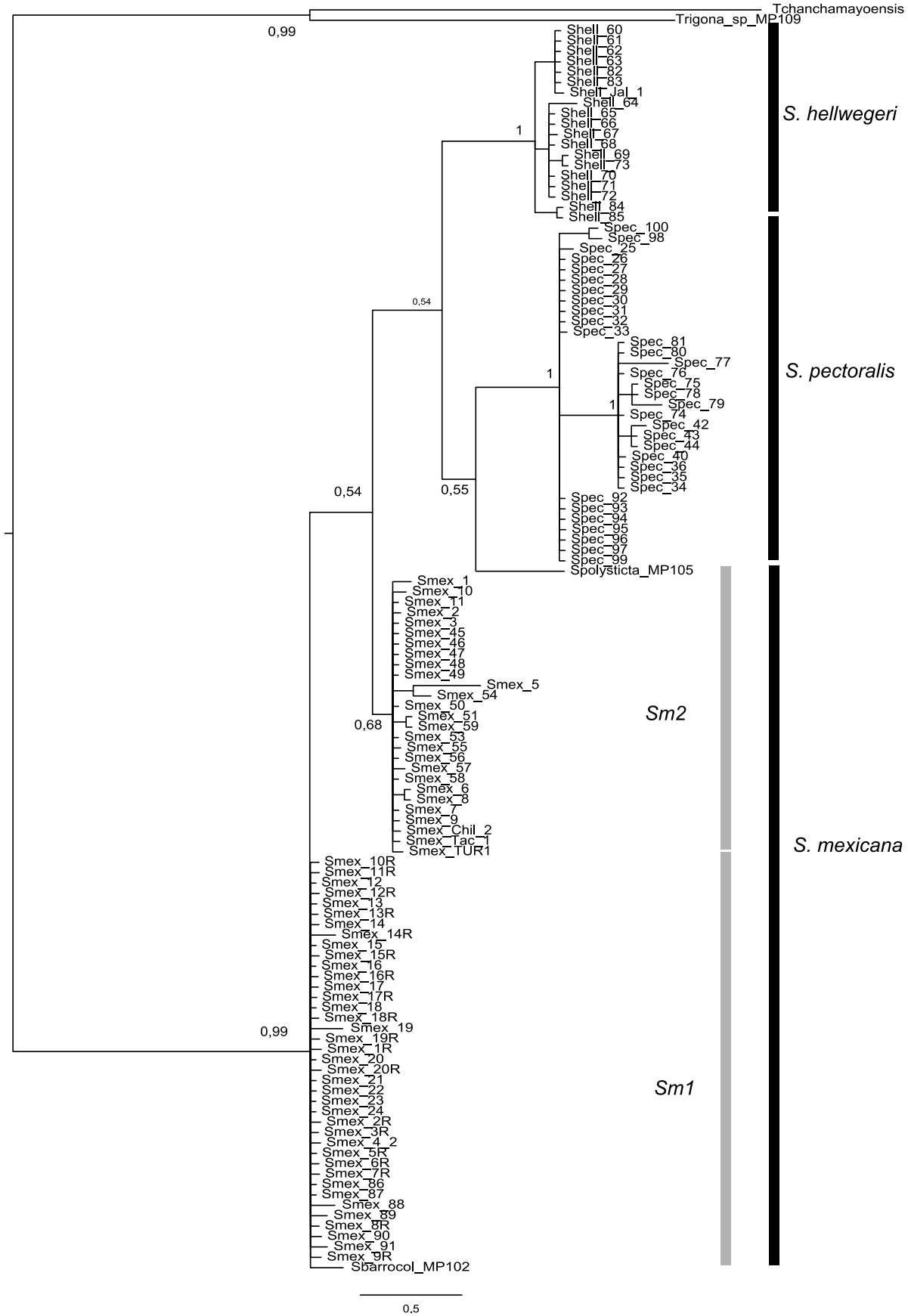
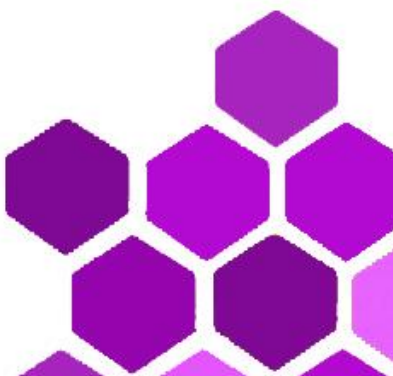


Figure 3. Bayesian phylogeny of the concatenated mitochondrial and nuclear markers. Any posterior probability value lower than 0.9 was removed.

Conclusions



GENERAL CONCLUSIONS

This thesis has explored the pattern of differentiation within the species *S. hellwegeri*, *S. mexicana* and *S. pectoralis* distributed in Mexico and northern Guatemala. Our results of the geometric morphometrics and molecular analyses have pointed the existence of cryptic lineages within this species. The genus *Scaptotrigona* is more diverse than expected, but different aspects of its diversity, distribution and biology remain to be studied. Furthermore, this work has shown the impact of the management on these bees. Therefore, we recommend establishing programs to conserve the biodiversity of Mexican stingless bees to improve and help the ancient practice of the meliponiculture.

Chapter 1. Barcoding stingless bees: genetic diversity of the economically important genus *Scaptotrigona* in Mesoamerica

1. DNA barcoding is a quick and easy technique that provides trustful species identification of stingless bee genus *Scaptotrigona* distributed in Mexico.
2. This tool is also efficient in the reassignment of misidentified individuals to the species they belong to.
3. The intraspecific divergence in *S. mexicana* (1.90 %) exceedes the limit of intraspecific variation (1.58 %) suggesting the existence of cryptic species or genetic lineages within *S. mexicana.*
4. The phylogeny supports the morphological hypothesis stating that *S. mexicana* is evolutionarily closer to *S. hellwegeri* than to *S. pectoralis.*
5. Conservation programs to protect their habitats should be implemented to protect the biodiversity of the genus. Specifically, strategies for avoiding the exchange of *S. mexicana* colonies among coasts should be also developed.

Chapter 2. Shedding light on the biodiversity of Mesoamerican stingless bees: geometric morphometrics and microsatellite analyses of *Scaptotrigona mexicana* and *S. pectoralis* (Apidae: Meliponini)

6. Molecular and morphological approaches provided a similar resolution to discriminate between evolutionary units.
7. Both tools supported the hypothesis of different evolutionary units within *S. mexicana* from Pacific (*Sm1*) and Atlantic (*Sm2*) coasts, but no differentiation within *S. pectoralis*.
8. Despite of the support for the existence of different evolutionary units in *S. mexicana* our results are not conclusive of an speciation event. Even though, we suggest treating them as separate units.
9. Management policies must be developed to maintain the diversity within the two lineages of *S. mexicana*.
10. We recommend to place meliponaries with *S. pectoralis* colonies near feral areas to increase the genetic diversity and promote outbreeding.

Chapter 3. Multilocus species delimitation in Mesoamerican stingless bees supports the existence of cryptic species in the genus *Scaptotrigona* (Apidae: Meliponini)

11. The multilocus coalescence-based analysis has suggested the existence of four species within the Mexican *Scaptotrigona.*
12. Bayesian species delimitation analysis has good resolution to delimitate species even in datasets with low signal.
13. The divergences within *S. hellwegeri* are not enough for consider two species but, on the other hand, there is enough genetic divergence to point the existence of cryptic lineages in *S. mexicana*.
14. Further analysis with samples from the entire distribution in Mexico and Central America must be done to confirm the presence of two species.
15. The population of *S. mexicana* from Chiapas must be treated as a different species to prevent the gene flow.

16. We recommend to establish conservation strategies regulating beekeeping practices and enhancing pollinator-friendly habitats.

Annexes

ANNEXE I: CURRICULUM VITAE

CURRICULUM VITAE

Miguel Hurtado Burillo

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Date of birth: 02/01/1986 – Spanish nationality – Full driver's license

EDUCATION

- **Master Degree in Teaching Compulsory Secondary and Pre-university Education (Biology and Geology)**
  International University of La Rioja, Spain, 2013-2014

- **Master Degree in Biodiversity Management in Mediterranean Environments**
  University of Murcia, Murcia Spain, 2009-2010

- **Degree in Environmental Sciences (5-year Science degree)**
  University of Murcia, Murcia, Spain, 2004-2009

PUBLICATIONS

- "Estudio de microsatélites en las abejas sin aguijón *Melipona colimana* y *M. beecheii* de Mesoamérica"
  Hurtado-Burillo Miguel, Martínez Jacinto, May-Itzá William de Jesús, Quezada-Euán José Javier G., De la Rúa Pilar. Archivos de Zootecnia, 63, 145-151. 2014

- "Barcoding stingless bees: genetic diversity of the economically important genus *Scaptotrigona* in Mesoamerica"
  Hurtado-Burillo Miguel, Ruiz Carlos, May-Itzá William de Jesús, Quezada-Euán José Javier G., De la Rúa Pilar. Apidologie, 44, 1–10. 2013

- "Phylogenetic analysis of stingless bees from genus *Scaptotrigona* using COX1 and ITS1 markers"
  Hurtado-Burillo Miguel, Ruiz Carlos, May-Itzá William de Jesús, Quezada-Euán José Javier G., De la Rúa Pilar. Memorias del VII Seminario Mesoamericano sobre Abejas Nativas. Cuetzalan (México). pp. 123-126. 2011

CONGRESS PARTICIPATION

- "Effectiveness of molecular markers for the phylogeny of the Mesoamerican stingless bee genus *Scaptotrigona"* for **Eurbee 6**, Murcia (Spain). September 2014.

- "Characterization of plant phenolic metabolites in honeybee (*A. mellifera*) hives (honey, beewax, pollen and propolis) treated and untreated against *Varroa destructor*" for **Apimondia**, Kiev (Ucrania). September 2013.

- "Setting up of toxicity experiments of botanical extracts and essential oils to treat *Apis mellifera* against *Varroa destructor*" for **II Iberian Beekeeping Congress**, Guadalajara (Spain). October 2012.

- "Setting up of tests for acaricidal effect of botanical extracts against *Varroa destructor*" for **Eurbee 5**, Halle (Germany). September 2012

- "Barcoding and phylogeny of stingless bees genus *Scaptotrigona* of Mesoamerica" for **I Iberian Animal Systematic Congress (CISA)**, Madrid (Spain). January 2012.

- "Hymenoptera pollinators identification through DNA barcoding" for **III Congress of the Evolutionary Biology Spanish Society**, Madrid (Spain). November 2011

- "Phylogenetic analysis of stingless bee genus *Scaptotrigona* using COX1 and ITS1 markers" for **VII Mesoamerican Seminary about native bees**, Puebla (Mexico). May 2011

- "Biodiversity analyses genus *Scaptotrigona* in Mexico through barcoding" for **I Workshop "Introduction to Research"** for Biology students, Murcia (Spain). May 2011

- "DNA barcoding reveals genetic variation within three Neotropical stingless bee species (genus *Scaptotrigona*)" for **Eurbee 4**, Ankara (Turkey). September 2010

PROFESSIONAL EXPERIENCE

Process group chemistry at Quidel Corporation (San Diego, CA, USA)
11/10/2014 – 01/28/2015

- Chemistry manufacturing
- Tests running
- Buffer fabrication
- Spectrometer usage
- Pipetting
- Use of pH meter, balance, hand tools, misc. lab equipment

Researcher under the BEEDOC project at CEBAS-CSIC (Murcia, Spain)
04/2012 – 02/2013

- Sampling bees and mites
- Setting up experiments to treat *Apis mellifera* against *Varroa destructor*
- Characterization of plant phenolic metabolites in honeybee

OTHER PROFESSIONAL EXPERIENCES

- 2014. Member of the Organizing Committee of the 6th European Conference of Apidology (EURBEE) at the University of Murcia

- 2012. Course “Phylogenies and Genealogies of DNA: Reconstruction and Applications”. University of Barcelona.

- 2012. Seminar “Biological control of plagues. Learning from Nature to get healthier foods”. Guest speaker. “Pollinators in agriculture: benefits and threats”

- 2012. Seminar about auxiliary fauna at Iberian Southeast. Guest speaker. “Honey bee (*Apis mellifera*), a millennialally of agriculture”

- 2012. Member of the Organizing Committee of the Workshop “Honey bee genetics” at the University of Murcia

- 2011- 2014. Collaborator in theoretical and practical lessons:
  - “Molecular Ecology Techniques” at Environmental Science´ Degree (lab practicum and seminars)
  - “Beekeeping” at Veterinary Medicine´ Degree (practical field work in the apiary and seminars)

- 2010- Currently. Translator of abstracts from English to Spanish in the *Journal of Apicultural Research*

- 2010. Research grant project “FP00-274 PHYLOGENY AND ANIMAL EVOLUTION”

- 2010. Participation in Research Projects:
  - MUTUAL “Mutualisms and bees in tropical countries: risks and rescue for biodiversity and agriculture” (4293 “Fondo de Cooperación Internacional en Ciencia y Tecnología UE – Mexico” (FONCICYT)
  - “Conservation of stingless bees in Mexico (Hymenoptera: Meliponini): identification of cryptic species and genetic diversity indicators” (SEP CONACYT 103341-Z).

- 2010. Curricular practices in the Regional Park of Sierra Espuña (Murcia, Spain).

PROFESSIONAL SKILLS AND COMPETENCES

- Languages:
  - Spanish (mother tongue),
  - English (Official School of Languages, Advanced Level),
  - Valencian (Junta Qualificadora de Coneixements de València, Intermediate level).
- High user level computer skills: Good command of Microsoft Office™ tools (Word™, Excel™ and PowerPoint™) and Adobe PhotoShopTM.
- Intermediate-high level specific software skills: MEGA, MrBAYES, PAUP, Beast, Bioedit, Model Test, Seqman, DNAsp, FigTree, TaxonDNA, Tracer, LaserGene DNAstar, Arlequin, GenAlex, MorphoJ, TpsDig.
- Phylogenetic and population genetics analyses
- DNA extraction and purification
- PCR technique
- Electrophoresis in acrylamide and agarose gels, ethidium bromide stains
- DNA sequencing and sequence editing
- Microsatellites
- Geometric morphometrics
- Sampling bees and mites

- Good Manufacturing Practices (GMP)
- Adaptability to work in an international environment. Motivation, responsibility, efficiency and diligence.
- Equipment used:
  - Micropipetes (simple and multichannel)
  - Optic microscope
  - Stereomicroscope
  - Thermo cycler
  - Autoclave
  - Gel documentation
  - Heather
  - Centrifuge
  - Magnetic agitator
  - Quantifier of DNA (Nanodrop)

ANNEXE II: ARTICLE AUTHOR

ESTUDIO DE MICROSATÉLITES EN LAS ABEJAS SIN AGUIJÓN *MELIPONA COLIMANA* Y *M. BEECHEII* DE MESOAMÉRICA

STUDY OF MICROSATELLITE MARKERS IN STINGLESS BEES *MELIPONA COLIMANA* AND *M. BEECHEII* FROM MESOAMERICA

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PALABRAS CLAVE ADICIONALES

Diversidad genética. Deforestación. Meliponicultura. México. Cuba.

ADDITIONAL KEYWORDS

Genetic diversity. Deforestation. Meliponiculture. Mexico. Cuba.

RESUMEN

En este estudio, se ha analizado por primera vez la variabilidad de los *loci* de microsatélites en la especie silvestre de abejas sin aguijón *Melipona colimana*. Los datos obtenidos se han comparado con los de una población insular de la especie domesticada *M. beecheii*. Los resultados del genotipado de los individuos de *M. colimana*, indican que la secuencia de los *loci* de microsatélites está conservada dentro del género *Melipona*. Los parámetros poblacionales estudiados (número de alelos y valores de heterocigosidad) no mostraron diferencias significativas entre las dos especies estudiadas, estando ambos dentro del rango observado en otras especies del género. Estos valores fueron inferiores en la población insular de *M. beecheii* con respecto a otras poblaciones continentales de la misma especie analizadas previamente, lo cual coincide con lo observado en estudios previos de otros organismos insulares. La especie silvestre *M. colimana* es susceptible a los efectos de la deforestación, por ello se han comparado parámetros poblacionales de colmenas situadas en una zona deforestada y en otra conservada. La diversidad poblacional no ha mostrado diferencias significativas, probablemente debido a que las perturbaciones del medio estudiado son recientes y aún no se han reflejado en la diversidad genética de estos insectos.

SUMMARY

In this study, variability of microsatellite *loci* has been for the first time analyzed in the wild stingless bee *Melipona colimana*. Data have been compared with those obtained from an insular population of the managed species *M. beecheii*. Genotyping results in *M. colimana* demonstrate that microsatellite *loci* sequences are conserved within the genus *Melipona*. Population parameters such as number of alleles and heterocigosity values, were not significantly different between the two studied species, both being within the observed range in other *Melipona* species. Values of the insular *M. beecheii* population were lower than in previously studied continental populations of the same species, in agreement with the results obtained in other island organisms. Wild *M. colimana* colonies are susceptible to deforestation effects, so that, we have compared the genetic parameters of colonies from a deforested area with those of a conserved area. Population diversity was not significantly different, may be due to recent environmental perturbations that are not yet reflected in the genetic diversity of these insects.

INTRODUCCIÓN

Las abejas sin aguijón son insectos eusociales (Apidae: Meliponini) que se distribuyen por las zonas tropicales del plane-

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ta, conociéndose alrededor de 500 especies de 42 géneros diferentes (Michener, 2007; Camargo y Pedro, 2008). El género *Melipona* abarca unas 40 especies presentes en la zona tropical americana (Michener, 2007), entre las que se encuentran *Melipona colimana* Ayala, 1999 y *M. beecheii* Bennett, 1831. *M. colimana* es endémica de la provincia mexicana de Jalisco, aparece en bosques de pino-encino a más de 1000 metros de altitud, en donde habita en los troncos de los árboles y no es manejada para la producción de miel (Quezada-Euán, 2005). Sin embargo, *M. beecheii* es el meliponino más común en México con una amplia distribución a lo largo de las dos costas y la península de Yucatán, así como en Belice, Cuba y Jamaica, aunque su distribución por el Caribe podría estar influida por su uso en la meliponicultura. A diferencia de *M. colimana*, esta especie es explotada desde épocas precolombinas para la producción de miel, cera y polen, siendo manejadas en jobones o cajas especialmente diseñadas para ello.

Las abejas sin aguijón juegan un papel esencial en la producción primaria de los ecosistemas al ser polinizadoras de multitud de especies vegetales (Roubik, 1989). Su importancia ecológica radica también en la especificidad que presentan determinadas plantas para ser polinizadas por ellas: aproximadamente un 33% de las plantas de selvas tropicales son exclusivamente visitadas por estas abejas (Wilms *et al*., 1996). Además son efectivas polinizadoras en invernaderos (Kakutani *et al*., 1993). A causa de su necesidad de árboles maduros, vivos y huecos, para hacer sus nidos (González-Acereto, 2008), de su reducida capacidad de dispersión, debida a la corta distancia de vuelo de las obreras y el escaso movimiento de sus enjambres (Imperatriz-Fonseca y Engels, 1990; Roubik, 2006), las especies silvestres son especialmente susceptibles de extinción en zonas sometidas a amenazas de origen antrópico como la fragmentación, destrucción y degradación de los hábitats, el uso de herbicidas y pesticidas que reducen la disponibilidad de plantas silvestres y con ello su fuente de alimento (Kerr *et al*., 2001). Por ello, es fundamental evaluar la diversidad genética de las poblaciones ya que su disminución conlleva una reducción del potencial adaptativo de las especies. Entre las herramientas moleculares, los microsatélites han sido usados en estudios de genética de poblaciones de abejas sin aguijón (Borges *et al*., 2010; Quezada-Euán *et al*., 2007, 2012). Lopes *et al*. (2010) demostraron que el uso de microsatélites específicos dio una mayor frecuencia de polimorfismo y de alelos por locus en dos especies del género *Melipona* que al usar marcadores diseñados para otras especies del mismo género. Igualmente Viana *et al*. (2011) pusieron en marca el análisis de microsatélites en cuatro especies de *Melipona* usando cebadores diseñados para otras especies del mismo género.

El objetivo del presente estudio es poner en marcha el análisis de marcadores microsatélites en la especie silvestre *M. colimana*. Para probar su eficacia se compararon los resultados con los obtenidos en la especie manejada *M. beecheii*. De esta especie ya se tienen datos sobre la variabili-

Tabla I. *Información sobre los muestreos de* M. colimana *y* M. beecheii. (Sampling information of *M. colimana* and *M. beecheii*).

Especie	Localidad	Colonias
M colimana		
	Tecalitlán (Jalisco)	24
	San Isidro (Jalisco)	6
	Total	30
M beecheii		
	Nueva Paz	10
	San Nicolás	4
	San José	8
	Jaruco	4
	Consolación Sur	2
	Jagüey Grande	2
	Total	30

dad de los mismos *loci* de microsatélites en poblacionales continentales (Quezada-Euán *et al*., 2007) por lo que se ha usado como referencia. Una vez que se constató la efectividad de estos marcadores en la especie, se compararon parámetros poblacionales obtenidos en colonias de *M. colimana* situadas en una zona recientemente deforestada con los de colonias de una zona conservada, para evaluar el efecto de dicha actuación en la diversidad genética de esta especie.

MATERIAL Y MÉTODOS

Se han estudiado un total de 60 colonias silvestres de *M. colimana* de dos áreas del estado de Jalisco, una conservada (Tecalitlán) y la otra deforestada (San Isidro), más otras 30 colonias manejadas de *M. beecheii* de Cuba **(tabla I, figura 1)**. *M. colimana* presenta una distribución restringida a áreas específicas de la provincia biogeográfica del Eje Volcánico Transversal (Yañez-Ordoñez, 2008) por lo que es difícil encontrar colonias de esta especie. Sin embargo, *M. beecheii* es la especie más común de su género en Mesoamérica y tiene una amplia distribución (Ayala, 1999). En ambos casos, se muestrearon abejas obreras adultas del interior del nido de cada colmena y se conservaron en etanol absoluto a –20 ºC.

La extracción de ADN se realizó a partir

Figura 1. *Localidades de muestreo marcadas con círculos negros.* (Sampling sites labelled with black dots).

de dos patas traseras de un ejemplar por colonia. Para la amplificación de los *loci* de microsatélites se realizaron dos reacciones múltiples de PCR. En la primera reacción (R1) se amplificaron conjuntamente los *loci* T4-171 y T7-5 (Paxton *et al*., 1999), y en la segunda (R2) Mbi254, Mbi259, Mbi278, Mbi28 y Mbi201 (Peters *et al*., 1998). Las reacciones de amplificación se prepararon con un volumen total de 12,5 µL con el reactivo PCR beads Pure Taq™ Ready-To-Go™ (GE Healthcare, Buckinghamshire, Reino Unido). El programa de amplificación fue de 95 ºC durante 5 minutos de desnaturalización inicial, 30 ciclos de 30 segundos a 96 ºC, 30 segundos a 55 ºC (R1) o 57 ºC (R2), y 30 segundos a 72 ºC, y una elongación final de 10 minutos a 72 ºC.

Los productos de PCR se visualizaron por electroforesis capilar en un secuenciador ABI® 3730 DNA (Applied Biosystems, Foster City, CA, EEUU) y con un estándar de tamaño interno (Servei Central de Suport a la Investigació Experimental de la Universidad de Valencia, España). El tamaño de los fragmentos de amplificación se determinó con el programa GeneMapper® v4.0 (Applied Biosystems, Foster City, CA, EEUU). El nivel de polimorfismo fue evaluado mediante parámetros poblacionales (R: rango del tamaño de los alelos de los *loci* de microsatélites, A: número de alelos, Ho: heterocigosidad observada, He: heterocigosidad esperada) obtenidos con el programa GenAlex v6.41 (Peakall y Smouse, 2006). Para comprobar la presencia de alelos nulos en cada uno de los *loci* se usó el programa Micro-checker (Van Oosterhout *et al*., 2004).

Dado el diferente número de ejemplares analizados en cada una de las dos áreas de muestreo de *M. colimana*, se llevó a cabo un análisis de rarefacción, para estimaciones objetivas de la riqueza alélica (Leberg, 2002), con el programa HP-RARE 1.0 (Kalinowski, 2005). Una vez obtenidos los datos se comprobó si existían diferencias estadísticamente significativas con la prueba t de Student (http://www.physics.csbsju.edu/stats/t-test_bulk_form.html).

RESULTADOS Y DISCUSIÓN

Los parámetros de diversidad por *locus* obtenidos en las dos especies del género *Melipona* estudiadas se muestran en la **tabla II**. Todos los *loci* fueron polimórficos excepto Mbi259, que mostró una sola alternativa alélica de 177 pares de bases (pb) en *M. beecheii* y 180 pb en *M. colimana*. En

Tabla II. *Parámetros de diversidad por locus obtenidos para* M. colimana *y* M. beecheii. (Diversity parameters per *locus* obtained for *M. colimana* and *M. beecheii*).

	M. colimana				*M. beecheii*			
Locus	R	A	H_o	H_e	R	A	H_o	H_e
T4-171	95-105	6	0,640	0,656	93-95	2	0,615	0,426
T7-5	66	1	0,000	0,000	72-78	3	0,583	0,570
Mbi201	152-158	4	0,280	0,550	152-158	3	0,500	0,558
Mbi254	203-230	4	0,615	0,723	188-203	4	0,733	0,713
Mbi259	180	1	0,000	0,000	177	1	0,000	0, 000
Mbi278	84-87	2	0,038	0,038	72-123	4	0,346	0,332
Mbi28	86-113	4	0,273	0,622	89-104	4	0,241	0,276
Media		3,143	0,264	0,370		3,000	0,431	0,411

R: rango del tamaño de los alelos de los *loci* de microsatélites; A: número de alelos; Ho: heterocigosidad observada; He: heterocigosidad esperada.

esta última especie, el *locus* T7-5 también mostró un único alelo de 66 pb. Los valores más altos de riqueza alélica por *locus* se observaron en *M. colimana* en los *loci* T4-171 (6), Mbi201, Mbi254 y Mbi28 (4), mientras que en *M. beecheii* el mayor valor (4) correspondió a los *loci* Mbi254, Mbi278 y Mbi28. En cuanto a la media de riqueza alélica para todos los *loci* se obtuvieron valores similares en ambas especies (3,143 en *M. colimana* y 3,000 en *M. beecheii*, diferencia no significativa, p= 0,87). Estos valores están incluidos dentro del rango de variación observado en otras especies de *Melipona*: desde 1,67 en *M. rufiventris* (Lopes *et al*., 2010) hasta 3,78 obtenido en *M. bicolor* (Peters *et al*., 1998), especie para la que fueron diseñados parte de los cebadores utilizados.

Los valores de heterocigosidad esperada por *locus* fueron mayores en *M. beecheii* (0,276-0,713) que en *M. colimana* (0,038-0,723) aunque no significativamente diferentes (p= 0,80). La heterocigosidad media esperada (H_e) de la población insular de *M. beecheii* (0,411) es algo menor que la obtenida en las poblaciones continentales de *M. beecheii* estudiadas por Quezada-Euán *et al*. (2007) de México (0,535) y Costa Rica (0,711). Esto es debido, probablemente, al hecho de que las poblaciones estudiadas en este trabajo son insulares y éstas suelen presentar valores más bajos de heterocigosidad tal y como se ha observado en poblaciones insulares de la abeja de la miel *A. mellifera* (De la Rúa *et al*., 2001).

Los bajos valores observados en *M. colimana* pueden deberse a que los cebadores usados no son específicos de esta especie; ya que los cebadores Mbi fueron diseñados en *M. bicolor* y T4-17 y T7-5 en *Scaptotrigona postica*. Estos resultados coinciden con los de Lopes *et al*. (2010) en los que se obtuvieron valores de riqueza alélica y heterocigosidad más altos en poblaciones de *M. rufiventris* y *M. mondury* analizadas con cebadores específicos que cuando se usaron cebadores diseñados en *M. bicolor*. Otra consecuencia del uso de cebadores no específicos puede ser la presencia de alelos nulos, tal y como se ha observado en el *locus* Mbi28 en la especie *M. colimana*. Estos alelos aparecen cuando se produce una mutación en la secuencia de nucleótidos contigua al microsatélite, lo que impide la unión de los cebadores a su secuencia complementaria (Chapuis y Estoup, 2006) y, en consecuencia, la amplificación del fragmento (Callen *et al*., 1993). Alternativamente, la detección de alelos nulos en este *locus* en concreto podría deberse a un error analítico dado el reducido número de muestras analizadas. En cualquier caso, la eficacia obtenida en la amplificación indica que las secuencias de estos *loci* de microsatélites están conservadas en las especies del género *Melipona*, ya que junto con *M. colimana*, son seis las especies (*M. bicolor* en Peters *et al*., 1998; *M. beecheii* en Quezada-Euán *et al*., 2007; *M. yucatanica* en May-Itzá *et al*., 2010; *M. rufiventris* y *M. mondury* en Lopes *et al*., 2010) en las que la amplificación de estos mismos *loci* y el genotipado de los individuos es posible. Se puede concluir por tanto que, si bien se pueden usar cebadores no

Tabla III. *Riqueza alélica por locus para los dos tipos de colonias de* M. colimana *obtenida tras la rarefacción.* (Allelic richness per locus for the two types of *M. colimana* colonies after rarefaction).

Locus	Zona deforestada	Zona conservada
T4-171	4	4
T7-5	1	1
Mbi201AAG	3	3
Mbi254AAG	1	1
Mbi259AAG	2	2
Mbi278AAG	1	1
Mbi28AAG	4	3
Media	2,293	2,070

específicos en estudios de especies filogenéticamente próximas, los resultados deben ser cuidadosamente analizados, para evitar la inclusión de aquellos *loci* que puedan mostrar alelos nulos en los paneles usados para determinar la diversidad genética.

La pérdida de diversidad genética en meliponinos como consecuencia de la degradación de sus hábitats ha sido demostrada en estudios anteriores. Lopes *et al.* (2010) observaron un descenso de la heterocigosidad en *M. rufiventris* y *M. mondury* a causa del reducido número de colonias encontradas como consecuencia de la destrucción y fragmentación de sus ecosistemas naturales. Otros estudios revelaron que la fragmentación de los hábitats afecta en mayor medida a los individuos especialistas que a los generalistas (Kitahara y Fujii, 1994), como es el caso de las abejas sin aguijón (Zayed *et al.*, 2005). En el caso de *M. colimana,* al no ser una especie explotada para la producción, sus poblaciones están sujetas a los efectos de la deforestación y de la destrucción de su hábitat. Aún así, en este estudio no se observaron diferencias significativas en la riqueza alélica por *locus* tras el análisis de rarefacción **(tabla III)** entre las colmenas situadas en la zona deforestada y la conservada, ya que en el momento del muestreo, la zona deforestada apenas llevaba dos años siendo talada (Macías, 2008). El hecho de que la degradación de los hábitats de *M. colimana* sea tan reciente podría explicar los valores similares en ambas zonas, ya que el tiempo transcurrido no es suficiente para que se aprecie el efecto sobre la pérdida de diversidad genética.

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