Phylogenetics as a tool for evolutionary studies: evolution of the leafcutter bees and chemical ecology of bee-flower relationships

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Thesis defended on
26th June 2017

IMPRIMATUR POUR THESE DE DOCTORAT

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Neuchâtel, le 21 août 2017 Le Doyen, Prof. R. Bshary

Remerciements

Une thèse de doctorat ressemble probablement pour tous ceux qui s'y frottent à l'ascension d'une montagne. Pour moi cette montagne était l'équivalent de l'Everest, et j'avais besoin d'un guide. Mon superviseur de thèse, Christophe Praz, a joué ce rôle de tout son cœur, et je ne saurais le remercier assez de m'avoir mené là où moi-même je ne pensais pas pouvoir arriver.

Il y a de nombreuses personnes qui m'ont aussi aidé tout au long du chemin, parfois en travaillant avec moi sur certains points de ma thèse. Un immense merci à vous en particulier, Dimitri Bénon, Anne-Marie Labouche et Romain Piault. Merci à Gaétan Glauser et Matteo Lucchetti pour toute l'aide pour les analyses chimiques. Un grand merci à l'équipe du Jardin Botanique de Neuchâtel qui m'a accueilli et aidé pour la réalisation de mes élevages.

Je tiens à remercier chaleureusement les membres de mon comité de thèse pour leur bienveillance et leur intérêt pour cette étude, mais aussi pour leurs nombreuses questions et commentaires qui ont aidé à améliorer ce document.

Je ne suis pas arrivé sur cette voie par un complet hasard. Un petit caillou m'a fait sortir des ornières du chemin tranquille que je m'étais donné de suivre il y a une quinzaine d'années. Cette voie alternative s'est révélée extrêmement plus difficile, mais incommensurablement plus riche. Ce petit caillou, ce battement d'ailes d'un papillon, c'est toi, Flo Chardo, et je t'en suis infiniment reconnaissant.

Merci à ma famille et à mes amis pour leur soutien, et plus que tout, merci à toi, Marie, pour ton appui indéfectible durant cette période parfois mouvementée. Mon humeur n'a pas toujours été au beau fixe, loin s'en faut, mais tu as bravé la tempête et tu m'as aidé à tenir le cap. Merci.

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Abstract of the thesis

Bees are present in almost all terrestrial ecosystems and are of vital importance for the pollination of wild flowers and cultivated crops. Despite being well investigated compared to many other insects, numerous open questions remain on their taxonomy, phylogeny and ecology. The present thesis aims at filling some of the gaps in knowledge concerning the evolutionary and natural history of bees. In addition, it aims at obtaining a better understanding of their complex relationships with flowers. Throughout the thesis, phylogenetics is used as a tool for examining an observed pattern in an evolutionary context. In the first chapter, the evolutionary history and the biogeography of the second largest bee genus worldwide is examined; in the second and third, pollen chemical ecology is investigated in two plant lineages each exhibiting multiple transitions in their pollination biology.

In the first chapter of this thesis, we inferred a phylogeny based on five nuclear genes for the bee tribe Megachilini, one of the most diverse and widely distributed tribe of bees. This tribe includes the large and cosmopolitan genus *Megachile*, or the leafcutting bees, as well as two cleptoparasitic lineages - "cuckoo" bees that do not build their own nests but hide their eggs into the nest cells of other bees. We use our phylogenetic framework to examine the evolutionary history of these bees, reconstruct their biogeography and propose a new classification. In addition, we test a new methodological approach for the reconstruction of nearly fully-resolved phylogenetic trees using DNA barcodes, approximately 600 bp long sequences of the mitochondrial gene cytochrome oxidase. Our results indicate that the two cleptoparasitic lineages form a monophyletic group nested within the nest-building genus *Megachile*, thus suggesting a single origin of cleptoparasitism in this bee clade. Biogeography reconstruction analyses suggest a center of origin in the Paleotropical area some 30 million years ago and numerous colonization events of the other geographic regions. Contrary to expectations, there were very few long-distance dispersal events and the current distribution of this large bee tribe can largely be explained by geodispersal between adjacent continents. When used alone, DNA barcodes had little phylogenetic signal: only nodes < 5 million years old could be recovered in such analyses; however, the addition of a single nuclear gene was enough to recover a majority of nodes up to 20 million years old. Lastly, we propose a

revised classification at the generic and subgeneric levels for this important group of bees.

In the second part of this thesis (chapters 2 and 3), we focus on bee-pollinated plant clades and examine a little studied floral trait: the presence of secondary defense compounds in the pollen. While numerous studies have examined the ecological significance of nectar secondary compounds, pollen secondary compounds represent a nearly unexplored field of research. Because bee-flower relationships are likely centered on pollen, not nectar, they may strongly be impacted by pollen chemistry. In chapter two, we focus on the plant family Boraginaceae and on its western Palearctic members. This plant family represents an important resource for solitary bees, bumblebees as well as honeybees. We survey the alkaloids in the pollen of 23 species and use comparative phylogenetic analyses to test hypotheses on their evolutionary origin. Second, we examine the possible impact of these alkaloids on solitary bees in bioassays where natural pollen and nectar provisions of three solitary bees (one specialist, two generalists) were supplemented with realistic levels of pollen alkaloids. Eggs of these three species of bees were transplanted onto these provisions and their development was examined. These bioassays indicated that two of the three species, one generalist and one specialist, failed to develop on provisions supplemented with realistic levels of alkaloids. This result demonstrates for the first time that pollen secondary compounds can impact bee development and thus ultimately bee-flower relationships. Based on our comparative phylogenetic analyses, we postulate that the presence of alkaloids in the pollen of some species of Boraginaceae is not directly linked to bees, but rather that alkaloids are part of the chemical defense of the reproductive tissues of the flower. However, six independent lineages exhibiting a sophisticated, bee-adapted morphology had significantly lower levels of alkaloids in the pollen than other species of Boraginaceae. We postulate that in these species, pollen may be considered a reward to pollinating bees, while in other species pollinated by nectar-seeking visitors, pollen does not act as a reward. This important difference allows us to re-examine the complex nature of bee-flower relationships in this plant family.

The focus of chapter three is another plant clade where bees are important pollinators, the Neotropical plant genus *Sinningia* in Gessneriaceae. This genus is a remarkable

model group as it exhibits strongly defined pollination syndromes (mostly hummingbirds and bees), and as there has been numerous, independent switches between these syndromes. As in chapter two, we survey all the secondary metabolites of the pollen and ask the question whether pollen secondary chemistry differs among the different syndromes. Based on our results of chapter two, our general hypothesis is that bee-pollinated species exhibit lower levels of defense chemicals than other species. The only secondary compounds identified in the pollen were saponins. In agreement with our hypothesis, saponin concentrations were lower in bee-pollinated species than in bird-pollinated species. We discuss several hypotheses explaining this pattern: the saponins may deter pollen-collecting insects in plants pollinated by nectar-feeding visitors, given that saponins have known deterrent, antifeedant and insecticidal properties; alternatively, the saponins may play another, hitherto unrecognized function, related for example to pollen adherence to bird pollinators or for the growth of the pollen tube. Future work is needed to examine these hypotheses.

Keywords: Bees, phylogenetics, Megachile, bee-flower relationships, pollen, secondary compounds, pollination, Boraginaceae, Gesneriaceae.

Résumé de la thèse

Les abeilles sont présentes dans presque tous les écosystèmes terrestres et ont une importance vitale tant pour la flore sauvage que pour les cultures. Bien que les abeilles aient été très étudiées par rapport à d'autres groupes d'insectes, de nombreuses questions restent ouvertes quant à leur taxonomie, phylogénie et écologie. Cette thèse a pour but de compléter nos connaissances concernant l'histoire évolutive et naturelle des abeilles. De surcroit, elle vise à mieux comprendre leurs relations complexes avec les fleurs. A travers cette thèse, la phylogénétique est utilisée comme outil pour examiner les phénomènes dans un contexte évolutif. Dans le premier chapitre, la phylogénétique nous sert à examiner l'histoire évolutive et la biogéographie du second plus grand genre d'abeilles au monde ; dans les deuxième et troisième chapitres, l'écologie chimique du pollen est examinée dans deux lignées de plantes montrant toutes deux de multiples transitions dans leur écologie de pollinisation.

Dans le premier chapitre de cette thèse, nous avons produit une phylogénie sur la base de cinq gènes nucléaires pour la tribu des Megachilini qui se situe parmi les groupes d'abeilles les plus divers et les plus largement distribués. Cette tribu inclut le grand genre cosmopolite *Megachile*, appelé aussi abeilles coupeuses de feuilles, ainsi que deux lignées cleptoparasites (abeilles-coucous) qui ne construisent pas leurs propres nids, mais pondent leurs œufs dans des cellules faites par d'autres abeilles. Nous utilisons notre trame phylogénétique pour examiner l'histoire évolutive de ces abeilles, reconstruire leur biogéographie et proposer une nouvelle classification. De plus, nous testons une nouvelle approche méthodologique pour la reconstruction d'arbres phylogénétiques presque entièrement résolus en utilisant les barcodes ADN, constitués de séquences d'environ 600 pb du gène mitochondrial cytochrome oxidase. Nos résultats suggèrent que les deux lignées cleptoparasites forment un groupe monophylétique niché au sein de lignées construisant des nids, ce qui suppose une origine unique du cleptoparasitisme dans cette tribu. Les analyses de reconstruction biogéographique suggèrent un centre d'origine dans l'aire paléotropicale il y a de cela 30 millions d'années et de nombreux événements de colonisation des autres aires biogéographiques. Contrairement à nos attentes, très peu de dispersions longue-distance ont eu lieu, si bien que la distribution actuelle de cette grande tribu d'abeilles

peut amplement être expliquée par de la géodispersion entre continents adjacents. Lorsqu'ils ont été utilisés seuls, les barcodes ADN ont peu de signal phylogénétique : seuls les nœuds de moins de 5 millions d'années ont pu être retrouvés dans de telles analyses ; cependant, l'addition d'un seul gène nucléaire était suffisant pour retrouver la majorité des nœuds d'un âge allant jusqu'à 20 millions d'années. Dernièrement, nous proposons une classification révisée aux niveaux générique et sous-générique pour cet important groupe d'abeilles.

Dans la seconde partie de cette thèse (chapitres 2 et 3), nous nous concentrons sur les clades de plantes pollinisées par les abeilles pour examiner un trait floral peu étudié : la présence de composés chimiques secondaires dans le pollen. Alors que de nombreuses études ont examiné l'importance écologique des composés secondaires du nectar, les composés secondaires du pollen représentent un domaine presque inexploré. Puisque les relations plante-abeille sont probablement centrées sur le pollen et non le nectar, elles pourraient être fortement influencées par la chimie du pollen. Dans le chapitre 2, nous nous concentrons sur les plantes de la famille des Boraginaceae et sur ses membres ouest-paléarctiques. Cette famille de plantes représente une importante ressource pour les abeilles solitaires et les bourdons autant que pour les abeilles mellifères. Nous examinons les alcaloïdes du pollen de 23 espèces et utilisons une analyse phylogénétique comparative pour tester des hypothèses sur leur origine évolutive. Deuxièmement, nous examinons l'impact possible de ces alcaloïdes sur les abeilles solitaires dans des bioessais où des provisions de pollen et nectar de trois de ces espèces (une spécialiste, deux généralistes) ont été supplémentées à des concentrations naturelles d'alcaloïdes du pollen. Des œufs de ces trois espèces d'abeilles ont été transplantées sur ces provisions et leur développement a été examiné. Ces bioessais ont montré que deux de ces trois espèces, une généraliste et une spécialiste, n'ont pas pu se développer sur les provisions supplémentées. Ce résultat démontre pour la première fois que la chimie secondaire du pollen peut avoir un impact sur le développement des abeilles solitaires et par conséquent sur les relations plante-abeille. Sur la base de notre analyse phylogénétique comparative, nous postulons que la présence d'alcaloïdes dans le pollen de certaines espèces de Boraginaceae n'est pas directement liée aux abeilles, mais plutôt que les alcaloïdes font partie de la défense chimique des tissus reproductifs de la fleur. Cependant, six lignées indépendantes disposant de fleurs

morphologiquement sophistiquées et adaptées aux abeilles ont des taux d'alcaloïdes du pollen significativement moins élevés que les autres espèces de Boraginaceae. Nous postulons que pour ces espèces, le pollen peut être considéré comme une récompense aux abeilles pollinisatrices, alors que pour d'autres espèces pollinisées par des visiteurs cherchant du nectar, le pollen ne peut pas être considéré comme une récompense. Cette différence importante nous permet de réexaminer la nature complexe des relations plante-abeille dans cette famille de plantes.

Le focus du chapitre trois est un autre clade de plantes où les abeilles sont d'importants pollinisateurs : le genre néotropical *Sinningia* (Gesneriaceae). Ce genre est un groupe modèle remarquable car il possède des syndromes de pollinisation fortement marqués (principalement colibri et abeille), et car il y a eu de nombreuses transitions indépendantes entre ces syndromes. Comme pour le chapitre deux, nous explorons les métabolites secondaires du pollen pour examiner si la chimie secondaire diffère entre les deux principaux syndromes. Sur la base de nos résultats du chapitre deux, notre hypothèse générale est que les espèces pollinisées par les abeilles montrent des taux de molécules défensives moins élevées que les autres espèces. Les seuls composés chimiques secondaires identifiés dans le pollen étaient des saponines. En accord avec notre hypothèse, les concentrations de saponines étaient inférieures dans le pollen des espèces pollinisées par les abeilles que dans celui des espèces pollinisées par les colibris. Nous discutons plusieurs hypothèses pour expliquer cet résultat : les saponines peuvent repousser les insectes collecteurs de pollen chez les plantes pollinisées par des visiteurs à nectar, étant donné que les saponines ont un effet repoussant, anti-appétant et insecticides reconnus ; alternativement, les saponines pourraient jouer une autre fonction jusqu'à présent non-reconnue, en lien par exemple à l'adhérence du pollen sur les oiseaux pollinisateurs ou pour la croissance du tube pollinique. Des travaux supplémentaires seront nécessaires pour examiner ces hypothèses.

Mots-clés: Abeilles, phylogénétique, Megachile, relations plantes-abeilles, composés secondaires du pollen, pollinisation, Boraginaceae, Gesneriaceae.

General introduction

To the great majority of the public, bees are represented by a single species: the honeybee. During my PhD thesis, whenever I told people that I was working on bees, they immediately wanted to know if I was keeping hives. Their next question was: “do you know why bees are disappearing?“ I usually answered that this question strongly depends on the kind of bees: the honeybee *Apis mellifera*, or the approximately 600 species of wild bees found in Switzerland, or 20’000 species of wild bees around the world (Ascher & Pickering 2017)? The usual response to my answers were raising eyebrows and widening eyes, but definitely a strong interest and more questions about bees.

Even though most people are not aware of the amazing diversity of the bees, the widespread concerns about bee decline are representative of a recent awareness of a worldwide problem (Vogel 2017). Some of the reasons for this decline may be the same for honeybees and wild bees, but others may not. For example, honeybees suffer increasing parasitism by varroa mites, an acarid that does not affect most bee species as it is related to social behavior. Varroa mites are transmitted through contact between adults and their offspring and such contact does not occur in solitary species, which constitute the great majority of all bees. Yet many other parasites and pathogens, which affect wild or/and solitary bee species, have been transported through the world by human activities and increasingly affect wild bee populations (see Goulson *et al.* 2015). The destruction of floral resources is another major problem for wild bees and to some extend for honeybees. Wild bee communities undergo strong changes in species composition and diversity following habitat loss (Bommarco *et al.* 2010). But is there a common cause for the disappearance of both wild and domesticated bees?

The “windshield phenomenon” (Vogel 2017) describes the fact that people driving a car in the 80’s remember that it was impossible keep the car clean on a hot summer day, as it always got maculated by lots of insects. This happens only rarely nowadays. More and more scientists try to measure this global decline of insects, a difficult task due to the lack of former quantitative studies (Michener 2007; Vogel 2017). The drastic simplification of agricultural landscapes, the disappearance of fallow land or hedges, and the general use of fertilizers in meadows and grasslands have massively increased food production, yet strongly eroded biodiversity and decreased resources for wild bees and managed honeybees. In addition, many insects feed on plants and are therefore highly exposed to the recent evolution of agricultural methods and massive use of various pesticides, especially neonicotinoids. This new class of pesticides is commonly used to coat seeds and acts systemically in plant tissues. Nectar and pollen of wild flowers occurring near treated crop area were found to contain neonicotinoids as well (Goulson *et al.* 2015). Moreover, synergetic effects have been demonstrated for several pesticides (see Goulson *et al.* 2015). As bees rely solely on floral resources (Grimaldi 1999; Michener 2007) and are therefore very much exposed to pesticides, it is likely that those synthetic compounds also bear a responsibility in their disappearance. Only a small positive note can be heard, the slow down of the decrease, or sometimes recovery of bee species biodiversity in Northern Europe (Carvalheiro *et al.* 2013).

What is the impact of such biodiversity loss? What importance may insects, and especially bees, have for humanity? Animal pollinators, among which wild bees are the most important (Grimaldi 1999; Michener 2007; Willmer *et al.* 2017), are responsible for the pollination of about one third of all crops and therefore human food production (Kremen *et al.* 2007). The yearly cost of worldwide insect pollination services was estimated to 153 billion Euros (Gallai *et al.* 2009). This food production is therefore endangered by the massive decline of insects, which may reach a decline of 84% in Europe (Williams 1994). Some studies also suggested that field productivity began to decline in parallel with the use of pesticides; such productivity loss might be due to the loss of pollinators (Vogel 2017). A compensation for the loss of natural pollinators through the rearing of more honeybees is also challenging as they too suffer heavy colony declines in Europe and North America. Moreover, wild pollinators have been shown to be the main contributors to crop pollination, even in the presence of high densities of honey bees (Garibaldi *et al.* 2011). The role played by wild insects in crop pollination demonstrates the importance of natural habitats, since most wild insects cannot survive in intensive agricultural landscapes.

What are bees ?

Recent phylogenetic analyses place the origin of bees among a paraphyletic assemblage referred to as the Crabronidae (Debevec *et al.* 2012), a group of digger wasps, or sphecid wasps. Recent phylogenomic studies have confirmed this finding and place the Pemphredoninae and Philanthinae as sister to bees (Peters *et al.* 2017; Branstetter *et al.* 2017). Interestingly, Philanthinae mainly search flowers for their preys, which may consist of diverse groups of pollinating insects. Consequently, the origin of the bees probably results from a shift from prey-hunting to pollen-collecting in the wasp-like, common ancestor of all bees. This change in diet probably contributed to the massive radiation of bees (Cardinal & Danforth 2013) and also to the rapid and large diversification of flower morphologies in the angiosperms in the early mid-cretaceous (Grimaldi 1999; Danforth *et al.* 2004; Cardinal & Danforth 2013). The fact that over 20’000 species of bees arose from a clade composed today of around 2’200 hunting wasp species (Branstetter *et al.* 2017) further reinforces the idea that the transition to pollen-feeding habit must have been a key innovation accounting for this immense radiation. Bees have become the most abundant pollinators in many ecosystems, and ironically, bees are now the main preys of their ancestors the Philanthine wasps (Branstetter *et al.* 2017).

Most bees nest in sand or existing cavities in the soil, where they dig (if necessary) and create brood cells (Michener 2007). Nests in the soil may be the ancestral state in bees, as imagined by Engel (Engel 2001), a reasonable hypothesis if we consider ground-nesting Philanthinae to be the sister clade to all bees. However an ancestor nesting in pre-existing cavities, as observed in Pemphredoninae, cannot be excluded. Unlike philanthine wasps, bees often line their cells with a secretion produced using their Dufour’s gland (Cane 1983; Hefetz 1987). This lining prevents water and pathogenic microorganisms from entering the cell. In their evolution, bees also started to use foreign materials to line and coat, close, or construct their nests (Michener 2007; Amiet & Krebs 2012). The Megachilidae use highly diverse materials to built their nests: mud in mason bees (*Osmia*) or dauber bees (*Chalicodoma*); resin in *Heriades* or in the Anthidiine bees; masticated plant material in other *Osmia* species; trichomes in other Anthidiine bees; leaf discs in leafcutting bees (*Megachile*). Whether build in the soil or in above-ground substrate, and lined with secretions or with exogenous material, the brood cells host the growing larvae. As food provisions for the larvae, most bees accumulate a mixture of nectar, pollen and sometimes oils. The honeybees are an exception, as their larvae do not feed on pollen and nectar: adult workers consume pollen and nectar to produce a protein-rich, glandular secretion called “brood food”. This secretion is progressively fed to the larvae with some addition of nectar and pollen (Winston 1991). In solitary bees, each cell is usually closed after the deposition of one egg per provision, so that there is no contact between adults and offsprings, unlike in eusocial species which provide brood care. Usually, the nesting female produces provisions of different sizes depending on whether it will produce a male or a female. In the case of *Osmia cornuta*, *O. bicornis* and *Chelostoma rapunculi*, the species used in our bioassays in chapter 2, the three bees nest in cavities where they produce an alignment of several cells. The first cells produced are often bigger and host females, while the last cells contain less provision and will lead to the development of males. As haplo-diploid organisms, female Hymenoptera can determine the sex of their offspring by respectively fertilizing or not the egg. The provision, once enclosed with the egg, is sufficient for the complete development of the larvae. In solitary bees, the egg hatches after two or three days to give birth to a small, legless larva that will eat the mixture of pollen and nectar. After consumption of the entire provision, the last larval instar will turn into a pupa and stay in diapause until transformation into a newly formed adult, which often emerges only in the following year.

The evolution of bees

Several studies have examined the phylogeny of all bees (Danforth et al. 2006, Cardinal and Danforth 2013, Branstetter et al. 2017, Peters et al. 2017) or of selected bee families (e.g., Cardinal et al. 2010, Litman et al. 2011, Almeida & Danforth 2009). This high-level phylogenetic framework has provided numerous insights into early bee evolution and diversification (e.g., Litman et al. 2011, Cardinal & Danforth 2013), the evolution of cleptoparasitism (Cardinal et al. 2010, Litman et al. 2013) or bee biogeography (e.g., Almeida et al. 2011). However, most bee genera have not been the subject of detailed phylogenetic studies. Yet such low-level phylogenetic studies are much needed, as they enable studies on the evolution of certain traits such as eusociality (Danforth et al. 2003) or host-plant specialization (Sedivy et al. 2008) within restricted bee clades. Therefore, future phylogenetic studies of bees should focus on selected genera, especially those large genera lacking a phylogenetic framework (*Andrena*, *Megachile*, *Eucera*), given that a backbone phylogeny is now available for the main families and tribes. In contrast to numerous other bee clades, the Megachilini are not restricted to Mediterranean or temperate climates, but reach particularly high diversity in the tropics. In particular, the bee faunas of South America, Tropical Africa and Tropical Asia include a large number of *Megachile* species. This pan-tropical richness is particular among bees, and several hypotheses can be formulated to account for this pattern. First, the tribe Megachilini may have originated in tropical regions at the end of the Cretaceous or during the Palaeocene, at a comparatively warm geological time where regions with tropical conditions were still connected by land bridges through the North Atlantic (Davis et al. 2002; Praz & Packer 2014). In agreement with this hypothesis, some Eocene (35-50 mya) fossil leaves bear circular incisions that have been attributed to the leafcutting bees (reviewed in Wedmann *et al.* 2009); the leafcutting species are likely a derived clade within Megachilini (Litman et al. 2011), thus rendering a Cretaceous or Palaeocene age of the tribe possible. Second, the origin of the tribe may be in tropical regions after the disappearance of North Atlantic land bridges, and the pantropical distribution of Megachilini may be explained by long-distance dispersal events. Long-distance dispersal events have been postulated to be more likely in wood-nesting than in ground-nesting bee clades (Fuller *et al.* 2005, Praz *et al.* 2008; Sedivy *et al.* 2013). Third, Megachilini bees may be a recent clade that has colonized all biogeographic regions through geodispersal between adjacent continents, for example from Asia to North America through Beringia, and then from North America to South America through the Isthmus of Panama. To evaluate these alternative hypotheses, a sound phylogenetic framework combined with dating analyses are necessary. Such a comprehensive study is presented in the first chapter of this thesis.

Bees and flowers

Since their origin around 120 million years ago (Cardinal & Danforth 2013, Branstetter *et al.* 2017, Peters *et al.* 2017), bees have developed an intimate relationship with the angiosperms. As mentioned above, bees are a major group of pollinators. However, they differ from other pollinators in one important aspect: they do not visit flowers only for their own energy requirements, but to provision brood cells for their larvae. The quantitative pollen requirements of bees can be extremely high and some species need the entire pollen content of more than 1000 flowers to rear a single larva (Müller *et al.* 2006). Not only does pollen collected for bee reproduction represent a significant loss for the plant, but pollen-collecting bees often possess specialized transporting structures and behaviors that prevent pollen deposition on other flowers (Westerkamp 1996; Thorp 2000). Therefore pollen visitation by bees may represent a conflict of interest between bees and flowers: to maximize their own reproduction, bees must collect as much pollen as possible in an efficient way; flowers, on the other hands, must ensure that at least part of this pollen is used for pollination. Consequently, plants may either attract nectar-visiting pollinators (including nectar-foraging bees) instead of pollen collecting bees, or develop mechanisms to prevent excessive pollen losses to the bees.

Floral morphology is probably the main way used by plants to reduce pollen losses, to select the most-efficient visitors and sometimes to manipulate the behavior or position of the visitors. Morphological adaptations to minimize pollen losses to bees are numerous: many plant species in several families conceal or protect the pollen in specialized structure to prevent bees from directly grooming the pollen off the anters. For example, Fabaceae have enclosed anthers in a specific structure, the keel, that only some bees can open (Westerkamp 1997); Lamiaceae developed bilabiate nototribic (anthers placed on the dorsal side of the pollinator) flowers (Westerkamp & Classen-Bockhoff 2007) and deposit the pollen on the back of pollinators, reducing the ability of bees to collect large quantities of pollen; Solanaceae and many other plant families have evolved poricidal anthers that need to be sonicated (buzzed) by bees to release the pollen (Buchmann 1985; De Luca & Vallejo-Marín 2013).

It has been hypothesized that the proto-bee (common ancestor of all bees) foraged on flowers which did not possess these specific adaptations to the bees (Litman *et al.* 2011), possibly resembling a flower of *Ranunculus,* with exposed anthers and a radial symmetry. Bilateral symmetry may have partly evolved in response to pollination by bees. This pattern is mirrored by the floral preferences and evolution in the

Megachilidae family (Litman *et al.* 2011): basal members forage on open flowers with a radial symmetry, while numerous derived lineages restrict their foraging on bilateral flowers that are highly adapted to the bees, notably Fabaceae. An example are the species of the genus *Megachile*, the genus investigated in chapter 1 of this thesis, a comparatively recent genus that is tightly associated with flowers of the Fabaceae family.

These highly exclusive flower forms and concealment of floral resources led to many further adaptations in both plants and pollinators. Numerous plant species do not produce nectar but rather mimic other, nectar-producing flowers; they take advantages of errors made by foraging insects. However such deceptive plants, including several well-studied examples among the orchids, must be less numerous than the flowers they mimic in order to keep their advantage (Bronstein *et al.* 2006; Renner 2006). On the insect side, cheating is also frequent. For example, short-tongued bumblebees often pierce holes at the basis of a long, tubular corolla to obtain nectar; they access a restricted resource but do not contribute to pollination. A similar behavior is regularly observed during pollen collection. Bees act as pollen thieves when they reach the anthers and remove pollen without pollinating the flower. This case is frequent with small bees landing directly on the anthers without touching the stigmata (Thorp 2000).

Yet many plant families neither hide their pollen nor visibly manipulate bee position through sophisticated floral morphologies. Their vulnerability to pollen losses may be compensated by other parameters, such as pollen physical and chemical properties (Sedivy et al. 2011). Egg exchanges among pollen provisions of different bee species specialized to various flowers, including specialist and generalist, revealed that pollen was not an easy-to-use resource, in contrast to earlier views. Indeed, development time and success varied highly across different bee species and pollen types (Praz *et al.* 2008; Sedivy *et al.* 2011). Several hypotheses have been formulated to explain the low nutritive value of some pollen types: 1) Some essential compounds such as sterols, which bees require for the production of certain hormones (Blum 1985) but cannot synthesize, may be lacking in some pollen types and thus may prevent larval development in bees not specialized to these types 2) The thickness of the pollen grain wall, or the lack of certain enzymes in the gut of certain bee species, may impede nutriment uptake 3) Toxic secondary compounds in the pollen may have a detrimental effect on the larvae (Roulston & Cane 2000; Praz *et al.* 2008). In chapters 2 and 3 of this thesis, we examine the latter hypothesis: first, we investigate whether pollen secondary chemicals impact the larval development of solitary bees. In addition, we ask whether the profile and concentrations of secondary compounds in the pollen are correlated with pollination syndromes.

Objectives of this thesis

In the present thesis, we address two very different topics: the first part focuses on the evolutionary history of the leafcutter bees; the other examines bee-flower relationships and the role of pollen secondary chemistry. Both however were investigated on the basis of phylogenetic hypotheses. As mentioned by Dobzansky: “Nothing in biology makes sense except in the light of evolution”. Whether examining biogeography patterns in bees or comparing alkaloid levels in pollens, both questions cannot be investigated without explicitly taking into account the phylogenetic relationships between the species investigated. For this reason, in all three chapters, a phylogenetic hypothesis between the species examined is reconstructed and used as a backbone to trace the evolution of characters or assess if characters are constraint by evolutionary relatedness or ecological drivers.

In the first chapter, we aim to solve what has been described by Michener (2007) as one of the five large, taxonomical problems in bees: the evolutionary relationships within the very diverse tribe Megachilini.

In the second chapter, we explore the bee-flower relationships in the plant family Boraginaceae. In particular, we compare the secondary chemistry of the pollen and examine if these chemicals have an impact on bee development, and whether their presence can be explained by the spectrum of their pollinators. The purpose of the third chapter is partly similar to the second chapter, but it focuses on a tropical system, the hummingbird- and bee-pollinated plant genus *Sinningia*.

Chapter 1

Foreword

As Michener points out in his master work "The Bees of the World" (Michener 2007), the "bible" for anyone working on bee biology or systematics, the tribe Megachilini is one of the few taxonomically problematic clades, especially its highly specious genus, *Megachile*. Michener (2007) evaluates several proposals to split the heterogeneous genus *Megachile* in several smaller genera to finally reluctantly withdraw to the original classification of one large genus (Michener 2007). He noted however, that the morphological and behavioral diversity within this genus was huge and corresponded to the diversity observed in other bee clades traditionally split into several genera. For example, two broad series may be distinguished based on nesting biology, the dauber bees, which use mud or resin for the construction of their nests, and the leafcutters, which use leaf discs to build their brood cells. These groups have sometimes been recognized as two distinct genera, but the morphological segregation of both is challenging. Michener's conclusion was thus that future work may lead to a revised classification for this bee group, but that such classification should rely on sound phylogenetic hypotheses.

The aim of this chapter is to conduct such a phylogenetic study for this important group of bees. Within this thesis, this work aims at understanding the diversity pattern within one of the main bee clades, a cosmopolitan group that accounts for a significant portion of the pollinator community in most ecosystems: *Megachile* bees are the main pollinators of flowers of the Fabaceae plant family throughout the world. In addition, this work allows for the establishment of a new classification for this important group of bees.

Chapter 1

Manuscript

The following manuscript was published in *Molecular Phylogenetics and Evolution* under the reference:

Trunz, V., Packer, L., Vieu, J., Arrigo, N., & Praz, C. J. (2016). Comprehensive phylogeny, biogeography and new classification of the diverse bee tribe Megachilini: Can we use DNA barcodes in phylogenies of large genera? Molecular Phylogenetics and Evolution, 103, 245–259. http://doi.org/10.1016/j.ympev.2016.07.004

Molecular Phylogenetics and Evolution 103 (2016) 245–259

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Comprehensive phylogeny, biogeography and new classification of the diverse bee tribe Megachilini: Can we use DNA barcodes in phylogenies of large genera?

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ARTICLE INFO

Article history:
Received 19 February 2016
Revised 23 June 2016
Accepted 7 July 2016
Available online 8 July 2016

Keywords:
Bees
Megachile
Coelioxys
Species-level phylogeny
Phylogenetic signal
Taxon sampling

ABSTRACT

Classification and evolutionary studies of particularly speciose clades pose important challenges, as phylogenetic analyses typically sample a small proportion of the existing diversity. We examine here one of the largest bee genera, the genus *Megachile* – the dauber and leafcutting bees. Besides presenting a phylogeny based on five nuclear genes (5480 aligned nucleotide positions), we attempt to use the phylogenetic signal of mitochondrial DNA barcodes, which are rapidly accumulating and already include a substantial proportion of the known species diversity in the genus. We used barcodes in two ways: first, to identify particularly divergent lineages and thus to guide taxon sampling in our nuclear phylogeny; second, to augment taxon sampling by combining nuclear markers (as backbone for ancient divergences) with DNA barcodes. Our results indicate that DNA barcodes bear phylogenetic signal limited to very recent divergences (3–4 my before present). Sampling within clades of very closely related species may be augmented using this technique, but our results also suggest statistically supported, but incongruent placements of some taxa. However, the addition of one single nuclear gene (LW-rhodopsin) to the DNA barcode data was enough to recover meaningful placement with high clade support values for nodes up to 15 million years old. We discuss different proposals for the generic classification of the tribe Megachilini. Finding a classification that is both in agreement with our phylogenetic hypotheses and practical in terms of diagnosability is particularly challenging as our analyses recover several well-supported clades that include morphologically heterogeneous lineages. We favour a classification that recognizes seven morphologically well-delimited genera in Megachilini*:* *Coelioxys*, *Gronoceras*, *Heriadopsis*, *Matangapis*, *Megachile*, *Noteriades* and *Radoszkowskiana*. Our results also lead to the following classification changes: the groups known as *Dinavis*, *Neglectella*, *Eurymella* and *Phaenosarus* are reestablished as valid subgenera of the genus *Megachile*, while the subgenus *Alocanthedon* is placed in synonymy with *M. (Callomegachile)*, the subgenera *Parachalicodoma* and *Largella* with *M. (Pseudomegachile)*, *Anodonteutricharaea* with *M. (Paracella)*, *Platysta* with *M. (Eurymella)*, and *Grosapis* and *Eumegachile* with *M. (Megachile)* (new synonymies). In addition, we use maximum likelihood reconstructions of ancestral geographic ranges to infer the origin of the tribe and reconstruct the main dispersal routes explaining the current, cosmopolitan distribution of this genus.

1. Introduction

Molecular phylogenies are available for a number of bee clades (reviewed in Danforth et al., 2013) and have contributed to the resolution of many long-standing controversies in bee systematics (e.g. Danforth and Ji, 2001; Praz et al., 2008; Almeida and Danforth, 2009; Cardinal et al., 2010; Rasmussen and Cameron, 2010; Praz and Packer, 2014; Romiguier et al., 2016). Together, these studies are contributing to the development of stable classifications and provide a framework for examining patterns of diversification (e.g., Hines, 2008; Litman et al., 2011, 2013), plant-bee coevolution (e.g., Sedivy et al., 2008), social evolution (e.g., Schwarz et al., 2007) and historical biogeography (e.g., Almeida et al., 2011; Chenoweth and Schwarz, 2011; Praz and Packer,

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http://dx.doi.org/10.1016/j.ympev.2016.07.004
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2014). For a few large bee clades, including most of the largest bee genera, however, no phylogenetic hypothesis is yet available, and classifications remain largely conjectural. Nearly ten years ago, Michener (2007: 120) listed five large complexes of problematic taxa, "for which the current generic classification is arbitrary and will probably be revised in the near future". Of these five groups, three have been examined using molecular markers (Praz et al., 2008 for the osmiine complex; Almeida and Danforth, 2009 for the *Leioproctus* and *Lonchopria*-group; and, among others, Gibbs et al., 2012 for the various lineages of the genus *Lasioglossum*). The present paper examines a fourth problematic group, the bees currently placed in the genus *Megachile* in the tribe Megachilini.

Four genera are currently recognized in this speciose tribe: *Coelioxys*, *Megachile*, *Noteriades* and *Radoszkowskiana*. The genus *Noteriades* includes only a few little-known African and Oriental species (Griswold and Gonzalez, 2011). *Coelioxys* and *Radoszkowskiana* are cleptoparasites, principally of species belonging to the genus *Megachile* (Michener, 2007). The former is distributed worldwide and includes more than 400 species (Ascher and Pickering, 2016; see Rocha-Filho and Packer, in press, for a subgeneric treatment of this genus), while the latter includes only four species limited to the Palearctic (Schwarz, 2001). With more than 1400 species (Ascher and Pickering, 2016), the cosmopolitan genus *Megachile* is among the largest bee genera worldwide and represents a significant proportion of most bee faunas, from tropical to temperate regions. Unlike most bee lineages, *Megachile* have their maximal diversity in tropical regions. Numerous species cut leaf discs that they use to line their brood cells in the ground or in existing cavities, and are commonly referred to as the leafcutter bees. Other *Megachile* species do not cut leaves but rather build brood cells with resin or mud mixed with salivary secretions (Kronenberg and Hefetz, 1984) in cavities, in the ground, or sometimes exposed on the surfaces of stone or wood (references in Michener, 2007). While some authors have referred to these species as either resin or mason bees (e. g., O'Toole and Raw, 1991), we find these terms confusing as both are also used for Anthidiini and Osmiini, respectively. We prefer to follow Eardley (2012) and refer to these species as "dauber" bees.

The relationships among the main megachiline lineages remain largely unclear, with the exception of the genus *Noteriades*, which has been demonstrated to be the sister group to all other Megachilini based on analyses of molecular (Praz et al., 2008; Litman et al., 2011) and morphological data (Gonzalez et al., 2012). The phylogenetic position of the two cleptoparasitic genera *Coelioxys* and *Radoszkowskiana* is unclear both in terms of whether they are derived from within the genus *Megachile* (Litman et al., 2011, 2013) and whether they are sister taxa (Litman et al., 2011, 2013; Rocha-Filho and Packer, in press). Thus, it remains unknown whether cleptoparasitism has evolved once or twice within the tribe (Rozen and Kamel, 2007, 2008; Litman et al., 2013).

Michener (2007) recognizes 56 subgenera within *Megachile*. While acknowledging that the diversity observed in this genus is larger than that seen in other tribes that are divided into numerous genera (e.g. the Eucerini, Osmiini and Anthidiini), Michener refrains from recognizing distinct genera because of the morphological intergradation among the diverse lineages. Instead, he assembles the subgenera in three groups, which correspond to the genera recognized by some authors, and broadly mirror the species' nesting biology. Michener's Group 1 includes the leafcutter species, in which the female mandible mostly has a "cutting edge", a blade-like structure between some mandibular teeth. Group 2 is made of the dauber lineages, which mostly lack mandibular cutting edges and use resin or mud to build their brood cells. Group 3 only includes the subgenus *Creightonella*, whose species exhibit an intermediate morphology between the other two groups and use a combination of resin or mud and leaf discs as nesting materials. The morphological separation of these three groups is difficult (Michener, 2007): Group 2 is likely a paraphyletic assemblage from which Group 1 and possibly the cleptoparasitic genera arose (Litman et al., 2011); and distinction between *Creightonella* and the other groups is "about as weak as that between Groups 1 and 2" (Michener, 2007: 554). Nesting biology and associated mandibular structure (presence or absence of cutting edges) do not always differ clearly by group. Most members of Groups 1 and 3 are leafcutters but cutting edges are reduced or absent in some lineages of Group 1; moreover, some subgenera included in Michener's Group 2 have distinct cutting edges, such as *Chelostomoda* and *Mitchellapis*. At least the former uses leaf discs to close its nests (Iwata, 1976). Other morphological characters used to segregate these three groups are equally ambiguous (Michener, 2007: 556).

Group 1, 2 and 3 correspond to the genera *Megachile*, *Chalicodoma* and *Creightonella* of some authors (Michener, 1965; Pasteels, 1965; Tkalců, 1969). In addition, some group 2 subgenera such as *Gronoceras* and *Thaumatosoma* have sometimes been given generic rank (e. g., Cockerell, 1935; King and Exley, 1985). The proposal of Mitchell (1980) to further divide Michener's group 1 into five genera does not appear practical (Michener, 2007: 555) and is not further discussed here. Michener (2007) suggested splitting his heterogeneous Group 2 into the following five genera: *Matangapis*, *Mitchellapis*, *Megella*, *Chelostomoides* ("including [as subgenera] *Callomegachile*, perhaps *Gronoceras* and *Thaumatosoma*"), and *Chalicodoma*. Based on cladistic analyses of morphological characters, Gonzalez (2008) (see also Engel and Gonzalez, 2011; Gonzalez and Engel, 2012) suggested a classification that breaks up Group 2 into three genera: *Matangapis*, *Chalicodoma* (including as subgenera, among others, *Pseudomegachile*, *Gronoceras* and *Callomegachile*) and *Thaumatosoma* (including the heriadiform subgenera, such as *Hackeriapis*, *Chelostomoides* and *Maximegachile*).

The purpose of the present study is to unravel the relationships within Megachilini, especially among the subgenera of the diverse genus *Megachile*, lay the foundations for a sound classification of the group and obtain insights into their biogeographic history. We sequenced five nuclear genes for more than 100 species of *Megachile* representing most subgenera, members of all other genera of Megachilini and representatives of all other megachiline tribes. To further refine our taxon sampling in the genus *Megachile*, we also use information from trees based on DNA-barcodes (the 658 bp fragment of the mitochondrial gene cytochrome oxidase I; Hebert et al., 2003) to pinpoint particularly divergent species and to break-up long branches within subgenera. We examine the phylogenetic signal of DNA barcodes and evaluate the possibility of combining our nuclear dataset with DNA barcodes (see Kjer et al., 2014, for a similar approach applied to the caddisfly genus *Chimarra*). We use our nuclear dataset as a backbone to infer old divergences (e.g., between subgenera and species-groups) and DNA barcodes to augment taxon sampling within more recent clades. We discuss the potential benefits and pitfalls of using DNA barcodes in combination with nuclear genes to reconstruct densely sampled phylogenies.

2. Materials and methods

2.1. Taxon sampling

In total, 127 species were included in our phylogenetic analyses of nuclear genes (Table S1). As outgroup taxa, we included representatives of the subfamilies Lithurginae and Pararhophitinae (following the classification of Gonzalez et al., 2012), as well as representatives of all tribes of the subfamily Megachilinae (Aspidosmiini, Dioxyini, Anthidiini, Osmiini) and of the genera *Ochreri-*

ades and *Afroheriades* because of their ambiguous position within Megachilinae (Praz et al., 2008; Litman et al., 2011). Within Megachilini, we included one species each of the genera *Noteriades* and *Radoszkowskiana*, eight species of the genus *Coelioxys*, and 106 species of the genus *Megachile*. In addition, we obtained sequences of one nuclear gene (28S) using next generation sequencing (see below) for two important subgenera (*Matangapis* and *Heriadopsis*). Of the 58 subgenera of *Megachile sensu lato* currently recognized (Michener, 2007, with updates by Durante and Cabrera, 2009; Engel and Baker, 2006; Engel and Gonzalez, 2011; Gonzalez et al., 2010; Gonzalez and Engel, 2012; Raw, 2006), eleven could not be included: *Austrosarus*, *Dactylomegachile*, *Neocressoniella*, *Platysta*, *Ptilosaroides*, *Rhyssomegachile*, *Schrottkyapis*, *Trichurochile* and *Zonomegachile* in Michener's group 1 and *Cestella* and *Schizomegachile* in Michener's group 2. For subgenera present on several continents, we included members from each biogeographic area whenever possible.

In addition, we used trees based on 1783 mitochondrial DNA barcodes originating from all continents to further guide our taxon selection. The majority of these barcodes were generated at the Canadian Centre for DNA barcoding with material from the Packer bee collection; we also used all published barcodes from the BOLD platform (extracted January 2016; data from Sheffield et al., 2009; Magnacca and Brown, 2012; Schmidt et al., 2015). All barcodes used are available on BOLD (www.boldsystems.org). We retrieved these DNA barcodes, produced NJ trees using the BOLD platform and selected one barcode per BIN (a proxy for biological species: Ratnasingham and Hebert, 2013), favouring the longest and best quality sequences and ignoring sequences less than 500 bp. In total 460 DNA barcodes were retained and aligned. The resulting barcode matrix was used to produce trees to augment our taxon sampling. Preliminary analyses of this matrix using maximum likelihood and Bayesian inference were particularly time consuming and yielded trees with very low support values for most nodes; we therefore used neighbour joining trees to guide our taxon sampling. These trees were a surprisingly good match with the existing subgeneric classification: most existing subgenera formed single clusters. However, several large subgenera were divided among clusters. We ensured that each of these clades was represented by at least one taxon in our nuclear matrix. In total, 20 species were added to our initial dataset based on their position in trees inferred from DNA barcodes only: four species of the subgenus *Callomegachile* (taxa with voucher numbers 162, 164, 193, 319; see Table S1 and Fig. 1); three species each of *Megachile* s. str. (40, 339, 750) and *Xanthosarus* (427, 741, 744); two species each of *Chelostomoides* (202, 786), *Eutricharaea* (318, 645) and *Pseudomegachile* (24, 1134); and one species each of the subgenera *Aethomegachile* (168), *Chalicodoma* (43), *Leptorachis* (205) and *Sayapis* (199).

2.2. DNA sequencing

DNA was extracted from one leg, and the rest of the specimen was preserved as a voucher. Voucher specimens are deposited in the Praz Collection at the University of Neuchatel and in the Packer collection at York University, except if stated otherwise (Table S1). We obtained DNA using phenol-chloroform extractions (following Praz et al., 2008) or DNA extraction kits (Nucleospin tissue, Macherey-Nagel). PCR-reactions were performed with Hotstart GoTaq polymerase (Promega) in a Biometra T1 thermocycler following standard protocols (Praz et al., 2008), with a blank sample as a negative control. PCR products were examined visually using agarose gel electrophoresis and purified enzymatically with a mix of exonuclease and FastAP thermosensitive alkaline phosphatase (Fermentas). Sequencing reactions were performed using BigDye Terminator v3.1 technologies (Applied Biosystems). Sequencing products were purified with Sephadex gel columns (GE Healthcare Life Sciences) and analysed using an ABI-3130 sequencer at the Genetic Diversity Centre at ETH Zürich.

2.3. Genes analysed

We sequenced fragments of five nuclear genes used in previous studies of Megachilidae (Litman et al., 2011): the four protein-coding genes elongation factor 1-α (hereafter EF), LW-rhodopsin (Opsin), conserved ATPase domain (CAD), sodium potassium adenosine triphosphatase (NAK), and the ribosomal gene 28S. To amplify each of these genes, we used the primer sites selected by Litman et al. (2011) and slightly modified each internal primer to optimize amplification in Megachilini based on available sequences from Litman et al. (2011). Primer sequences are given in Table S2. For the amplification of the 658 bp DNA barcoding fragment of COX1 (hereafter COX1), we used the primers LepF1 and LepR1 (Hebert et al., 2004) with standard protocols from the Canadian Centre for DNA Barcoding (http://www.ccdb.ca/resources.php). We used next-generation sequencing techniques to obtain sequences of 28S for two taxa for which fresh specimens were not available: *Megachile (Matangapis) alticola* (one specimen collected in 1992) and an undescribed species of *M. (Heriadopsis)* (one specimen collected in 2008, with highly degraded DNA). Both are important subgenera as they are the only species of *Megachile* having an arolium on all or some legs (Michener, 2007). The library preparation was made with a custom protocol (modified from Bronner et al., 2014) derived from the standard Illumina protocol. Details on the lab protocol and on the bioinformatics are given in the supplementary material.

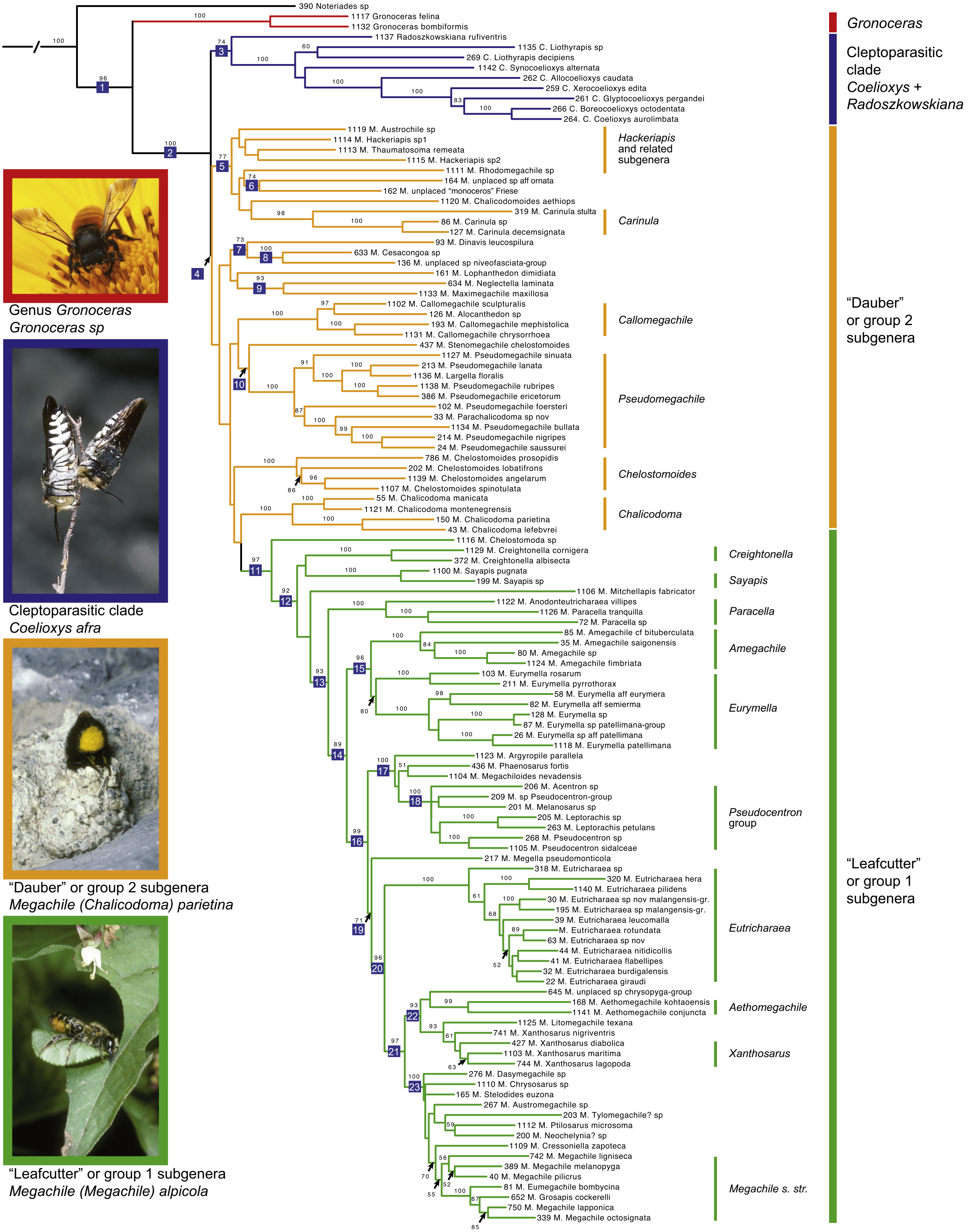
2.4. Editing and aligning of sequences

Chromatograms were trimmed, assembled and edited using Geneious R6 (Kearse et al., 2012). Alignments were performed in Mafft (Katoh and Standley, 2013). 28S sequences were aligned with consideration to secondary structure using the iterative refinement method Q-INS-i, implemented in Mafft (Katoh and Toh, 2008). Alignments for each gene were examined visually and corrected when necessary in Geneious. The coding sequence of each protein-coding gene was converted to amino-acid sequence to ensure that no stop codons were found. Introns of EF and Opsin were relatively conserved and were aligned using Mafft and corrected manually in Geneious; ambiguously aligned regions were removed. The small intron of CAD, however, proved too variable to be aligned and was excluded. Each gene fragment was tested for heterogeneous base composition using the Chi-square test implemented in a beta version of Paup 4.0 (Swofford, 2002) kindly made available by D. Swofford. For protein-coding genes, the three nucleotide positions were tested separately. The third codon positions of NAK (hereafter NAK3) and COX1 were significantly heterogeneous, so we performed analyses both with and without these partitions. The different genes were concatenated into a single matrix for final analyses using Geneious.

2.5. Maximum likelihood analyses

2.5.1. Single gene analyses

We first performed analyses of each gene separately using maximum likelihood (ML) inference. Protein-coding genes were separated into three or four partitions corresponding to the three nucleotide positions and the introns, if applicable. ML-analyses were performed in RAxML version 8 (Stamatakis, 2014) on the CIPRES server (Miller et al., 2010), performing 1000 bootstrap replicates and applying a GTR model with a gamma distribution (G) to each partition. For all analyses performed in this study,



Genus *Gronoceras*
Gronoceras sp

Cleptoparasitic clade
Coelioxys afra

"Dauber" or group 2 subgenera
Megachile (Chalicodoma) parietina

"Leafcutter" or group 1 subgenera
Megachile (Megachile) alpicola

Fig. 1. Best tree found in maximum likelihood analyses of the matrix with significantly heterogeneous partitions removed (third codon positions of NAK and COX1), with the introns, partitioned by codon position; number above branches represent bootstrap support values (only values ⩾ 50%) based on 1000 bootstrap replicates. Outgroup taxa have been removed and the branch leading to the ingroup has been shortened for better graphic representation. Numbers in blue squares indicate 23 nodes discussed in the text for which no taxonomic name is available. Pictures by Dino Martins (*Gronoceras* sp.), Andreas Müller (*Coelioxys afra*, *Megachile parietina*) and Albert Krebs (*Megachile alpicola*).

including Beast analyses, we decided not to use the combination of a proportion of invariant sites and a gamma distribution because both parameters model rate heterogeneity in different ways (see discussion in the RAxML v8.2.X Manual, page 59).

2.5.2. Concatenated dataset

We concatenated the coding sequence of each nuclear gene, the nuclear introns, and COX1, and performed ML analyses on six different matrices: with and without COX1, with and without the introns, with and without significantly heterogeneous partitions. Each dataset was analysed under two different partitioning regimes: by codon position and following the more complex partitioning regime suggested by PartitionFinder v. 1.1.0 (Lanfear et al., 2012), with 9–11 partitions, depending on whether NAK3, COX1 or the introns were included. Details on these analyses are given in the supplementary material. We repeated each analysis with the 28S sequences of *Matangapis* and *Heriadopsis* added to the concatenated matrix.

2.6. Bayesian analyses

Bayesian analyses were run in BEAST 1.8 (Drummond et al., 2012) with the same six matrices used for the ML analyses; we also applied two distinct partitioning regimes: by codon as in ML analyses, with both introns combined because initial analyses with two intron partitions yielded poor convergence of the tree likelihood parameter for the intron of Opsin; and by gene, with five partitions (or six, if COX1 was included). More complex models, such as the partitioning regime suggested by PartitionFinder (see above), resulted in poor convergence for numerous parameters and were thus not retained. These twelve Beast analyses were run without fossil calibration for 100 million generations. Additional details on these analyses are presented in the supplementary material. Maximum clade credibility trees were computed after removal of an appropriate burn-in (usually 20% of the trees) using TreeAnnotator (Drummond et al., 2012).

2.7. Divergence time estimation and biogeographic reconstruction

We repeated one selected BEAST analysis including two calibration points to estimate divergence times in our phylogeny. Only one fossil can confidently be attributed to Megachilini, *Megachile glaesaria* from Dominican amber (15–20 mya; Engel, 1999). However, its exact phylogenetic placement within Megachilini is unclear (Engel, 1999) and this fossil would at best be used as a stem group calibration point for the subgenus *Chelostomoides* given its similarity to that subgenus. Given the long branch leading to *Chelostomoides*, spanning 8–21 mya in our final dated phylogeny, this fossil is of little use for calibrating our tree. We thus used two calibration points from Cardinal and Danforth (2013): the age of the root of the tree, corresponding to the age of the node uniting Lithurginae, Pararhophitinae and Megachilinae (this node was recovered with maximal support values in phylogenetic studies of the Megachilidae: Litman et al., 2011) and the age of the node uniting Megachilini + Osmiini; applying a prior to the age of Megachilini was not appropriate given the considerably smaller taxon sampling within this tribe in Cardinal and Danforth (2013). We placed normal priors with the following values on the ages of these nodes: mean 74, stdev 7, initial value 74 for the root; and mean 57, stdev 5, initial value 57 for the node uniting Osmiini + Megachilini. These prior distributions correspond to the means and confidence intervals found for these nodes in analysis 3 of Cardinal and Danforth (2013: Table S6). We also compared analyses with and without an additional calibration point corresponding to trace fossils putatively attributed to leafcutter bees. Fossil leaves bearing circular incisions, hereafter “fossil leafcuttings”, have been reported by various authors (reviewed in Wedmann et al., 2009), but attribution to the genus *Megachile* is only hypothetical. These fossil leafcuttings are of various ages, from the Miocene to the Paleocene (Wedmann et al., 2009). We compared analyses with and without a calibration point corresponding to the oldest of these fossils (ignoring the single questionable Paleocene fossil), which originated from Messel deposits (47 mya; Wedmann et al., 2009). We applied a prior distribution (lognormal distribution, mean of 1, Stdev of 1, zero offset of 47 Mya; 95% HPD: 47–61 Mya) to the most recent common ancestor (hereafter MRCA) of all lineages having a cutting edge in the female mandible.

Probabilistic inference of ancestral range reconstruction was performed using Biogeobears (Matzke, 2013, 2014) to infer the centre of origin of the tribe and to examine the main dispersal routes explaining the cosmopolitan distribution of the genus *Megachile*. We ran the BEAST analysis twice independently, each for 100 million generations, computed a maximum clade credibility tree from both runs after removal of an appropriate burn-in, and removed non-Megachilini taxa using the “drop-tip” function of the ape package in R (Paradis et al., 2004). We recognized the following six biogeographical regions: Nearctic (A), Neotropical (B), Afrotropical (C), Palaearctic (D), Oriental (E) and Australian (F), following Fig. 1a of Rueda et al. (2013) for delimitations of these regions. Maximum range size was set to two and the possible ranges were the following: A, AB, AD, B, C, CD, CE, D, DE, E, EF, F. We allowed the Oriental and Afrotropical regions to be adjacent (see Fig. 2) given the great faunal similarities of these two zones in bees (Michener, 2007), and the fact that numerous megachiline lineages (e.g., among others, the genus *Noteriades*) are distributed in both regions but are absent from the Palearctic. The coding for each terminal (Fig. 2; see supplementary material) reflects the geographic distribution of the entire subgenus or of the species-group that each terminal represents; a few exceptions are detailed in the supplementary material. We implemented only one time period in the DEC model of Biogeobears (thus with dispersal probabilities constant throughout the tree; see the supplementary material for a justification); dispersal probabilities were set to 1.0 between all adjacent regions. We also explored a model incorporating founder-event speciation (or, speciation through long-distance dispersal; Matzke, 2014), the DEC + J model implemented in Biogeobears. The nests of wood-nesting *Megachile* may be carried over water barriers as has been suggested for other twig-nesting bees (Fuller et al., 2005) and thus long-distance dispersal events between non-adjacent geographic regions are theoretically possible. In the DEC + J analyses, the dispersal probabilities were set to 1.0 between adjacent regions (AB, AD, CD, CE, DE, EF), and 0.1 between non-adjacent regions.

2.8. Phylogenetic signal of DNA barcodes and combined nuclear + barcode analyses

We used our phylogenetic framework to examine the phylogenetic signal of DNA barcodes (COX1) and to evaluate the possibility of augmenting taxon sampling by combining DNA barcodes with a nuclear “backbone” phylogeny. We selected all well-supported (bootstrap support > 95% in all ML analyses, posterior probability 1.0 in all Bayesian analyses), recent clades representing either subgenera, or species-groups within subgenera in our nuclear phylogeny; older clades, that is, clades grouping several subgenera were excluded, with the exception of the genus *Coelioxys*, which was included as one clade. We analysed a matrix including only the DNA barcodes for the species included in the nuclear phylogeny (taxa with missing COX1 sequence were excluded) using both ML and Bayesian inferences (three partitions modelled with a GTR + gamma model for the ML analyses and a TN93 + G model with empirical base frequency for the Bayesian analyses). The sup-

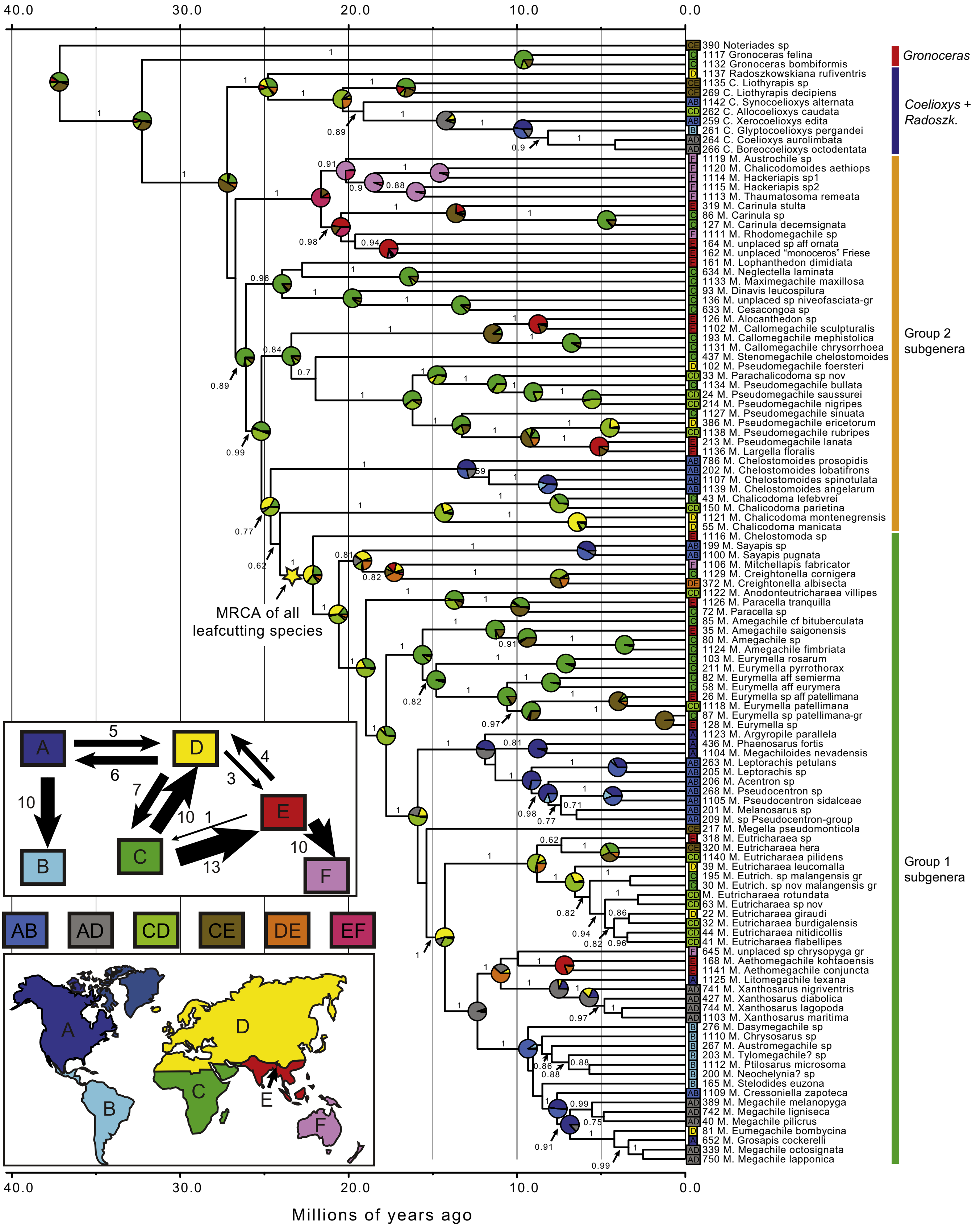


Fig. 2. Maximum clade credibility tree found in the dated BEAST analysis of the matrix with significantly heterogeneous partitions removed (third codon positions of NAK and COX1), with the introns, without the calibration point corresponding to the fossil leafcuttings (yellow star), partitioned by codon position. The pie-charts on the nodes represent the ancestral range reconstructions performed under the DEC model in Biogeobears; the six geographic ranges A–F are indicated on the map; colour codes for polymorphic ranges are indicated above the map. The arrows on the diagram above the map represent all exchanges suggested by the most likely reconstruction in the DEC model; the width of the arrow is proportional to the number of exchanges documented, which are indicated by numbers above the arrow.

port values for these clades were computed by filtering trees in PAUP (either the 1000 bootstrap trees in ML analyses, or all posterior trees after removal of 20% burn-in in Bayesian analyses). To evaluate variation in the phylogenetic signal of COX1 over time, these values were plotted against node age, as determined in our dating analysis in BEAST. We also examined the support for these clades when the barcodes were used in combination with a nuclear backbone: starting with our complete nuclear + COX1 matrix, we randomly selected one species from each of the clades; for these species, the full nuclear + COX1 matrix was retained; for the other species in these clades, the nuclear matrix was deleted (thus leaving only COX1); for all other taxa (i.e., taxa not included in these clades), the full nuclear + COX1 matrix was retained. The resulting matrix was analysed using ML and Bayesian inference applying our preferred model and partitioning strategy (see below). Again, the support values for these clades were computed by filtering trees in Paup. The same procedure was repeated twice more with the same clades, leaving one nuclear marker (either 28S or LW-Rhodopsin, respectively) in addition to the mitochondrial barcode; both 28S and Rhodopsin have been used as a nuclear markers to delineate species in insects (e.g., Blaimer and Fisher, 2013; Kjer et al., 2014).

Lastly, we concatenated our nucleotide matrix (including the three codon positions of COX1) with a matrix including 350 additional mitochondrial DNA barcodes of the genus *Megachile sensu lato*; these 350 barcodes correspond to the 460 barcodes mentioned above (see section entitled "Taxon sampling") minus those already represented in our dataset. Because the focus was on the genus *Megachile*, *Coelioxys* and *Radoszkowskiana* were removed, as well as taxa with missing barcode sequences. This supermatrix was subjected to Bayesian analyses, partitioning the matrix into codon position and using the substitution models mentioned above, except that we enforced a strict clock model for COX1.

3. Results

3.1. Phylogenetic analyses

After sequence editing and alignment, the concatenated, nuclear matrix included 5480 aligned bp (EF: 887, 146 bp in the intron; Opsin: 794, 122 in introns; CAD: 879; NAK: 1488; 28S: 1432). In addition, the barcode fragment of COX1 was 654 bp long. Missing data of all nucleotide sites amounted to 6.9% (Table S1, with Genbank accession numbers). Although trees were little resolved in individual gene analyses, no conflicting topology was supported by high (>70%) bootstrap support (hereafter BS) values among the different gene trees (introns included). ML-analyses of the six different matrices all yielded highly congruent topologies (Table S3). The exclusion of NAK3 had virtually no impact on tree topology (see details in Table S3), but resulted in a higher number of nodes supported by values above 50% within Megachilini (90 versus 87 nodes). Exclusion of the introns did not significantly alter the topology or the number of nodes with BS > 50% (in both cases 90 nodes), although it resulted in a slight decrease in support values for a majority of the nodes (Table S3). The addition of COX1, either with or without third codon positions resulted in nearly identical trees, although with one node less supported by BS values > 50% (89 nodes in both cases). For analyses of these six matrices, partitioning regime had virtually no effect on tree topology or nodal support values (Table S3). Based on the number of nodes supported, and because we prefer to exclude significantly heterogeneous partitions, our preferred analysis was performed on a dataset with the introns, without both the significantly heterogeneous third positions of NAK and COX1 (see methods, Section 2.4, editing and aligning of sequences), with 6 partitions (Fig. 1). Bayesian analyses performed in BEAST yielded trees that were highly similar to those found in ML analyses (Table S3; Fig. S1). Differences between the 12 Bayesian analyses and support values for taxonomically important nodes are given in Table S3.

3.2. Phylogenetic relationships within Megachilini

Important clades that do not have a taxonomic name are numbered from 1 to 23 for ease of reference (Fig. 1, S1; Table S3). All phylogenetic analyses recovered the tribe Megachilini as a monophyletic group with maximal support values (BS 100%, PP 1.0), with the genus *Noteriades* sister to all other Megachilini (clade 1; Table S3). Outgroup taxa have been removed from the trees shown in all figures. In all analyses, the subgenus *Gronoceras* branched next and was sister to all remaining Megachilini with maximal support values (clade 2; Table S3), including the cleptoparasitic genera *Coelioxys* and *Radoszkowskiana.* Both cleptoparasitic genera formed a monophyletic group (hereafter the cleptoparasitic clade; clade 3) in most analyses (Table S3); support for this clade was moderate to high (BS up to 83% and PP 1.0; Table S3), whereas support for any alternative placement was weak (BS < 50% in ML analyses; PP less than 0.5 in Bayesian analyses). The cleptoparasitic clade was sister to all other Megachilini (except *Noteriades* and *Gronoceras*) in most analyses (clade 4; Table S3), although support for this relationship was weak. The inclusion of both subgenera of *Megachile* having arolia (*Heriadopsis* and *Matangapis*, represented only by 28S sequences) resulted in slightly different topologies and lower support values for nodes among the early diverging branches of Megachilini: the positions of *Noteriades* and *Gronoceras* were unchanged, but clade 4 was no longer recovered; the subgenera *Heriadopsis* and *Matangapis* were not closely related to any other lineage of *Megachile* but appeared within the cleptoparasitic clade (Fig. S2), although support for this relationship was below 50% (Fig. S2).

After the divergence of the aforementioned groups, we are left with most members of Michener's Group 2, which are the paraphyletic dauber bees from among which the leafcutter bees arose. The dauber bees are made up of a number of clades as follows: **A.** A well-supported clade (clade 5) comprising several morphologically heterogeneous lineages: one Asian lineage (clade 6; Table S3) which included taxa currently placed in the subgenus *Callomegachile* (the *ornata*-species group) and a currently unnamed species (*M. monoceros* Friese 1903, preoccupied name); both lineages were sister groups in all analyses (clade 6; Table S3); the group of species known as *Carinula*, currently considered to belong to the subgenus *Callomegachile*; and most of those Group 2 subgenera known from Australia (*Hackeriapis*, *Austrochile*, *Thaumatosoma*, *Rhodomegachile* and *Chalicodomoides*). **B.** A well-supported clade (clade 7) that included the subgenus *Dinavis* (currently synonymized under *Pseudomegachile* from which it was distant in the molecular results), the subgenus *Cesacongoa* and the *niveofasciata*-species group (currently included in the subgenus *Chalicodoma).* **C.** A well-supported clade (clade 9) composed of the subgenera *Neglectella* (currently synonymized under *Pseudomegachile* from which it was well separated in our results) and *Maximegachile*. The position of the subgenus *Lophanthedon* was unstable; this subgenus was either sister to clade 5, 7, or 9. **D.** The subgenus *Callomegachile*, excluding *Carinula* and the species of clade 6, but including *Alocanthedon*, which rendered *Callomegachile* paraphyletic. **E.** The subgenus *Pseudomegachile*, including *Largella* and *Parachalicodoma*, both nested within *Pseudomegachile*. The position of the subgenus *Stenomegachile* was variable, although it was mostly found as sister to *Pseudomegachile*, with weak support (clade 10 in Table S3 and Fig. 1). **F.** The subgenus *Chelostomoides*. **G.** The subgenus *Chalicodoma*.

All leafcutting subgenera including three Group 2 subgenera (*Chelostomoda*, *Mitchellapis* and *Megella*), the lone member of Michener's Group 3 (*Creightonella*), and all of Michener's Group 1, formed a well-supported monophyletic group (BS > 86%; PP 1.0; Clade 11). In all analyses, *Chelostomoda* was sister to all other leafcutting subgenera (clade 12), with high support values (BS > 89%; PP 1.0). The subgenera *Creightonella*, *Sayapis*, and *Mitchellapis*, considered as intermediate between group 1 and group 2 by Michener (2007), were among the earliest diverging lineages of group 1.

Within the rest of group 1, the subgenus *Paracella* was the first branch, the second was a well-supported clade (clade 15) formed by the subgenera *Amegachile* and *Eurymella*. The five subgenera of the *Pseudocentron*-group (see Michener, 2007) formed a well-supported clade (clade 18) itself forming a well-supported clade (clade 17) along with the subgenera *Argyropile*, *Megachiloides* and *Phaenosarus*. The latter, placed in synonymy with *Xanthosarus* by Michener (2000) was not closely related to that subgenus in our analyses. Within clade 19, the subgenus *Megella* branched first, although its placement as sister to the rest of clade 19 (i.e., clade 20) was not strongly supported (Table S3). The large and diverse subgenus *Eutricharaea* was monophyletic after exclusion of *Eurymella*, except for the Australian species group allied to *M. chrysopyga*, currently placed in *Eutricharaea* but which was part of a distinct clade (clade 22) formed by *Aethomegachile*, *Xanthosarus* and *Litomegachile*. The subgenus *Megachile* was part of a poorly resolved clade (Clade 23), which also included many South American subgenera. Both *Grosapis* and *Eumegachile* were nested within the subgenus *Megachile s. str*; this paraphyly of *Megachile s. str* was highly supported in all analyses.

3.3. Dating and biogeographic analyses

Results from the BEAST analysis without the calibration point of the fossil leafcuttings are presented in Fig. 2. The inclusion of the fossil leafcuttings as a calibration point had a major impact on all inferred ages. For example, the ages (with 95% confidence interval) of the root (Pararhophitinae + Lithurginae + Megachilinae) were 87.4 mya (79.2–95.5) and 69.1 mya (58.2–90.0), respectively, with and without this calibration point. The stem age of Megachilini varied from 61.5 to 36.9 mya, while the age of the MCRA of all species having a leafcutting edge (thus the node chosen for the calibration with the fossil leafcuttings; yellow star in Fig. 2) varied from 47.6 to 22.0, the former value mirroring exactly the youngest portion of the prior distribution enforced on this particular node. In spite of these differences, the likelihood values of both analyses were comparable (−55′628.96 and −55′627.18, with and without this calibration point, respectively).

Inferred ancestral ranges found in the DEC model (LnL – 272.34) for important nodes are presented in Fig. 2. The DEC model suggested ancestral ranges including the Afrotropical and Oriental regions for the MCRA of Megachilini, of clade 2, and for most splits within Michener's Group 2 (Fig. 2). For early splits within Group 1 the DEC model suggested either an Afrotropical origin or a Palearctic origin, or an ancestor distributed over both.

According to the most likely reconstruction inferred in the DEC model, there were thirteen dispersal events from the Afrotropic to the Oriental region (Fig. 2); ten dispersal events each from the Nearctic to the Neotropic and from the Afrotropic to the Palearctic; seven from the Palearctic to the Afrotropic, six from the Palearctic into the Nearctic and five in the other direction; four from the Oriental region to the Australian region; three from the Palearctic to the Oriental region, one from the Oriental region to the Afrotropic; and none from the Oriental region to the Palearctic and from the Neotropic to the Nearctic (although see discussion).

In contrast, the DEC + J model (Fig. S3) allowed long-distance dispersal between non-adjacent regions, that is, long-distance dispersal over vast oceanic distances (>1000 km). This model had substantially higher likelihood (LnL – 246.49); ancestral range reconstructions for the tribe and for most nodes within Group 2 also included the Afrotropical and Oriental regions (Fig. S3); however, reconstructions for early splits within Group 1 favoured an Afrotropical origin. Other differences were mostly minor (Fig. S3). In contrast to the DEC model in which the only dispersal route allowed into the Nearctic was from the Palearctic, the Nearctic was colonized three times through long-distance dispersal events, twice from the Afrotropic (MRCA of *Chelostomoides* and of clade 17) and once from the Oriental region (MRCA of *Sayapis*).

3.4. Use of DNA barcodes in combination with a nuclear backbone

Thirty-four recent, well-supported clades were retained for the examination of the phylogenetic signal of COX1; they are indicated in Fig. S1. Of these 34 clades, the phylogenetic placement of only three was recovered with BS values > 75% in ML analyses (Fig. 3, closed circles) of the mitochondrial DNA barcodes only. Crown ages of these three clades were between 0.8 and 4.2 million years according to our dating analyses. By 5 million years all BS values were below 50% (Fig. 3). Of all 34 clades, 12 were recovered in the best tree while 22 were not and thus placement of their species in the barcode tree contradicted results of our nuclear phylogenies. Incongruent placements were never supported by >50% BS values. Results were largely similar in Bayesian analyses of the barcode data: 12 clades were recovered with values above 0.5; support mostly dropped to below 0.5 PP after 4 million years. Twenty taxa had a contradictory placement compared to our final phylogenetic hypothesis, and support for the incongruent placement was commonly above 0.5 (13 of the 20 contradictory placements) and even up to 0.95 (one case) and 0.98 (one case). Having a nuclear backbone for all species outside these 34 clades and one randomly selected species within each clade did not improve the phylogenetic signal of DNA barcodes: support dropped to below 50% BS values beyond a divergence time of approximately 4 million years, and 22 clades were not recovered in the best tree.

This failure of accurate placement of taxa based only on barcode data was easily overcome if only one nuclear marker (LW-Rhodopsin) was added (Fig. 3, open circles): all 34 clades were

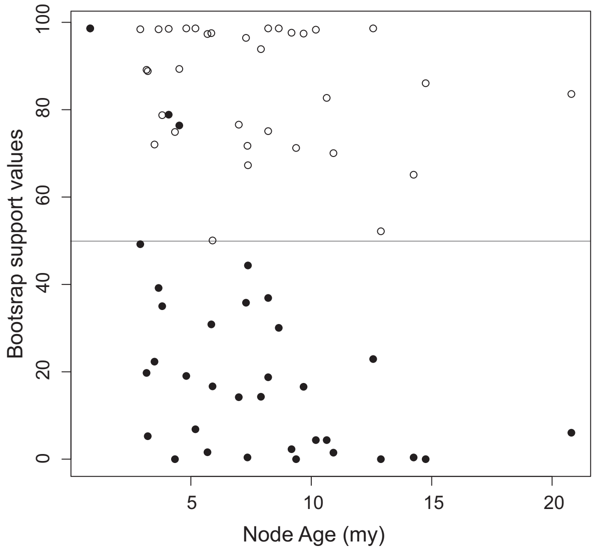


Fig. 3. Support values (bootstrap support in maximum analyses with 1000 bootstrap replicates) for 34 well established clades in maximum likelihood analyses of DNA barcodes (COX1) only (closed circles) or DNA barcodes + the nuclear marker LW-Rhodopsin (open circles). Values are plotted against the crown age of the clade in our BEAST analysis.

recovered in ML analyses with BS values above 50% and only four nodes had BS values below 70% (Fig. 3). In Bayesian analyses, one clade was not recovered in the best tree; four further nodes had PP between 0.6 and 0.85; all other nodes (30) had PP above 0.95. Having a nuclear backbone for all species outsides these 34 clades and for one randomly selected species within each of them resulted in trees highly similar to those derived from analyses of the full nucleotide matrix, with each of the 34 clades recovered as a monophyletic group and placed in agreement with our phylogenetic analyses of the complete nuclear dataset. 28S was less informative than LW-Rhodopsin when analysed in combination with COX1: only 31 clades were recovered in ML analyses, six of them with support below 50% BS.

Our combined analysis including 413 taxa (outgroup excluded) is presented in Fig. S4. In this analysis, all terminals had a barcode sequence; 99 ingroup terminals had a full nuclear matrix (in red in Fig. S4), 12 had only one nuclear marker, LW-Rhodopsin (in blue), and 302 terminals had only the DNA barcode (black). While not all nodes are fully resolved, all subgenera (as reassigned in the present study, see discussion and Table 2) appear as monophyletic and most of them have high statistical support, with the exception of *Hackeriapis* (see below, discussion: subgeneric classification). Morphological examination of all terminals with barcode sequences only, for which the vouchers were available, confirmed their molecular placement. A list of 34 barcodes not placed in sampled clades is given in the supplementary material; most of them are unidentified species.

4. Discussion

Our phylogenetic analysis provides the first comprehensive phylogeny of the species-rich bee tribe Megachilini, with a particularly dense taxon sampling that covers most of the diversity observed within the tribe: all genera and 81% of currently recognized subgenera of the genus *Megachile*, including all three of Michener's groups. A large majority of the clades are well supported and many of these clades represent morphologically well-defined species-groups or subgenera. However, the relationships among these well-defined groups, and especially among the different lineages of Michener's Group 2 subgenera are more difficult to resolve, rendering classification attempts particularly challenging.

Nevertheless, our phylogeny provides meaningful insights into the evolution of nesting biology within the tribe. First, both cleptoparasitic lineages were found to form a monophyletic group (Clade 3) in the majority of our analyses. Support for this clade was moderate to high, whereas support for any alternative placement was weak in all cases. Based on larval anatomy and nesting biology of *Radoszkowskiana rufiventris* and of some species of *Coelioxys*, Rozen and Kamel (2008) suggested that the ancestor of both genera shared a cleptoparasitic ancestor. An open question is the ancestral mode of parasitism: Rozen and Kamel (2008) suggested a similar mode of parasitism (the host egg is killed by the modified first larval instar) in *Radoszkowskiana* and *C. (Allocoelioxys)*, but not in *C. (Liothyrapis) decipiens*, with a larval anatomy and behaviour more similar to other members of *Coelioxys* (the host larva is killed by the modified third larval instar). Second, our analyses recover a well-supported clade that includes all leafcutting members of the tribe (clade 11), including taxa from Michener's Groups 2. A topology that groups the leafcutting subgenus *Chelostomoda* with Michener's group 1 has not been suggested before; it indicates that the sophisticated ability to cut circular leaf discs has evolved only once within the tribe.

4.1. Generic classification of the Megachilini

Our phylogeny does not support recent, morphology-based classification proposals to break up Michener's group 2 subgenera into several genera (Gonzalez, 2008; Engel and Gonzalez, 2011; Gonzalez and Engel, 2012). In particular, our results strongly indicate that the elongate ("hoplitiform" or "heriadiform") subgenera, placed into one genus in Gonzalez (2008), are scattered among lineages with a more robust body form. Some superficially similar, heriadiform subgenera such as *Chelostomoides* or *Hackeriapis*, long-thought to be closely related (see biogeographic analyses below; Michener, 1979, 2007) do not form a monophyletic group in our analyses and uniting these subgenera is not appropriate. An elongate body form is most likely an adaptation to nesting in narrow cavities (Praz et al., 2008; Sedivy et al., 2013; Rozen et al., 2015).

Table 1
Four classification proposals for the tribe Megachilini; genera under each proposal are presented in bold, with the estimated included number of species (based on Ascher and Pickering, 2016) indicated in parentheses. Morphologically heterogeneous genera which would be particularly challenging to identify under each proposal are indicated with an asterisk.

Proposal	Number of genera	Agreement with phylogeny	Diagnosability	Changes compared to actual classification	Included genera
1	2	Good	Good	Major	***Noteriades*** (16), ***Megachile*** [incl. *Coelioxys* and *Radoszkowskiana*] (2014)
2	7	Moderate; monophyly of *Megachile* not recovered in all analyses	Good	Minor	***Noteriades*** (16), ***Gronoceras*** (12), ***Matangapis*** (1), ***Heriadopsis*** (2), ***Coelioxys*** (475), ***Radoszkowskiana*** (4), ***Megachile*** [clade 4] (1520)
3	16	Good (placement and status of *Cestella* unknown)	Poor	Major	***Noteriades*** (16), ***Gronoceras*** (12), ***Matangapis*** (1), ***Heriadopsis*** (2), ***Coelioxys*** (475), ***Radoszkowskiana*** (4), ***Dinavis***∗ [clade 7, incl. *Cesacongoa* and the *niveofasciata*-gr] (7), ***Lophanthedon*** (5), ***Thaumatosoma***∗ [clade 5] (117), ***Maximegachile***∗ [clade 9; incl. *Neglectella*] (13), ***Callomegachile*** (98), ***Stenomegachile*** (4), ***Pseudomegachile*** (73), ***Chalicodoma*** (61), ***Chelostomoides*** (34), ***Megachile***∗ [incl. *Chelostomoda, Creightonella, Mitchellapis* and *Megella*] (1117)
4	20	Good (placement and status of *Cestella* unknown)	Acceptable	Major	***Noteriades*** (16), ***Gronoceras*** (12), ***Matangapis*** (1), ***Heriadopsis*** (2), ***Coelioxys*** (475), ***Radoszkowskiana*** (4), ***Dinavis*** (2), ***niveofasciata***-gr (4), ***Cesacongoa*** (1), ***Lophanthedon*** (5), ***Thaumatosoma***∗ [clade 5] (117), ***Maximegachile*** (3), ***Neglectella*** (10), ***Callomegachile*** (98), ***Pseudomegachile*** (73), ***Stenomegachile*** (4), ***Chalicodoma*** (61), ***Chelostomoides*** (34), ***Chelostomoda*** (22), ***Megachile*** (1095)

Table 2
New classification proposed for the bee tribe Megachilini. Genera are indicated in bold; new synonymies are indicated in parenthesis.

Genus ***Noteriades***
Genus ***Gronoceras***
Genus ***Matangapis***
Genus ***Heriadopsis***
Genus ***Radoszkowskiana***
Genus ***Coelioxys***
Genus ***Megachile***

Group 1 subgenera		Group 2 subgenera
Acentron	*Melanosarus*	*Austrochile*
Aethomegachile	*Mitchellapis*[a]	*Carinula*
Amegachile	*Moureapis*	*Callomegachile* (incl. *Alocanthedon*)
Argyropile	*Neochelynia*	*Cesacongoa* (=*Cuspidella*)
Austromegachile	*Neocressoniella*	*Cestella*
Austrosarus[c]	*Paracella* (incl. *Anodonteutricharaea)*	*Chalicodoma*
Chelostomoda[a]	*Phaenosarus*	*Chalicodomoides*
Chrysosarus	*Pseudocentron*	*Chelostomoides*
Creightonella[b]	*Ptilosaroides*	*Dinavis*
Cressoniella	*Ptilosarus*	*Hackeriapis*
Dactylomegachile[c]	*Rhyssomegachile*	*Lophanthedon*
Dasymegachile	*Sayapis*	*Maximegachile*
Eurymella (incl. *Platysta*)	*Schrottkyapis*	*Neglectella*
Eutricharaea	*Stelodides*[c]	*Pseudomegachile* (incl. *Largella* and *Parachalicodoma*)
Leptorachis	*Trichurochile*	*Rhodomegachile*
Litomegachile	*Tylomegachile*	*Schizomegachile*
Megachile s. str (incl *Grosapis and Eumegachile)*	*Xanthosarus*	*Stenomegachile*
Megachiloides	*Zonomegachile*[c]	*Thaumatosoma*
Megella[a]		

[a] Subgenera included in group 2 in Michener (2007).
[b] Lone subgenus of group 3 in Michener (2007).
[c] Placed in synonymy with *Megachile (Chrysosarus)* by Gonzalez (2013).

Based on the phylogenetic results presented here, we envision four possible classifications for the tribe Megachilini (Table 1): **(1)** Recognition of only two genera in Megachilini: *Noteriades* and *Megachile*, the latter including the cleptoparasitic genera *Radoszkowskiana* and *Coelioxys*, which would be downgraded to subgeneric level within a heterogeneous genus *Megachile* (clade 1 in Fig. 1); **(2)** Recognition of seven genera: *Noteriades*, *Gronoceras*, *Heriadopsis*, *Matangapis*, *Coelioxys, Radoszkowskiana* and *Megachile*, the latter including all other subgenera currently assigned to the genus *Megachile* (clade 4 in Fig. 1); *Heriadopsis* and *Matangapis* are recognized as distinct genera because of their tentative phylogenetic position (Fig. S2), the presence of an arolium and their straight-forward morphological diagnosis from all other genera of the Megachilinae; **(3)** Sixteen genera, with the seven genera recognized under proposal 2, plus the following: *Thaumatosoma* (clade 5), *Dinavis* (clade 7), *Lophanthedon*, *Maximegachile* (clade 9, thus with *Neglectella* included as a subgenus of *Maximegachile*), *Stenomegachile*, *Callomegachile*, *Pseudomegachile*, *Chelostomoides*, *Chalicodoma*; in this scheme the genus *Megachile* would include all leafcutting subgenera, including *Chelostomoda* (Clade 11); **(4)** Twenty genera, with the morphologically heterogeneous clades 7, 9 and 11 further divided (see Table 1) to improve morphological diagnosis of the different genera.

The establishment of new classifications (see Vences et al., 2013 for a careful review) should balance practical aspects (diagnosis of the groups; number of species; homonymies) with phylogenetic considerations. In the case of large and diverse groups such as Megachilini, accounting for these two aspects proves extraordinarily challenging. The advantages and disadvantages of our four classification proposals are summarized in Table 1. Proposals 1 (two genera), 3 (16 genera) and 4 (20 genera) are those best in agreement with our phylogenetic hypotheses: each of the resulting genera would be well supported and the classification might have greater stability. However, there are practical drawbacks to these proposals. The first proposal suggests downgrading the cleptoparasitic lineages (the genera *Coelioxys* and *Radoszkowskiana*) to subgenera within a large and heterogeneous genus *Megachile*. Our results indeed suggest that the cleptoparasitic lineages are derived from nest-building lineages of *Megachile senso latu*, given the well-supported position of the subgenus *Gronoceras* as the sister group to all other Megachilini (with the exception of *Noteriades*). A similar approach has been adopted for some bee groups (e.g., *Bombus* and the social parasite species included in the subgenus *Psithyrus*: Williams, 1994; Cameron et al., 2007; the cleptoparasitic *Hoplitis*: Sedivy et al., 2013). However, this approach is not desirable for Megachilini because of the strong morphological differences between *Megachile* and *Coelioxys* and the high number of species within both genera. Including both groups in the same genus would result in many homonymies and would likely not gain acceptance among bee taxonomists. The third proposal (16 genera) would also result in genera whose monophyly is well established, but whose diagnosis is challenging. Of the 14 non-parasitic genera recognized, four would be difficult to delimit using morphological criteria (Table 1). Particularly problematic are clades 5, 7 and 9, as they include morphologically heterogeneous species and are, to our knowledge, not supported by a single diagnostic morphological character. Modifying this proposal to end up with morphologically well-defined genera would result in 20 genera (proposal 4), of which a few would still be difficult to diagnose (most notably *Thaumatosoma*, Clade 5), at least under current morphological knowledge.

Our favoured classification, given our phylogeny and the poor current knowledge of the African and Asian faunas, is our proposal two, which results in the fewest changes to the present classification, reflects our phylogeny, and is practical in term of diagnosis. Consequently, *Gronoceras*, *Heriadopsis* and *Matangapis* are recognized here as valid genera of the tribe Megachilini in addition to the genera *Radoszkowskiana*, *Coelioxys, Noteriades* and *Megachile*, resulting in the following combinations (based on Ascher and Pickering, 2016): *Gronoceras africanibia* (Strand, 1912), *G. angolensis* (Cockerell, 1935), *G. armipygata* (Strand, 1911), *G. bombiformis* (Gerstäcker, 1857), *G. catulus* (Cockerell, 1910), *G. chapini* (Cockerell, 1935), *G. cincta* (Fabricius, 1781), *G. felina* Gerstäcker, 1857, *G. mabirensis* (Cockerell, 1937), *G. mutuala* (Strand, 1912), *G. praetexta* (Vachal, 1910), *Heriadopsis striatulus* Cockerell, 1931, *H. whiteanus* (Cameron, 1905) and *Matangapis alticola* (Cameron, 1902). The main drawback of this classification is the lack of strong support for clade 4, ie, the genus *Megachile* as established here, thus raising concerns about the stability of this classification. Once the African and Asian faunas are better described, it may be meaningful to adopt a classification closer to our proposals 3 or 4. Conversely, if more phylogenetic data confirms the monophyly of clade 4, we would strongly favour a classification close to our proposal 2. The new proposed classification is presented in Table 2.

4.2. Subgeneric classification

Our phylogeny strongly supports the following modifications at the subgeneric level. The groups known as *Carinula*, *Dinavis*, *Neglectella*, *Eurymella* and *Phaenosarus* are here reestablished as valid subgenera within the genus *Megachile*. These groups have been placed in synonymy with various subgenera of *Megachile* (Michener, 2000), but consistently emerged as distinct clades with high support values in all analyses. A list of the species included in these groups is provided by Pasteels (1965) and Mitchell (1980). The subgenus *Alocanthedon* is newly synonymized with the subgenus *Callomegachile*, and the subgenera *Largella* and *Parachalicodoma* are synonymized under the subgenus *Pseudomegachile* (new synonymies). Gonzalez et al. (2010) indicated that the subgenus known as *Platysta* was derived from *Eurymella*, which they kept in synonymy with the subgenus *Eutricharaea*. Although our sampling did not include any member of *Platysta*, we agree, based on morphological examination of both of its species (C. Praz, unpublished) with their treatment and place *Platysta* as a junior synonym of *Eurymella* (new synonymy). Michener (2000) placed the group known as *Anodonteutricharaea* (type species *M. lanigera*, included in Fig. S4) in synonymy with the subgenus *Eutricharaea*; in our analyses, *Anodonteutricharaea* (represented by *M. villipes*) was either sister to the subgenus *Paracella* (ML analyses; Fig. 1) or nested within *Paracella* (Fig. S4) and we place *Anodonteutricharaea* in synonymy with *Paracella* (new synonymy). Based on their phylogenetic position in all our analyses, *Eumegachile* and *Grosapis* are newly synonymized with the subgenus *Megachile* (new synonymies). The subgenus *Aethomegachile*, described from a single species from Thailand (Engel and Baker, 2006) appears to be a large Oriental group, in line with a recent treatment (Ascher et al., 2016). This subgenus was represented in our nuclear phylogeny by *M. conjuncta* and *M. kohtaoensis*, and several additional species clustered with them in the combined barcode + nuclear phylogeny (e.g., *M. laticeps* and *M. remota*; Fig. S4). The subgenus *Hackeriapis* was polyphyletic in our analyses (Fig. 1; Fig. S4); eventually several Australian, Group 2 subgenera may be united into a single subgenus, but we refrain from doing so given the poor resolution within clade 5 in our analyses. Lastly, our analyses also highlight several groups of species currently not placed into any existing subgenus: the *niveofasciata*-group of species (currently included in the subgenus *Chalicodoma*); the *ornata*-group of species (and *M. monoceros* Friese 1903, which showed affinities to this group), currently included in the subgenus *Callomegachile*; and the Australian group known as the *chrysopyga*-group of species, currently included in the subgenus *Eutricharaea*. All these groups appear distantly related to the subgenera in which they are currently placed.

4.3. Dating analyses

Our dating analyses indicate that the inclusion of the leafcutting trace fossils as a calibration point greatly altered ages throughout the trees. The ages inferred in analyses with this calibration point were nearly twice as old as those found in analyses without it. They actually correspond to analysis 1 (i.e., without prior distribution on the root) of Cardinal and Danforth (2013), while analyses without this calibration corresponded to their analysis 3 (with prior distribution on the root). In the absence of other fossils within Megachilini (see comments above regarding *Megachile glaesaria*), it is hard to evaluate our two alternative dating analyses. We favour the analysis without inclusion of these leafcutting trace fossils, for the following reasons. First, the assumption that these leaf incisions have been made by *Megachile* bees is only hypothetical given that many other insects produce arcuate excisions with sharp and cuspate margins (Labandeira, 2002: 50). In our opinion, and based on observations of excisions made by extant leafcutting bees, excisions can be confidently attributed to leafcutting bees only if they are oblong, cuspidate; leaves bearing only small, circular incisions are often found in habitats without leafcutting bees (C. Praz, pers. observation) and are likely made by other herbivorous insects. All Eocene fossil leafcuttings are rather small and circular and none bear the typical, oblong shape made by leafcutting bees. Second, dating analyses including the leafcutting fossils are less in keeping with the broader picture of hymenopteran phylogeny. Ronquist et al. (2012) suggested a crown age for Apoidea (thus the node including the bees and the apoid wasps) of 150 my, in line with Cardinal and Danforth's analysis 3. In contrast, our analyses with the leafcuttings included as a calibration point are in line with Cardinal and Danforth's analysis 1, which recovers a crown age for Apoidea of 181 my. Additionally, analyses without the leafcutting fossils are in line with our biogeographic scenario (detailed below) and the general fossil record for megachilid bees, in particular the absence of Megachilini from Eocene amber in spite of the widespread use of resin in early diverging lineages. In summary, we do not believe these fossils are indicative of the existence of leafcutting Megachilini in the Eocene.

4.4. Biogeography

Ancestral range inference under both the DEC and the DEC + J models suggests that the tribe Megachilini and most lineages of the dauber bees (Fig. 2, S3) originated either in the Afrotropic or as a widespread ancestral taxon that spanned both the Afrotropic and the Oriental region. The preferred biogeographical reconstruction under both the DEC and DEC + J models suggests a higher number of dispersal events from the Afrotropic to the Oriental (14) than between any other pair of geographic regions. This high dispersal confirms the statement of Michener (1979: 331) that the oriental faunal region "is inhabited by a depauperate African bee fauna". In *Megachile*, most groups present in both regions are indeed more diverse in the Afrotropic than in the Oriental region. Exchanges appear to have been nearly uninterrupted between both regions from at least 27.2 mya to the present, e.g., at the base of clade 2 (crown age 27.2 mya), and more recently, from the Afrotropic to the Oriental zone within *Eurymella* (crown age 14.78), *Callomegachile* (crown age 11.34) and *Amegachile* (crown age 11.26), and in the other direction at least within *Carinula* (crown age 13.59). The most recent documented exchanges between the Afrotropical and Oriental regions are found in *Eurymella* (0.82 and 3.79 my). For all these exchanges, it is likely that geodispersal over the Arabian Peninsula, and then through southern Iran and Pakistan, was possible under less arid climatic conditions (Michener, 1979; see Parker, 2009).

Both the DEC and the DEC + J model also agree on the mode of colonization of the Australian faunal region, namely, from the Oriental zone. Colonization of the Australian region is found three times in our phylogeny, although at least six further, more recent colonization events are implied in clades present in Northern Australia (*Coelioxys* and the subgenera *Callomegachile*, *Amegachile*, *Chelostomoda*, *Creightonella* and *Eutricharaea*) for which no Australian specimens were sampled. In the latter six cases, colonization was probably comparatively recent as the Australian members are morphologically similar to (or even conspecific with) species present in the Oriental zone (Michener, 1965, 1979; see also Rocha-Filho, 2016 for Australian *Coelioxys*). The earliest arrival of *Megachile* bees into the Australian region is found at the MRCA of a group of 5 (or 6) Australian subgenera of clade 5: *Hackeriapis*, *Thaumatosoma*, *Rhodomegachile*, *Chalicodomoides* and *Austrochile*; *Schizomegachile* also likely belongs to this group (Michener, 1965: 199). These subgenera colonized Australia once or more (phylogenetic relationships within clade 5 were poorly resolved) some 21 mya from the Oriental zone and considerable diversification occurred within these lineages in the temperate area of Australia. This time period is close to the Oligocene-Miocene boundary, which is posterior to the collision of the Australian plate with the Asian plate (around 25 mya: Hall, 2002), and which coincides with a period of global cooling and increase in the Antarctic Ice Sheet (Beddow et al., 2016). This would have reduced the oceanic barriers to dispersal from Asia to Australia. Our dating analyses with the calibration point corresponding to the fossil leafcuttings suggest an older age for clade 5 (40.5 mya), less in line with faunal exchanges between Asia and Australia. Fuller et al. (2005) and Chenoweth and Schwarz (2011) present and discuss several hypotheses for how allodapine bees may have reached Australia. The allodapine genus *Braunsapis* originated in Africa ca. 20.7 mya (26.1–17.7), dispersed into Asia 16.5 mya (20.5–13.3) and then into Australia 8.8 mya (11.9–7.0) (Fuller et al., 2005). This genus is still restricted to non-temperate regions of Northern Australia (Fuller et al., 2005). In contrast, the exoneurine genera of Allodapini (*Exoneura*, *Exoneurella*, *Brevineura* and *Inquilina*) are restricted to the temperate areas of southern Australia and are derived from a single, older dispersal into Australia (42–34 mya) (Chenoweth and Schwarz, 2011). Three scenarios for how exoneurine bees may have reached Australia were presented and discussed (Chenoweth and Schwarz, 2011): from southern Asia, as in *Braunsapis*; through a long-distance dispersal event from Africa through the Indian Ocean (Schwarz et al., 2006); or from Africa via Antarctica, also through long-distance dispersal. The biogeographic scenario inferred here for *Megachile* is interesting as it appears intermediate between the cases of *Braunsapis* (distributed in the north of Australia and likely diverged from an Oriental clade) and of the exoneurine genera (distributed in the south of Australia): Australian lineages of clade 5 are particularly diverse in temperate, southern Australia, but are inferred to have reached Australia from the Oriental Region, suggesting secondary adaptation to temperate climate. It is worth noting that inferred divergence times may substantially differ across studies: Chenoweth and Schwarz's inferred age for the crown age of Allodapini was 48.8 my (95% confidence interval 42–56), while the age for the same node was inferred to be 38 my (29–49) in analysis 3 of Cardinal and Danforth (2013), upon which our age estimates are based. Consequently the arrival of clade 5 *Megachile* into Australia may be contemporary to the arrival of the exoneurine bees.

The New World was colonized at least five times independently by non-parasitic Megachilini. These five colonization events are found at the base of the following clades: (1) the subgenus *Chelostomoides* (crown age 13.8 my, stem age 24.6 my), which has its centre of diversity in the Madrean region of North America and few species in northern South America; (2) the subgenus *Sayapis* (crown age 5.84, stem age 19.17), equally diverse in North and South America (Michener, 2007); (3) clade 17 (crown age 11.9, stem age 15.3), restricted to the New World, with early diverging lineages (*Argyropile*, *Phaenosarus*, *Megachiloides*) predominantly or exclusively found in the Nearctic region; clade 17 also includes a particularly large and diverse group of predominantly South American lineages, the *Pseudocentron* group of subgenera (clade 18, with 150 described species; Ascher and Pickering, 2016). (4) the clade containing the subgenera *Litomegachile* (Nearctic) and *Xanthosarus* (Holarctic), with crown age 5.65 and stem age 7.69; (5) clade 23 (crown age 8.51, stem age 12.3), with several predominantly Neotropical lineages and one Holarctic subgenus (*Megachile* s. str); phylogenetic relationships within clade 23 were not confidently established.

Regarding the dispersal routes associated with these five instances of colonization of the New World, both biogeographic models explored here suggest distinct scenarios. In the DEC model, colonization of the New World was only possible from the Palearctic, which resulted in ancestral ranges including the Palearctic for all clades from which the New World lineages were issued. In the DEC + J model, long-distance dispersal was possible and this model suggested that three of the five colonizations of the New World were achieved through long-distance dispersal, twice from the Afrotropic and once from the Oriental region. In contrast to other studies (see especially Almeida et al., 2011), the cases of long-distance dispersal events revealed in our DEC + J models are not strongly favoured over alternate scenarios. The first of these events was found at the base of *Chelostomoides*. This subgenus consistently nests in wood and its closed nests may have been carried over long distances but our data do not firmly exclude alternate scenario as the phylogenetic position of *Chelostomoides* was not confidently settled within Group 2. The second long-distance dispersal from Africa inferred under the DEC + J model is even more doubtful because it involves primarily ground-nesting lineages (Neff and Simpson, 1991; Sheffield et al., 2011, and references therein) at the base of clade 17. Although clade 17 includes a diverse and predominantly neotropical clade, (clade 18, the *Pseudocentron*-group), early-diverging lineages are found in temperate climates of North America and thus dispersal from the temperate Palearctic region appears more likely. The DEC + J model also suggested a dispersal event from the Oriental region into the New World at the MRCA of *Sayapis*. As discussed above, the relative phylogenetic placement of *Sayapis*, *Mitchellapis* and *Creightonella* were only poorly supported, and again, ancestral state reconstructions were ambiguous for these nodes.

All New World lineages of *Megachile*, including the subgenera *Sayapis*, *Chelostomoides*, *Litomegachile* and the early diverging lineages of clade 17 (subgenera *Argyropile*, *Phaenosarus*, *Megachiloides*), are present in North America as far north as southern Canada (Ascher and Pickering, 2016), with mean annual temperature (MAT) around 10° C (Hijmans et al., 2005). The faunal boundaries between the Oriental and Palearctic zones are not clear-cut within group 1 *Megachile*; many Oriental, subtropical subgenera are present in southern Japan or in central China, thus in the Palearctic region in areas with MAT around 12 °C. This pattern is especially pronounced in the leafcutting subgenera (*Chelostomoda*, *Amegachile*, *Megella, Aethomegachile*), the group within which most dispersal events into the New World have occurred. Wolfe (1994) suggested a MAT as high as 12 °C at paleolatitude 60°N (the latitude of Beringia) between 20 and 14 mya. Consequently the eastern Palearctic, subtropical region harbouring a particularly diverse megachiline fauna was likely more extensive in the Early Miocene and may have offered a corridor through Beringia into the New World. These ages and climatic conditions are perfectly in keeping with the oldest dates of arrival of Megachilini into the New World and suggest that lineages currently restricted to latitudes around 40°N and MAT 12 °C could have migrated through Beringia before 14 mya. Several additional exchanges between the Palearctic and the Nearctic regions are likely found in the Holarctic subgenera *Xanthosarus* and *Megachile* (crown ages 5.65 and 6.05 my, respectively); these two subgenera have their centre of diversity in boreal climates and thus are expected to have been able to cross Beringia after 14 mya. In keeping with conclusions inferred for lycaenid butterflies (Vila et al., 2011), Beringia appears to have acted as a climate-regulated dispersal route for bees.

4.5. Use of barcodes in phylogenetic studies

Our attempts to use DNA barcodes as a tool in phylogenetic reconstruction yield mixed results. On the one hand, our approach to using DNA barcodes to guide taxon sampling was promising: it highlighted several members of the heterogeneous subgenera *Callomegachile* (numbers 162, 164) and *Eutricharaea* (number 645), which, based upon our analyses of the entire DNA dataset and future morphological research, will likely eventually constitute new subgenera. In the case of the diverse and polyphyletic subgenus *Callomegachile*, our combined nuclear + barcode analysis (Fig. S4) also enabled the placement of the type species of *Callomegachile* (*M. mystaceana*) with high support values, facilitating future systematic treatment and reclassification of this group of bees.

However, the addition of COX1 to our nuclear data (ML analyses 5 and 6) hardly affected bootstrap support, and if it did, it mostly lowered support values (Table S3), confirming that this marker is of very limited utility for old divergences (e.g., Kjer et al., 2001, 2014; Mueller, 2006; Klopfstein et al., 2010; Ekrem et al., 2010). Moreover, our attempts to recover 34 confidently established clades with a matrix of the DNA barcodes demonstrate that COX1 alone contains very little phylogenetic signal beyond an approximate age of 4 my (Fig. 3). A few isolated clades with older crown ages were recovered in Bayesian analyses, but these placements were accompanied by many contradictory placements with equally high posterior probabilities. Such contradictory placements are particularly worrisome with respect to the use of barcodes in phylogenetics, as they are difficult to distinguish from reliable phylogenetic signal. There are several issues with relying on a single mitochondrial marker for phylogenetic placement, such as saturation in the phylogenetic signal, biased compositional frequencies (e.g., Timmermans et al., 2016), or, worse, horizontal transfer of mitochondria between distantly related species (Nicholls et al., 2012; Klopfstein et al., 2016). Our limited data do not enable us to distinguish between these hypotheses; however, base composition of the third nucleotide position of COX1 was strongly biased in our dataset, with a GC content ranging from 0.8% to 22.9%; all erroneous placements with support above 0.8 in Bayesian analyses involved the grouping of unrelated taxa with similarly biased base composition.

In conclusion, we do not recommend the use of COX1 alone in phylogenetics; however, the approach of combining a solid backbone inferred with a nuclear dataset with two markers used as a barcode (e.g., COX1 and LW-Rhodopsin or ribosomal DNA) has great potential (see Kjer et al., 2014). Given the limitations in the use of COX1 to delineate species due to mitochondrial introgression, heteroplasmy (Magnacca and Brown, 2010), or within-species divergences not associated with morphological divergences (e.g., Huemer et al., 2014; Kekkonen et al., 2015; Schmidt et al., 2015), having two barcoding markers, one mitochondrial and one nuclear would offer numerous advantages both for species delineation and for enabling their placement in a phylogenetic framework (Kjer et al., 2014).

5. Conclusion

Our study is a first step towards a phylogeny-based classification of the Megachilini yet further work is needed. First, our dataset should be completed for missing subgenera, especially from Michener's group 2: *Heriadopsis* and *Matangapis,* whose position was not well established here, and *Cestella* and *Schizomegachile*. Second, other phylogenetic markers, such as ultraconserved elements (Faircloth et al., 2014) may help settle relationships among the various lineages of the dauber bees. Once the generic classification is better established, much work remains to be undertaken at the subgeneric level: the subgeneric placement of many *Megachile* species remains unclear, especially in the diverse tropical faunas. Our approach of combining a core nuclear phylogenetic backbone with DNA barcodes represents a novel application of increasingly abundant barcode data, providing a testable hypothesis for their phylogenetic placement. In our study, over 400 species of *Megachile* (Fig. S4), more than a quarter of the known species diversity, were placed in a phylogenetic context.

Acknowledgement

CP wishes to express his deep gratitude to Maximilian Schwarz for his continuous support and for putting his large collection at our disposal for ongoing studies on the genus *Megachile*. We thank all people listed in Table S1 who contributed specimens for this study, in particular, Andreas Müller, Claudio Sedivy, Sam Droege (US Geological Survey), Denis Michez, Jakub Straka, Achik Dorchin, Terry Griswold (USDA ARS Bee Biology & Systematics Laboratory), Jerry Rozen and John Ascher (American Museum of Natural History), Philipe Sagot, Peter Kwapong, Alana Pindar and the parataxonomists associated with the TIGER project. Nicolai de Silva and Natalia Veiga were largely responsible for processing Thai and Argentinean bees for barcoding respectively. LP's sampling in Chile was supported by the Natural Sciences and Engineering Research Council of Canada through research and discovery grants, the National Geographic Society and his own salary. Sequencing was funded by the University of Neuchatel (among other, through an "Overhead" grant to CP) and the Linnean Society (grant to VT), and was performed at the Genetic Diversity Center of ETH Zurich. We thank Stephan Risch and Connal Eardley for identifying many specimens, Nadir Alvarez and Tomasz Suchan for assistance with the NGS data, and Jessica Litman and two anonymous reviewers for numerous comments on this manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.07.004.

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Supplementary material - Trunz et al. 2016

Methods

1. NGS data

We used next-generation sequencing techniques to obtain sequences of 28S for two taxa for which fresh specimens were not available: *Megachile (Matangapis) alticola* (one specimen collected in 1992) and an undescribed species of the subgenus *Heriadopsis* (one specimen collected in 2008, with highly degraded DNA). Both are important subgenera as they are the only species of *Megachile* having an arolium on all or some legs (Michener, 2007). To ensure the quality of these sequences, we performed two independent extractions using in total two legs from each specimen and constructed and sequenced libraries from each extraction independently. In addition, we included as additional quality control two pinned specimens for which we had obtained 28S sequences using Sanger sequencing techniques, *Radoszkowskiana rufiventris* and *M. (Lophanthedon) dimidiata*. Lastly, we designed several primer pairs to amplify short fragments of 28S in Megachilini, sequenced the weak PCR products obtained for *Heriadopsis* (no product was obtained for *Matangapis*) and compared these short sequences with the NGS sequences.

The library preparation was made with a custom protocol (modified from Bronner et al., 2014) derived from the standard Illumina protocol. The input DNA was analysed with a Fragment Analyser (Advanced Analytical) and Qbit Fluorometric Quantification (Life Technologies). No fragmentation step was performed because of the already degraded state of the DNA for *Matangapis* and *Heriadopsis*; sonication was used as a fragmentation method for the specimens of *Radoszkowskiana* and *M. dimidiata* included as positive controls. We did not amplify the 28S regions using conventional PCR but simply performed whole genome sequencing. The high number of copies of the ribosomal DNA ensures sufficient coverage and bypass the problem of PCR amplifications. Blunted-end-repair was performed using T4 Polymerase, followed by a purification step using QIAquick MinElute colums (Qiagen). Paired adapters were then added by ligation using T4 DNA ligase (New England Biolabs), and the resulting product was purified using QIAquick MinElute colums. The adapters were then filled in using Bst polymerase (New Englands Biolab) and the samples were purified and size-selected using AMPure XP beads (Beckman Coulter). Finally, the libraries were amplified using Fusion Hst Hight-fidelity DNA polymerase (Thermo Scientific) and analysed on an Illumina HiSeq 2500 platform at the University of Lausanne.

The conserved portions of the ribosomal DNA (but not the variable portions of ITS1 and ITS2) could be retrieved easily via reference-based assembly. We used MITObim v1.7 (Hahn et al*.,* 2013) to assemble the 1500 bp fragment of 28S used in our phylogeny. MITObim reconstructs full-length sequences via successive read baiting and mapping steps, initiated from a reference sequence. This algorithm accommodates high levels of sequence divergence, so that using two different references (i.e. 28S from *Noteriades* - the sister clade to all other Megachilini, or *Gronoceras bombiformis*) had no impact on the final results. We assembled each of the six samples (two extractions each for *Matangapis* and *Heriadopsis; Radoszkowskiana* and *M. dimidiata*) with both references and performed phylogenetic analyses with each of the fragments. We validated the obtained NGS results using conventional Sanger sequencing for *Radoszkowkiana*, *Lophanthedon* and the short fragments of 28S obtained for *Heriadopsis*. The sequences of both independent extractions of *Heriadopsis* and *Matangapis* were 100% identical, and all NGS sequences were 100% identical to the sequences obtained using Sanger sequencing. We thus concluded that our NGS approach to sequencing 28S was adequate and that the sequences obtained for *Heriadopsis* and *Matangapis* were reliable.

2. Phylogenetic analyses

2.1. Maximum likelihood

We performed ML analyses on the following six concatenated matrices: the entire nuclear matrix without COX1 (analysis 1); the entire nuclear matrix with COX1 (analysis 2); the nuclear matrix, without COX1, under exclusion of the significantly heterogeneous partition NAK3 (analysis 3); the latter without the introns (analysis 4); the matrix without NAK3, with the introns, with the three codon positions of COX1 (analysis 5); and the same matrix without the significantly heterogeneous third codon position of COX1 (analysis 6). Each of these six analyses was conducted under two different partitioning regimes: by codon position (analyses 1-6a; three partitions for the three codon positions of the nuclear genes, one partition for 28S, two partitions for the introns of EF and Opsin, and two or three partitions for the three codon positions of COX1); and following the more complex partitioning regime suggested by PartitionFinder v. 1.1.0 (Lanfear et al., 2012), with 9-11 partitions, depending on whether NAK3, COX3 or the introns were included (analyses 1-6b; p1: CAD pos1, EF pos1, NAK pos1; p2: EF pos2, NAK pos2; p3: EF pos3, EF Intron, p4: CAD pos2, Opsin pos1, Opsin pos2; p5: CAD pos3, Opsin pos3; p6, NAK pos3; p7: 28S; p8: Opsin Intron; p9: COX1 pos1; p10: COX1 pos2; p11: COX1 pos3).

2.2. BEAST analyses

We implemented the following parameters in our BEAST analyses: the Megachilinae were constrained to be monophyletic to ensure appropriate rooting of the tree; the tree prior was set to "speciation: Yule process"; and trees and parameters were sampled every 10,000 generations. Each partition had a separate substitution model: we started with a GTR + G for each partition, and simplified the model iteratively if convergence and high ESS values (>300) were not obtained for any of the parameters. We used Tracer (Rambaut et al., 2015) to examine trace files, determine an appropriate burn-in, assess convergence of each parameter and calculate ESS values. Our final analysis implemented a GTR + G for each partition, except a GTR + I for the second codon positions in the analyses partitioned by codon; and a GTR + G for all partitions in the analyses partitioned by gene (except a GTR + I for NAK if its third codon position was excluded). Finding an appropriate substitution model for COX1 was challenging and we had to implement a TN93 + G model with empirical base frequencies for all codon positions of COX1 (and for the single COX1 partition in the analyses partitioned by gene) to obtain parameter convergence. In analyses partitioned by codon, we implemented a lognormal uncorrelated clock model linked across all partitions, with the following parameters: lognormal, uncorrelated clock model. Priors for the UCLD.mean parameter were as follows: lognormal distribution, logmean = 0, log Stdev = 1, initial value = 1. We allowed each partition to evolve under its own substitution rate by adding a rate multiplier (".mu" parameter) manually in the .xml file. These rate multipliers were linked as a compound parameter ("allMus"). If COX1 was included, it had its own clock model and three (or two, if its third codon position was excluded) additional rate multipliers, linked as a distinct compound parameter ("COX.allMUS"). In analyses partitioned by gene, we implemented an independent clock model, and thus no rate multiplier, to each partition, given that the mean rate for each partition was estimated (except one, which was set to 1.0).

3. Biogeography

Our biogeography analyses were performed with dispersal rates that are constant throughout the tree. Although rates have likely varied over time, we did not explore a time-stratified model for the following reasons. Faunal exchanges between the Oriental region and Australia have likely been possible after the collision of the Australian plate with the Asian plate some 25 mya (Hall, 2002). The oldest dispersal event between these two regions occurred some 21 mya, in line with the value mentioned above. Similarly, the Bering Land Bridge was uninterrupted from at least 20 to 8 my (Milne, 2006); yet faunal exchanges must have been possible after the opening of the Bering Strait given that some boreal species of *Megachile* are holarctic (e.g., *M. (Megachile) lapponica*). The oldest dispersal between the Palearctic and the Nearctic has likely occurred between 24.6 and 13.8 my in *Megachile*, in line with the age mentioned above. Thus setting two or more time periods for these rates, and for the others, appears of little utility for our data.

The coding for each terminal (Fig. 2) reflects the geographic distribution of the entire subgenus or of the species-group that each terminal represents; a few exceptions are detailed here. The subgenus *Maximegachile* is primarily African, but there are regular records from southern Palearctic (Sinai, Israel); this subgenus was coded CD. Similarly, the subgenus *Megella* includes one Afrotropical species and one Oriental species, the latter entering the Palearctic region in Japan and possibly Northern China; this subgenus was coded CE as only the Oriental species could be sampled in our phylogeny and a maximum of two zones was allowed in the analysis. Within the subgenus *Chalicodoma*, our sampling included four Palearctic species (*montenegrensis, manicata*, *lefebvrei* and *parietina*) but no Afrotropical species. In a comprehensive, unpublished phylogeny of the subgenus *Chalicodoma* (Praz et al., in prep), the entirely Palearctic *montenegrensis* group (*montenegrensis*, *manicata*) branched first, then the Afrotropical species (not sampled in the present study), which were sister to a clade composed of the *lefebvrei* and *parietina* groups. To reflect this pattern with the three terminals sampled in the present phylogeny, we coded *montenegrensis* and *manicata* as Palearctic, *lefebvrei* as Afrotropical (although it is predominantly Palearctic) and *parietina* as Palearctic and Afrotropical. For the subgenus *Paracella*, three species are sampled in our phylogeny (*villipes*, from the Palearctic, an unidentified African species, and *M. tranquilla* from the Oriental region), with *villipes* sister to the other two in final analyses. However, this subgenus is very diverse and morphologically particularly heterogeneous in the Afrotropical zone, suggesting an African origin. We therefore coded *villipes* as both Afrotropical and Palearctic for our analyses.

Results

List of DNA barcodes unplaced in combined nuclear + barcodes analyses

Sequences from BOLD: the first characters represent the sequence ID and can be used to search for sequences or specimen information on BOLD.

BOWGH559-12|CCDB-14515-H01|Megachile AUS11|COI-5P
HYQTB217-12|gvc17720-1L|Megachile turneri|COI-5P
KBOFTH545 10HM404334B04746 G02 THAMegachile Megachile sp thai nds
BEZAF253-11|CCDB-15278 F03|Megachile|COI-5P
2BEEAF359 10HQ93280006711G02 KENMegachile
BOWMT428 10CCDB 09854 D12Megachile
BOTWA1973-12|CCDB-15259-G04|Megachile ARGZ02|COI-5P
GBAH9155-14|Megachile anthracina|COI-5P|KF861940
8BOWMT380 10CCDB 09853 H11Megachile flavihirsuta
BOFTW522-10|06708E01-BRA|Megachile laeta|COI-5P
BOFTW125 08B1401 C09Megachile sp LP 2
BEECF652 11CCDB 09863 G10Megachile
BOTWA1298-11|CHL-14509-66|Megachile|COI-5P
ABBOL053-15|Megachile kyotensis|COI-5P
BOWGF2188-12|CCDB-15274
BEECF675-11|CCDB-12076
BOWMT012 10CCDB 09669 A12Megachile
BOTWA578 1109806A08 TTMegachile
BOFTW535-10|06708F02-BRA|Megachile
GBAH8045-14|Megachile sp|COI-5P|KC853380
BBBEE1297-11|BB3115|Megachile
9BOFWM453 09HM404480B03749G01 GTMMegachile MEX17
BBBEE1225-11|BB2826|Megachile
BOFTW083 08B1397 H01Megachile sp LP 6
1BWTWO518 09CCDB 03773 D07Megachile COL05
3BEECA921 07CHL 0070Megachile flammiventris
BOFTW082 08B1397 G12Megachile sp 8
BOTWA1712-12|CCDB-15260-A05|Megachile
BOFTW041 08B1397 D07Megachile sp 6
8BOTWC151 10HQ937376CCDB 10006 E08Megachile sp3
BOWGH561-12|CCDB-14515-H03|Megachile AUS13|COI-5P
MSAPB1062-12|MSAPB CAN003|Megachile
1MSAPB111-11AAO014Megachile_albomarginata
BOWGH553-12|CCDB-14515-G07|Megachile AUS1|COI-5P

Sequences from Genbank:

Megachile (Neocressoniella) anthracina (KF861940 and KT960845)

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Table S1. Taxa included in the phylogenetic analyses, with locality data, voucher depository, and Genbank accession numbers. Brackets around the accession numbers for COX indicate that a published sequence from another specimen of the same species has been used.

Voucher	Name	Locality	Legit	Collection	Genbank accession numbers					
Number					EF	Opsin	CAD	NAK	28S	COX
-	Pararhophites orobinus	(from Genbank)			HQ995679	HQ995749	HQ995823	HQ995922	HQ996018	missing
-	Lithurgus chrysurus	(from Genbank)			EU851523	EU851629	HQ995837	HQ995934	HQ996031	KJ836731
-	Aspidosmia volkmanni	(from Genbank)			HQ995702	HQ995774	HQ995851	HQ995946	HQ996043	missing
-	Aglaoapis tridentata	(from Genbank)			EU851524	EU851630	HQ995844	HQ995939	HQ996036	KJ837073
-	Ochreriades fasciatus	(from Genbank)			EU851590	EU851696	HQ995909	HQ996001	HQ996098	EU863057
-	Hoplitis adunca	(from Genbank)			EU851572	EU851678	HQ995908	HQ996000	HQ996097	KJ839331
-	Chelostoma florisomne	(from Genbank)			EU851546	EU851652	HQ995905	HQ995997	HQ996094	JQ677598
-	Anthidium punctatum	(from Genbank)			EU851525	EU851631	EU851420	KU976186	KX060904	KJ839528
-	Afroheriades primus	(from Genbank)			EU851532	EU851638	HQ995902	HQ995995	HQ996092	missing
-	Noteriades sp	(from Genbank)			EU851589	EU851695	HQ995900	HQ995993	HQ996090	missing
1132	Gronoceras bombiformis	South Africa, Waterpoort	B. Danforth	Cornell University	HQ995733	HQ995806	HQ995886	HQ995979	HQ996076	KX580317
1117	Gronoceras felina	South Africa, Waterpoort	B. Danforth	Cornell University	KX428308	KX428054	KX428224	KX428392	KX428149	(BOLD BEZAF022-11)
432	Heriadopsis sp nov	Central African Republic, Bangui	J. Halada	Praz	Missing	Missing	Missing	Missing	KX580316	Missing
129	Matangapis alticola	Indonesia, Pujungan	U. Rosichon	Packer	Missing	Missing	Missing	Missing	KX580315	Missing
262	C. Allocoelioxys caudata	Iran, Persepolis	Sedivy, Praz, Monfared	Praz	KX428309	KX428055	KX428225	KX428393	KX428150	KX580318
266	C. Boreocoelioxys octodentata	USA, NY, Ithaca	C. Praz	Praz	KX428310	KX428056	KX428226	KX428394	KX428151	KX580319
264	C. Coelioxys aurolimbata	Greece, Kypariossos	C. Praz	Praz	KX428311	KX428057	KX428227	KX428395	KX428152	KX580320
261	C. Glyptocoelioxys pergandei	Chile, N Caldera	J. Litman	Praz	KX428312	KX428058	KX428228	KX428396	KX428153	KX580321
269	C. Liothyrapis decipiens	Uzbekistan, Khiva	C. Praz	Praz	KX428313	KX428059	KX428229	KX428397	KX428154	KX580322
1135	C. Liothyrapis sp	Thailand, Thung Salaeng Luang NP	Tiger project	Packer	KX428314	KX428060	KX428230	KX428398	KX428155	BOLD COFC009-10
1142	C. Synocoelioxys alternata	USA, NY, Ithaca	B. Danforth	Cornell University	GU245004	AF344591	KX428231	GU245164	GU244852	(BOLD COFC359-11)
259	C. Xerocoelioxys edita	USA, AZ, Wilcox	Michez & Praz	Praz	KX428315	KX428061	KX428232	KX428399	KX428156	(BOLD BEECC416-08)
1137	Radoszkowskiana rufiventris	Egypt, Tel el Kebir	J. Rozen	AMNH	HQ995747	HQ995821	HQ995901	HQ995994	HQ996091	(BOLD BUSA017-05)
206	M. Acentron sp	Argentina	Packer Lab	Packer	KX428316	KX428062	KX428233	KX428400	KX428157	BOLD BOTWA1309-11
1141	M. Aethomegachile conjuncta	Thailand, Chiang Mai	C. Sedivy	Praz	HQ995720	HQ995793	HQ995871	HQ995964	HQ996061	KX580323
168	M. Aethomegachile kohtaoensis	Thailand	Tiger project	Packer	KX428317	KX428063	KX428234	KX428401	KX428158	BOLD BOWGF321-09
126	M. Alocanthedon sp	Thailand, Khao Khitchakut NP	S. Charoenchal	Packer	KX428318	KX428064	KX428235	KX428402	KX428159	KX580324
85	M. Amegachile cf bituberculata	Central African Republic, Sibut	J. Halada	Praz	KX428319	KX428065	KX428236	KX428403	KX428160	(BOLD BOWGF2624-12)
1124	M. Amegachile fimbriata	South Africa, E Waterpoort	B. Danforth	Cornell University	HQ995721	HQ995794	HQ995872	HQ995965	HQ996062	KX580325
35	M. Amegachile saigonensis	Thailand, Chiang Mai	C. Sedivy	Praz	KX428320	KX428066	KX428237	KX428404	KX428161	(BOLD SBGB039-03)
80	M. Amegachile sp	Central African Republic, Bangui	J. Halada	Praz	KX428321	KX428067	KX428238	KX428405	KX428162	KX580326
1122	M. Anodonteutricharaea villipes	UZ, Bukara, Gazli	C. Praz	Cornell University	HQ995731	HQ995804	HQ995883	HQ995976	HQ996073	KX580327
1123	M. Argyropile parallela	USA, AZ, Portal	C. Praz	Cornell University	HQ995722	HQ995795	HQ995873	HQ995966	HQ996063	KX580328
1119	M. Austrochile sp	Australia, WA, Leonora	B. Danforth	Cornell University	HQ995723	HQ995796	HQ995874	HQ995967	HQ996064	KX580329
267	M. Austromegachile sp	Argentina	A. Espindola	Praz	KX428322	KX428068	KX428239	KX428406	KX428163	KX580330
1131	M. Callomegachile chrysorrhoea	South Africa, Vivo	B. Danforth	Cornell University	KX428323	KX428069	KX428240	KX428407	KX428164	KX580331
193	M. Callomegachile mephistolica	Kenya	Packer Lab	Packer	KX428324	KX428070	KX428241	KX428408	KX428165	BOLD BOWMT413-10
1102	M. Callomegachile sculpturalis	USA, Ithaca	C. Praz	Praz	HQ995724	HQ995797	HQ995875	HQ995968	HQ996065	(BOLD ABBOL043-15)
127	M. Carinula decemsignata	Ghana, Central region	P. Kwapong	Packer	KX428325	KX428071	KX428242	KX428409	KX428166	(BOLD BOWGH606-12)

86	M. Carinula sp	Central African Republic, Bangui	J. Halada	Praz	KX428326	KX428072	KX428243	KX428410	KX428167	Missing
319	M. Carinula stulta	Thailand, Chiang Mai	C. Sedivy	Praz	KX428327	KX428073	KX428244	Missing	Missing	KX580332
633	M. Cesacongoa sp	Tanzania, Pwani, Chalinze	WJ Pulawski	Bee Lab, Logan	KX428328	KX428074	KX428245	KX428411	KX428168	Missing
43	M. Chalicodoma lefebvrei	Greece, Peloponnese, Kotronas	C. Praz	Praz	KX428329	KX428075	KX428246	KX428412	KX428169	(BOLD BOWGH394-12)
55	M. Chalicodoma manicata	Israel, Mt-Hermon	Sedivy & Praz	Praz	KX428330	KX428076	KX428247	KX428413	KX428170	KX580333
1121	M. Chalicodoma montenegrensis	Greece, Ano Dhiakopto	Sedivy & Praz	Praz	KX428331	KX428077	KX428248	KX428414	KX428171	(BOLD BOWGH408-12)
150	M. Chalicodoma parietina	Italy, Gargano	S. Gerber	Praz	EU851530	EU851636	HQ995876	HQ995969	HQ996066	(BOLD FBAPB415-09)
1120	M. Chalicodomoides aethiops	Australia, WA, Marble Bar	Bee Lab, Logan	Bee Lab, Logan	HQ995725	HQ995798	HQ995877	HQ995970	HQ996067	Missing
1116	M. Chelostomoda sp	Thailand, Chiang Mai	C. Sedivy	Praz	HQ995726	HQ995799	Missing	HQ995971	HQ996068	KX580334
1139	M. Chelostomoides angelarum	USA, NV, Wheeler Well	Danforth lab	Cornell University	HQ995727	HQ995800	HQ995878	GU245163	GU244851	(BOLD BCII071-10)
202	M. Chelostomoides lobatifrons	Mexico	Packer lab	Packer	KX428332	KX428078	KX428249	KX428415	KX428172	BOLD BOFWM515-10
786	M. Chelostomoides prosopidis	USA, AZ	C. Praz	Praz	KX428333	KX428079	Missing	Missing	Missing	(BOLD BEECB828-07)
1107	M. Chelostomoides spinotulata	USA, AZ, Portal, Rucker Canyon	C. Praz	Praz	HQ995728	HQ995801	HQ995879	HQ995972	HQ996069	(BEECC124-08)
1110	M. Chrysosarus sp	Argentina, Jujuy Co., Paso de Jama	Bee lab, Logan	Bee Lab, Logan	HQ995729	HQ995802	HQ995880	HQ995973	HQ996070	KX580335
372	M. Creightonella albisecta	Italy, Basilicata, Sant'Arcangelo	Trunz, Litman, Praz	Praz	EU851529	EU851635	HQ995881	HQ995974	HQ996071	(BOLD GBACU326-12)
1129	M. Creightonella cornigera	South Africa, Limpopo Prov.,	B. Danforth	Cornell University	KX428334	KX428080	KX428250	KX428416	KX428173	KX580336
1109	M. Cressoniella zapoteca	USA, AZ, Cochise Co.,	C. Praz	Cornell University	HQ995730	HQ995803	HQ995882	HQ995975	HQ996072	KX580337
276	M. Dasymegachile sp	Chile, Atacama, Caldera	J. Litman	Praz	KX428335	KX428081	KX428251	KX428417	KX428174	KX580338
93	M. Dinavis leucospilura	RSA, Karoo	J. Halada	Praz	KX428336	KX428082	KX428252	KX428418	KX428175	KX580339
81	M. Eumegachile bombycina	Russie, Ordynskiy	D. Michez	Praz	KX428337	KX428083	KX428253	KX428419	KX428176	KX580340
58	M. Eurymella aff eurymera	Central African Republic, Bangui	J. Halada	Praz	KX428338	KX428084	KX428254	KX428420	KX428177	KX580341
82	M. Eurymella aff semierma	Central African Republic, Bangui	J. Halada	Praz	KX428339	KX428085	KX428255	KX428421	KX428178	KX580342
1118	M. Eurymella patellimana	Oman, Sur	D. Michez	Praz	HQ995732	HQ995805	HQ995884	HQ995977	HQ996074	KX580343
211	M. Eurymella pyrrothorax	Kenya	Packer Lab	Packer	KX428340	KX428086	KX428256	KX428422	KX428179	BOLD BEEAF300-10
103	M. Eurymella rosarum	Malawi, Viphya Mts, Chikangawa	A. Freidberg	Praz	KX428341	KX428087	KX428257	KX428423	KX428180	KX580344
128	M. Eurymella sp	Thailand	Tiger project	Packer	KX428342	KX428088	KX428258	KX428424	KX428181	KX580345
26	M. Eurymella sp aff patellimana	Thailand, Chiang Mai Prov.	C. Sedivy	Praz	KX428343	KX428089	KX428259	KX428425	KX428182	Missing
87	M. Eurymella sp patellimana-group	Central African Republic, Sibut	J. Halada	Praz	KX428344	KX428090	KX428260	KX428426	KX428183	KX580346
32	M. Eutricharaea burdigalensis	Armenia, Ghor Vidap	SW Droege	Praz	KX428345	KX428091	KX428261	KX428427	KX428184	KX580347
41	M. Eutricharaea flabellipes	Greece, Peloponnese, Kotronas	C. Praz	Praz	KX428346	KX428092	KX428262	KX428428	KX428185	KX580348
22	M. Eutricharaea giraudi	Italy, Piemonte, Susa	Litman & Praz	Praz	KX428347	KX428093	KX428263	KX428429	KX428186	KX580349
320	M. Eutricharaea hera	Thailand, Chiang Mai Prov.	C. Sedivy	Praz	KX428348	KX428094	KX428264	KX428430	KX428187	KX580350
39	M. Eutricharaea leucomalla	Greece, Peloponnese, Mycenae	C. Praz	Praz	KX428349	KX428095	KX428265	KX428431	KX428188	KX580351
44	M. Eutricharaea nitidicollis	Iran	M. Soraya	Praz	KX428350	KX428096	KX428266	KX428432	KX428189	KX580352
1140	M. Eutricharaea pilidens	Switzerland, Weiach	A. Müller	Praz	EU851531	EU851637	HQ995885	HQ995978	HQ996075	(BOLD FBAPB418-09)
318	M. Eutricharaea sp	Thailand, Chiang Mai Prov.	C. Sedivy	Praz	KX428351	KX428097	KX428267	KX428433	KX428190	KX580353
195	M. Eutricharaea sp malangensis-gr.	South Africa	L. Packer	Packer	KX428352	KX428098	KX428268	KX428434	KX428191	BOLD BEZAF523-11
63	M. Eutricharaea sp nov	Iran, Kalus	Sedivy, Praz & Monfared	Praz	KX428353	KX428099	KX428269	KX428435	KX428192	KX580354
30	M. Eutricharaea sp nov malangensis-gr.	Oman, Al Hamra	Michez & Patiny	Praz	KX428354	KX428100	KX428270	KX428436	KX428193	KX580355
652	M. Grosapis cockerelli	Mexico, Chih., Gpe. y Calvo	Erika Esquivel	Praz	KX428355	KX428101	KX428271	KX428437	KX428194	KX580356
1114	M. Hackeriapis sp1	Australia, WA, Peak Charles NP	B. Danforth	Cornell University	KX428356	KX428102	KX428272	KX428438	KX428195	KX580357
1115	M. Hackeriapis sp2	Australia, WA, Coolgardie	B. Danforth	Cornell University	HQ995734	HQ995807	HQ995887	HQ995980	HQ996077	KX580358
1136	M. Largella floralis	Thailand, Phetchabun Nam Nao NP	Tiger project	Packer	HQ995735	HQ995808	HQ995888	HQ995981	HQ996078	(BOLD BOWGH510-12)
263	M. Leptorachis petulans	USA, NY, Suffolk Co, Gardinera Island	RG Goelet	Praz	KX428357	KX428103	KX428273	KX428439	KX428196	(BOLD BEECA146-06)
205	M. Leptorachis sp	Argentina	Packer Lab	Packer	KX428358	KX428104	KX428274	KX428440	KX428197	BOLD BOTWA1303-11
1125	M. Litomegachile texana	USA, NY, Ithaca	C. Praz	Praz	HQ995736	HQ995809	HQ995889	HQ995982	HQ996079	(BOLD BBHYL303-10)

161	M. Lophanthedon dimidiata	Thailand	Packer Lab	Packer	KX428359	KX428105	KX428275	KX428441	KX428198	BOLD BOFTH385-09
1133	M. Maximegachile maxillosa	South Africa, Mount Rupert	J. Litman	Praz	HQ995737	HQ995810	HQ995890	HQ995983	HQ996080	(BOWGF1159-10)
750	M. Megachile lapponica	Russie, Mondy	D. Michez	Praz	KX428360	KX428106	KX428276	Missing	Missing	KX580359
742	M. Megachile ligniseca	Switzerland, Eclepens	C. Praz	Praz	KX428361	KX428107	KX428277	Missing	Missing	KX580360
389	M. Megachile melanopyga	Switzerland, VS, Hohtenn	A. Müller	Praz	HQ995738	HQ995811	HQ995891	HQ995984	HQ996081	(FBAPB412-09)
339	M. Megachile octosignata	Italy, Abruzzo, Assergi, San Pietro	Trunz, Litman & Praz	Praz	KX428362	KX428108	KX428278	Missing	Missing	KX580361
40	M. Megachile pilicrus	Greece, Peloponnese, Kardamyli	C. Praz	Praz	KX428363	KX428109	KX428279	Missing	Missing	KX580362
1104	M. Megachiloides nevadensis	USA, UT, Wayne Co, South Torrey	C. Praz	Praz	HQ995739	HQ995812	HQ995892	HQ995985	HQ996082	KX580363
217	M. Megella pseudomonticola	South Korea, Chungnam	Tripotin	Praz	KX428364	KX428110	KX428280	KX428442	KX428199	Missing
201	M. Melanosarus sp	Argentina	Packer Lab	Packer	KX428365	KX428111	KX428281	KX428443	KX428200	BOLD BOFTW081-08
1106	M. Mitchellapis fabricator	Australia, NSW, Wodonga	B. Danforth	Cornell University	HQ995740	HQ995813	HQ995893	HQ995986	HQ996083	Missing
634	M. Neglectella laminata	Tanzania, Morogoro	WJ Pulawski	Bee Lab, Logan	KX428366	KX428112	KX428282	KX428444	KX428201	Missing
200	M. Neochelynia? sp	Mexico	Packer Lab	Packer	KX428367	KX428113	KX428283	KX428445	KX428202	BOLD BOWMT020-10
72	M. Paracella sp	Central African Republic, Sibut	J. Halada	Praz	KX428368	KX428114	KX428284	KX428446	KX428203	Missing
1126	M. Paracella tranquilla	Thailand, Chiang Mai	C. Sedivy	Praz	KX428369	KX428115	KX428285	KX428447	KX428204	KX580364
33	M. Parachalicodoma sp nov	Israel, Ein Yahav	Sedivy & Praz	Praz	KX428370	KX428116	KX428286	KX428448	KX428205	Missing
436	M. Phaenosarus fortis	USA, South Dakota, Jackson Co	J. Devallez	Praz	KX428371	KX428117	KX428287	KX428449	KX428206	KX580365
1105	M. Pseudocentron sidalceae	USA, AZ, Wilcox	C. Praz	Praz	HQ995741	HQ995814	HQ995894	HQ995987	HQ996084	KX580366
268	M. Pseudocentron sp	Chile, Coquimbo Reg	J. Litman	Praz	KX428372	KX428118	KX428288	KX428450	KX428207	KX580367
1134	M. Pseudomegachile bullata	SA, Northern Cape, Campbell	J. Litman	Praz	KX428373	KX428119	KX428289	KX428451	KX428208	KX580368
386	M. Pseudomegachile ericetorum	France, Ardèche	C. Praz & J. Litman	Praz	HQ995742	HQ995815	HQ995895	GU245165	GU244853	(BOLD FBHAP343-09)
102	M. Pseudomegachile foersteri	Israel, En Zetim	L. Friedman	Praz	KX428374	KX428120	KX428290	KX428452	KX428209	Missing
213	M. Pseudomegachile lanata	Oman, Al Quiwair, Muscat	Michez & Patiny	Praz	KX428375	KX428121	KX428291	KX428453	KX428210	KX580369
214	M. Pseudomegachile nigripes	Egypt, Sharkia, Houssania	M. Shebl & Kamel	Praz	KX428376	KX428122	KX428292	KX428454	KX428211	Missing
1138	M. Pseudomegachile rubripes	Uzbekistan, Khiva	C. Praz	Praz	KX428377	KX428123	KX428293	KX428455	KX428212	(BOLD BOWGF623-09)
24	M. Pseudomegachile saussurei	Iran, Persepolis	Sedivy, Praz, Monfared	Praz	KX428378	KX428124	KX428294	KX428456	KX428213	(BOLD BWONE359-09)
1127	M. Pseudomegachile sinuata	South Africa, Limpopo Prov.	B. Danforth	Cornell University	KX428379	KX428125	KX428295	KX428457	KX428214	Missing
1112	M. Ptilosarus microsoma	Trinidad, El Dorado, Caura Valley	Bee Lab, Logan	Bee Lab, Logan	HQ995743	HQ995816	HQ995896	HQ995988	HQ996085	Missing
1111	M. Rhodomegachile sp	Australia, W, Tom Price	B. Danforth	Cornell University	HQ995744	HQ995817	HQ995897	HQ995989	HQ996086	KX580370
1100	M. Sayapis pugnata	USA, NY, Schuyler Co.	Danforth lab	Cornell University	AY585147	HQ995818	DQ067196	HQ995990	HQ996087	(BOLD BBHYL312-10)
199	M. Sayapis sp	Argentina	Packer Lab	Packer	KX428380	KX428126	KX428296	KX428458	KX428215	BOLD BOFTW077-08
209	M. sp Pseudocentron-group	Argentina	Packer Lab	Packer	KX428381	KX428127	KX428297	KX428459	KX428216	BOLD BOFTW304-10
165	M. Stelodides euzona	Chile	Packer Lab	Packer	KX428382	KX428128	KX428298	KX428460	KX428217	BOLD BOTWC598-10
437	M. Stenomegachile chelostomoides	Kenya, E of Mwingi	Snizek	Praz	KX428383	KX428129	KX428299	KX428461	KX428218	KX580371
1113	M. Thaumatosoma remeata	Australia, WA, Laverton	B. Danforth	Cornell University	HQ995745	HQ995819	HQ995898	HQ995991	HQ996088	Missing
203	M. Tylomegachile? sp	Argentina	Packer Lab	Packer	KX428384	KX428130	KX428300	KX428462	KX428219	BOLD BOFTW079-08
162	M. unplaced “monoceros” Friese	Thailand	Tiger project	Packer	KX428385	KX428131	KX428301	KX428463	KX428220	BOLD BOFTH397-09
164	M. unplaced sp aff ornata	Thailand	Tiger project	Packer	KX428386	KX428132	KX428302	KX428464	KX428221	BOLD BOTV027-11
645	M. unplaced sp chrysopyga-group	Western Autralia, Pardoo	F.D.Parker, M.E.Irwin	Bee Lab, Logan	KX428387	KX428133	KX428303	KX428465	KX428222	KX580372
136	M. unplaced sp niveofasciata-group	RSA, Kimberly	J. Litman	Praz	KX428388	KX428134	KX428304	KX428466	KX428223	KX580373
427	M. Xanthosarus diabolica	Turkey, Adana, Hamidiye	J. Straka	Praz	KX428389	KX428135	KX428305	Missing	Missing	KX580374
744	M. Xanthosarus lagopoda	Italy, S.Giovanni Rotondo	J. Litman & C. Praz	Praz	KX428390	KX428136	KX428306	Missing	Missing	(BOLD FBAPB392-09)
1103	M. Xanthosarus maritima	Uzbekistan, Bukara	C. Praz	Praz	HQ995746	HQ995820	HQ995899	HQ995992	HQ996089	KX580375
741	M. Xanthosarus nigriventris	Switzerland, VS, Bovernier	C. Praz	Praz	KX428391	KX428137	KX428307	Missing	Missing	(BOLD BCHYM421-13)
-	M. rotundata	(from Genbank)	-	-	XM_003705302	XM_003705921	XM_012287299	XM_012290255	Missing	BOLD FBHAP356-09

438	Gronoceras mutuala	Central African Republic, NE Nota	J. Halada	Praz	-	KX428138	-	-	-	KX580376
637	M. Hackeriapis sp 15	Australia, W, Auski Rd House	F.D. Parker, M.E.Irwin	Bee Lab, Logan	-	KX428139	-	-	-	KX580377
641	M. Hackeriapis sp 19	Australia, W, Mt. Augustus NP	F.D. Parker, M.E.Irwin	Bee Lab, Logan	-	KX428140	-	-	-	KX580378
642	M. Hackeriapis sp. 6	Australia, W, Great Northern HWY	F.D. Parker, M.E.Irwin	Bee Lab, Logan	-	KX428141	-	-	-	KX580379
635	M. aurifrons	Australia, W, Marble bar	F.D. Parker, M.E.Irwin	Bee Lab, Logan	-	KX428142	-	-	-	KX580380
640	M. Hackeriapis sp. 18	Australia, W, Auski Rd House	F.D. Parker, M.E.Irwin	Bee Lab, Logan	-	KX428143	-	-	-	KX580381
296	M. riyadhensis	Israel, Avenat, Rt 90	Freidberg	Praz	-	KX428144	-	-	-	KX580382
843	M. Eurymella sp	Kenya, Coast Province, Mida Creek	L. Packer D. Martins	Packer	-	KX428145	-	-	-	BOLD BOWGF2711-13
847	M. sp Pseudocentron-group	Argentina Entré Rios El Palmar	A. Taylor	Packer	-	KX428146	-	-	-	BOLD BOTWA1971-12
845	M. ventrisi	Kenya, Lake Bogoria Lakeshore	L. Packer D. Martins	Packer	-	KX428147	-	-	-	BOLD BEEAF565-11
841	M. Megachile sp	Russia, Primorksy Reg. Terekhovka	M. Proshchalykin	Packer	-	KX428148	-	-	-	BOLD EPALB028-10

Table S2. Sequences of all primers used in this study and PCR conditions for each primer pair.

Primer	**Sequence (5' to 3')**
Elongation factor	
HaF2For	GGG YAA AGG WTC CTT CAA RTA TGC
Intron2Rev-meg	GAA AAT CCT CCG GTG GAA AC
F2Rev-meg	AAT CAG CAG CAC CCT TRG GTG G
LW-Rhodopsin	
OpsinFora	AAT TGY TAY TWY GAG ACA TGG GT
OpsinFor5	GCG TGC GGC ACC GAY TAC TTC
OpsinRev6-meg	GCC ARY GAY GGG AAY TTC T
OpsinRev3-meg	GCY AGT TTA CAC TCK GCR CT
CAD	
CADFor4	TGG AAR GAR GTB GAR TAC GAR GTG GTY CG
CADFor2-meg	GAT GGG ATC TRG GRA AAT TTC
CADRev1-meg	GCC ATC RCT TCT CCT ACR CTC TTC AT
CADRev4a	GGC CAY TGN GCN GCC ACY GTG TCT ATY TGY TTN ACC
NAK	
NAKFor1	GGY GGT TTC GCS WTG YTG YTG TGG ATC GG
NAKFor2-meg	GCR TTC TTC TCV ACR AAY GCY GTY GAR GG
NAKRev1a-meg	CCR ATC ARG AAG ATA ACC GCG TCY AAC CAR TG
NAKRev2	ACC TTG ATR CCG GCY GAW CGG CAC TTG GC
NAKFor3-Meg	CAR ATC ATC GAR GCC GAY AC
28S	
A	CCC CCT GAA TTT AAG CAT AT
Mar	TAG TTC ACC ATC TTT CGG GTC CC
Bel	AGA GAG AGT TCA AGA GTA CGT G
D4	GTT ACA CAC TCC TTA GCG GA
COI	
LepF1	ATT CAA CCA ATC ATA AAG ATA T
LepR1	TAA ACT TCT GGA TGT CCA AAA A
PCR conditions	
Elongation factor	
HaF2For / Intron2Rev-meg	5' 94°C // [30" 94°C / 30" 57°C / 30" 72°C] (35x) // 7' 72°C
HaF2For / F2Rev-meg	5' 94°C // [45" 94°C / 45" 58°C / 1'15" 72°C] (35x) // 7' 72°C
LW-Rhodopsin	
OpsinFora / OpsinRev3-meg	5' 94°C // [45" 94°C / 45" 58°C / 45" 72°C] (35x) // 7' 72°C
OpsinFor5 / OpsinRev6-meg	5' 94°C // [45" 94°C / 45" 58°C / 45" 72°C] (35x) // 7' 72°C
CAD	
CADFor4 / CADRev1-meg	5' 94°C // [30" 94°C / 30" 57°C / 30" 72°C] (35x) // 7' 72°C
CADFor2-meg / CADRev4a	5' 94°C // [30" 94°C / 30" 57°C / 30" 72°C] (35x) // 7' 72°C
NAK	
NAKFor1 / NAKRev1a-meg	5' 94°C // [45" 94°C / 45" 58°C / 45" 72°C] (35x) // 7' 72°C
NAKFor2-meg / NAKRev2	5' 94°C // [45" 94°C / 45" 58°C / 1'15" 72°C] (35x) // 7' 72°C
28S	
A / Mar	5' 94°C // [1' 94°C / 1' 58°C / 1'30" 72°C] (35x) // 7' 72°C
Bel / D4	5' 94°C // [1' 94°C / 1' 58°C / 1'30" 72°C] (35x) // 7' 72°C
COI	
LepF1 / LepR1	1' 94°C // [1' 94°C / 1'30" 45°C / 1'15" 72°C] (5x) // [1' 94°C / 1'30" 51°C / 1'15" 72°C] (30x) // 5' 72°C

Table S3. Nodal support for taxonomically important nodes or nodes discussed in the text. All subgenera of *Megachile* s. l. not included in this table are supported by values above 95% (maximum likelihood) or of 1.0 (Bayesian analyses). Values represent bootstrap support values and posterior probability values, for maximum likelihood and Bayesian analyses, respectively; they are presented for analyses of six different matrices (1 and 7, entire nuclear dataset without COX1; 2 and 8, entire nuclear dataset with COX1; 3 and 9, nuclear dataset without the third position of NAK, without COX1; 4 and 10, as 3 and 9, without the introns; 5 and 11, as 3 and 9, with COX1; 6 and 12, as 5 and 11, without the third codon position of COX1). In each cell, left values (analyses 1-12a) correspond to the analyses of the matrix partitioned by codon (Maximum likelihood analyses and Bayesian analyses); right values (1-12b) correspond to the analyses partitioned by the regime suggested by PartitionFinder (maximum likelihood) or partitioned by gene (Bayesian analyses).

	Maximum likelihood						Bayesian analyses					
	no COX1	with COX1	no NAK3 no COX1	no NAK3, no COX1, no introns	no NAK3 with COX1	noNAK3 no COX1 nt3	no COX1	with COX1	no NAK3 no COX1	no NAK3, no COX1, no introns	no NAK3 with COX1	noNAK3 no COX1 nt3
Clade/Taxon	1a/1b	2a/2b	3a/3b	4a/4b	5a/5b	6a/6b	7a/7b	8a/8b	9a/9b	10a/10b	11a/11b	12a/12b
Megachilini	100/100	100/100	100/100	100/100	100/100	100/100	1/1	1/1	1/1	1/1	1/1	1/1
1	94/95	99/99	96/94	95/95	99/99	96/96	1/1	1/1	1/1	1/1	1/1	1/1
2	100/100	100/100	100/100	100/100	100/100	100/100	1/1	1/1	1/1	1/1	1/1	1/1
3	<50/-	<50/81	68/69	59/60	83/81	74/72	0.62/-	1/0.99	0.92/0.99	0.98/0.94	1/1	1/0.99
Coelioxys	100/100	100/100	100/100	100/100	100/100	100/100	1/1	1/1	1/1	1/1	1/1	1/1
4	<50/-	-/-	<50/<50	<50/<50	-/-	<50/-	<0.5/-	-/0.99	0.64/-	<0.5/0.55	-/0.95	0.5/<0.5
5	83/84	72/70	78/74	65/66	69/70	77/78	1/1	1/1	1/1	1/1	1/1	1/1
6	64/63	54/56	73/71	65/63	59/56	74/73	0.85/-	0.98/0.7	0.99/1	0.98/0.99	0.96/1	0.94/0.98
7	86/92	80/84	95/98	94/96	81/84	73/80	1/1	1/1	1/1	1/1	1/1	1/1
8	100/100	100/100	100/100	99/100	100/100	100/100	1/1	1/1	1/1	1/1	1/1	1/1
9	94/96	95/96	96/96	94/94	94/96	93/95	1/1	1/1	1/1	1/1	1/1	1/1
10	<50/<50	-/<50	55/61	52/58	-/<50	<50/<50	0.84/-	-/-	0.89/0.87	0.88/0.87	-/-	-/-
11	86/88	94/95	98/99	98/99	97/95	97/98	1/1	1/1	1/1	1/1	1/1	1/1
12	97/97	94/89	94/94	92/88	91/89	92/91	1/1	1/1	1/1	1/1	1/1	1/1
13	78/77	97/89	53/-	62/52	94/89	93/92	1/1	1/1	-/-	-/-	1/1	1/1
14	95/93	93/75	77/73	73/72	74/75	89/89	1/1	1/1	1/1	1/1	1/0.99	1/1
15	98/98	99/98	96/98	97/96	98/98	96/96	1/1	1/1	1/1	1/1	1/1	1/1
Eurymella	97/97	95/92	85/84	88/86	89/92	80/78	1/1	1/1	0.94/0.96	0.86/0.96	0.84/0.97	0.77/0.98
16	89/94	99/99	95/97	92/94	99/99	99/99	1/1	1/1	1/1	1/1	1/1	1/1
17	100/100	100/100	100/100	100/100	100/100	100/100	1/1	1/1	1/1	1/1	1/1	1/1
18	99/100	99/100	100/100	100/100	100/100	100/100	1/1	1/1	1/1	1/1	1/1	1/1
19	75/69	75/61	68/73	<50/<50	58/61	71/72	0.73/0.56	0.85/0.86	-/-	<0.5/-	0.72/-	0.57/-
20	96/96	99/97	94/93	59/53	97/97	96/97	1/1	1/1	1/1	0.66/<0.5	1/1	1/1
21	97/97	98/99	95/96	98/99	98/99	97/98	1/1	1/1	1/1	1/1	1/1	1/1
22	92/92	83/92	92/92	99/97	92/92	93/94	1/1	1/1	1/1	1/1	1/1	1/1
Xanthosarus	80/84	75/73	79/82	75/86	71/73	61/62	1/1	1/1	1/1	1/1	1/1	1/1
23	100/100	100/99	99/99	99/99	99/99	100/100	1/1	1/1	1/1	1/1	1/1	1/1
Megachile	91/92	82/67	71/71	<50/<50	64/67	55/-	1/1	1/1	1/1	<0.5/-	0.92/1	0.93/0.88

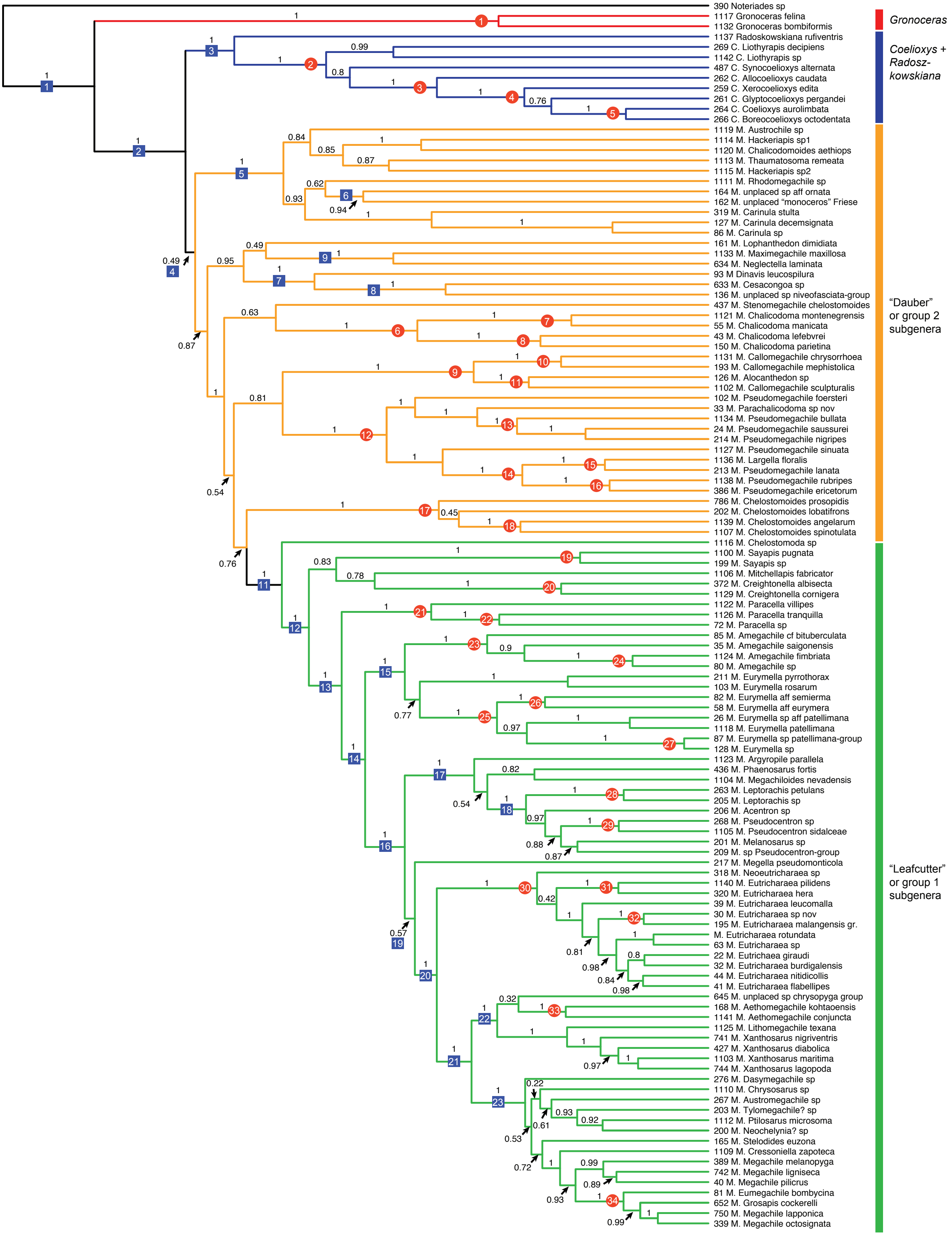
Figure S1. Maximum clade credibility tree found in the dated BEAST analysis of the matrix with significantly heterogeneous partitions removed (third codon positions of NAK and COX), with the introns, without the calibration point corresponding to the fossil leafcuttings, partitioned by codon position. Outgroup taxa have been removed. Values above branches represent posterior probabilities. Numbers in blue squares indicate 23 nodes discussed in the text for which no taxonomic name is available, numbers in red circles indicate the 34 clades selected for examination of the phylogenetic signal of mitochondrial DNA barcodes.

Figure S2. Best tree found in our preferred maximum analyses (entire matrix without the third codon positions of NAK and COX, partitioned by codon) with two additional species (*Matangapis monticola* and an undescribed species of *Heriadopsis*) represented only by the 1500 bp fragment of 28S obtained using next generation sequencing techniques. Outgroup taxa have been removed. Values above branches represent bootstrap support based on 1000 bootstrap replicates.

Figure S3. Maximum clade credibility tree found in our preferred BEAST analysis (entire matrix without the third codon positions of NAK and COX, partitioned by codon, without internal calibration of the fossil leafcuttings) with ancestral ranges inferred by the DEC + J analysis in Biogeobears.

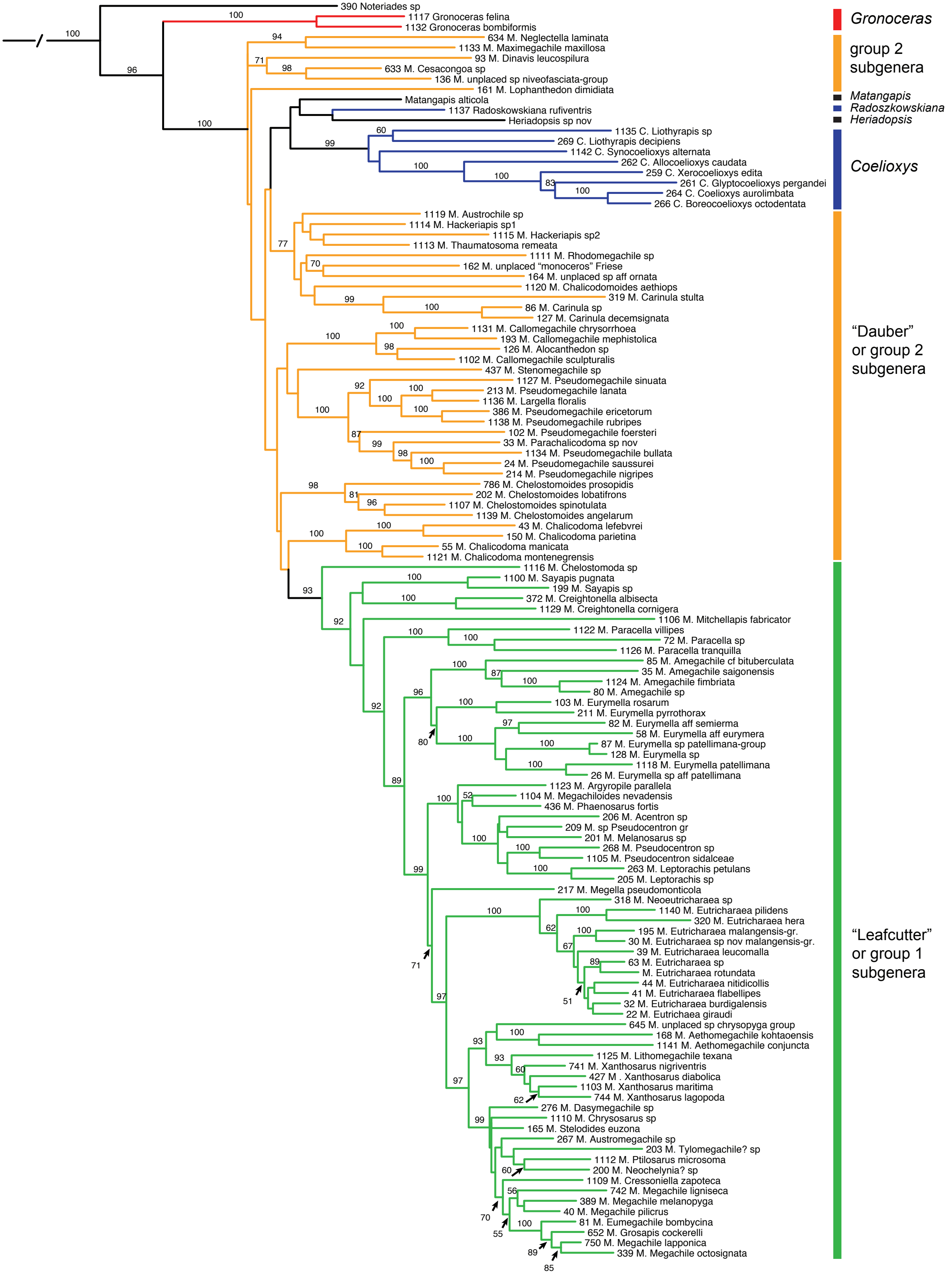
Figure S4. Majority rule consensus tree found in BEAST analyses of the supermatrix combining the nuclear dataset (as a backbone phylogeny) with mitochondrial barcodes. All terminals include the mitochondrial barcode sequence; 99 terminals (in red) additionally include the full nuclear matrix; 12 terminals (in blue) have only the nuclear gene LW-Rhodopsin in addition to the mitochondrial barcode; and 302 terminals (in black) only have the mitochondrial barcode.

Fig. S1



390 Noteriades sp
1117 Gronoceras felina
1132 Gronoceras bombiformis
1137 Radoskowskiana rufiventris
269 C. Liothyrapis decipiens
1142 C. Liothyrapis sp
487 C. Synocoelioxys alternata
262 C. Allocoelioxys caudata
259 C. Xerocoelioxys edita
261 C. Glyptocoelioxys pergandei
264 C. Coelioxys aurolimbata
266 C. Boreocoelioxys octodentata
1119 M. Austrochile sp
1114 M. Hackeriapis sp1
1120 M. Chalicodomoides aethiops
1113 M. Thaumatosoma remeata
1115 M. Hackeriapis sp2
1111 M. Rhodomegachile sp
164 M. unplaced sp aff ornata
162 M. unplaced “monoceros” Friese
319 M. Carinula stulta
127 M. Carinula decemsignata
86 M. Carinula sp
161 M. Lophanthedon dimidiata
1133 M. Maximegachile maxillosa
634 M. Neglectella laminata
93 M Dinavis leucospilura
633 M. Cesacongoa sp
136 M. unplaced sp niveofasciata-group
437 M. Stenomegachile chelostomoides
1121 M. Chalicodoma montenegrensis
55 M. Chalicodoma manicata
43 M. Chalicodoma lefebvrei
150 M. Chalicodoma parietina
1131 M. Callomegachile chrysorrhoea
193 M. Callomegachile mephistolica
126 M. Alocanthedon sp
1102 M. Callomegachile sculpturalis
102 M. Pseudomegachile foersteri
33 M. Parachalicodoma sp nov
1134 M. Pseudomegachile bullata
24 M. Pseudomegachile saussurei
214 M. Pseudomegachile nigripes
1127 M. Pseudomegachile sinuata
1136 M. Largella floralis
213 M. Pseudomegachile lanata
1138 M. Pseudomegachile rubripes
386 M. Pseudomegachile ericetorum
786 M. Chelostomoides prosopidis
202 M. Chelostomoides lobatifrons
1139 M. Chelostomoides angelarum
1107 M. Chelostomoides spinotulata
1116 M. Chelostomoda sp
1100 M. Sayapis pugnata
199 M. Sayapis sp
1106 M. Mitchellapis fabricator
372 M. Creightonella albisecta
1129 M. Creightonella cornigera
1122 M. Paracella villipes
1126 M. Paracella tranquilla
72 M. Paracella sp
85 M. Amegachile cf bituberculata
35 M. Amegachile saigonensis
1124 M. Amegachile fimbriata
80 M. Amegachile sp
211 M. Eurymella pyrrothorax
103 M. Eurymella rosarum
82 M. Eurymella aff semierma
58 M. Eurymella aff eurymera
26 M. Eurymella sp aff patellimana
1118 M. Eurymella patellimana
87 M. Eurymella sp patellimana-group
128 M. Eurymella sp
1123 M. Argyropile parallela
436 M. Phaenosarus fortis
1104 M. Megachiloides nevadensis
263 M. Leptorachis petulans
205 M. Leptorachis sp
206 M. Acentron sp
268 M. Pseudocentron sp
1105 M. Pseudocentron sidalceae
201 M. Melanosarus sp
209 M. sp Pseudocentron-group
217 M. Megella pseudomonticola
318 M. Neoeutricharaea sp
1140 M. Eutricharaea pilidens
320 M. Eutricharaea hera
39 M. Eutricharaea leucomalla
30 M. Eutricharaea sp nov
195 M. Eutricharaea malangensis gr.
M. Eutricharaea rotundata
63 M. Eutricharaea sp
22 M. Eutrichaea giraudi
32 M. Eutricharaea burdigalensis
44 M. Eutricharaea nitidicollis
41 M. Eutricharaea flabellipes
645 M. unplaced sp chrysopyga group
168 M. Aethomegachile kohtaoensis
1141 M. Aethomegachile conjuncta
1125 M. Lithomegachile texana
741 M. Xanthosarus nigriventris
427 M. Xanthosarus diabolica
1103 M. Xanthosarus maritima
744 M. Xanthosarus lagopoda
276 M. Dasymegachile sp
1110 M. Chrysosarus sp
267 M. Austromegachile sp
203 M. Tylomegachile? sp
1112 M. Ptilosarus microsoma
200 M. Neochelynia? sp
165 M. Stelodides euzona
1109 M. Cressoniella zapoteca
389 M. Megachile melanopyga
742 M. Megachile ligniseca
40 M. Megachile pilicrus
81 M. Eumegachile bombycina
652 M. Grosapis cockerelli
750 M. Megachile lapponica
339 M. Megachile octosignata
Gronoceras
Coelioxys + *Radosz-kowskiana*
“Dauber” or group 2 subgenera
“Leafcutter” or group 1 subgenera

Fig. S2


100
390 Noteriades sp
100
1117 Gronoceras felina
Gronoceras
1132 Gronoceras bombiformis
94
634 M. Neglectella laminata
group 2 subgenera
1133 M. Maximegachile maxillosa
71
93 M. Dinavis leucospilura
98
633 M. Cesacongoa sp
96
136 M. unplaced sp niveofasciata-group
161 M. Lophanthedon dimidiata
Matangapis alticola
Matangapis
1137 Radoskowskiana rufiventris
Radoszkowskiana
Heriadopsis sp nov
Heriadopsis
100
60
1135 C. Liothyrapis sp
269 C. Liothyrapis decipiens
99
1142 C. Synocoelioxys alternata
262 C. Allocoelioxys caudata
100
Coelioxys
259 C. Xerocoelioxys edita
100
83
261 C. Glyptocoelioxys pergandei
100
264 C. Coelioxys aurolimbata
266 C. Boreocoelioxys octodentata
1119 M. Austrochile sp
1114 M. Hackeriapis sp1
1115 M. Hackeriapis sp2
77
1113 M. Thaumatosoma remeata
1111 M. Rhodomegachile sp
70
162 M. unplaced “monoceros” Friese
164 M. unplaced sp aff ornata
1120 M. Chalicodomoides aethiops
99
319 M. Carinula stulta
100
86 M. Carinula sp
127 M. Carinula decemsignata
100
1131 M. Callomegachile chrysorrhoea
100
“Dauber” or group 2 subgenera
193 M. Callomegachile mephistolica
98
126 M. Alocanthedon sp
1102 M. Callomegachile sculpturalis
437 M. Stenomegachile sp
1127 M. Pseudomegachile sinuata
92
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213 M. Pseudomegachile lanata
100
1136 M. Largella floralis
100
100
386 M. Pseudomegachile ericetorum
1138 M. Pseudomegachile rubripes
87
102 M. Pseudomegachile foersteri
99
33 M. Parachalicodoma sp nov
98
1134 M. Pseudomegachile bullata
100
24 M. Pseudomegachile saussurei
214 M. Pseudomegachile nigripes
98
786 M. Chelostomoides prosopidis
81
202 M. Chelostomoides lobatifrons
96
1107 M. Chelostomoides spinotulata
1139 M. Chelostomoides angelarum
100
43 M. Chalicodoma lefebvrei
100
150 M. Chalicodoma parietina
100
55 M. Chalicodoma manicata
1121 M. Chalicodoma montenegrensis
1116 M. Chelostomoda sp
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1100 M. Sayapis pugnata
93
199 M. Sayapis sp
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1129 M. Creightonella cornigera
92
1106 M. Mitchellapis fabricator
1122 M. Paracella villipes
100
100
72 M. Paracella sp
1126 M. Paracella tranquilla
85 M. Amegachile cf bituberculata
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35 M. Amegachile saigonensis
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1124 M. Amegachile fimbriata
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80 M. Amegachile sp
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103 M. Eurymella rosarum
92
211 M. Eurymella pyrrhothorax
97
82 M. Eurymella aff semierma
58 M. Eurymella aff eurymera
80
100
100
87 M. Eurymella sp patellimana-group
128 M. Eurymella sp
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1118 M. Eurymella patellimana
26 M. Eurymella sp aff patellimana
89
1123 M. Argyropile parallela
100
52
1104 M. Megachiloides nevadensis
436 M. Phaenosarus fortis
206 M. Acentron sp
209 M. sp Pseudocentron gr
100
201 M. Melanosarus sp
100
268 M. Pseudocentron sp
1105 M. Pseudocentron sidalceae
100
263 M. Leptorachis petulans
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205 M. Leptorachis sp
217 M. Megella pseudomonticola
318 M. Neoeutricharaea sp
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100
1140 M. Eutricharaea pilidens
320 M. Eutricharaea hera
62
100
195 M. Eutricharaea malangensis-gr.
“Leafcutter” or group 1 subgenera
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30 M. Eutricharaea sp nov malangensis-gr.
39 M. Eutricharaea leucomalla
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89
63 M. Eutricharaea sp
M. Eutricharaea rotundata
44 M. Eutricharaea nitidicollis
41 M. Eutricharaea flabellipes
51
97
32 M. Eutricharaea burdigalensis
22 M. Eutrichaea giraudi
645 M. unplaced sp chrysopyga group
100
168 M. Aethomegachile kohtaoensis
93
1141 M. Aethomegachile conjuncta
93
1125 M. Lithomegachile texana
60
741 M. Xanthosarus nigriventris
427 M . Xanthosarus diabolica
97
1103 M. Xanthosarus maritima
62
744 M. Xanthosarus lagopoda
276 M. Dasymegachile sp
99
1110 M. Chrysosarus sp
165 M. Stelodides euzona
267 M. Austromegachile sp
203 M. Tylomegachile? sp
1112 M. Ptilosarus microsoma
60
200 M. Neochelynia? sp
1109 M. Cressoniella zapoteca
56
742 M. Megachile ligniseca
389 M. Megachile melanopyga
70
40 M. Megachile pilicrus
100
81 M. Eumegachile bombycina
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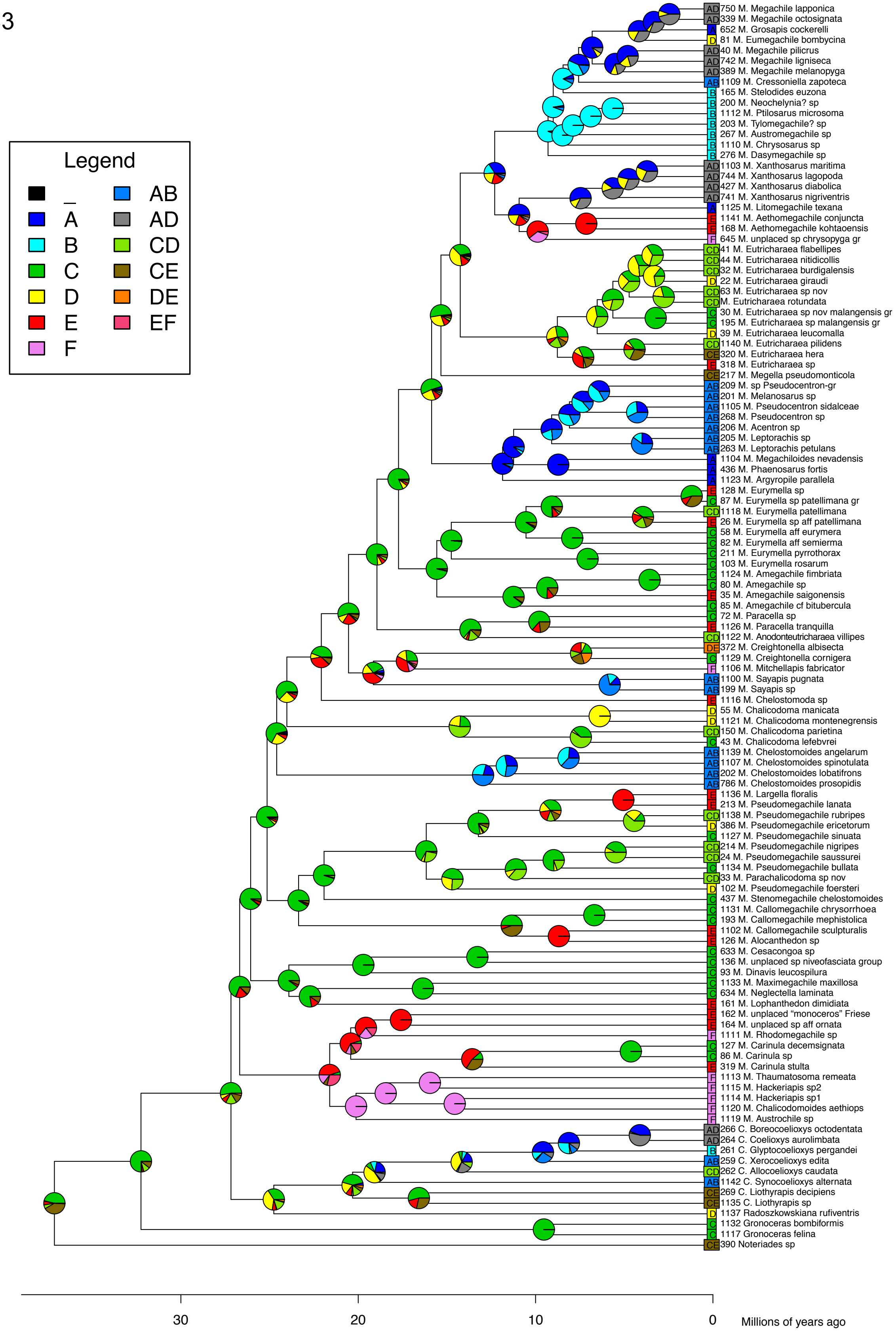
652 M. Grosapis cockerelli
750 M. Megachile lapponica
89
339 M. Megachile octosignata
85

Fig. S3



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AD 742 M. Megachile ligniseca
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B 165 M. Stelodides euzona
B 200 M. Neochelynia? sp
B 1112 M. Ptilosarus microsoma
B 203 M. Tylomegachile? sp
B 267 M. Austromegachile sp
B 1110 M. Chrysosarus sp
B 276 M. Dasymegachile sp
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AD 744 M. Xanthosarus lagopoda
AD 427 M. Xanthosarus diabolica
AD 741 M. Xanthosarus nigriventris
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E 1141 M. Aethomegachile conjuncta
E 168 M. Aethomegachile kohtaoensis
F 645 M. unplaced sp chrysopyga gr
CD 41 M. Eutricharaea flabellipes
CD 44 M. Eutricharaea nitidicollis
CD 32 M. Eutricharaea burdigalensis
D 22 M. Eutricharaea giraudi
CD 63 M. Eutricharaea sp nov
CD M. Eutricharaea rotundata
C 30 M. Eutricharaea sp nov malangensis gr
C 195 M. Eutricharaea sp malangensis gr
D 39 M. Eutricharaea leucomalla
CD 1140 M. Eutricharaea pilidens
CE 320 M. Eutricharaea hera
E 318 M. Eutricharaea sp
CE 217 M. Megella pseudomonticola
AB 209 M. sp Pseudocentron-gr
AB 201 M. Melanosarus sp
AB 1105 M. Pseudocentron sidalceae
AB 268 M. Pseudocentron sp
AB 206 M. Acentron sp
AB 205 M. Leptorachis sp
AB 263 M. Leptorachis petulans
A 1104 M. Megachiloides nevadensis
A 436 M. Phaenosarus fortis
A 1123 M. Argyropile parallela
E 128 M. Eurymella sp
C 87 M. Eurymella sp patellimana gr
CD 1118 M. Eurymella patellimana
E 26 M. Eurymella sp aff patellimana
C 58 M. Eurymella aff eurymera
C 82 M. Eurymella aff semierma
C 211 M. Eurymella pyrrothorax
C 103 M. Eurymella rosarum
C 1124 M. Amegachile fimbriata
C 80 M. Amegachile sp
E 35 M. Amegachile saigonensis
C 85 M. Amegachile cf bitubercula
C 72 M. Paracella sp
E 1126 M. Paracella tranquilla
CD 1122 M. Anodonteutricharaea villipes
DE 372 M. Creightonella albisecta
C 1129 M. Creightonella cornigera
F 1106 M. Mitchellapis fabricator
AB 1100 M. Sayapis pugnata
AB 199 M. Sayapis sp
E 1116 M. Chelostomoda sp
D 55 M. Chalicodoma manicata
D 1121 M. Chalicodoma montenegrensis
CD 150 M. Chalicodoma parietina
C 43 M. Chalicodoma lefebvrei
AB 1139 M. Chelostomoides angelarum
AB 1107 M. Chelostomoides spinotulata
AB 202 M. Chelostomoides lobatifrons
AB 786 M. Chelostomoides prosopidis
E 1136 M. Largella floralis
E 213 M. Pseudomegachile lanata
CD 1138 M. Pseudomegachile rubripes
D 386 M. Pseudomegachile ericetorum
C 1127 M. Pseudomegachile sinuata
CD 214 M. Pseudomegachile nigripes
CD 24 M. Pseudomegachile saussurei
C 1134 M. Pseudomegachile bullata
CD 33 M. Parachalicodoma sp nov
D 102 M. Pseudomegachile foersteri
C 437 M. Stenomegachile chelostomoides
C 1131 M. Callomegachile chrysorrhoea
C 193 M. Callomegachile mephistolica
E 1102 M. Callomegachile sculpturalis
E 126 M. Alocanthedon sp
C 633 M. Cesacongoa sp
C 136 M. unplaced sp niveofasciata group
C 93 M. Dinavis leucospilura
C 1133 M. Maximegachile maxillosa
C 634 M. Neglectella laminata
E 161 M. Lophanthedon dimidiata
E 162 M. unplaced “monoceros” Friese
E 164 M. unplaced sp aff ornata
F 1111 M. Rhodomegachile sp
C 127 M. Carinula decemsignata
C 86 M. Carinula sp
E 319 M. Carinula stulta
F 1113 M. Thaumatosoma remeata
F 1115 M. Hackeriapis sp2
F 1114 M. Hackeriapis sp1
F 1120 M. Chalicodomoides aethiops
F 1119 M. Austrochile sp
AD 266 C. Boreocoelioxys octodentata
AD 264 C. Coelioxys aurolimbata
B 261 C. Glyptocoelioxys pergandei
AB 259 C. Xerocoelioxys edita
CD 262 C. Allocoelioxys caudata
AB 1142 C. Synocoelioxys alternata
CE 269 C. Liothyrapis decipiens
CE 1135 C. Liothyrapis sp
D 1137 Radoszkowskiana rufiventris
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CE 390 Noteriades sp
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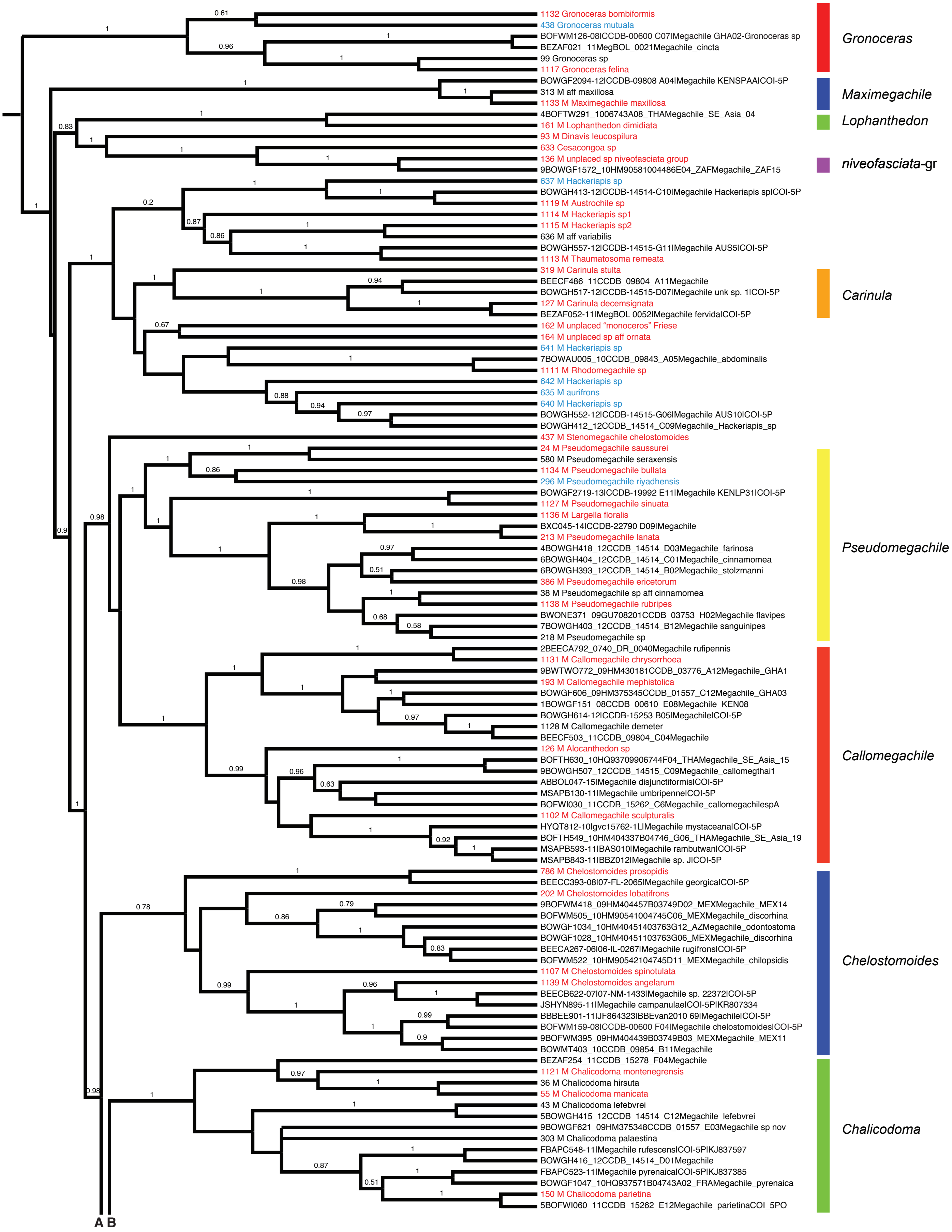
Fig. S4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1115 M Hackeriapis sp2
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164 M unplaced sp aff ornata
641 M Hackeriapis sp
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635 M aurifrons
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43 M Chalicodoma lefebvrei
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FBAPC523-11|Megachile pyrenaica|COI-5P|KJ837385
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Maximegachile
Lophanthedon
niveofasciata-gr
Carinula
Pseudomegachile
Callomegachile
Chelostomoides
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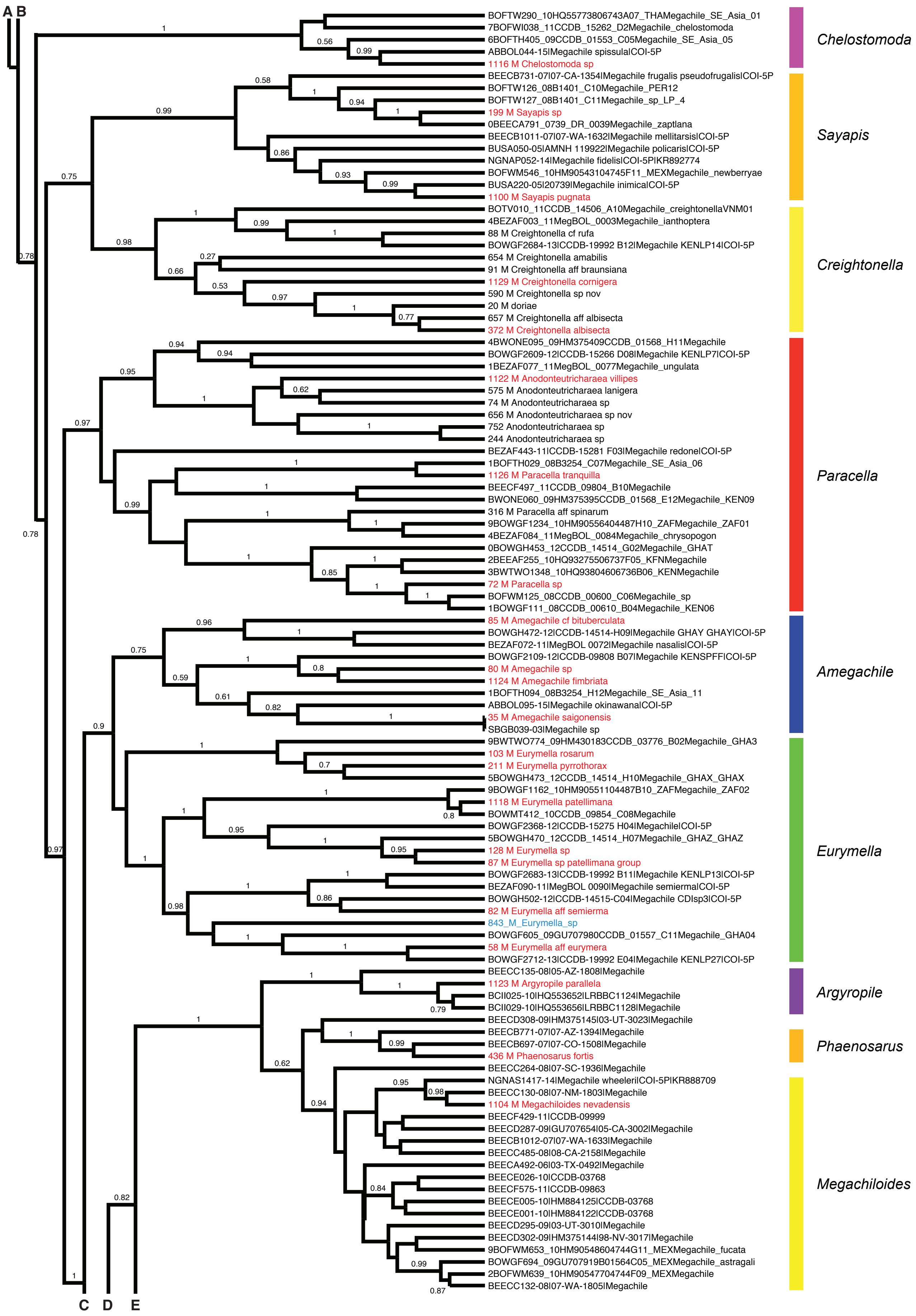
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1129 M Creightonella cornigera
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590 M Creightonella sp nov
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20 M doriae
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657 M Creightonella aff albisecta
372 M Creightonella albisecta
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Phaenosarus
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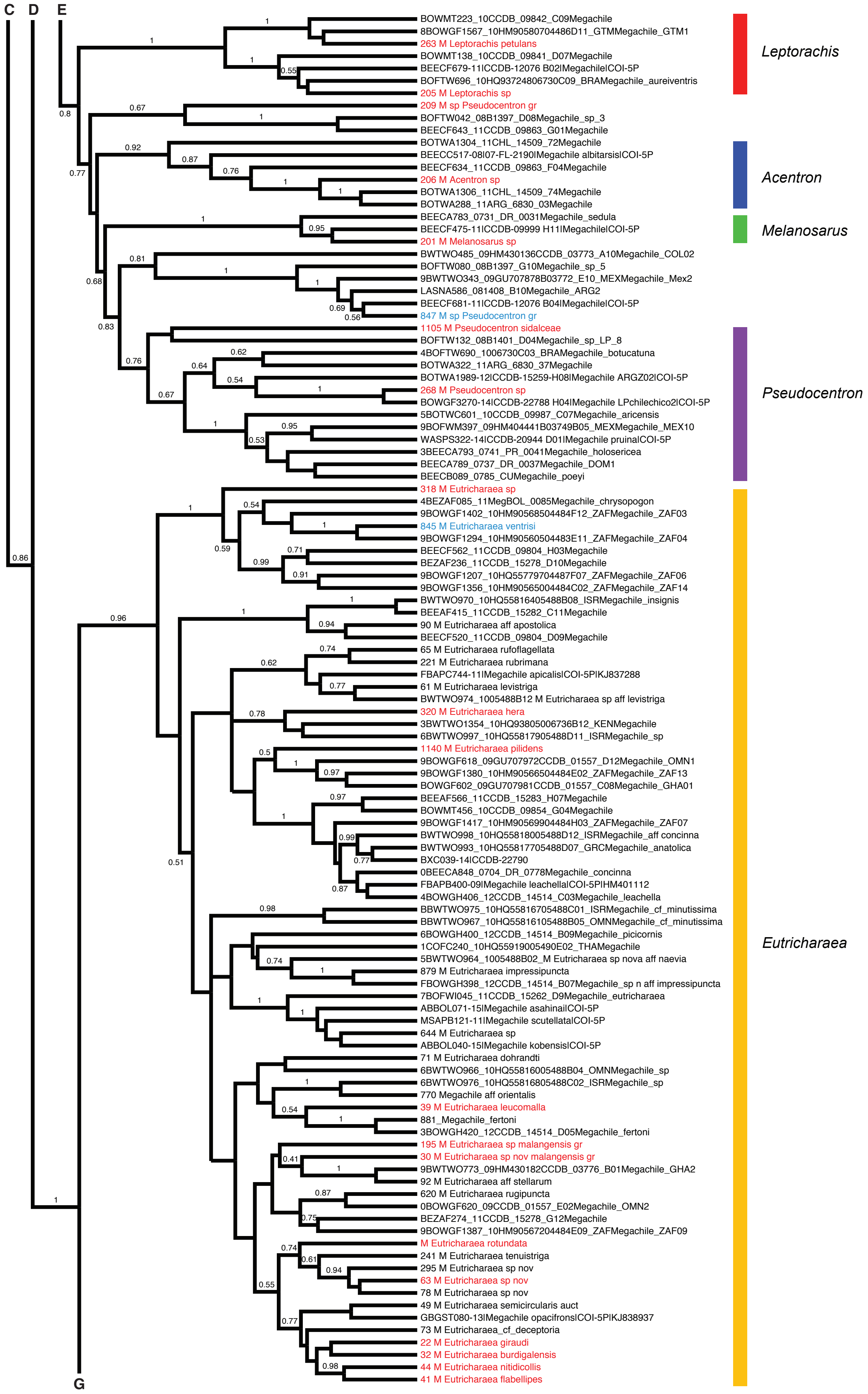
D
E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Acentron
Melanosarus
Pseudocentron
Eutricharaea
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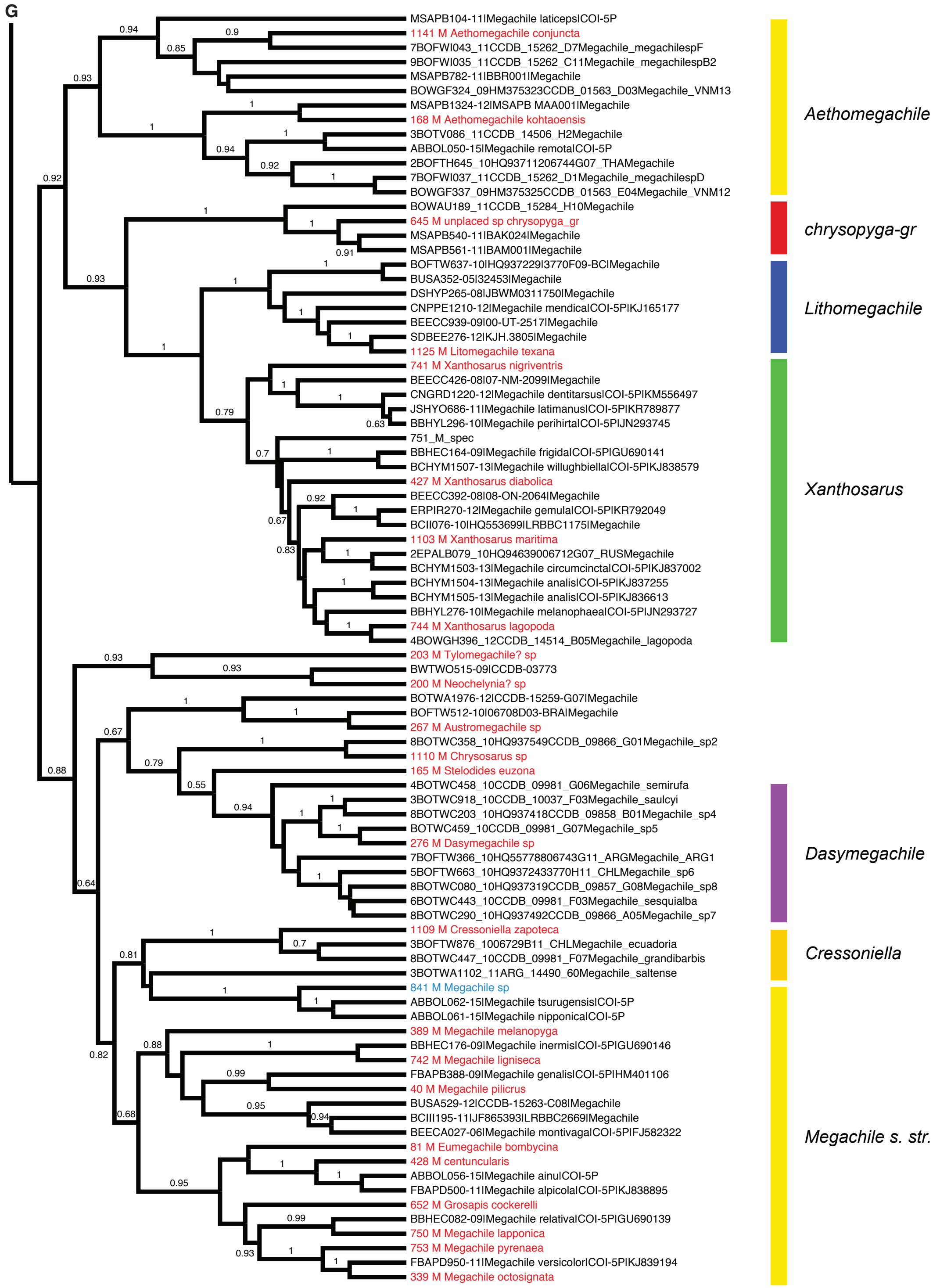
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Chapter 2

Foreword

Pollen has long been considered as a universal, high quality food for bees. Only recently was this nutritional value and digestibility tested through exchanges of pollen provisions among different bee species, each specialized to a different spectrum of host-plants (Praz *et al.* 2008). These feeding experiments revealed that pollen types coming from different plant species are unequally digestible for bee larvae. The underlying mechanisms remained unknown and several hypotheses were formulated (Praz *et al.* 2008), among others that some pollen diets lacked specific essential nutrients, or that pollen toxins were underlying the mortality. My interest was to test the latter hypothesis, namely that secondary compounds in pollen were toxic for bee larvae.

After a few inconclusive explorations of the pollen chemistry of some Asteraceae, we decided to focus on Boraginaceae, notable for their pyrrolizidine alkaloids and their wide range of flower morphologies. Fortunately, many species and genera were accessible in Switzerland, in nature but also in the botanical gardens of Geneva and Neuchâtel. To complete our sampling, I organized two field trips with my friend and colleague Dimitri Bénon.

View of the site were we found nests of Ochreriades fasciatus and of Hofferia schmiedeknechti on the Golan heights, Israel.

These trips enabled us to sample the pollen from numerous additional genera of Boraginaceae, for example in Tenerife, where we scrupulously observed pollinators of the famous “Tajinaste rojo” (*Echium wildpretii*) during several days, and in Israel where we sampled the pollen of numerous genera of Boraginaceae.

These trips were also an occasion to get familiar with the natural history of bees in general. In Israel for example, we could discover for the first time nests of two little-known bee lineages: the nests of the enigmatic bee *Ochreriades fasciatus* and of the heriadine bee *Hofferia schmiedeknechti*. These discoveries lead to two descriptive papers placed at the end of this thesis: (Rozen *et al.* 2015) for *Ochreriades* (Appendix 1) and (Mueller & Trunz 2014) for *Hofferia* (Appendix 2),

To bee or not to bee? Chemical ecology and the complex nature of bee-flower relationships in the family Boraginaceae

Based on: Vincent Trunz[1], Matteo Luchetti[1,2], Dimitri Bénon[1], Christina Kast[2], Gaetan Glauser[3] and Christophe J. Praz[1], in preparation.

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Abstract

The evolutionary origin of secondary compounds in floral rewards is an important field of research. While numerous studies have examined the ecological and evolutionary significance of nectar secondary compounds, the impact of pollen secondary compounds on pollinators remains little explored. Using the Western Palearctic Boraginaceae as a model system, we examine the following questions. First, do pollen secondary compounds, at realistic concentrations, may account for the previously documented failure of larvae of several solitary bee species to develop on provisions of Boraginaceae pollen. To test this hypothesis, we supplemented natural provisions of three species of megachilid bees with field realistic amounts of PAs extracted from *Echium vulgare*. In perfect agreement with bioassays previously conducted for these species, larvae of two species failed to develop on PA-supplemented provisions. Second, we measured pollen PAs in 23 representative Boraginaceae species and applied phylogenetic comparative methods to examine the evolutionary origin of pollen PAs in Boraginaceae. We show that PA concentrations in the pollen are variable and likely represent a trait under selection. However, PA concentrations were not higher in species with accessible pollen than in those species with hidden anthers, rejecting previous hypotheses on the possible origin of pollen defence compounds. In addition, our data do not support the view that pollen PA may trigger specialization in bees. In contrast, we demonstrate that six phylogenetically unrelated genera presenting sophisticated morphological adaptations to pollen-collecting bees had significantly lower PA levels in the pollen than the other species. In these plants, we postulate that pollen-collecting bees are legitimate pollinators, unlike in most other species of Boraginaceae where nectar foraging insects (including bumblebees and solitary bees) are the legitimate pollinators. We used our results to re-examine the complex nature of bee-flower relationships in general.

Introduction

The majority of the flowering plants are pollinated by insects (Grimaldi & Engel 2005; Ollerton *et al.* 2011), and among insects bees are the most important pollinating group in most biomes (Michener 2007; Willmer *et al.* 2017) While the ecological importance of bees has been demonstrated (e.g., Biesmeijer 2006; Garibaldi *et al.* 2013; Willmer *et al.* 2017), the relationships between bees and flowers are subtle and not entirely mutualistic (Westerkamp 1996). In striking contrast to most other pollinating insects, female bees do not primarily visit flowers for their own nutritive requirements, but for the provisioning of larval cells (Michener 2007). Beside copious amounts of nectar, bees require enormous quantities of pollen for the rearing of their larvae (Schlindwein *et al.* 2005; Müller *et al.* 2006). Consequently, female bees are particularly efficient at harvesting pollen (reviewed in (Westerkamp & Classen-Bockhoff 2007), which may conflict with the flower's pollination success: they specifically "attack" anthers and actively groom the pollen with their legs, in contrast to other pollinators onto which the pollen is passively deposited during nectar visits; pollen-collecting females often restrict their foraging to flowers in the male phase, thus avoiding flowers with receptive stigmas (Müller 1996b); or they pack the pollen in specialized pollen-collecting structures (the "scopa"), thereby preventing its deposition onto the stigma (Thomson 1986; Westerkamp 1996). The efficient removal of pollen by bees may not only decrease the pollination efficiency of bees but may also erode pollen transfer efficiency during succeeding visits by other insects (Thomson & Thomson 1992).

Several studies have documented the considerable impact of 120 millions years of active pollen collection by female bees on the evolution of floral morphology in the angiosperms (reviewed by (Westerkamp & Classen-Bockhoff 2007): many flowers in unrelated families conceal their pollen in narrow floral tubes (Müller 1995), in the upper lip (Müller 1996a; Thorp 2000), in the keel (Westerkamp 1997b), or in poricidal anthers, the latter having evolved in 72 angiosperms families (Buchmann 1983). In all these cases, only some bees equipped with specialized morphological features (e.g., (Müller 1995; 1996a; Thorp 2000) or having evolved specific foraging behaviours such as buzzing (Buchmann 1983; 1985; Thorp 2000; De Luca & Vallejo-Marín 2013) can harvest the pollen. Whether these bees with specialized pollen collecting traits are efficient pollinators of theirs host plants, remains to be investigated in most cases (Müller 1995; Schlindwein 2004).

Morphology is only one way used by plants to modulate the spectrum of their floral visitors. In addition, pollination systems and more generally plant-insect interactions are largely mediated by plant chemical compounds, in particular floral volatiles and secondary compounds (Lucas-Barbosa *et al.* 2011; Rusch *et al.* 2016). Given the adaptive nature of secondary compounds and their major impact on plant-insect interactions, we postulate that much can be learned from the field of chemical ecology in examining the complex nature of bee-flower relationships. While the impact of secondary compounds in nectar on pollinators has received particular attention (Adler 2000; Adler & Irwin 2005; Elliott *et al.* 2008; Irwin & Adler 2008; Adler & Irwin 2012) little is known on the impact of pollen secondary compounds on bee-flower interactions (Irwin *et al.* 2014); yet bee-flower interactions are likely centred on pollen, not nectar (Müller *et al.* 2006; Michener 2007). Two lines of evidence suggest that pollen chemistry may in fact play a much more important role in shaping bee-flower interactions than hitherto recognized. First, pollen has been shown to contain particularly large amounts of secondary compounds compared to nectar or other plant tissues (London-Shafir *et al.* 2003; Boppré 2011; Gosselin *et al.* 2013; Lucchetti *et al.* 2016). Second, while early studies have largely assumed that pollen was an easy-to use protein source for bees, growing evidence suggests that larvae of both generalist and specialist bees fail to develop on some diets of non-host pollen (Williams 2003; Praz *et al.* 2008; Sedivy *et al.* 2011; Haider *et al.* 2014; Bukovinszky *et al.* 2017), pointing to some protective properties of the pollen. However, an effect of pollen metabolites on larval survival has never been demonstrated to our knowledge; in fact one study recently dismissed the hypothesis that the main toxic compound found in the pollen of *Ranunculus* flowers, ranunculin, was underlying the failure of some bee species to develop on pure *Ranunculus* pollen (Sedivy *et al.* 2012).

The evolutionary explanation for the presence of defensive compounds in the pollen remains unclear (Dobson & Bergstrom 2000; Irwin *et al.* 2014). On the one hand, defensive chemical compounds are regularly found in high amounts in reproductive organs to prevent destructive flower feeding by herbivores (Hartmann & Zimmer 1986;

Dobson & Bergstrom 2000; Frölich *et al.* 2007; see also Dobson and Bergström 2000, and references therein). The high concentrations of these compounds in the anthers and in the pollen may thus simply reflect the comparatively high levels of these compounds in the reproductive organs of the flowers. Yet the spectrum and concentrations of defensive compounds in the pollen may be distinct from that in other floral parts (Dobson & Bergstrom 2000; Gosselin *et al.* 2013). As suggested by Dobson and Bergström (2000), the presence of deterrent chemicals in non-pollen parts of the flower may reduce the need for the presence of defensive compounds in the pollen. Consequently, the pollen of flowers that are dependent on pollen-foraging insects may exhibit lower levels of defensive compounds than non-pollen floral parts. Alternatively, plants with exposed pollen that is readily available to non-pollinating, pollen-feeding insects are expected to produce defensive compounds in the pollen (Dobson & Bergstrom 2000). This hypothesis was further developed by Sedivy et al. (2011), which noted that all pollen types found to have protective properties originate from flowers with freely accessible pollen. Following this hypothesis, pollen secondary compounds may specifically deter pollen-feeding insects from flowers legitimately pollinated by nectar-visiting insects.

On the other hand, flower volatiles, which have been suggested to have their evolutionary origin in flower defence (Pellmyr & Thien 1986), likely serve as attractant to pollinators (Bergstrom *et al.* 1995). Foraging naive females of *Chelostoma florisomne*, a solitary bee specialized to the plant genus *Ranunculus*, uses the main pollen volatile, protoanemonin, to recognize its host (Dobson & Bergstrom 2000). Yet paradoxically protoanemonin has both poisonous and deterrent effects on herbivores (Bergstrom *et al.* 1995 and references therein). In addition, pollen secondary compounds may trigger host-plant specialization in bees, which may be advantageous from the flower's perspective. Chemically protected pollen may not be suitable for generalist bees and would thus represent a reliable source for pollen specialists (Müller & Kuhlmann 2003). Flowers of the genus *Aconitum* have higher amounts of alkaloids in their pollen than in other plant tissues (Gosselin *et al.* 2013), and they are the exclusive host plants of the two only pollen-specialist bumblebees known so far, *B. consobrinus* in Scandinavia and *B. gestaeckeri* in the Alps (Goulson 2003). Thostesen & Olesen (1996) have demonstrated that *B. consobrinus* was the only quantitatively important pollinator of

Aconitum in Scandinavia. Lastly, pollen secondary chemistry may be advantageous to some bees in that they may reduce pathogen or parasite loads, as has been demonstrated for nectar secondary compounds (Manson *et al.* 2010; Richardson *et al.* 2015; Spear *et al.* 2016).

In this study, we focus on the plant family Boraginaceae to examine the evolution of pollen secondary compounds and their impact on bees. Boraginaceae represent an important pollen source for both specialist and generalist bees in Europe (Westrich 1989; Müller 2017) and elsewhere (Forrest *et al.* 2011; Gotlieb *et al.* 2014) and references therein). They exhibit strikingly varied floral morphologies probably tightly linked to their pollination biology (Cohen 2013). In addition, both a comprehensive phylogeny of the family (Cohen 2013) and accounts on the pollination biology of several species (references in Table 1) are available. Lastly, most Boraginaceae species investigated so far contain high levels of the toxic secondary metabolites pyrrolizidine alkaloids (hereafter called PAs) either in the roots, the leaves or in the floral parts, including the pollen (Lucchetti *et al.* 2016). The pollen of one species, *Echium vulgare*, has been shown to be unsuitable for larvae of several wild bee species not specialized onto this plant genus (Praz *et al.* 2008; Sedivy *et al.* 2011). *Echium vulgare* pollen contains very high amino acid content (Somerville & Nicol 2006), but also particularly high levels of PAs (Boppré 2011; Lucchetti *et al.* 2016), pointing to the possibility that PAs, and not lack of nutrients, underlie the high mortality of the larvae.

	Species	Sample provenance	Natural distribution	Floral morphology description	Main visitors	Known oligolectic bee (species/genera)	Mean alkaloids in pollen (µg/mg)
Echium-type	*Echium aculeatum*	Tenerife, Spain	Tenerife	Funnel-shaped corolla-tube, stamen protruding from the corolla	Anthophila, others unknown	13 / 2	0.02
	Echium bonnetii	Tenerife, Spain	Canary Islands	Funnel-shaped corolla-tube, stamen protruding from the corolla	Anthophila, others unknown	13 / 2	2.14
	Echium virescens	Tenerife, Spain	Tenerife	Funnel-shaped corolla-tube, stamen protruding from the corolla	Anthophila, others unknown	13 / 2	0.39
	Echium vulgare	Neuchâtel, Bern and Valais, Switzerland	Mediterranean region, occidental Asia	Funnel-shaped corolla-tube, stamen protruding from the corolla	Hymenoptera, Lepidoptera, Coleoptera, Diptera	13 / 2	4.68
	Echium wildpretii	Tenerife, Spain	Tenerife	Funnel-shaped corolla-tube, stamen protruding from the corolla	Anthophila, Lepidoptera, Diptera, birds	13 / 2	0.12
	Moltkia suffruticosa	Botanical garden Geneva, Switzerland	Italy	Tubular corolla, stamen protruding from the corolla	Unknown	0 / 0	0.34
Pulmonaria-type	*Anchusa italica*	Botanical garden Neuchâtel, Switzerland	Central and southern Europe, occidental Asia ; northern Africa	Tubular corolla with scales, anthers hidden in the corolla	Anthophila	3 / 3	3.17
	Anchusa officinalis	Botanical garden Neuchâtel, Switzerland	Central Europe	Tubular corolla with scales, anthers hidden in the corolla	Anthophila	3 / 3	1.80
	Buglossoïdes purpurocaerulea	Botanical garden Geneva, Switzerland	Southern Europe, occidental Asia	Tubula corolla, anthers hidden in the corolla	Anthophila, Diptera	0 / 0	0.00
	Cynoglossum montanum	Mt Hermon, Israel	Mediterranean region (montanous)	Tubular corolla with scales, anthers hidden in the corolla	Unknown	0 / 0	0.14
	Heliotropium sp.	Negev desert, Israel	Unknown	Short tubular corolla, anthers hidden in the corolla	Unknown	7 / 4	4.98
	Lithodora oleifolia	Botanical garden Neuchâtel, Switzerland	Spain	Tubular corolla with scales, anthers hidden in the corolla	Anthophila, Lepidoptera, Diptera	2 / 2	0.99
	Myosotis sylvatica	Fribourg, Switzerland	European region	Homogamous, hidden anthers	Coleoptera, Diptera, Hymenoptera	0 / 0	3.28
	Nonea erecta	Botanical garden Geneva, Switzerland	Eastern Europe, central and occidental Asia	Homogamous, hidden anthers	Unknown	0 / 0	0.06
	Pulmonaria obscura	Vaud, Switzerland	Europe	Tubula corolla, anthers hidden in the corolla	Anthophila, Diptera	0 / 0	0.00
Borago-type	*Borago officinalis*	Botanical garden Neuchâtel, Switzerland	Central and southern Europe, Asia Minor, northern Africa	Scatter-cone-blossoms	Anthophila	0 / 0	0.03
	Cerinthe glabra	Fribourg, Switzerland	Europe, occidental Asia	Scatter-cone-blossoms	Anthophila	2 / 2	0.90
	Cerinthe major	Botanical garden Neuchâtel, Switzerland	Mediterranean region, occidental Asia	Scatter-cone-blossoms	Anthophila	2 / 2	0.35
	Cerinthe minor	Botanical garden Geneva, Switzerland	South-East Europe, occidental Asia	Scatter-cone-blossoms	Anthophila	2 / 2	0.13
	Onosma helvetica	Valais, Switzerland	Meridional Europe	Scatter-cone-blossoms	Anthophila	5 / 3	0.19
	Podonosma orientalis	Jerusalem, Israel	Eastern mediterranean region	Scatter-cone-blossoms	Unknown	1 / 1	0.18
	Symphytum officinale	Fribourg, Switzerland	Eurasia	Scatter-cone-blossoms	Anthophila	0 / 0	0.00
	Trichodesma africana	Negev desert, Israel	Africa and Asia	Scatter-cone-blossoms	Anthophila	2 / 2	0.02

Table 1: Investigated Boraginaceae species with geographic distribution, flower morphology, known visitors and total PA concentration. In violet are represented *Echium*-type flowers, in orange *Pulmonaria*-type flowers, in blue the intermediate *Moltkia suffruticosa* and in green *Cerinthe*-type flowers. Information has been compiled from (Knuth 1908; Olesen 1979; Philipp & Schou 1981; Corbet *et al.* 1988; Dukas & Dafni 1990; Ahmed *et al.* 1995; Oberrath *et al.* 1995; Teppner 1996; Goulson *et al.* 1998; Oberrath & Bohning-Gaese 1999; Bennett 2003; Dupont & Skov 2004; Brys *et al.* 2008; Ferrero *et al.* 2011; Nocentini *et al.* 2012) and our personal observations.

Specifically, we survey and quantify the levels of PAs found in the pollen of the main genera of Boraginaceae found in Europe. Using *Echium vulgare* as a model system, we examined the effect of pollen PAs on bee larval development to determine whether pollen PAs, at realistic levels, can impact the development of bees and thus, ultimately, bee-flower relationships. We also complement our knowledge on the visitor spectrum of some selected, exemplary Boraginaceae species, specifically to quantify the proportion of pollen-collecting and nectar-collecting individuals among between bee visitors. Using a phylogenetic framework, we asked whether the presence or absence of secondary compounds in the pollen is associated in Boraginaceae with specific morphological types; whether we find a significant relationship between pollen PAs, the spectrum of floral visitors and bee foraging behaviour (pollen *versus* nectar collection); and whether genera with high concentrations of PAs in the pollen host more specialized bees than genera without PAs in the pollen. Our general hypothesis is the following: if toxic pollen metabolites have their evolutionary origin as a trigger to pollinator specialization, we expect bee-specialized Boraginaceae species to have higher levels of toxic compounds than generalized species. Alternatively, if toxic pollen metabolites act as defence against floral herbivores or against pollen robbers, we expect lower levels of metabolites in specialized bee flowers than in generalized species. We integrate all the results to summarize what is known about the complex nature of bee-flower relationships in Boraginaceae.

Material and methods

1. Plant species examined and pollination biology

We selected 23 species of Boraginaceae representing 16 genera (of 24 in Europe) and the main floral morphological types in the family in the Western Palearctic (Table 1); importantly, these 23 species sample all lineages of the *Borago*-type (see below, results section), which present striking morphological adaptations for bees. Information on the pollination biology for the investigated species, including the spectrum of visitors and the number of specialized bee species, was compiled from the literature (Table 1). We further determined the floral visitors of three exemplary species representing each of the three morphological types in the western Palearctic (see below, results section, and

Fig. 1): *Echium vulgare, Pulmonaria obscura* and *Cerinthe glabra*. For these three species, we identified and counted in 10-minutes periods all floral visitors on one plant (or on a few agglomerated individuals) until at least 500 visits were recorded. Bees were identified to genera or species, other insects to family. Honeybees were not recorded since their presence was largely dependent on the presence of hives; no honeybee was observed on *Pulmonaria* and *Cerinthe*, but numerous honeybees, mostly collecting nectar, were observed on some patches of *Echium vulgare*. For bees we additionally recorded the gender of each individual and whether the females were collecting pollen and nectar, or only nectar; our observations indicate that pollen-only visits are rare on these three Boraginaceae.

Lastly, we examined pollen presentation mechanisms in two selected Boraginaceae species lying at both ends of the morphological spectrum, *Echium vulgare* and *Cerinthe glabra*. In large natural populations of both plant species (Visp, Valais, Switzerland for *E. vulgare*; Bounavaux, Fribourg, Switzerland for *C. glabra*), we bagged a total of 50 individual flowers of each species. Flowers were bagged early in the morning and buds reaching the opening time (when a hole appears at the apex of the flower bud) were selected. No more than one flower per individual plant was selected. Ten bagged flowers were collected after 0.5 (*E. vulgare* only), 1, 2, 3, 4, and 5 hours. At collection time, each flower was placed in 1ml ethanol 70% in a microcentrifuge tube and shacked by hand for 30 seconds to release the pollen available at the moment (Müller *et al.* 2006). The flower was then removed and placed into a new tube. All tubes were placed open in an oven at 60 °C for 24 hours to evaporate all the liquid. The dried samples were resuspended in 100µl Ethanol, vortexed 30 seconds and sonicated for 2 minutes to release the remaining pollen from the anthers. We estimated pollen concentrations in 10µl aliquots by counting pollen grains in eight squares of 0.1 µl using a haemocytometer.

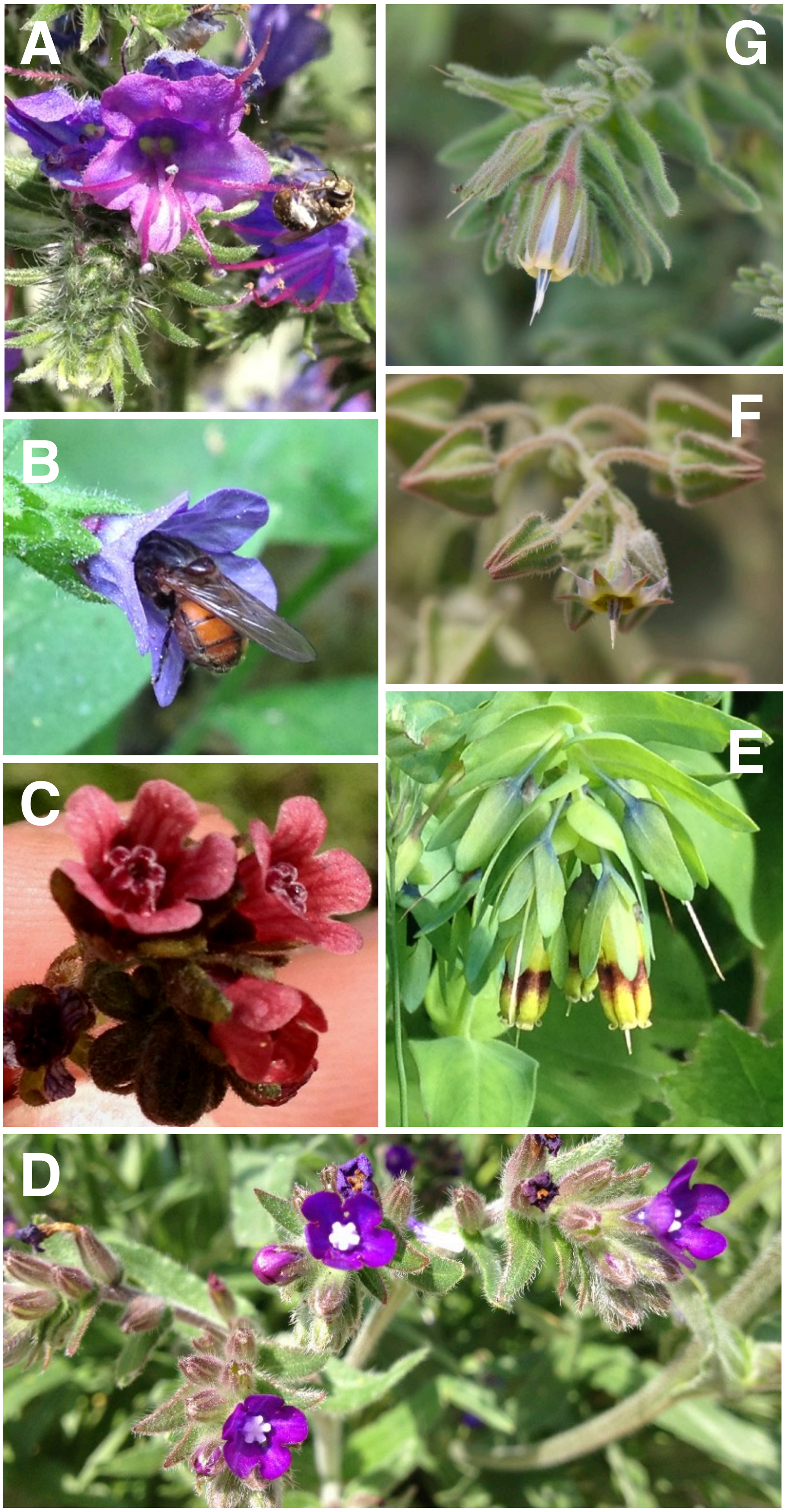

Fig. 1: Variability of floral morphology in Boraginaceae. Flowers of (a) *Echium vulgare* (b) *Pulmonaria obscura* (c) *Nonea erecta* (d) *Anchusa officinalis* (e) *Cerinthe glabra* (f) *Trichodesma africana* (g) *Podonosma orientalis*. *Echium*-type flowers: (a); *Pulmonaria*-type flowers: (b), (c) and (d); *Borago*-type flowers: (e)(f) and (g). Credits: Dimitri Bénon and Vincent Trunz

2. PAs quantification in pollen

For each of the 23 selected species we collected by hand a minimum of 1 mg of pollen from the anthers using precision tweezers; the pollen was stored in microcentrifuge tubes at -80 °C until analysis. For each sample, 1 mg of pollen was weighed using a Mettler Toledo precision scale. 100µl of extraction solvent made of water, methanol (MeOH) and formic acid (FA) (70:29.5:0.5) and 6-7 glass beads (2 mm diameter) were added and the PAs were extracted for 4 minutes at 30 Hz in a Retsch MM300 grinder. We verified that this treatment crushed all pollen grains with an optical microscope. The samples were then centrifuged for 4 minutes at 14'460 g, the supernatant was transferred into vials for LC-MS analysis and diluted 5x with the same solvent. PA profiling was performed by ultra-high pressure liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS) according to the protocol of Lucchetti et al (2016). The system used was an Acquity UPLC™ coupled to a Synapt G2 QTOF (Waters, Milford, USA) and was controlled by Masslynx v4.1. Separation was carried out on an Acquity UPLC BEH C18 column (50x2.1 mm, 1.7 µm particle size) at a flow rate of 0.4 mL/min and a temperature of 30°C. A binary gradient consisting of water+0.05% formic acid (solvent A) and acetonitrile+0.05% formic acid (solvent B) was applied as follows: 5-40% B in 4 min, 40-100% B in 2 min, holding at 100% B for 3 min, reequilibration at 5% B for 1.5 min. Injection volume was 1 µL. Detection by QTOFMS was performed in electrospray positive mode over a mass range of 50-600 Da. Scan time was 0.4 s, capillary voltage 2.8 kV, cone voltage +30 V, desolvation gas temperature and flow 350°C and 800 L/h, respectively. Accurate mass measurements were achieved by infusing a 400 ng/mL solution of leucine-enkephalin throughout the run as internal calibrant. Prior to the analysis, the instrument was calibrated externally using a 0.5 mM solution of sodium formate. PAs were manually extracted from the total ion chromatograms. Peaks corresponding to molecular formulae containing a single nitrogen atom were considered to be potential PAs and further explored. PAs were characterized by comparison with available standards or existing literature and databases (Table S1). In total, 44 PAs were identified or partially identified. Relative quantification was performed using the Quanlynx software.

3. Statistical analyses and phylogenetic comparative analyses

We specifically tested three hypotheses with respect to the concentrations of PAs in the pollen: 1. Plant species with hidden anthers (that is, those species in which pollen collection is only possible for bees equipped with specialized morphological features) have lower PA concentrations in the pollen than plant species with exposed anthers (Dobson & Bergstrom 2000; Sedivy *et al.* 2011). 2. Plant species exhibiting specialized "bee" morphology (*Borago*-type; see below) have lower concentrations of PAs in the pollen than other plant species. 3. Considering the 16 genera investigated, there is a positive correlation between average PA concentrations in the pollen and the number of solitary bees species specialized to these genera. Since in the case of Boraginaceae the vast majority of oligolectic bees specialize to genera and not to species, we reduced our sampling to the generic level, averaging within a genus the PA values of all species investigated.

In each case we first tested our data using statistical tests that did not incorporate the phylogenetic relationships between the species. We used parametric tests when the variables were normally distributed and non-parametric tests otherwise. Second, we used the package MVmorph (Butler & King 2004; Clavel *et al.* 2015) to perform multivariate phylogenetic comparative methods for hypotheses 1 and 2. This package allows the evaluation of different hypotheses on the evolution of traits in a phylogenetic framework: whether the evolution of a trait follows a pure "drift" model of evolution (Brownian motion); or whether the evolution of a trait is influenced by both drift and selection (Ornstein-Uhlenbeck model). In the latter case, several scenarios can be evaluated: all species investigated may be affected equally by selection (that is, Ornstein-Uhlenbeck model with one optimum); or conversely, two or more groups made a priori are affected differently by selection (Ornstein-Uhlenbeck model with two or more optima). In all cases, models are compared using "Akaike Information Criterion" values (hereafter AIC); a difference of two log-units is commonly taken as a significance threshold. For hypothesis three, we used the "pgls" tool of the CAPER package in R (Orme 2013) to perform a correlation analysis between PA levels in the pollen and the number of specialized bees for each plant genus taking into account phylogenetic non-independence.

To infer a phylogeny of the investigated taxa, we downloaded available sequences from Genbank (Cohen 2013). Sequences were aligned with Mafft (Katoh 2002) and maximum likelihood inference in RAxML (Stamatakis 2014) was used to reconstruct trees. The phylogenetic tree is given in Fig. S1.

4. Effect of pollen PAs on bee larval development

PAs isolation in *Echium vulgare*

To test the effect of pollen PAs on the development and mortality of solitary bee larvae, we developed a protocol for the isolation of PAs from the inflorescence (described in Lucchetti *et al.* 2016), given that the PAs found in the pollen were also found in the corollas. We isolated PAs from 1550 g of inflorescence of *Echium vulgare* collected in Northern Switzerland. In short, inflorescences were collected in liquid nitrogen, lyophilized and grinded. The resulting dry material was extracted in methanol during 24 hours, filtrated and the methanol evaporated. Chlorophyll was removed by filtration after dissolution of the dry extract into 40:60:0.5 MeOH: water: FA. The PAs were reduced to their tertiary form using zinc dust and extracted using an aqueous ammonia/chloroform solution. The solution containing the PAs was separated in different fractions by semi-preparative HPLC coupled with UV-detector. PA-containing fractions were recovered, evaporated at 40°C and lyophilized. The obtained powder was tested for purity with LC-MS and UPLC-MS.

PAs quantification and supplementation in pollen provisions

We obtained pure provisions of *E. vulgare* from three cells of *Hoplitis adunca* from artificial nests collected in Zurich, Switzerland. We quantified the PAs found in the pollen and nectar provisions using the protocol detailed above for PA quantification in pollen. We then specifically tested whether the PAs contained in *E. vulgare* pollen account for the previously documented failure of two solitary bee species (*Chelostoma rapunculi* and *Osmia bicornis*; Praz *et al.* 2008; Sedivy *et al.* 2011) to complete larval development on pure *Echium* pollen and nectar provisions. We included a third species closely related to *O. bicornis*, *O. cornuta*, which was shown to be able to develop on *Echium* provisions (Sedivy *et al.* 2011). In both these previous studies, eggs of *C. rapunculi* or the *Osmia* species were transferred onto pure *E. vulgare* provisions obtained from nests of the *Echium* specialist bee *Hoplitis adunca*. We obtained eggs and provisions of the *Osmia* species and *Chelostoma rapunculi* from both outdoors and encaged rearings in Neuchatel. Closed nests in bamboo stems were brought to the lab, opened and unhatched eggs were removed with a spatula and temporarily stored in petri dishes. For each bee species, the necessary amount of pollen and nectar provision was obtained from these nests and thoroughly mixed in a petri dish, weighed and supplemented with known amounts of PAs in 50% ethanol aqueous solution. Control provisions were prepared from the same provision mix, adding 50% ethanol aqueous solution. PAs concentrations (reduced forms only) in the treatment were calculated to match the mean concentrations of echimidine, acetylechimidine and echivulgarine (+ N-oxide forms) found in the natural provisions of *H. adunca*. We recovered and froze 2 mg of provision from each treatment at beginning (egg stage), middle (larval development stage) and end (cocoon stage) of the bioassays to determine the stability of the PAs concentrations throughout the experiment.

Artificial cells and larval development

As nest substitute, we provided artificial brood cells made of blocks of beech wood (2 x 2 x 4 cm) drilled to be opened both at the top and the front side (Sedivy et al. 2011). Hole dimensions were of 8 x 20 mm for *O. cornuta*, 6 x 20 mm for *O. bicornis*, and 4 x 15 mm for *C. rapunculi*. Each cell was coated in histological paraffin. For each treatment, the number of replicates was between 14 and 30; in each cell, we placed enough provision to support larval development of each species, namely 600 mg, 400 mg and 65 mg, for *O. cornuta*, *O. bicornis* and *C. rapunculi*, respectively. Given that we had enough eggs but were limited by PAs and thus by provisions, we placed three eggs per cell to ensure that at least one larva would hatch in each cell. After a few days, only one larva was kept in each cell and the others removed. We closed the cells with cover slips and paraffin film. We placed the cells at room temperature in cardboard boxes to keep the larvae in the dark. Relative humidity was maintained high to prevent dehydration of the pollen provisions. Death and cocoon spinning were reported during larval development. After five (*Osmia* species) or eleven (*Chelostoma rapunculi*) months, we opened the cocoons to assess whether metamorphosis was complete and whether the imago was fully developed and alive.

Statistical treatment

Statistical analyses were performed in R. Survival in the larval bioassays was tested with pairwise treatment comparisons of the proportions of living adults using Chi-Square tests with Monte-Carlo resampling. For the pollen release experiments, confidence intervals for proportions of pollen grains released over time were obtained using bias-corrected and accelerated bootstrap. All statistical tests are two-sided with an alpha level set to 5%. In all analyses, the coefficient for confidence interval estimation was set to 95%. *P*-values ≤ 0.05 were considered significant.

Results

1. Plant species examined and pollination biology

Floral morphology and floral visitors

The floral morphology of the Boraginaceae is diverse (see Fig. 1 and Cohen 2013), but the western Palearctic species investigate fall into three main morphological types. 1. ***Echium*-type**. In all *Echium* species investigated, the flower consists of an open funnel-shaped corolla with extruding anthers; the flower is open laterally and the anthers and style are placed in the inferior part of the corolla so that the ventral side of visiting pollinators contacts both stamens and style. 2. ***Pulmonaria*-type**. In numerous species such as *Pulmonaria*, *Heliotropium*, *Myosotis* and *Anchusa*, the corolla forms a more or less narrow tube in which the anthers are hidden. The flower is mostly directed upwards and serves as a small landing platform for nectar-seeking visitors; more rarely, the flower is hanging down. The flower of *Moltkia suffruticosa* is somehow intermediate between the *Echium* and *Pulmonaria* types, with a tubular corolla but with the anthers extruding and exposed. 3. ***Borago*-type**. In a third, distinct type found in genera such as

Borago, *Onosma* or *Symphytum*, the corolla forms a pendulous tube and is thus directed inferiorly, and the anthers are large and united medially around the style; this floral type is referred to as the *Borago* type by (Faegri 1986) and as the "scatter-cone-blossoms" by (Teppner 1996). Of note, the six genera of this floral type sampled here likely represent six independent origins of this sophisticated morphology.

We reviewed the literature on the floral visitors of all selected Boraginaceae species (Table 1). *Echium* type-flowers are visited by a vast array of pollinators, including bumblebees, honeybees and solitary bees; among the latter, both generalist and specialist species are known as visitors (Westrich 1989; Sedivy *et al.* 2013). In our surveys in Switzerland, *Echium vulgare* was visited by bees (74.5%) and Lepidoptera (23.5%). Among bees, bumblebee workers were the most abundant visitors (51.9% of the total visitors, 67.9% of the bees), followed by female solitary bees (26.1% of the bees) and male solitary bees (5.7% of the bees). The bumblebee workers mostly visited *Echium vulgare* for nectar only (73.1%), while females of solitary bee mostly visited the flowers for both pollen and nectar (86.4%).

Müller (1995) has convincingly demonstrated that only bees with morphological adaptations are able to extract the pollen from flowers of the *Pulmonaria* type. Most of these species are visited by a broad array of nectar-foraging insects (Table 1). Our surveys of the visitors of *Pulmonaria obscura* reveal that the visitors were Diptera (79.8%) and bumble bee workers (20.2%); all of the bumblebee visits were nectar visits. In contrast, *Borago*-type species of the Boraginaceae are almost exclusively visited by bees (Knuth 1908; Teppner 1996; Bennett 2003; Teppner 2011). In our surveys, *Cerinthe glabra* was almost exclusively visited by pollen-collecting bumblebees (both queens and workers); nectar visits by bumblebees were less frequent (30% of total visits). No other visitor could be observed during our observations.

Oligolectic bees are known to specialized on all three types of flowers (Table 1): there are, for example, numerous known specialists on *Echium*, *Heliotropium*, *Anchusa*, *Onosma* and *Cerinthe* in the West Palearctic region (Westrich 1989; Teppner 1996; 2011; Sedivy *et al.* 2013; Müller 2017; see further references in Table 1). It has to be stressed that the compilation of a list of specialist bees on each genus is an approximate task: the host-plant spectrum of most bees in Southern Europe has not been investigated in detail; several bee species are specialists on Boraginaceae but collect pollen from several genera (e.g., *Andrena symphyti*, oligolectic on Boraginaceae but collecting pollen from the genera *Onosma*, *Cerinthe* and *Symphytum*; (Teppner 2011), or are generalist with only a preference for Boraginaceae (e.g., *Osmia pilicornis*: Prosi *et al.* 2016). Nevertheless, our compilation reflects what is currently known on the specialist bees visiting these genera and is probably a realistic estimate of the number of specialist bees on each genus.

Pollen presentation in *Cerinthe glabra* and *Echium vulgare*

Significant differences were observed between the anther opening of *Echium vulgare* and *Cerinthe glabra* (fig. 2). At opening time, less than 1% of the pollen could be extracted from *C. glabra*, but already 22% of the pollen was released from *E. vulgare* anthers, and 67% after one hour (2% in *C. glabra*). After five hours, 95% of *E. vulgare* pollen was free and less than 7% in *C. glabra*. Teppner (1996) reports that the anthers of *Cerinthe* need three days to release their entire content and (Nocentini *et al.* 2012) reports that liberation of *C. major* pollen is rapid during the second day of anthesis.

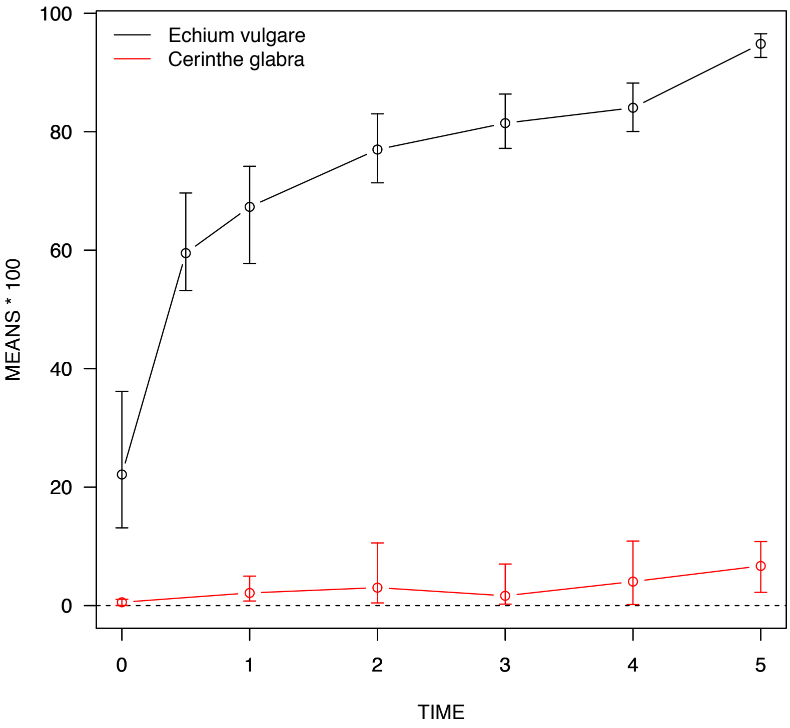

Fig. 2: Pollen presentation (in %) in *E. vulgare* and *C. glabra* after opening of the flower (t0 = beginning of petals separation and time of first possible visits by pollinators), with confidence intervals.

2. Presence of PAs in the pollen in relation to floral morphology

We were able to detect 43 different PAs in the pollen of the species investigated (Table S1). Many PAs were present either in a reduced or N-oxide form, or both. Total PA concentrations in pollen of *Echium*-type flowers varied between 0.023 µg/mg (*Echium aculeatum*) and 4.68 µg/mg (*Echium vulgare*; see table 1); between 0.003 µg/mg (*Pulmonaria obscura*) and 4.98 µg/mg (*Heliotropium* sp.) for *Pulmonaria*-type flowers and between 0.0034 µg/mg (*Symphytum officinale*) and 0.91 µg/mg (*Cerinthe glabra*) for *Borago*-type flowers. *Echium vulgare* pollen contained four main PAs, mainly in their N-oxide form: echimidine-N-oxide, acetylechimidine-N-oxide, acetylvulgarine-N-oxide and echivulgarine-N-oxide.

In conducting comparative analyses of the PAs concentrations in the pollen, we were limited by the number of species investigated. Preliminary analyses using the MVmorph package suggested that there was not enough information in our dataset (according to the Monte Carlo-based approach developed by Boettiger et al. 2012(Boettiger *et al.* 2012)) to compare more than two groups. For this reason, we separated our species into two groups for testing hypotheses one and two. Our data do not support hypothesis one, namely that species with exposed pollen have higher PAs concentration in the pollen than species with hidden pollen. We compared PAs concentrations according to two groupings: in the first, we compared the species of the *Pulmonaria* group (with anthers hidden in the corolla) with all other species (*Echium* and *Borago* groups). In the second, we compared the species with protruding anthers (*Echium* group, *Moltkia*) with all other species. The rational for conducting these two different tests is that in species of the *Borago* group, the pollen is somehow hidden in the corolla but still accessible for bees without specialized morphology. In both cases, the PA concentration in the pollen was not significantly different across groups (Table 2). To test hypothesis 2, namely whether species with a specialized bee morphology had lower PA concentrations in the pollen than the other species, we compared the species of the *Borago* group with the other species. In this case, our data significantly support our hypothesis: comparative phylogenetic analyses MVmorph significantly rejected model in which only drift explained the evolution of PA concentrations (Brownian Motion; AIC: 210.517; Table 2) and favored a model incorporating both drift and selection. A model incorporated two optima for the two groups recognized (AIC 203.623) was favored over a model incorporating only one optimum (AIC 205.171). However, the difference between the same two groups was not significant when tested using a ANOVA (Table 2). Lastly, no significant correlation was detected between the PA concentrations in the pollen and the number of bee species specialized to each genus, whether phylogenetic independence was taken into account (P = 0.0933) or not (P = 0.053) (Table 2).

a. Hypothesis 1: pollen alkaloids concentrations are high in species with exposed pollen

	Anova (without phylogeny)			**Comparative phylogenetic analyses (AICc)**		
	Df	F	P	BM	OU	OUM
Anthers hidden (*Pulmonaria*-type) versus others	1	0.3493785	0.5605	210.517	214.7364	212.9639
Anthers protruding (*Echium, Moltkia*) versus others	1	1.172	0.291	210.517	214.736	214.706

b. Hypothesis 2: pollen alkaloids concentrations are low in flowers with specialized bee morphology

	Anova (without phylogeny)			**Comparative phylogenetic analyses (AICc)**		
	Df	F	P	BM	OU	OUM
Borago-type versus others	1	2.913	0.102	210.517	205.171	203.623

c. Hypothesis 3: species with high pollen alkaloid concentrations host many specialist bees

	Anova (without phylogeny)			**Anova (accounting phylogeny)**		
	Df	F	P	Df	F	P
Correlation between alkaloid concentration in the pollen and number of specialist bee species	14	4.458	0.053	14	3.242	0.0933

Table 2: Results of the statistical analyses on the concentrations of alkaloids in the pollen in Boraginaceae. Three hypotheses are tested (a-c; see text for details)

3. Effect of pollen PAs on bee larval development

The three main PAs (N-oxide and tertiary forms put together) found in pollen and nectar provisions of *H. adunca* provisions were echimidine (0.139 µg/mg, SD=0.037), acetylvulgarine (0.018 µg/mg, SD=0.03) and echivulgarine (0.361 µg/mg, SD=0.031); the proportion of the N-oxide forms relative to the tertiary forms were 5.5%, 90.2% and 36.53%, respectively for these three alkaloids (see Table 3). In our bioassays, we supplemented the bee provisions with exactly the concentrations of the total PAs in equivalent tertiary form, the form in which we extracted all alkaloids. It is assumed that the N-oxide form is converted to the tertiary form during the digestive process in insects (Lindigkeit *et al.* 1997; Hartmann 1999; Macel 2010). Hereafter, PA-supplemented provisions are referred to as the treatment.

	Echimidine	**Echimidine-N-oxide**	**Acetylvulgarine**	**Acetylvulgarine-N-oxide**	**Echivulgarine**	**Echivulgarine-N-oxide**
***Echium vulgare* pollen**	0.022	0.554	0.026	0.718	0.094	1.817
	0.68%	17.15%	0.80%	22.22%	2.91%	56.24%
Echium vulgare* pollen + nectar provision made by *H. adunca	0.131	0.008	0.002	0.016	0.229	0.132
	25.34%	1.46%	0.35%	3.18%	44.22%	25.46%

Table 3: concentrations of the main PAs in the pollen collected directly from flowers of *Echium vulgare* (first line) and in Echium vulgare pollen + nectar provisions originating from nests of *Hoplitis adunca* (second line). Values are in mg/g and the proportion (%) of the tertiary (reduced) to the N-oxide forms of each compound are given.

Adult survival in *Osmia cornuta* did not significantly differ between control and treatment (P = 0.471; Table 4); all larvae reached the cocoon stage (N=15 for both control and treatment), and 13 (control) and 15 (treatment) individuals reached the adult stage. Although no significant difference in larval mortality was found before the cocoon stage in *O. bicornis*, highly significant differences in adult mortality were observed between treatment (N = 30) and control (N = 29; $P < 0.001$). Mortality in the control was 10 %, whereas all bees died in the treatment. Of the 21 individuals able to spin a cocoon in the treatment, 14 were found dead as larvae and 7partly transformed into pupae, the abdomen still showing larval morphology. Lastly, all individuals of *Chelostoma rapunculi* from the control developed into adults, but none in the treatment (0 of 15 individuals). Mortality occurred either prior to cocoon spinning (2 of 15 individuals), as larvae in the finished cocoon (10 of 15) or partly transformed into a pupa in the cocoon (3 individuals). In summary, our bioassays using PA-supplemented provisions exactly mirror results based on bioassays where the same three species were forced to develop on pure *Echium* provisions: *Osmia cornuta* was able to successfully develop on *Echium* provisions, but neiter *O. bicornis* nor *C. rapunculi* (Praz *et al.* 2008; Sedivy *et al.* 2011).

Bee species	Diet	No. Bees	No. Survivors	Survivors (%)	*P*
Osmia cornuta	Control	15	15	100.0	0.471
	Treatment	15	13	86.7	
Osmia bicornis	Control	29	26	89.7	< 0.001
	Treatment	30	0	0.0	
Chelostoma rapunculi	Control	15	15	100.0	< 0.001
	Treatment	15	0	0.0	

Table 4: Comparison of the survival of larvae of three solitary bee species on control (natural provisions supplemented with solvent only) and on provisions supplemented with field realistic concentrations of PAs (treatment). P refers to the P value with pairwise treatment comparisons of the proportions of living adults using Chi-Square tests with Monte-Carlo resampling.

Discussion

The evolutionary and ecological significance of pollen secondary compounds remains a largely unexplored field of research (Irwin *et al.* 2014). It remains unclear whether these compounds, at realistic doses, impact the fitness of pollinators, and whether their presence in the pollen is an adaptive response to pollinators or merely a pleiotropic effect of other traits related to plant defence. Using the plant family Boraginaceae as a model group, our study is the first comprehensive study that examines this question.

Our first important finding is that these compounds, at realistic doses, may have significant, detrimental effects on larval development, demonstrating for the first time that pollen secondary compounds may impact the fitness of solitary bees. Numerous previous studies have demonstrated that pollen was not an easy-to-use protein source for bees (Williams 2003; Praz *et al.* 2008; Sedivy *et al.* 2011; Haider *et al.* 2014; Bukovinszky *et al.* 2017), although a direct effect of defence compounds present in the pollen on larval development has only been hypothesized. Our experiments clearly suggest that the previously documented failure of *Osmia bicornis* and of *C. rapunculi* to develop on pure *Echium* provisions is due to the high PA contents of the pollen of *Echium vulgare* (Praz *et al.* 2008; Sedivy *et al.* 2011). In our experiments, PAs had no significant effect on the development of *Osmia cornuta*, a species that can develop on pure *Echium vulgare* provisions (Sedivy *et al.* 2011). It is possible, as hypothesized by Sedivy et al. (2011) that this species has developed physiological adaptations to cope with these secondary compounds.

Based on our survey of pollen PAs across numerous Boraginaceae species, we tentatively predict that the pollen of species with levels comparable to *Echium* may represent a toxic resource for bees, namely *Anchusa italica*, *Heliotropium* sp and *Myosotis sylvatica*. A surprising result in the light of previous larval tests was the low amounts of PAs in the pollen of *Borago officinalis*, given that larvae of *O. bicornis* have recently been shown to suffer high mortality on pure pollen and nectar provisions of *B. officinalis* (Bukovinszky *et al.* 2017). The comparatively early larval mortality of *O. bicornis* on *B. officinalis* pollen led Bukowinszky et al. (2017) to hypothesize an acute negative (possibly toxic) effect of this pollen type on larval development. Yet both the absence of PAs (and of any other putative toxic metabolite) in the pollen of *B. officinalis*, and the comparatively late mortality of larvae of *O. bicornis* and of *C. rapunculi* on PA-supplemented provisions, suggest that other factors must underlie larval mortality of *O. bicornis* on *B. officinalis* pollen. Nutrient deficiency is one possible explanation (Praz *et al.* 2008); another may be the unsuitable consistency of the pollen provisions originating from *B. officinalis* for *O. bicornis*: the natural provision of *O. bicornis* is unique among *Osmia* for being very dry and powdery, and this species forage on plants producing very little nectar (Haider *et al.* 2014); or an inability of *O. bicornis* larvae to extract the nutrients from the pollen grains of *B. officinalis*.

The presence in the pollen of defence compounds at concentrations that negatively impact pollinators is a paradoxical situation. Sedivy et al. (2011) have hypothesize that "the high quantitative pollen requirements of bees might have selected for protective properties of the pollen"; they further mention that pollen types found so far to possess unfavorable properties originate from flowers with an open morphology, exposed anthers and freely accessible pollen that can easily be harvested by any flower-visiting bee (Sedivy *et al.* 2011: 723). Our comparative study of the Boraginaceae allows for a critical examination of this hypothesis since both open flowers with exposed anthers (e.g., *Echium vulgare*) or tubular flowers with hidden anthers (e.g., *Myosotis,* *Heliotropium*) are found in this plant family. Contrary to expectations, we found no association between pollen accessibility and PA content in the Boraginaceae; some species with particularly small corolla and entirely hidden anthers (e.g., *Myosotis*, *Heliotropium*) had high PA contents, while other did not (e.g., *Pulmonaria*). Thus in the Boraginaceae a link between the accessibility of the anthers and the levels of defence in the pollen could not be found; a similar conclusion has been reached for the Fabaceae(Haider *et al.* 2014).

Alternatively, pollen secondary compounds may trigger specialization in bee-pollinated plants, which may result in specific bee-flower interactions and efficient pollen transfer. Our data were ambiguous in this respect as there was a marginally significant association between PA content and the number of specialist bees (P=0.0933 when phylogenetic non-independence was taken into account). A few genera hosting large numbers of specialist bees (*Echium*, *Heliotropium*) had high PA concentrations in the pollen, largely driving the marginally significant trend in our data; however, these are also speciose genera that have their centre of diversity in the Mediterranean area, where maximal diversity and abundance of bees are observed. Other genera hosting numerous specialist bees (e.g. *Onosma*, also a large genus with diversity centre in the Mediterranean region) had low levels of PA in the pollen, while the large, mountainous genus *Myosotis*, nearly unvisited by bees in Europe (Westrich 1989; Müller 2017) had high levels of PAs. Thus in spite of a weak trend, no conclusive evidence allows us to link the levels of PAs in the pollen with the number of specialized bees.

In contrast, species with a specialized bee-morphology had significantly lower PA levels in the pollen than other Boraginaceae species. When phylogeny was not incorporated in the model, the difference in the PAs was not significant, but phylogenetic comparative analyses did favour a model with two optima (a low PA value, 2.0 µg/mg for species of the *Borago* type; and a higher value, 14.7 µg/mg for the other species). This difference between both types of analysis may be due to the high variance in the PA levels in the flowers of the *Pulmonaria* type or within *Echium*. Yet the five sampled genera of the *Borago* type represent five independent phylogenetic origins of this sophisticated morphology, likely accounting for the significant differences in PA levels observed when phylogeny is incorporated into the model.

Based on these results, we postulate the following evolutionary origin of pollen PAs in Boraginaceae. In species of the *Pulmonaria* type, pollen PAs represent a variable trait likely little influenced by interactions with pollinators. The high levels of PAs in the pollen of *Myosotis sylvatica* is a good illustration of this hypothesis: the corolla in this plant is very small and the anthers particularly well concealed. No solitary bee in Central Europe is able to harvest the pollen from this common plant genus (Westrich 1989; Müller 2017). Although mountain honeys frequently contain trace amounts of *Myosotis* pollen, *Myosotis* pollen is nearly absent from honeybee-collected pollen (Christina Kast, unpublished results), suggesting that honeybees use this plant genus for nectar but not for pollen. Given the very difficult accessibility of *Myosotis* pollen, and given that no European species of bee is known to specialize on *Myosotis* or merely to exploit this plant fenus for pollen in spite of the abundance of this genus, the high PA levels in the pollen of *Myosotis* are unlikely to be an adaptation to deter pollen-collecting insects or to trigger pollinator specialization. Rather, pollen PAs in *Myosotis* may have its evolutionary origin in the defence of reproductive tissues against other herbivores. In species of the *Pulmonaria* type, we postulate that the legitimate pollinators are nectar-seeking insects (e.g., in *Myosotis*, *Pulmonaria*, *Heliotropium*). In *Anchusa* access to the nectar is rendered difficult by complex, hair-like structures that partially obstruct the corolla and the visitors are likely essentially nectar-seeking solitary bees or bumblebees. Only few specialized bees, likely representing a minority of the visitors have the morphological adaptations necessary to extract pollen from *Anchusa* species. In short, in species of the *Pulmonaria* type, the floral reward is essentially nectar, not pollen.

In contrast, all six genera of the *Borago* type had low levels of PAs in the pollen; in these species, the morphology of the anthers is perfectly adapted for pollen-collecting bees: the flower is pendulous and thus the pollinators must be hanging down to visit the flower, preventing the majority of non-bee visitors to access floral resources. Moreover, the pollen is released progressively over a period of 1-3 days (Fig. 2; Teppner 2011), so that at a given time only a small fraction of the pollen is available to bees. Bees are thus forced to repeatedly visit the flowers to gather pollen, enhancing the probability of pollen deposition on the stigma. We postulate that in species of the *Borago* type, unlike in the other investigated species of Boraginaceae, both pollen and nectar may be considered a reward to the legitimate pollinators, bees. In agreement with this hypothesis, selection has likely decreased the levels of defensive compounds in the pollen of these species.

Species of *Echium* are particular diverse both in their morphology, pollination biology and, according to our study, the concentrations of PAs in the pollen. Our observations suggest that the legitimate pollinators of *Echium vulgare* are nectar-foraging bumblebees. In this plant species, the anthers and style are particularly long and come into contact with the ventral side of bumblebees (Rademaker & De Jong 1997). The three central European species of *Hoplitis* oligolectic on *Echium* are likely not the most efficient pollinators of their host plant. During pollen visits, the females place their abdominal scopa on the anthers and come into contact with the style (V. Trunz, unpublished observations), although in this protandrous flower the stigma is likely not receptive during the male (pollen-producing) phase. During nectar visits, *Hoplitis* bees land on the corolla and may deposit some pollen accidentally, although when gathering nectar their comparatively short body is placed deeply in the corolla and often fails to contact the long stigma. Other, Mediterranean species of *Echium* such as *E. judaense* (not samples in our study) have shorter anthers and stigma. The latter species is abundant in arid areas of Israel, where bumblebees are largely absent and where most visitors are solitary bees. Interestingly, we detected only trace amounts of PAs in the pollen of *E. wilpretii*, a large species of the Canary Islands. Field observations indicate that the majority of the floral visitors (excluding the very numerous honeybees, which are not native to the Canary Islands) are pollen-collecting, solitary bees of the genera *Eucera* and *Anthophora* (V. Trunz, unpublished results). Possibly, *Echium* represents an exception in Boraginaceae: in the temperate species *E. vulgare*, the legitimate pollinators are nectar-foraging insects, mostly bumblebees. In constrast, in Mediterranean species, solitary bees are the main visitors and pollinators; whether their pollination efficiency differs between pollen and nectar visits needs to be investigated. The evolutionary origin of the PAs in the pollen of *Echium vulgare* remains unclear. We consider it unlikely that these PAs specifically deter pollen-collecting visitors, for the following reason: PAs are non-volatile compounds; while pollinators can taste alkaloids in the nectar, it is unlikely that bees can detect PAs in the pollen. *Hoplitis adunca*, a bee specialized to *Echium*, does not use PAs to locate and recognize its host, but other, volatile compound (Burger *et al.* 2010).

PAs and oligolectic bees

The assumption that specialized bees are better pollinators is likely a simplistic view in Boraginaceae. *Andrena symphyti,* a short-tongued bee specialized to several Boraginaceae genera, is likely not a good pollinator of *Onosma*: to gather nectar, this species punches holes at the base of the corolla (Teppner 2011), and during pollen visits, there is a "high probability that the [long] stigma may miss the bee" (Teppner 2011: 175). More generally, Boraginaceae species of the *Borago* type, thus with specialized "bee" morphology, do not host more specialist bees that other species. In constrast, *Echium vulgare* and the species of *Heliotropium* investigated host numerous specialist bees, yet in both cases, we postulate that these bees are neither the legitimate pollinators nor the most efficient pollinators. The three genera that host most specialist bees in the Boraginaceae (*Echium*, *Onosma* and *Heliotropium*) have the following common attributes: their maximal abundance and diversity is found in arid, Mediterranean habitats, where bees also reach maximal abundance. These genera likely represent reliable, predictable and abundant pollen resources for bees (Minckley *et al.* 2000), explaining more than any other factor why they host numerous specialist bees. We strongly think that the evolutionary origin of bee specialization to Boraginaceae is entirely unlinked to the pollination efficiency or the pollen chemistry.

Conclusion

Our results demonstrate for the first time a toxic effect of pollen secondary compounds on bee larvae. Yet we postulate that pollen PAs are only indirectly linked to bee-flower relationships. Their evolutionary origin is likely to be sought in the defence of reproductive parts against floral herbivores. In species where pollen, and not only nectar, is a reward to pollinators (species of the *Borago* type), we hypothesize that selection has acted to decrease PA-levels in the pollen. A similar conclusion was reached for the Ranunculaceae, where nectarless genera (thus species where pollen is the only reward to pollinators) had lower concentrations of the main pollen toxin, protoanemonin, than nectar-producing genera (Jürgens & Dötterl 2004). A more general conclusion of our study is that pollination by bees often confounds two very distinct behaviours: nectar collection, and pollen collection. Most bees visit flowers for both resources, yet their pollination efficiency likely strongly differs between both types of visits. Specific floral adaptations to bees may be considered as adaptations *against* bees (Westerkamp 1997a) if they merely restrict access to the pollen, as in the case of the species of the *Pulmonaria* type. In these species only few bees with morphological adaptations can utilize the pollen, and these bees are likely not legitimate pollinators. In contrast, specific adaptations that dispense the pollen in a progressive way likely maximise the pollination efficiency of pollen-foraging bees, and may thus be considered as adaptations *for* bees, as observed in species of the *Borago*-type in Boraginaceae.

Acknowledgements

We would like to thank Claudio Sedivy for the nests of *H. adunca* and *C. rapunculi*, Maryse Vanderplanck for help in the field, the Jardin botanique de Neuchâtel and especially Laurent Oppliger for the culture of *Echium* and *Campanula* and for hosting our encaged rearings. We also thank Anne-Marie Labouche for the figures and Romain Piault for his help with statistical analyses. This study was funded by the University of Neuchâtel and the Fondation Dr. Joachim de Giacomi.

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Supplementary materials

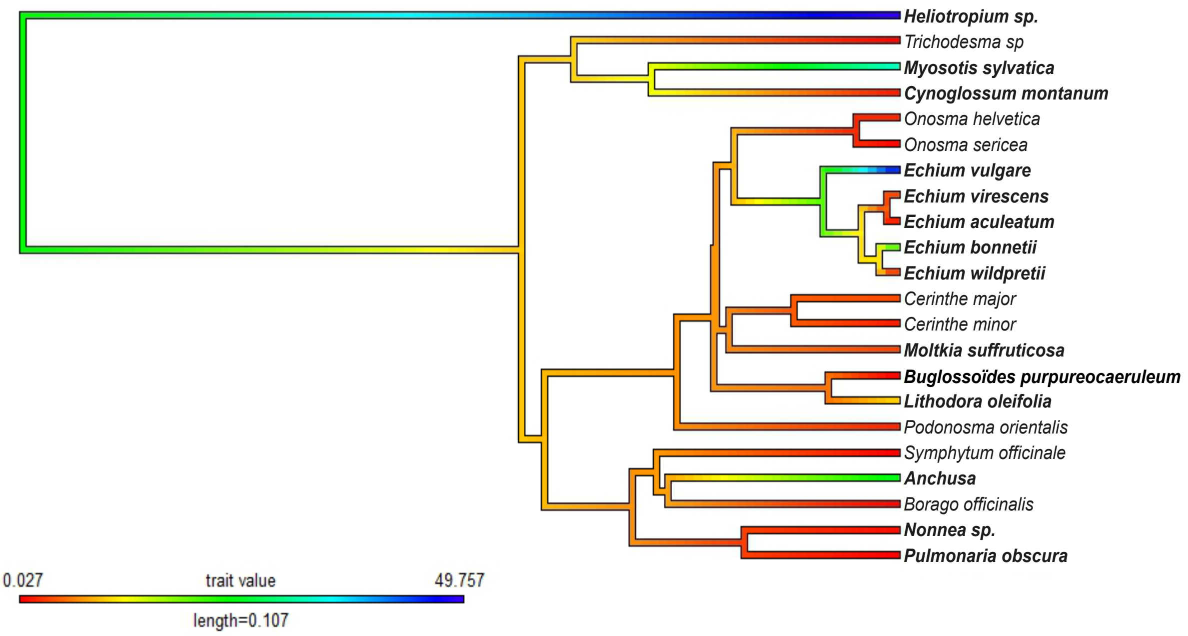
Table S1: PAs found in the pollen of the investigated species of Boraginaceae

N°	Name	RT (min)	m/z	Formula
1	Lycopsamine	1.16	300.181	C15H25NO5
2	Intermedine	1.13	300.181	C15H25NO5
3	Lycopsamine N-Oxide	1.28	316.175	C15H25NO6
4	Echimidine	2.32	398.218	C20H31NO7
5	Echimidine N-Oxide	2.32	414.212	C20H31NO8
6	Vulgarine N-Oxide	2.53	414.212	C20H31NO8
7	Acetylechimidine	2.81	440.228	C22H33NO8
8	Acetylvulgarine	2.94	440.228	C22H33NO8
9	Acetylechimidine N-Oxide	2.81	456.223	C22H33NO9
10	Acetylvulgarine N-Oxide	2.99	456.223	C22H33NO9
11	Echivulgarine	3.95	480.259	C25H37NO8
12	Echivulgarine N-Oxide	3.95	496.254	C25H37NO9
13	Unidentified alkaloid	1.57	386.18	C18H27NO8
14	Unidentified alkaloid	0.82	380.149	C22H21NO5
15	Unidentified alkaloid	2.24	398.181	C19H27NO8
16	Unidentified alkaloid	2.32	428.192	C20H29NO9
17	Unidentified alkaloid	1.09	358.149	C16H23NO8
18	Unidentified alkaloid	1.8	356.17	C17H25NO7
19	Unidentified alkaloid	2.45	398.181	C19H27NO8
20	Unidentified alkaloid	1.99	358.186	C17H27NO7
21	Unidentified alkaloid	1.3	302.197	C15H27NO5
22	Unidentified alkaloid	1.35	316.176	C15H25NO5
23	Echimidine N-oxide isomer	2.26	414.212	C20H31NO8
24	Uplandicin	1.86	358.187	C17H27NO7
25	Unidentified alkaloid	1.75	356.17	C17H25NO5
26	Retronecine 9-O-curassavate N-Oxide	1.68	330.192	C16H27NO6
27	Isomer of cpd 26	1.87	330.192	C16H27NO6
28	Unidentified alkaloid	2.4	372.202	C18H29NO7
29	Unidentified alkaloid	2.5	400.196	C19H29NO8
30	Unidentified alkaloid	2.12	416.229	C20H33NO8
31	Unidentified alkaloid	3.32	400.233	C20H33NO7
32	Unidentified alkaloid	1.51	302.197	C15H27NO5
33	Unidentified alkaloid	1.8	358.18	C17H27NO7
34	Acetylechimidin isomer	3.65	440.23	C22H33NO8
35	Europine	1.79	330.192	C16H27NO6
36	Europine N-Oxide	1.19	346.187	C16H27NO7
37	Lasiocarpine-N-Oxide	3	428.229	C21H33NO8
38	3-Acetyllasiocarpine N-Oxide	3.7	470.239	C23H35NO9
39	Unidentified alkaloid	1.42	318.19	C15H27NO6
40	Unidentified alkaloid	1.12	374.181	C17H27NO8
41	Unidentified alkaloid	1.67	416.192	C19H29NO9
42	Unidentified alkaloid	2.02	434.218	C19H31NO10
43	Unidentified alkaloid	2.34	352.176	C18H25NO6
44	Unidentified alkaloid	2.34	514.229	C24H35NO11
45	Unidentified alkaloid	2.21	372.202	C18H29NO7

Table S2: DNA sequences of Boraginaceae extracted from Genebank. “Cohen” refers to sequences obtained from the publication of Cohen (2013): Cohen, J. I. (2013): A phylogenetic analysis of morphological and molecular characters of Boraginaceae: evolutionary relationships, taxonomy, and patterns of character evolution. Cladistics, 30(2), 139–169.

Species \ Gene	**ITS**	**matK**	**ndhF**	**trnL-trnF**
Anchusa italica	Cohen	Cohen	Cohen	GQ285268.1
Anchusa officinalis	Cohen	Cohen	Cohen	Cohen
Borago officinalis	Cohen	Cohen	Cohen	Cohen
Buglossoides purpurocaerulea	Cohen	Cohen	Cohen	Cohen
Cerinthe glabra	Cohen	Cohen	no seq	no seq
Cerinthe major	Cohen	Cohen	Cohen	Cohen
Cerinthe minor	FJ763223.1	no seq	no seq	FJ763281.1
Cynoglossum montanum	FR715308.1	no seq	no seq	KC542517.1
Echium aculeatum	Cohen	Cohen	Cohen	Cohen
Echium bonneti	Cohen	Cohen	Cohen	Cohen
Echium virescens	EU048850.1	EU599693.1	EU599781.1	EU433601.1
Echium vulgare	Cohen	FJ827257.1	KF158018.1	Cohen
Echium wildpretti	Cohen	Cohen	Cohen	EU600039.1
Heliotropium sp. (aegyptiacum)	Cohen	Cohen	Cohen	Cohen
Lithodora oleifolia	FJ789869.1	FJ789905.1	no seq	EU044896.1
Moltkia suffruticosa	Cohen	Cohen	no seq	Cohen
Myosotis sylvatica	AY092935.1	Cohen	Cohen	no seq
Nonnea sp. (lutea)	Cohen	Cohen	Cohen	Cohen
Onosma helvetica	GU827176.1	no seq	no seq	no seq
Onosma sericea	FR718857.1	no seq	no seq	no seq
Podonosma orientalis	Cohen	Cohen	no seq	Cohen
Pulmonaria obscura	Cohen	Cohen	Cohen	Cohen
Symphytum officinale	no seq	JN896115.1	no seq	JQ041857.1
Trichodesma africana	Cohen	Cohen	Cohen	KC542568.1

Figure S1: Phylogenetic tree of the investigated Boraginaceae species with colour indication of the Alkaloid concentration (indicated in µg/g on the scale “trait value”) and its hypothetical evolution.



Chapter 3

Foreword

This chapter focuses on pollen chemistry, as chapter 2, but focuses on another plant family: the Gesneriaceae, and more specifically the genus *Sinningia.* This Neotropical plant genus exhibits very diverse floral morphologies and presents an archetype of the morphologies associated with different pollination syndromes (Hummingbird, bee, Lepidoptera and bat). In fact, the morphologies are so different among species with different pollination syndromes that these species may be considered as belonging to different genera.

Thanks to the collections of the Botanical Garden of Geneva and to two Gesneriaceae specialists, Matthieu Perret and Alain Chautems, we could sample the pollen from 31 of these tropical species; in addition a phylogeny including all these plants is available (Perret *et al.* 2003; 2007). Using this sampling and phylogenetic framework we further test the hypothesis that plants legitimately pollinated by pollen-collecting bees do not chemically protect their pollen, while species pollinated by nectar-foraging visitors may include high levels of chemical protection in their pollen. Working with this plant lineage provided a unique opportunity to test hypotheses on the evolution of pollen chemistry; however an important drawback was that natural pollinators of these plants are absent in Switzerland and thus neither bioessays nor field observations could be conducted to link the observed pattern to the physiology or behavior of the pollinators.

How pollen chemistry complements pollination syndromes: pollen secondary compounds in bee- and bird-pollinated flowers of the genus *Sinningia* (Gesneriaceae)

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Abstract

Pollination syndromes represent a suite of traits evolved as a response to a certain type of pollinators. They have been the focus of numerous studies in the past. Surprisingly, few groups of plants perfectly fit into the discrete syndromes expected based on theoretical considerations. Here we use a plant system with pronounced, strikingly different pollination syndromes, the plant genus *Sinningia* (Gessneriaceae), and examine one specific floral trait hitherto little examined: the secondary compounds present in the pollen. We compared the pollen chemistry of species exhibiting two different syndromes resulting from pollination by two distinct groups of pollinators, bees and hummingbirds. Previous phylogenetic work has revealed numerous shifts between these two syndromes. Our general hypothesis is that the pollen of plants pollinated by nectar-collecting visitors is not a reward to pollinators and thus that it may include chemical defensive compounds, unlike the pollen of species legitimately pollinated by pollen- and nectar- collecting bees. Multiple components analyses of all compounds found in the pollen suggest that the pollen of both pollination groups differ in their chemical composition. The only secondary defensive compounds isolated in the pollen were saponins, which have been shown to act as deterrent and antifeedant on insects. In agreement with our hypothesis, the concentrations of saponins in the pollen of bee-pollinated species were significantly lower than those in the pollen of bird-pollinated species. One possible explanation is that hummingbird pollinated plants have exposed anthers and thus that their pollen may be accessible to pollen-collecting bees, which may not contribute to pollination due to inappropriate morphology. Therefore, these plants may chemically protect their pollen to reduce pollen losses to bees. However this hypothesis should be verified up by both field observations detailing the spectrum and behavior of pollinators on both types of flowers, and bioassays examining the effect of the saponins on bee adults and larvae.

Introduction

It has long been recognized that floral traits reflect strong adaptive responses to selection by pollinators (reviewed in Waser 2006; Ollerton & Coulthard 2009). Unrelated plant species or lineages may converge to similar shape, colour or odours in response to selection by similar pollinators; this convergent evolution is the theoretical basis for what has been commonly referred to as the "pollination syndromes" (Vogel 1954). Surprisingly, in spite of the universal use by botanists of the concept of pollination syndromes, these syndromes have long remained untested. Ollerton *et al.* (2009) have attempted to match "ideal" floral traits, as derived from numerous literature sources, to "observed" plant communities using a multivariate statistical approach; surprisingly, only a minority of plant species fell into the discrete syndrome clusters expected based on the description of the syndromes; and the majority of the plants' main pollinators, as observed by direct field observations, could not be predicted based on their suite of phenotypic traits in two thirds of the plant species. The authors concluded that further research should focus on the response of individual floral traits to selection by flower visitors, including both legitimate pollinators and possible antagonists, while taking into account possible pleiotropic effects on other plant traits.

To achieve this, floral traits should be examined within a system of closely related plant species with known phylogenetic relationships and known visitors; ideally the selected system would exhibit numerous independent, sharp switches in the spectrum of pollinators. Such plant systems are rare in nature: they include for example the genus *Iochroma* in Solanaceae (Smith *et al.* 2008); *Oenothera* in Onagraceae (Raven 1979); *Silene* (Bernasconi *et al.* 2009) and the plant genus *Sinningia* in Gesneriaceae (Perret 2001; Perret *et al.* 2003). All of these plant systems have served as "evolutionary laboratories" to address pollination biology questions.

In this study, we examine one specific and little investigated plant trait, pollen chemistry, in relation with visitations by bees. The presence of secondary compounds in the pollen has received little attention and the ecological significance of these compounds remains largely unclear. The presence of these compounds may simply be a pleiotropic consequence of their presence in other floral tissues (Irwin *et al.* 2014);

alternatively, their presence may reflect an adaptive response to the type of pollinators. Our general working hypothesis is the following: pollen chemical composition varies according to whether pollen is offered as a reward to pollinators (legitimate pollination by pollen-collecting bees) or not (legitimate pollination by nectar-foraging visitors). Pollen-collecting bees may act as antagonists in systems where the legitimate pollinators are nectar foraging animals (birds or insects) by removing pollen from the systems and thus lowering the pollination efficiency during subsequent, legitimate visits (Wilson & Thomson 1991; Thomson & Thomson 1992; Lau & Galloway 2004). Consequently, we expect that the levels of defence compounds in the pollen will be lower in plants legitimately pollinated by bees than in those pollinated by nectar foraging animals such as birds or insects.

We use the genus *Sinningia* as a model system to examine this question. *Sinningia* species occur mainly in Neotropics and are most diverse in southeastern Brazil (Perret *et al.* 2007). These plants exhibit a wide array of ecologies and habitus and their flowers are mainly visited by hummingbirds and bees, but bats and moths are also reported (Perret *et al.* 2003). 81 species are known in the tribe Sinningiae: 76 species of the genus *Sinningia* species, in which six and eight species of the genera *Paliaviana* and *Vanhouttea*, respectively, are nested (Perret *et al.* 2007). The latter two genera are here considered to be synonyms of *Sinningia*, see Perret et al. (2007). In the tribe Sinningiae, 48 present a typical hummingbird pollination syndrome, 16 a bee pollination syndrome, four a bat pollination syndromes, one a moth pollination syndrome and seven are unknown. Independent pollinator shifts between hummingbird and bee pollination syndromes are frequent in *Sinningia* and both could represent the ancestral state of the clade. According to recent phylogenetic studies (Serrano-Serrano *et al.* 2017), the ancestor of Gesneroideae was insect-pollinated and shifts to hummingbird pollination occurred many times. Reversions to insect pollination syndromes were even more frequent in Gesneroideae; in the Sinningieae they occurred back to bee-pollination syndrome at least three to seven times (Perret *et al.* 2003), while transitions from bee to hummingbird one to five times. Numerous field observations have confirmed in several cases that species exhibiting one particular syndromes were largely visited by the corresponding group of pollinators (SanMartin-Gajardo & Sazima 2004; 2005). Hummingbird species are mainly red and tubular, usually with extruding or apparent anthers (see pictures in Perret 2001). Bee-pollinated species show varied colours, from yellow to pink or white, and have larger corollas with anthers positioned inside the corolla. Species presenting both the bee and hummingbird syndromes have nototribic anthers, that is, anthers placed in the upper part of the corolla and which contact the dorsal part of pollinators. The discrete nature of most pollination syndromes and their numerous shifts from hummingbird to bee and reverse make *Sinningia* an excellent and rarely equalled system to study plant-pollinator correlated features.

Secondary compounds in Gesneriaceae have been investigated in several studies (Verdan & Stefanello 2012 and references therein) but their ecological significance remains poorly known. Pollen especially has not been analysed so far and no account of its chemical composition is currently available. Nectar sugars concentrations were investigated and compared across different pollination syndromes but no significant difference between hummingbird and bee species was found. (Perret 2001), suggesting that flowers rely on other aspects such as morphology to modulate the spectrum of visitors.

The present study describes the secondary metabolites found in the pollen of these plants. We specifically address whether we can detect differences in the composition of the secondary metabolites between bee-pollinated species and bird-pollinated species. If a difference can be found, we try to identify the compounds responsible for this difference and test whether the presence and concentration of these compounds is associated with the sharp pollination syndromes observed in this plant genus.

Material and methods

Plant species examined and pollination biology

We collected and analysed the pollen of 31 *Sinningia* species (Table 1), nine presenting a bee pollination syndrome and 22 presenting a hummingbird pollination syndrome. All these species are cultivated in greenhouses of the Botanical Garden in Geneva. For each species we removed by hand a minimum of 1 mg of pollen from the anthers using precision tweezers and dried the pollen in microcentrifuge tubes for five days in a closed container with silica gel. We stored the samples at -80 °C until extraction.

Chemical extractions and LCMS analyses

For each sample, we weighed 1 mg of pollen using a Mettler Toledo precision scale. We added 100µl of pure methanol (MeOH) and 6-7 glass beads (2 mm diameter) and mixed the samples for 4 minutes at 30 Hz in a Retsch MM300 grinder. We verified that this treatment crushed all pollen grains with an optical microscope. The samples were then centrifuged for 4 minutes at 14'460 g, the supernatant was transferred into vials for LC-MS analysis.

Saponin profiling was performed by ultra-high pressure liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS). The system used was an Acquity UPLCTM coupled to a Synapt G2 QTOF (Waters, Milford, USA) and was controlled by Masslynx v4.1. Separation was carried out on an Acquity UPLC BEH C18 column (100 x 2.1, 1.7 µm particle size) at a flow rate of 0.4 mL/min and a temperature of 30°C. Injection volume was 2.5 µL. Detection by QTOFMS was performed in electrospray negative mode over a mass range of 50-1200 Da in the so-called MSe mode which alternates between low and high collision energies. Total scan time was 0.4 s, capillary voltage -2.5 kV, cone voltage -25 V, desolvation gas temperature and flow 350°C and 800 L/h, respectively. Accurate mass measurements were achieved by infusing a 400 ng/mL solution of leucine-enkephalin throughout the run as internal calibrant. Prior to the analysis, the instrument was calibrated using a 0.5 mM solution of sodium formate. Signals of saponins were manually extracted from the total ion chromatograms and identified based on their typical molecular formulae and fragmentation mass spectra.

Species	Syndrome	Sap1	Sap2	Sap3	Sap4	Sap5	Sap6	Sap7	Sap8	Sap9	Sap10	Sap11	Total
Sinningia aghensis	Bee	20	122	87	0	289	34	263	4833	177	3185	63	**9073**
Sinningia conspicua	Bee	183	1001	203	4314	11363	0	637	6400	1878	1913	93	**27985**
Sinningia eumorpha	Bee	1184	8466	2204	13225	26862	184	242	2045	361	9646	369	**64788**
Sinningia gerdtiana	Bee	4484	3138	876	8603	19702	0	142	4088	434	16743	154	**58366**
Sinningia guttata	Bee	3104	1535	965	4297	25285	0	186	895	248	3790	85	**40390**
Sinningia schiffneri	Bee	26113	6743	2943	7934	24814	73	904	3534	935	8527	632	**83152**
Sinningia speciosa	Bee	884	1345	213	11816	17588	0	67	275	257	8274	135	**40855**
Sinningia tenuifolia	Bee	254	2184	925	8404	31287	0	184	5082	170	870	89	**49451**
Sinningia villosa	Bee	2982	2108	2956	8065	39029	64	340	1278	579	5008	267	**62676**
Sinningia aff. reitzii	Hummingbird	6498	67583	18184	19002	26755	760	10355	19018	13458	2454	494	**184560**
Sinningia aggregata	Hummingbird	0	16860	5254	41291	66596	964	449	2554	1685	13393	719	**149764**
Sinningia bulbosa	Hummingbird	705	45806	11668	21226	36474	1722	1879	6707	3378	4954	600	**135120**
Sinningia bullata	Hummingbird	0	21208	8614	4695	17732	1294	2668	5076	1344	1274	355	**64261**
Sinningia calcarata	Hummingbird	207	797	387	1861	4309	0	7054	1205	1116	6973	16671	**40580**
Sinningia canescens	Hummingbird	791	9406	2817	8538	26687	250	1074	3966	1634	1306	380	**56850**
Sinningia carangolensis	Hummingbird	23	23763	466	15806	5080	1019	275	5082	6033	316	26	**57889**
Sinningia cardinalis	Hummingbird	722	26087	12214	14071	27265	1094	6829	1546	1883	1797	3861	**97368**
Sinningia cooperi	Hummingbird	867	11677	6111	10455	27347	929	6449	1927	1291	1421	2819	**71293**
Sinningia douglasii	Hummingbird	170	42715	14123	14367	32686	8262	872	2830	1305	529	118	**117977**
Sinningia glazioviana	Hummingbird	237	8037	2298	10267	23023	278	1508	5158	1832	8608	1444	**62691**
Sinningia iarae	Hummingbird	827	60888	28484	16947	34434	4900	3481	2782	2308	6549	1373	**162972**
Sinningia lanata	Hummingbird	117	319	613	2731	3507	0	7189	479	1008	4068	18254	**38285**
Sinningia lateritia	Hummingbird	427	30750	16280	15184	25646	3715	24365	3236	5430	1483	5869	**132384**
Sinningia leopoldi	Hummingbird	3079	46688	20085	7905	19689	11896	1884	24081	2578	3821	211	**141916**
Sinningia leucotricha	Hummingbird	76	35359	13753	11290	27929	2172	589	7494	1115	2193	103	**102073**
Sinningia lineata	Hummingbird	487	25352	4592	5789	10176	1026	9340	18722	7747	5611	740	**89583**
Sinningia macropoda	Hummingbird	105	9929	3980	2757	8163	2204	7747	7461	2349	2238	1799	**48734**
Sinningia macrostachya	Hummingbird	220	28637	9615	8181	13959	4632	1762	18782	3142	5403	556	**94888**
Sinningia reitzii	Hummingbird	562	30193	8078	11810	20295	3601	3215	6236	4072	679	88	**88828**
Sinningia sellovii	Hummingbird	379	5814	1213	10607	24707	584	442	776	2195	1796	285	**48797**
Sinningia sulcata	Hummingbird	101	2443	64	24397	8571	99	253	3065	2262	7941	270	**49465**

Table 1 : LC-MS peaks height (relative value) for the eleven saponins found in the 31 species of Sinningia investigated. These 31 species exhit two main pollination syndromes, either bee-pollination or hummingbird pollination.

Phylogenetic analyses

Correlation between phylogenetic evolution and saponin total concentration of bee versus humminbird species was tested using the R packages Phytools (Revell 2012) and mvMORPH (Clavel *et al.* 2015). We used this test to establish if a character evolved according to brownian motion (BM), one optimum (OU) or two optima (OUM). BM here represents a random evolution of the saponin concentrations in pollen, OU an evolution toward one optimal concentration and OUM two different optimal concentrations. A phylogenetic tree containing 27 of the 31 investigated species was supplied by Matthieu Perret (pruned from a tree presented in Perret *et al.* 2007).

Statistical treatment

In order to assess correlations between pollen chemistry and floral syndrome, we performed a principal component analysis (PCA) with a Kruskal-Wallis test and a Partial Least Squares Discriminant Analysis (PLS-DA) after Pareto scaling with Anova. PLS-DA was tested for overfitting using a leave-one-out cross-validation and a permutation test using 200 permutations. As saponins were amongst the most concentrated compounds and the most correlated with pollination syndromes, we performed further statistical analyses on them: Anova on total amount of saponins, PCA with Kruskal-Wallis tests. Technical triplicates of four species were performed and variation was assessed with t-tests to check for repeatability. A measure of the relative quality of each model in the correlation test between phylogenetic and secondary composition is given by the Akaike information criterion (AIC).

Results

LCMS analyses

No significant differences were found between technical replicates (Table S1), indicating that variability among samples collected from the same species was low. LCMS profiles of pollen across all species revealed 1025 markers. PCA of all species with all compounds as variables showed partial segregation between bee and hummingbird pollination syndromes (Fig. 1) although there was a weak overlap between both clusters of points; this separation was significant on the first axis (Kruskal-Wallis test df = 1, *P* = 0.00004) but not on the second (Kruskal-Wallis test df = 1, *P* = 1). PLS-DA separated bee and hummingbird syndromes more clearly (Fig. 2) and ANOVAs were significant on the first two axes (1: *P* < 0.0001; 2: *P* = 0.0007).

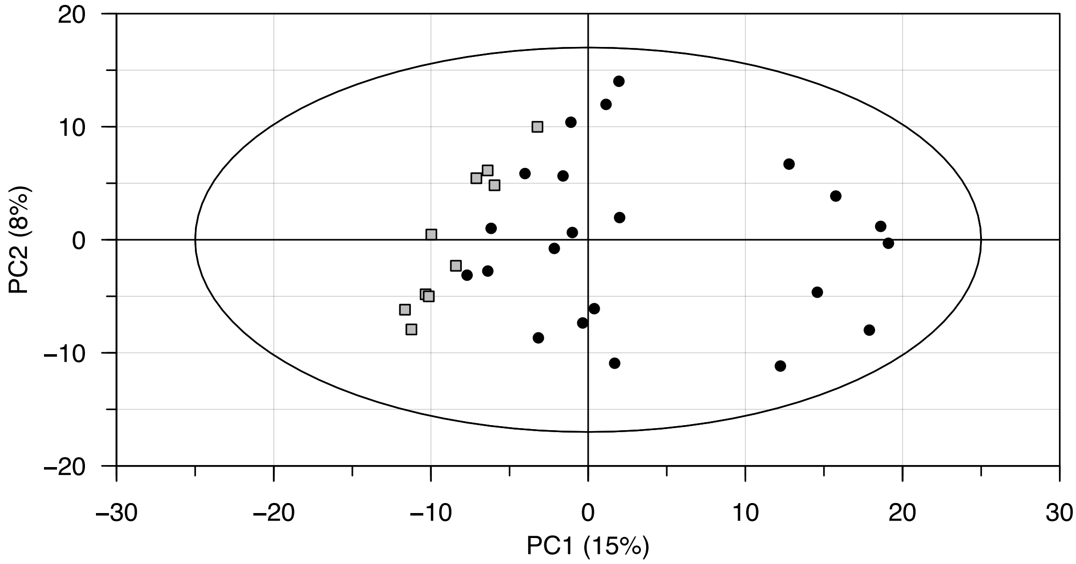

Fig.1: Principal component analysis (PCA) for all secondary compounds found in the pollen of the 31 species of *Sinningia*. The grey squares represent bee-pollination syndromes and the black circles represent hummingbird-pollination syndromes.

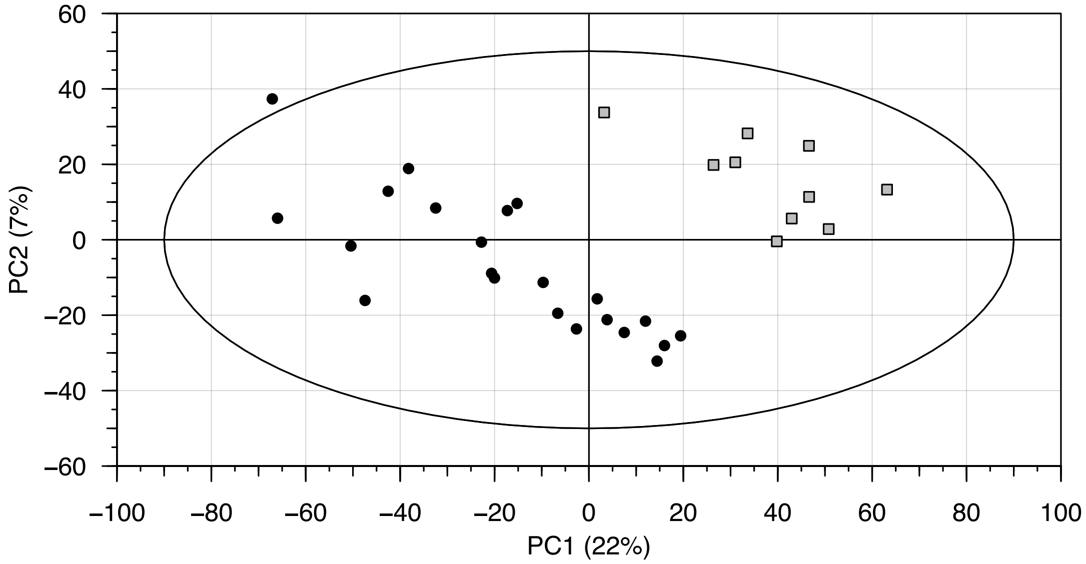

Fig.2: Partial least squares Discriminant Analysis (PLS-DA) (see text for details) for all secondary compounds found in the 31 species of *Sinningia*. The gray squares represent bee-pollination syndromes and the black circles represent hummingbird-pollination syndromes.

Among the 36 most discriminant markers in our PLS-DA, nine were saponins (Table S2); the other could not precisely be identified, but belong mainly to sucroses, amino-acids, flavonoids, spermidines, phospholipids and fatty acids. In total there were 11 different saponins (Table 1), thus the majority (nine of 11) of the saponins found were included in the most discriminant compounds (Table S1). Total concentrations of saponins were significantly lower for the *Sinningia* species with bee pollination syndrome than for those with hummingbird pollination syndrome (Fig. 3; *P* = 0.0076). PCA analysis of saponins contents shows significant differences on the first axis only (Fig. 4; Kruskal-Wallis test df = 1, *P* = 0.0004).

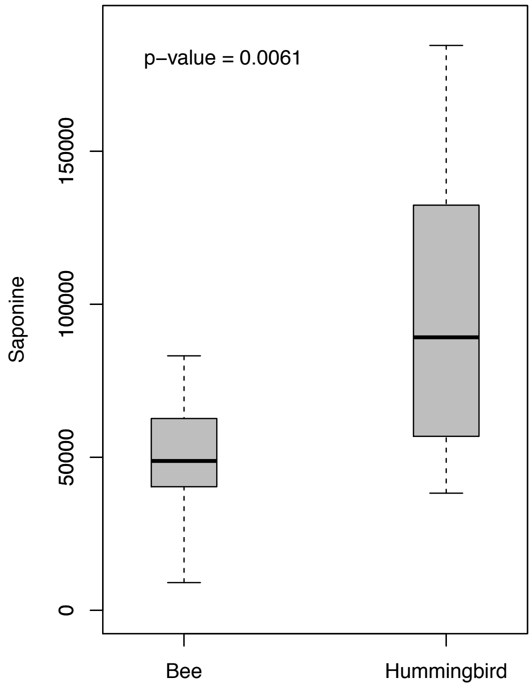

Fig.3: Boxplots showing the concentrations of saponins in the pollen for the species of *Sinningia* separated into two groups based on their pollination syndromes: hummingbird pollination and bee pollination.

Phylogenetic comparative methods favoured an evolutionary scenario with two optima (OUM; AIC -327.625) for bird- and bee-pollinated species; however, this scenario was not significantly better than a model in which pure drift (Brownian motion; AIC = -328.635) or only one optimum accross all species (OU; AIC = -328.325). A difference in two log units in the AIC values is commonly taken as evidence for one model over another. Fig.5 presents a phylogenetic tree of investigated *Sinningia* species and a hypothetical evolution of saponins concentrations in their pollen.

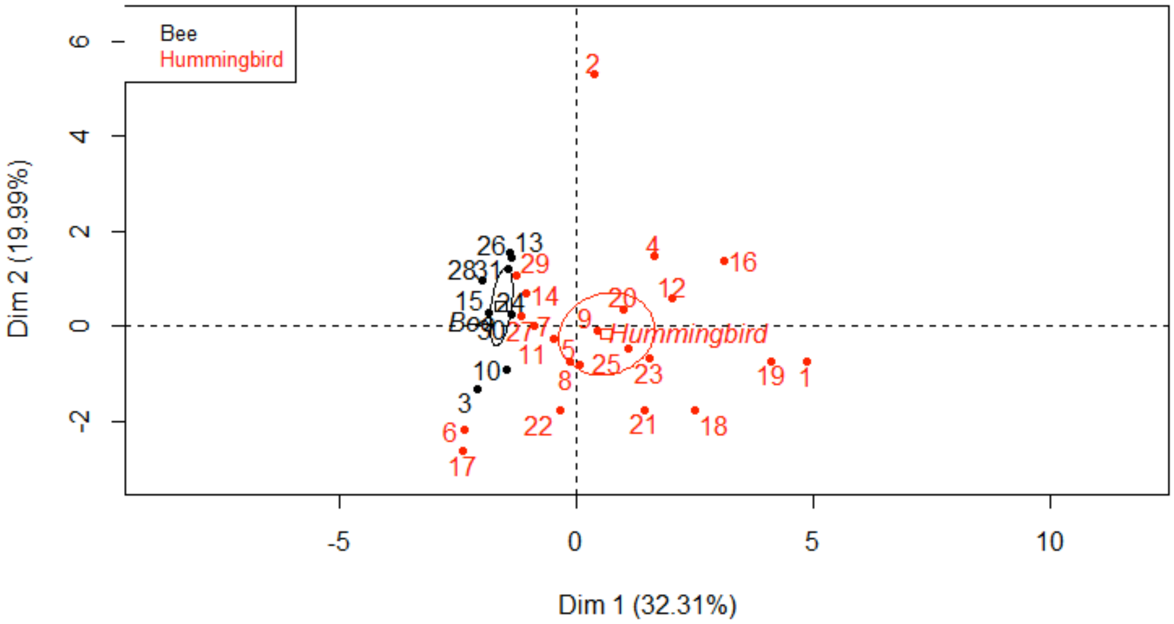

Fig.4: PCA representation of all saponins found in the 31 species of *Sinningia*.

Discussion

The significant difference between bee and hummingbird pollination syndromes on the first axis of the PCA considering all markers indicates that floral morphology, and thus the spectrum of pollinators (in our data bees or hummingbirds), was correlated with secondary chemistry. The levels of saponins were also significantly higher in hummingbird-pollinated species than in bee-pollinated species. Phylogenetic comparative methods favoured a model with two optima to trace the evolution of total saponin concentrations in the pollen, although the three investigated models (drift: BM, one single optimum for all species considered: OU, or two optima for the two groups: OUM) were not significantly different and thus our data remain somehow inconclusive. This low power of our data in a phylogenetic context is likely due to an insufficient sampling, since large and complete datasets are required for this kind of analyses (Beaulieu *et al.* 2012); we could obtain the pollen of only 31 *Sinningia* species out of a total of 81. Possibly, the lack of signal also indicates that the pattern observed is not independent from the phylogenetic signal, for example in the case where several bee-pollinated species with low saponin levels form a clade and thus can not be considered as multiple independent observations in analyses taking phylogenetic independence into account.

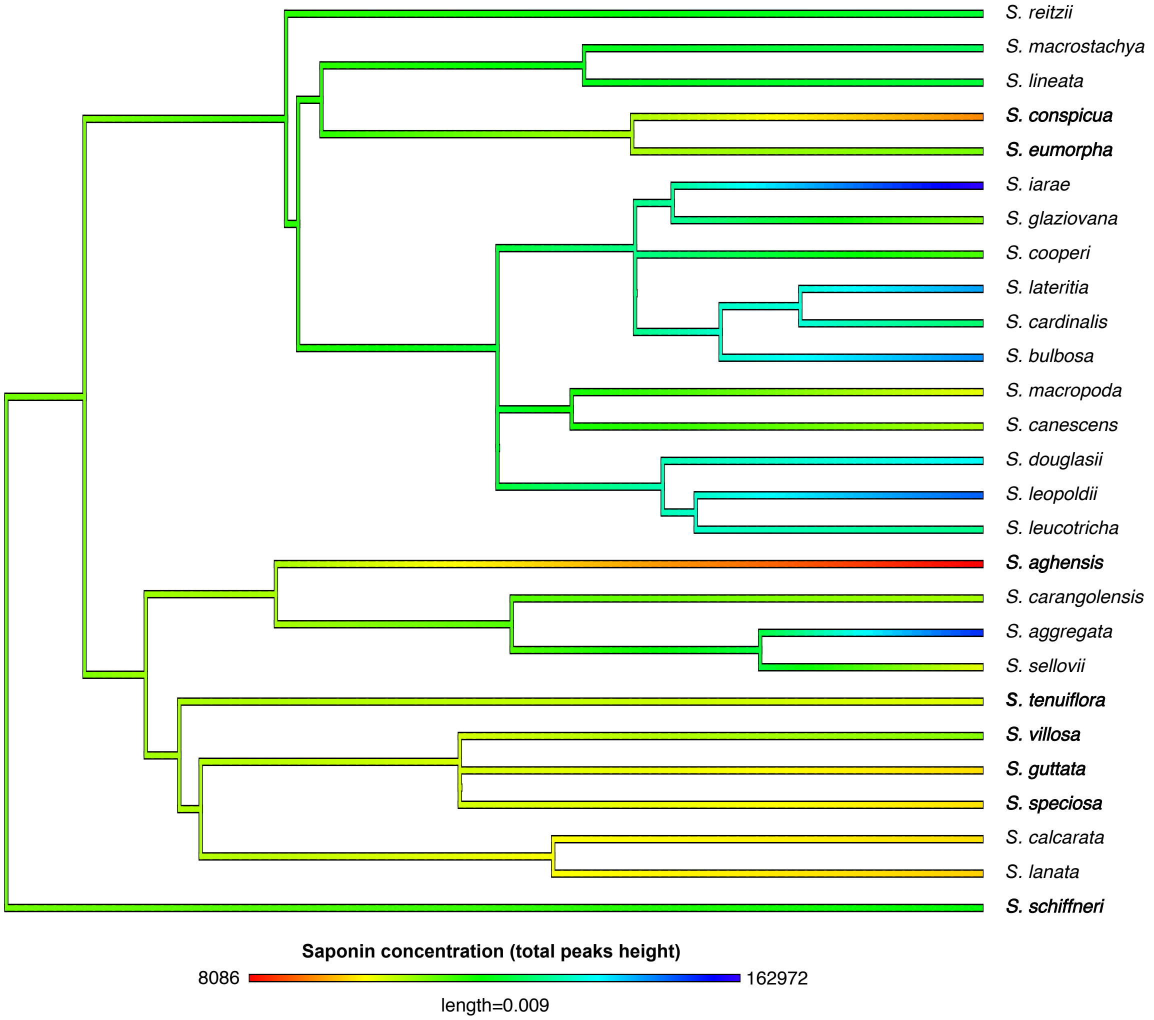

Fig.5: Phylogenetic tree based on maximum likelihood analysis with a possible evolutionary scenario of Saponins concentration. Sinningia with Bee-pollination syndromes are indicated in bold.

Nevertheless, saponins were the only putative defence compounds identified in the pollen. They were among the pollen compounds that were the most segregated between bee and hummingbird- pollinated species. Saponins are bioactive secondary compounds studied for their antifeedant effect and toxicity toward herbivores and especially insects (Goławska 2007; De Geyter *et al.* 2011; Faizal & Geelen 2013; Goławska *et al.* 2014). In addition, they show physical properties not directly linked to defence against herbivores (see below). Saponins can be found in many plant families (Faizal & Geelen 2013), not only in leafs, roots and flowers, but also in pollen (Wadhawan & Rao 1993). Toxicity of saponins on bees has rarely been tested but, when injected into the body of the solitary bee *Megachile rotundata*, they significantly increased mortality (Thorp & Briggs 1972). In addition, a deterrent and a toxic effect of saponins has been demonstrated in adult honeybees (Detzel & Wink 1993).

The first hypothesis that comes to mind to explain our results is that pollen saponins may have a deterrent effect on pollen collecting bees in species of *Sinningia* where the legitimate pollinators are hummingbirds. In addition, the pollen may have reduced nutritional values for larvae, which may in turn impact floral choices in bees (Praz et al. 2008; Sedivy et al. 2011; but see Bukovinszky et al. 2017). This hypothesis is in accordance with the general hypothesis that plants attracting nectar-feeding visitors must have exposed pollen to maximise pollen deposition, while pollen-collecting visitors on such plants may have detrimental effects on pollen transfer efficiency by removing abundant quantity of pollen and therefore erode pollen transfer efficiency of following visitors (Thomson & Thomson 1992; Dobson & Bergstrom 2000; Sedivy *et al.* 2011). Following this hypothesis, the abundant, exposed pollen of hummingbird-pollinated species of *Sinningia* would be chemically protected to reduce losses to illegitimate pollen-collecting visitors.

To further test this hypothesis, the following aspects should be examined. First, it would be important to document whether pollen-collecting visitors, notably pollen-collecting female bees, visit humming-bird pollinated species of *Sinningia*; if this is the case, the impact of such pollen collection could be quantified. A general hypothesis is that pollen-collecting bees do not transfer pollen but instead remove large quantities of pollen and thus decrease the pollen available to subsequent, legitimate (nectar-feeding) pollinators. 2. The effect of the saponins found in the pollen of *Sinningia* should be investigated on adult bees. Bioassays with feeding choices can be conducted using for example honeybees or bumblebees, both of which accept pollen presented artificially in petri dishes. Tests with other bees are more difficult as they only collect pollen from real flowers. 3. The impact of saponins on bee larvae may be investigated by supplementing realistic levels of saponins to natural pollen and nectar provisions of solitary bees. If the presence of saponins in the pollen has its evolutionary origin in deterring pollen robbers, especially pollen-collecting bees, we expect these compounds to have both deterrent effects on the adults and toxic effects on the larvae.

However, several alternative hypotheses can be formulated to explain the pattern observed. First, the higher concentrations of saponins in hummingbird-pollinated species may be due to a pleiotropic effect between saponins content and other floral traits, e.g. the red pigmentation of the corollas of hummingbird-pollinated species. To our knowledge, no association between the production pathways of these compounds has been suggested. Second, an alternative function of saponins in pollen has been suggested (Wadhawan *et al.* 1993): steroidal saponins have been suggested to have a hormone-like effect on the germination and growth of the pollen tube. Due to the very different floral morphology between bee- and hummingbird pollinated species of *Sinningia*, it is expected that the length of the style may vary between both syndromes; this would have an important impact on the chemistry of the pollen given that the length of the pollen tube is directly correlated with the length of the style. Third, as anthers of Hummingbird-pollinated species of *Sinningia* are extruded and therefore exposed to direct sunlight and UV, in contrast to bee-pollinated species, it cannot be excluded that saponins play an hitherto unrecognized protective role against DNA damages by radiation. To our knowledge no such function of saponins has been described, the UV protection generally being attributed to pigments like carotenoids and flavonoids (Lunau 1995). Fourth, the saponins are complex and diverse and they have many additional, physical properties (Güçlü-Üstündağ & Mazza 2007). For examples they show varied solubilities and can sometimes form foams. These physical properties could play a role in enhancing the adherence of pollen grains to visiting hummingbirds. In insect-pollinated flowers, the pollenkitt, an oily substance coating the pollen grains, plays this role by enhancing pollen stickiness (Pacini 2005). Possibly, pollination by birds may require different physical attributes than pollination by insects. It is unknown whether the saponins are present in the pollenkitt, thus at the surface of pollen, or inside the pollen grains; this would be a future testable hypothesis.

Acknowledgements

We would like to thank Alain Chautems, and the botanical Gardens of Geneva to allow us to collect pollen from their *Sinningia* collection. We also thank Dimitri Bénon for his great help with statistical analyses and figures.

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Supplementary tables

Table S1: technical triplicates of four LC-MS analyses with means, standard deviations and t-tests

Sample	Total peaks height	Mean	standard deviation	t-test
S. conspicua 1	20320			
S. conspicua 2	19067	**18545**	**2086**	**1**
S. conspicua 3	16247			
S. lineata 1	72889			
S. lineata 2	58704	**62755**	**8835**	**1**
S. lineata 3	56673			
S. cooperi 1	74652			
S. cooperi 2	72062	**73238**	**1311**	**1**
S. cooperi 3	72998			
S. cardinalis 1	68320			
S. cardinalis 2	69261	**68617**	**559**	**1**
S. cardinalis 3	68269			

Table S2: PLS-DA 36 most discriminant markers (among 1025 markers) arranged according to the first axis, with saponins highlighted (yellow).

Marker ID (retention time_molecular mass)	First axis	Second axis
4.23_562.3144	-0.219145	-0.0590817
3.76_649.3945	-0.214214	-0.222879
4.21_474.2617	-0.161153	-0.0597781
1.83_623.1974	-0.127552	0.0908683
4.13_562.3143	-0.127295	-0.0802152
2.20_630.2446	-0.126916	-0.214138
1.93_411.1552	-0.114736	0.12696
4.59_571.2880	-0.109857	0.105068
3.95_647.3789	-0.108968	-0.075692
1.85_579.1349	-0.107916	-0.0589247
2.10_665.2078	-0.10446	-0.178658
3.31_811.4462	-0.101861	-0.158295
4.84_540.3298	-0.0926534	0.1814
2.38_644.2602	-0.0867582	0.0887303
4.82_452.2775	-0.0843133	0.0986654
4.02_593.2722	-0.0808914	0.0678972
1.60_625.1401	-0.0794238	-0.206978
5.17_631.3842	-0.0773858	-0.06952
4.11_474.2617	-0.0738723	-0.0755045
2.20_652.2263	-0.0708888	-0.0809228
5.31_580.3606	-0.0670421	0.0190047
2.03_651.1921	-0.0624197	-0.0618424
4.23_1079.6281	-0.0566701	0.0221372
1.94_623.1973	-0.0549634	0.0426376
3.98_548.2988	-0.0534227	-0.0137417
2.95_665.3890	-0.0528572	-0.058182
4.51_633.3995	-0.0517735	-0.118196
5.28_492.3087	-0.0502802	0.0174845
1.85_647.1221	-0.0493294	-0.0335698
2.00_549.1243	-0.0474381	-0.0143554
4.34_793.4369	-0.0461109	-0.0707914
3.76_717.3807	-0.0451549	-0.0496044
4.61_391.2595	-0.044705	0.125602
4.22_991.5774	-0.0429628	0.0123675
3.59_649.3941	-0.0417991	-0.0662312

General discussion

Bees originated some 100-120 millions ago from a group of prey-hunting sphecid wasps (Branstetter et al. 2017, Peters et al. 2017). This transition in diet and life history has eventually given rise to an immense clade of pollinating insects. From their ancestral, presumably arid habitats (Litman et al. 2011), bees have colonized all terrestrial ecosystems, including temperate and tropical habitats. Their nesting biology has evolved, and, importantly, they have developed an intimate relationship with the flowering plants. Understanding the timing and pattern in the evolution of these transitions and in bee diversification is an important task. This thesis has focused on widely different aspects of bee evolution and natural history and has successfully filled several gaps. In particular, it has contributed to a better understanding of bee biogeography (chapter 1), nesting biology (Appendix 1, Appendix 2) and bee-flower relationships (chapters 2 and 3).

Phylogeny of the leafcutting bees: the recent rise of a cosmopolitan genus

Chapter 1 presents the first comprehensive phylogenetic study for a large and cosmopolitan bee clade, the genus *Megachile*. As mentioned in the introduction and more thoroughly in chapter 1, *Megachile* bees represent an important proportion of all bee faunas worldwide. Before our study, no phylogenetic hypothesis was available for the genus *Megachile* and the classification was entirely based on the study of morphological characters. Using DNA sequences of five nuclear genes and one mitochondrial marker (the gene segment commonly used for DNA barcoding) for 127 taxa, we provide a sound phylogenetic framework for this group. Moreover, using our nuclear dataset and the mitochondrial DNA barcodes of approximately 300 additional species, we present a phylogenetic tree that includes 413 taxa. These results demonstrate that the rapidly accumulating number of DNA barcodes, in addition to facilitating species identifications, may eventually be used for the reconstruction of densely sampled phylogenetic trees. However, the inclusion of one additional, nuclear marker would greatly enhance the phylogenetic signal of these DNA barcodes.

Using a time-calibrated phylogeny and ancestral range reconstructions, the biogeographic history of this bee clade is proposed. In the first (and still the only) review on the biogeography of the bees, Michener (1979) suggested that the current distribution of most lineages of *Megachile* could be explained by short-distance dispersal events between adjacent continents. Our results largely confirm Michener's hypothesis. South America and Australia were both colonized 10 times independently or more according to our preferred biogeography scenario; in each case, colonization was achieved from a neighboring geographic zone, the Nearctic for South America and Southeastern Asia for Australia. Michener (1979), however, mentioned one exception in the geographic pattern within *Megachile*, the Nearctic subgenus *Chelostomoides*, writing that this case was "noteworthy and not easy to explain". *Chelostomoides* is diverse in North and Central America, and morphologically "almost identical to the large subgenus *Hackeriapis* of Australia". In the phylogenetic hypothesis presented in chapter 2, *Hackeriapis* was only distantly related to *Chelostomoides*, but instead appeared nested within a clade of Australian or Oriental subgenera. Consequently, the superficial morphological resemblance between *Hackeriapis* and *Chelostomoides* does not appear to reflect common ancestry. This pattern was observed repeatedly within *Megachile* and demonstrates the strong limitations of morphological characters for the inference of phylogenetic hypotheses. Consequently, our phylogenetic study also led to numerous changes in the current classification.

In addition to filling some of the gaps in the knowledge of the evolution and classification of this bee genus, our study also suggests that much work remains to be done. The placement of the two cleptoparasitic genera *Coelioxys* and *Radoszkowskiana* was not firmly established in our study. In most analyses, they formed a monophyletic group that was sister to all subgenera of *Megachile* (*Gronoceras* excepted), yet this placement was only poorly supported. Our analyses suggest a succession of poorly supported nodes among several lineages of *Megachile* and the cleptoparasitic genera *Coelioxys* and *Radoszkowskiana*. Obtaining a better resolution at the base of this clade is more than of academic importance: if the cleptoparasitic genera consistently form the sister group to all other lineages of *Megachile*, then the current classification with one large non parasitic genus *Megachile* and two cleptoparasitic genera would be preferred. However, if the cleptoparasitic clade appears nested within nest-building lineages, then the genus *Megachile* would constitute a paraphyletic unit from which the cleptoparasitic genera (*Coelioxys* and *Radoszkowskiana*) are derived. More phylogenetic characters, ideally of genomic scale, should be assembled to yield better-supported phylogenetic hypotheses in order to further improve the classification of this important bee lineage.

Pollen: Resource or reward?

Results from chapters 2 and 3 have shed some light on a hitherto little examined floral trait, pollen secondary chemistry. In addition, these chapters provide detailed case studies of bee-flower relationships using two different systems, the Boraginaceae and Gesneriaceae plant families. The presence of secondary compounds in floral rewards is a paradoxical situation: secondary compounds mostly act as defense mechanisms against herbivores, yet floral nectars and sometimes pollen attract and reward pollinators and are thus expected to be free of defense compounds. Both nectar and pollen have been shown to contain secondary compounds; in particular these compounds may be found in particularly high concentrations in the pollen. The evolutionary significance of high concentrations of toxic compounds in the pollen remains largely unclear.

Chapter 2 and 3 address several questions related to this field of research. The first question that has been addressed in chapter 2 is whether, at realistic doses, the secondary compounds found in the pollen have an impact on the fitness of pollinators. Larvae of two species of solitary bees out of three species investigated were negatively impacted by the secondary compounds found in the pollen of *Echium*. This result demonstrates that pollen secondary compounds have the potential to strongly impact the fitness of pollinators, in particular solitary bees. Nearly 50% of all bee species are specialized on some flowers (Michener, 2007). The evolutionary explanation for such high rate of specialization remains debated (Sedivy et al. 2008). Our results strongly indicate that pollen chemistry may at least partly underlie such specialization, as specialization in insect herbivores can partly be explained by the presence of toxic compounds in plants. In addition to previous studies (Williams 2003, Praz et al. 2008, Sedivy et al. 2011, Haider et al. 2014), our results thus further indicate that pollen is not an easy-to-use protein source for bees, and that physiological adaptations are needed to digest it.

The second aspect that is examined in both chapter 2 and chapter 3 relates to the evolutionary explanation of the presence of secondary compounds in the pollen. As noted by Sedivy et al. (2011), "all pollen types experimentally found so far to possess unfavourable properties for bee larval development [...] originate from flowers with freely accessible pollen that can easily be harvested by any flower visiting bee." Sedivy et al. (2011) thus hypothesized that flowers with free anthers would exhibit high secondary compounds in the pollen, and flowers in which the anthers are concealed in specialized morphological structures would have low levels of secondary compounds in the pollen. Our results from chapter 2 provide a direct test of this hypothesis: the plant family Boraginaceae contains species with variable morphologies with respect to pollen presentation: in a few species, the anthers are exposed and the pollen thus readily available to floral visitors (e.g., *Echium*); in another group, the pollen is slightly hidden in the corolla but pollinators, in particular bees, without specialized morphologies are able to harvest the pollen (e.g., *Borago* or *Symphytum*); in the last group, the anthers are concealed in a narrow floral tube and bees without specialized morphological structures are not able to extract the pollen (e.g., *Myosotis*, *Heliotropium*). This plant family thus provides an outstanding model system to test the hypothesis that plants with readily accessible pollen have higher defensive compounds in the pollen than plants with morphologically protected pollen. Contrary to expectations, this hypothesis could not be verified: there were high variations in the amounts of secondary compounds both within the genus *Echium* (with freely accessible pollen) and within the group with hidden anthers.

Yet all species exhibiting a specialized bee-morphology (our *Borago*-type) exhibited lower levels of secondary compounds in the pollen that the other species. Similarly, in chapter 3, those species of Gesneriaceae relying on bees for their pollination also had lower levels of saponins than the other species. Consequently, a similar pattern in two widely different groups of plants enables the formulation of a new hypothesis: in generalized flowers (that is, attracting a broad spectrum of nectar-visitors, including nectar-foraging bees), the presence of secondary compounds in the pollen is likely more linked to the defense of floral tissues (corolla, anthers, style) against herbivores than to the relationships with pollinators. In such flowers, nectar is the reward mediating the relationship with pollinators, not pollen. However, in plant species relying on pollen-collecting bees for their pollination, selection acts to lower the levels of defensive compounds in the pollen. In such bee-dependent flowers, both pollen and nectar act as rewards and mediate the relationships with bees.

Interestingly, the floral morphology of bee-pollinated Boraginaceae, with pendulous flowers and large anthers joined medially, converges to the typical morphology of other specialized bee-flowers, the buzz-pollinated flowers found for example in the Solanaceae family. Such buzz-pollinated flowers do not produce nectar and strongly depend on pollen-collecting bees for their pollination. It is conceivable that future evolution of bee-pollinated genera of the Boraginaceae (*Onosma, Cerinthe* and *Borago*) will be the suppression of nectar for energetic restraints, while pollen becomes the only reward that can be slowly liberated and sprayed on buzzing bees. In fact several solitary bee species buzz these Boraginaceae flowers to gather the pollen (Sedivy et al. 2013). While no Boraginaceae is nectarless and strictly buzz-pollinated, the morphological pattern observed enables the formulation of hypotheses on the evolution of buzz pollination in the angiosperms (Dukas & Dafni 1990). Future work on the Boraginaceae may compare the quantities and the composition of nectar across several species exhibiting different floral morphologies and pollinator spectrum. A testable hypothesis could be that buzz-pollinated species produce less nectar than other species. Another important field of future research is the relative pollination efficiency of different types of visitors: are nectar-collecting bees able to pollinate those Boraginaceae species exhibiting specialized bee-morphology; conversely, it is conceivable that only pollen-collecting females are able to liberate the pollen and thus to pollinate the flower in some of these flowers. Lastly, it is possible that pollen-collecting bees act as pollen-thieves in those Boraginaceae species not adapted to the bees. In particular, the pollination efficiency of specialized bees equipped with morphological adaptations to extract the pollen from narrow floral tubes remains unclear.

The possibility that the pollen of those bee-pollinated species of *Sinningia* also acts as a reward remains to be tested. In those nototribic flowers, the stamens may contact the back of the bees during nectar visits. Whether bees primarily visit *Sinningia* species for pollen or for nectar remains to be examined. In addition, whether the bees are good pollinators during pollen visits needs to be established. Our result that the pollen of bee-pollinated *Sinningia* contains less potentially toxic saponins suggests that pollen is not toxic to bees, and thus possibly acts as a reward. Contrary to the case of the Boraginaceae, it is difficult to imagine further evolution of one of the bee-pollinated lineages of *Sinningia* towards complete reduction of nectar, as the typical buzz-pollination morphology observed in Boraginaceae is unknown in *Sinningia*. Convergent morphologies and high resemblance of all the bee-pollinated lineages of *Sinningia* rather indicates that an optimum of the floral morphology has been reached.

The subfamily Faboideae in the Fabaceae family also presents highly specialized flowers that are mainly pollinated by bees; this clade arose some 58 Mya (Wikstrom *et al.* 2001). Only the strength of bees can open the keel, a petals structure that conceals the anthers (Westerkamp 1997). Usually, a complex movement of the legs of the bees on the keel and the reaching for nectar with the head provokes the release (sometimes explosive) of either the pollen or the stamen (Westerkamp 1997). Some Faboideae genera, like *Ononis*, do not offer nectar (Rodríguez-Riaño *et al.* 1999); in these genera, self-pollination may occur without pollinators, but cross-pollination is probably mostly achieved by pollen-collecting bees. In Switzerland, *Ononis* species are very often visited by *Megachile* bees. These bees must visit other flowers for nectar.

Interestingly, experiments using two Fabaceae species indicated that the pollen of one of them was improper for larval development of three Osmiini species out of five (Haider *et al.* 2014). Although no chemical analysis of the pollen was performed, the authors suggested that the cyanogenic glycoside compounds found in the vegetative parts of some Fabaceae may account for this pattern. This result is in contradiction with our hypothesis that morphologically concealed pollen destined to be collected by bees and thus acting as a reward should be free of toxic compounds. Possibly, the relationships between bees and flowers are further tightened and narrowed by the presence of toxic compounds, which may drive specialization in bees. This hypothesis was also developed for another clade of bees, the genus *Hoplitis*, many of which specialized to Boraginaceae. Beside Boraginaceae, Pyrrolizidine alkaloids are found in *Crotalaria*, a Fabaceae species that hosts the polylectic bee *Hoplitis parana*, also collecting pollen on Boraginaceae (Sedivy *et al.* 2013). Sedivy et al. (2013) presented in what they referred to as the Boraginaceae-Fabaceae paradox, a scenario in which the use of a novel pollen source might be triggered by the presence of toxic compounds. This would explain the intriguing use of such different flower morphologies and its maintenance through time in a specious bee genus. This hypothesis however is somehow lessened by the fact that we found very low amounts of alkaloids in several Boraginaceae species (see chapter 2) that also host specialized *Hoplitis*. *Trichodesma* for example was found to contain almost no alkaloid in its pollen, but is the only host plant of the oligolectic *Hoplitis bassana*. Alkaloids therefore cannot fully explain the constraints that maintain *Hoplitis* spp. on Boraginaceae.

When specialist bees and specialist plants meet

Schlindwein (2004) hypothesize that bees and flowers shall specialize in parallel and develop a close relationship to obtain maximum pollination efficiency and bee reproductive success. How do we explain, then, that oligolectic bees are often specialists of generalist flowers and polylectic bees often forage on bee-specialist plants (Waser 2006 and references therein; Chapter 2)? In Boraginaceae, *Echium* is a good example of a highly generalist plant, visited by many different insects for nectar, but also by several oligolectic species of *Hoplitis* for pollen and nectar (Sedivy *et al.* 2013). *Echium* is the main Boraginaceae genus used by *Hoplitis*, and *H. adunca* makes provisions of pollen and nectar of pure *Echium* from three species: *E. vulgare, E. italicum* and *E. plantagineum* (Westrich 1989). The high levels of alkaloids found in the natural provisions of *H. adunca* (chapter 2) shows that this bee has physiological adaptations to cope with these toxic compounds. Its pollination efficiency however remains to be tested. The size of the flower and position of the anthers suggests that bumblebees are well-adapted and legitimate pollinators of *Echium vulgare*. Bumblebees mostly visit Echium for nectar and rarely for pollen (Chapter 2; Trunz, personal observations). *Hoplitis* species are much smaller and appear somehow undersized to visit the flowers of *Echium vulgare*, especially during nectar visitations, as they do not touch most of the the stamens and often not even the stigma, which is undersized and not receptive during the male phase of this proterandric plant (Trunz, personal observations). When *Hoplitis adunca* harvest pollen however, they use their legs to grab all stamen together (and the stile when its length is sufficient) and brush them on their ventral scopa, which often contains pollen from the same plant individual or from another plant. Whether this behavior efficiently cross-pollinates the flower remains to be determined, but in any case, the amounts of pollen lost in one visit is probably high. In the light of the toxicity of *Echium vulgare* pollen, the ability of *H. adunca* to cope with such chemicals has probably been an advantage, because this resource is expected to be less collected by other bees and thus to represent a competitor-free resource. In turn, the diverse spectrum of floral visitors must be an advantage for *Echium vulgare*, as such diversity also brings together very different behaviors. Bumblebees usually visit numerous flowers on the same plant during one visit, therefore favoring geitonogamy, but at the same time insuring and increasing the production of seeds. Other, occasional visitors such as diurnal sphingid moths or coleopterans may provide cross-pollination and long-distance gene flow to the system.

Conclusion and perspectives

Bee-flower relationships and their evolution through time are highly complex and much remains to be investigated. The impressive diversity of both plants and bees, and in particular the immense diversity of floral shape or pollen presentation mechanisms on the one hand, and of bee behaviors on the other, render the study of bee-flower relationships not only difficult, but also multi-layered, highly integrative and of course fascinating. Our study unraveled some aspect in this field of research, examining in particular the dual role of pollen - sometimes resource, sometimes reward - but it also brings many more questions. Are secondary compounds the sole factor underlying pollen toxicity and indigestibility? Is there a combination of factors in other plant families? Do pollen- and nectar-collecting bees differ in their pollination efficiency? It is hoped that some of these questions will be answered in the future, in order to gain an even more complete understanding of the relationships between bees and flowers.

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Appendix 1:

Mueller, A., & Trunz, V. (2014). Palaearctic osmiine bees of the genera *Hofferia* and *Stenoheriades* (Megachilidae, Osmiini): biology, taxonomy and key to species. *Zootaxa*, *3765*(2), 175–186. http://doi.org/10.11646/zootaxa.3765.2.5

Zootaxa 3765 (2): 175–186
www.mapress.com/zootaxa/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

http://dx.doi.org/10.11646/zootaxa.3765.2.5
http://zoobank.org/urn:lsid:zoobank.org:pub:C16A4648-C604-40A9-AAE2-C3357FD691AF

Palaearctic osmiine bees of the genera *Hofferia* and *Stenoheriades* (Megachilidae, Osmiini): biology, taxonomy and key to species

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Abstract

Hofferia and *Stenoheriades* are closely related, species-poor genera of the osmiine bees (Megachilidae). Analysis of female pollen loads and field observations indicate that species of both genera have a strong affinity to Asteraceae as pollen hosts. Both genera use insect burrows in dead wood as nesting site, and *Hofferia schmiedeknechti* was found to build cell walls and nest plug with resin partly mixed with small pebbles. The taxonomic revision of the Palaearctic *Hofferia* and *Stenoheriades* species revealed the existence of a still undescribed species in the Levant, *Stenoheriades levantica* spec. nov.. *Stenoheriades hofferi* (Tkalců, 1984) is synonymized with *S. coelostoma* (Benoist, 1935), which is distinct from *S. asiatica* (Friese, 1921), and *Heriades integra* Benoist, 1934, formerly considered a *Stenoheriades* species, is synonymized with *Osmia (Hoplosmia) scutellaris* Morawitz, 1868. Keys for the delimitation of *Hofferia* and *Stenoheriades* from the other Palaearctic osmiine bee genera and for the identification of the Palaearctic species are given.

Key words: Apiformes, host-plant choice, Hymenoptera, nesting behaviour

Introduction

Hofferia Tkalců and *Stenoheriades* Tkalců are species-poor genera of the osmiine bees (Megachilidae, Megachilinae, Osmiini), for which two and eleven species, respectively, have been described so far (Ungricht et al., 2008; Eardley & Urban, 2010; Ascher & Pickering, 2013). *Hofferia* is confined to the Palaearctic region, whereas *Stenoheriades* is distributed both in the Palaearctic and in the Afrotropical region (including Madagascar) with five and six described species, respectively (Ascher & Pickering, 2013).

Among the osmiine bees, *Hofferia* and *Stenoheriades* belong to the *Heriades* group (Michener, 2007; Praz *et al*., 2008). A recent molecular phylogenetic study of the Osmiini, which included one Palaearctic species each of *Hofferia* and *Stenoheriades*, revealed that the two genera are sister taxa (Praz *et al*., 2008). This sister-group relationship is supported by two morphological characters, which are usually not found in other taxa of the *Heriades* group, *i.e*. the transverse preapical ridge of male tergum 6 and the median impression on sternum 6, which bears rows of short and stiff bristles at its posterior end. The latter character, however, is lacking in *Stenoheriades eingeddica* Griswold, which is most closely related to southern African *Stenoheriades* species (Griswold, 1985, 1994), suggesting that the genus *Stenoheriades* might not be monophyletic in its current circumscription. In fact, *Stenoheriades* is divisible into four species groups, which considerably differ morphologically (Griswold, 1985).

The taxonomy of *Hofferia* is well settled, whereas the taxonomy of the Palaearctic representatives of *Stenoheriades* is in a poor state due to the rareness of most species. Over the last few years, a large number of *Hofferia* and *Stenoheriades* specimens from the Palaearctic region was investigated, which—in combination with the examination of the name-bearing type material from the Palaearctic—allowed the clarification of the taxonomy of the Palaearctic species and the assessment of floral preferences by microscopically analysing the pollen contained in the scopal brushes of collected females.

Accepted by C. Rasmussen: 7 Jan. 2014; published: 17 Feb. 2014

In the present publication, *Hofferia* and *Stenoheriades* are morphologically diagnosed, the current knowledge on their pollen hosts and nesting biology is summarized, the Palaearctic species are revised, one new *Stenoheriades* species is described and identification keys including all Palaearctic representatives are given. Morphological terminology follows Michener (2007) including definitions for body measurements. Measurements to the nearest 0.1mm or 0.5mm (for body length) were taken using an ocular micrometer on an Olympus VMT stereomicroscope. Photomicrographs were taken with the digital microscope Keyence VHX-2000. To assess the pollen hosts of the species, scopal pollen contents of all available females were analysed by light microscopy applying the method of Sedivy *et al*. (2008).

Morphological diagnosis of *Hofferia* and *Stenoheriades*

Both *Hofferia* and *Stenoheriades* strongly resemble species of the genus *Chelostoma* Latreille due to their slender and elongate body form. The following key, which is tailored to the Palaearctic representatives of the Osmiini, delimits *Hofferia* and *Stenoheriades* from *Chelostoma* and the other Palaearctic osmiine bee genera.

1 Segment 3 of the labial palpus continues the axis of segment 2, whereas segment 4 projects laterally. . . . . ***Chelostoma*** **group: genus** ***Chelostoma***
Chelostoma Latreille contains 45 described Palaearctic species. Body length 4–15mm. Body slender and elongate. *Females:* Mandible usually fringed with long hairs along upper margin. Labrum without tuft of long erect hairs. Clypeus without sharp preapical ridge. Sternum 6 apically without distinct spine-like projection. *Males:* Tergum 6 without preapical ridge. Tergum 7 with large basal depression or pit, its apical margin bidentate, tripartite, medially incised, truncated or rounded. Sternum 1 not prolonged. Sternum 2 with median projection of different shape. Posterior margin of sternum 5 usually with comb of bristles.

1* Both segments 3 and 4 of the labial palpus project laterally. 2

2 Basal zone of propodeum horizontal to faintly slanting, short, sharply ridged and with distinct transverse carina along its posterior margin. 3

2* Basal zone of propodeum of different inclination, length or structure, but usually not distinctly carinated posteriorly; if carinated, declivous part of tergum 1 not separated from horizontal part by raised carina. 5

3 Declivous part of tergum 1 separated from horizontal part by indistinct rounded ridge. Proboscis long, reaching beyond coxa of fore leg when folded together; total length of first and second segments of labial palpus almost as long as maximal length of mesosoma. Body length usually longer than 7.5mm. . . . . ***Heriades*** **group: genus** ***Hofferia***
Hofferia Tkalců contains 2 described Palaearctic species. Body length 7.5–10.5mm. Body slender and elongate, *Chelostoma*-like. *Females:* Head very robust, maximal width of genal area distinctly wider than compound eye. Mandible long and slender, not fringed with long hairs along upper margin (Figs. 4, 5). Labrum without tuft of long erect hairs. Apical half of clypeus bent at right angles and separated from basal half by sharp preapical ridge (Figs. 4, 5). Sternum 6 medioapically with distinct spine-like projection. *Males:* Tergum 6 with preapical transverse ridge, which is irregularly denticulate. Tergum 7 without large basal depression or pit, its apical margin truncated and projecting medially. Sternum 1 prolonged, basally bulging and apically with flat appendix of rectangular to quadrate shape (Figs. 6, 7). Sternum 2 without median projection. Sternum 5 covered with transversally oriented yellowish hairs; its apical margin without comb of bristles, laterally with tuft of yellowish-red hairs.

3* Declivous part of tergum 1 distinctly separated from horizontal part by sharp and raised carina. Proboscis short, not reaching coxa of fore leg when folded together; total length of first and second segments of labial palpus distinctly shorter than maximal length of mesosoma. Body length not exceeding 8mm. 4

4 *Females:* Clypeus medioapically with short and narrowly rectangular projection (Fig. 10) or more or less semicircular impression (Figs. 13, 14); if apical margin of clypeus is straight, interantennal area with two raised carinae (Fig. 8). *Males:* Tergum 6 with preapical transverse ridge, which is denticulate or has two median teeth (Figs. 9, 15). Tergum 7 strongly sclerotized, not hidden by tergum 6 and with two apical teeth (Figs. 9, 15). . . . . ***Heriades*** **group: genus** ***Stenoheriades***
Stenoheriades Tkalců contains 5 described Palaearctic species. Body length 5–8mm. Body slender and elongate, *Chelostoma*-like. Axilla never spined. *Females:* Labrum with preapical tuft of long erect hairs. Mandible basally strongly swollen in one species (Fig. 11). *Males:* Metasoma moderately curved. Tergum 6 with distinct lateral flaps. Sternum 1 never prolonged. Last antennal segment never flattened or broadened.

4* *Females:* Apical margin of clypeus more or less straight or shallowly emarginated along its whole width, with or without small tooth-like projections. Interantennal area without raised carinae. *Males:* Tergum 6 without preapical transverse ridge (except for *Heriades (Rhopaloheriades) clavicornis*). Tergum 7 weakly sclerotized and completely hidden by large tergum 6. . . . . ***Heriades*** **group: genus** ***Heriades***
Heriades Spinola contains 23 described Palaearctic species. Body length 4–8mm. Body moderately slender to moderately robust. Axilla spined in some species. *Females:* Labrum with preapical tuft of long erect hairs. Mandible basally never strongly swollen. *Males:* Metasoma strongly curved so that at most two sterna are exposed and apex of tergum 6 nearly contacts sternum 1 or 2. Tergum 6 without or with indistinct lateral flaps. Sternum 1 distinctly prolonged in one species. Last antennal segment flattened and broadened in one species.

5 Pronotal lobe with several minute transverse carinulae on its surface. *Females:* Labrum with preapical tuft of long erect hairs.

Males: Tergum 7 weakly sclerotized and completely hidden by large tergum 6.......... ***Heriades*** **group: genus** ***Protosmia***
Protosmia Ducke contains 22 described Palaearctic species. Body length 3.5–9mm. Body slender and elongate, *Chelostoma*-like, to rather robust. *Females:* Metasoma red-coloured in some species. *Males:* Sternum 1 prolonged, bulged or with distinct projection. Tergum 6 with lateral flaps. In some species, antenna modified and gena ventrolaterally with longitudinal groove.

5* Pronotal lobe usually without minute transverse carinulae on its surface. *Females:* Labrum without preapical tuft of long erect hairs. *Males:* Tergum 7 strongly sclerotized and not hidden by large tergum 6 (except for *Osmia iberica*), albeit sometimes small... ***Osmia*** **group: genera** ***Haetosmia, Hoplitis, Osmia, Wainia***
The genera *Haetosmia* Popov, *Hoplitis* Klug, *Osmia* Panzer and *Wainia* Tkalců contain about 500 described Palaearctic species. Body length 4–18mm. Very diverse in body shape, body colour and morphology.

Biology of *Hofferia* and *Stenoheriades*

All species of *Hofferia* and *Stenoheriades* have a strong affinity to Asteraceae as pollen hosts (see species accounts). Both *Hofferia mauritanica* (Lucas) and *H. schmiedeknechti* (Schletterer) are oligolectic on the subfamily Carduoideae. The only three pollen loads of *Stenoheriades maroccana* (Benoist) available so far contained pollen of the subfamily Cichorioideae, whereas the four other Palaearctic *Stenoheriades* species are probably all strictly specialized on the subfamily Asteroideae. Interestingly, all but one pollen loads of the three closely related species *Stenoheriades asiatica* (Friese), *S. coelostoma* (Benoist) and *S. levantica* spec. nov. (n = 69) only contained pollen grains of the *Anthemis* type, whereas the pollen loads of *S. eingeddica* Griswold (n = 10) consisted exclusively of pollen grains of the *Aster* type. This finding suggests that these *Stenoheriades* species might possibly restrict pollen harvesting to certain taxa within the Asteroideae.

Hofferia schmiedeknechti nests in beetle burrows in dead wood (Figs. 1–3; Tkalců, 1984), whereas the nesting biology of *H. mauritanica* is unknown. Several specimens of *Stenoheriades maroccana* and *S. eingeddica* were observed flying around dead wood (Benoist, 1928b; C. Praz and C. Sedivy, personal communication), indicating that insect burrows in dead wood are probably used by *Stenoheriades* species as nesting sites as well. The slender and elongate body of both *Hofferia* and *Stenoheriades* is likely an adaptation for nesting in such narrow linear cavities. The discovery of nests of *H. schmiedeknechti* in Israel in spring 2013 by V. Trunz and D. Bénon revealed for the first time that this species constructs both cell walls and nest plug with resin, which is partly mixed with small pebbles (Fig. 3). The nest building material of *Stenoheriades* species is still unknown. However, since all other genera of the *Heriades* group whose nesting behaviour is known use resin for nest construction (Müller, 2013), resin is probably also used by the *Stenoheriades* species to build their brood cells.

In most parts of their Palaearctic distribution area, species of *Hofferia* and *Stenoheriades* do not emerge before mid-May and are mainly active from end of May to July except for *S. eingeddica*, which flies in April (Israel) or October (Oman, Yemen), and *S. levantica*, which is active from mid-March to end of May.

Genus *Hofferia* Tkalců, 1984

Two species restricted to the Palaearctic region. The two species have an allopatric distribution with one species occurring in the Maghreb and the other species ranging from southeastern Europe to easternmost Turkey and from southern Turkey to the Levant.

Species accounts

Hofferia mauritanica (Lucas, 1849)

Chelostoma mauritanicum Lucas, 1849: 205. Type material: Holotype ♀, "lac Tonga" (Algeria).
Eriades obtusus Friese, 1897: 193. Type material: Lectotype ♂, by designation of Tkalců (1984), "Algeria", Museum für Naturkunde Berlin. Synonymy in Alfken (1914).

New records. ALGERIA: Tikjda Kabylie, 26. –28.6.1954; Constantine, 25.5.–16.6.2005; Constantine, 20.4.–23.5.2008; Guelma, 1.6.2008; Skikda, 2.6.2008; Mila, Ouledbazer, 18.4. –26.5.2013; Mila, Redjas, 1.6.2013. MOROCCO: Meknes, 16.–17.7.1931; Tizi-n-Tichka, 2.7.1987; Kenitra, 30.4.1990; Amouguer, 50km W Rich, 23.5.1995; Bhalil, 10km NW Sefrou, 28.5.1995; Oujda, Jbel Mahseur, 26.5.1996; Oujda, Oued Oussera, 29.5.1996; Ifrane env., 17.5.2003; Volubilis, 2.5km NW Moulay-Idriss, 2.6.2007. TUNISIA: Hammam-Liv, 22.5.1913; Tabarka, 4.7.1972; Zaghouan mountains, 1.–2.6.1993; 10km S Nefza, 18.4.2001.

Distribution. Northern Africa from Morocco to Tunisia.

Pollen hosts. Oligolectic on Carduoideae (Asteraceae) (based on 27 pollen samples from 11 different localities). Flower records: *Centaurea acaulis, C. calcitrapa, C. nicaensis, C. solstitialis, Galactites tomentosa, Onopordum illyricum, O. macracanthum* (label records).

Nesting biology. Unknown.

Hofferia schmiedeknechti (Schletterer, 1889)

Chelostoma schmiedeknechti Schletterer, 1889: 638. Type material: Holotype ♀, "Süd-Ungarn (Mehadia)" (Romania), Hungarian Natural History Museum Budapest. Type species of *Hofferia*.

Heriades tumida Benoist, 1928: 332. Type material: Holotype ♂, "Taurus" (Turkey), Muséum national d'Histoire naturelle Paris. Synonymy in Zanden (1990).

Heriades gibba Benoist, 1928: 333. Type material: Holotype ♂, "Taurus" (Turkey), Muséum national d'Histoire naturelle Paris. Synonymy in Zanden (1990).

New records. BULGARIA: Sozopol, 22.6.1963, 6.7.1977; Slancev Brjag, 25.7.1968, 20.6.1971, 1.–30.6.1972, 2.6.–7.7.2008; Pisatel, 6.7.1976; Biser-Harmanli, 28.6.1978; Albena, 6.7.1978; Varna-Vinica, 11.–21.7.1978; Ropotamo, 11.7.1979; Lozenec, 16.6.2008; 25km NE Svilengrad, 20.6.2008. GREECE, *Epirus:* Preveza, 25.6.1997; *Lesvos:* Sigri, 31.5.–2.6.2012; Vatousa, 4.6.2012; *Peloponnes:* Olympia, 4.–11.7.1979, 4.–6.6.1995, 27.5.1998; 17km SW Kalavrita, 1.6.1993; Sparta, Amyklai, 19.5.1995; Avia, Kalamata, 9.–10.6.1995, 26.6.1996; 5.5.2000; Diavolitsi, Karnasi, 12.6.1995; Ano Karnes (Likeos mountains), 17.6.1995; Chlemoutsi (Elis), 19.6.1995; Sparta, Menelaion, 5.6.1996; Hosiari, 8.6.1996; Samikon, 15.5.1997; Kotili (Likeos mountains), 21.–22.6.1997, 17.6.1998; Mavromati, Ithome, 24.6.1997, 2.6.1998; Mistras, Parori, 10.7.1997; Kalogria (Achaia), 14.–18.5.2000; Langada (Mani), 26.4.2001; 10km W Korinthos, 5.6.2005; Skoutari (Mani), 26.5.2006; Kato Loussi, 8.7.2006. ISRAEL AND PALESTINE: Banyas, 26.5.1991; Mt. Tabor, 28.5.1991; Mt. Meron, 30.5.1991; 40km NE Haifa, 1km E Hurfeish, 15.5.1996; Mt. Carmel, 29.5.2000; Golan, 1km NW Kefahr Hanasi, 23.5.2011; Golan, Lehavot HaBashan, 3.5.2013. JORDAN: North Shuna env., 29.–30.4.1996; Ajlun, 1.5.2006. SYRIA: Apamea, 29.4.1995. TURKEY, *Ankara:* around Camlidere, 17.6.2006; *Artvin:* Yusufeli/Ishan, 20.5.1993; *Aydin:* between Kuyucak and Buharli, 28.5.2005; *Bolu:* Ciller, 20km W Mudurnu; *Hakkari:* 10km N Uludere, 4.6.1980; 10km NE Oramar, 29.6.1985; 25km E Gözeldere, 22.6.2010; *Mersin:* Kilik. Taurus, Namrun, 16.6.–3.7.1979.

Literature records. MACEDONIA: Ohrid, 7.6.1965 (Zanden, 1989).

Distribution. Southeastern Europe (Bulgaria, Greece, Macedonia, southern Romania) to easternmost Turkey and southwards to the Levant (Syria, Jordan, Israel and Palestine).

Pollen hosts. Oligolectic on Carduoideae (Asteraceae) (based on 15 pollen samples from 13 different localities, the pollen content of three brood cells from the same nest and field observations; Tkalců, 1984); one pollen load contained pollen grains of the *Anthemis* type, suggesting that species of Asteroideae are occasionally exploited as pollen sources as well. Flower records: *Carduus acanthoides, C. thoermeri, Onopordum tauricum* (Tkalců, 1984); *Silybum marianum, Centaurea* spec. (personal observation).

Nesting biology. Beetle burrows in dead wood (Figs. 1–3; Tkalců, 1984). The nests are closed with a thick plug of plant resin mixed with small pebbles (Fig. 3); resin is also used for the construction of the thin walls that separate the linearly arranged brood cells.

Key to the species of *Hofferia*

Females

1 Preapical ridge between basal and apical half of clypeus medially with wide, rather deep and more or less triangular emargination (Fig. 4). Body length 8.5–10mm. . . . . ***Hofferia mauritanica***

1* Preapical ridge between basal and apical half of clypeus medially with two shallow and roundish emarginations separated by small triangular tooth (Fig. 5). Body length 8.5–10.5mm. . . . . ***Hofferia schmiedeknechti***

FIGURE 1–3. 1: Nesting site of *Hofferia schmiedeknechti* on the Golan Heights in northeastern Israel, near Lehavot HaBashan; several females nested in trunk and branches of this dead *Cupressus* tree (Photo D. Bénon). 2: Female of *Hofferia schmiedeknechti* entering her nest in a beetle burrow in a dead *Cupressus* tree (Photo D. Bénon). 3: Opened nest of *Hofferia schmiedeknechti* with three linearly arranged brood cells; the nest is plugged with dark plant resin of unknown origin mixed with small pebbles; the cell walls also consist of resin.

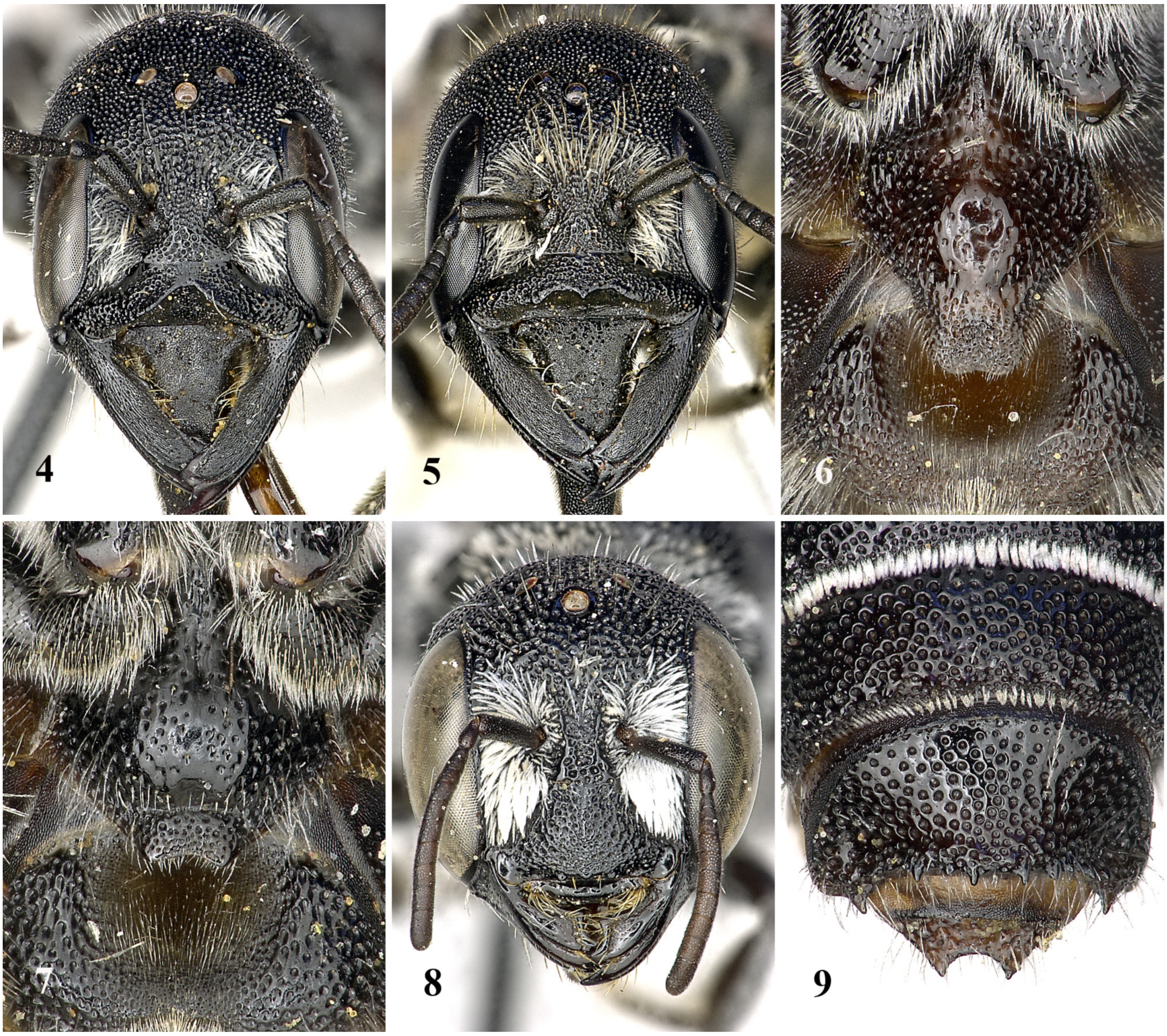

FIGURE 4–9. 4: *Hofferia mauritanica,* head of female. 5: *Hofferia schmiedeknechti,* head of female. 6: *Hofferia mauritanica,* sternum 1 of male. 7: *Hofferia schmiedeknechti,* sternum 1 of male. 8: *Stenoheriades eingeddica,* head of female. 9: *Stenoheriades eingeddica,* terga 5–7 of male.

Males

1 Medioapical appendix of sternum 1 as long as its basal width to slightly shorter (Fig. 6). Punctation of bulging part of sternum 1 medially very sparse (Fig. 6). Transversally oriented pilosity on sternum 5 rather loose and not tightly appressed to sternal surface. Body length 7.5–9.5mm. . . . . ***Hofferia mauritanica***

1* Medioapical appendix of sternum 1 half as long as its basal width to slightly longer (Fig. 7). Punctation of bulging part of sternum 1 medially often rather dense except for a narrow longitudinal zone (Fig. 7). Transversally oriented pilosity on sternum 5 dense and tightly appressed to sternal surface. Body length 7.5–10mm. . . . . ***Hofferia schmiedeknechti***

Genus *Stenoheriades* Tkalců, 1984

Eleven species distributed in the Palaearctic and the Afrotropical regions (including Madagascar) with five and six species, respectively. Six Afrotropical species are still undescribed (Michener, 2007). The Palaearctic distribution range of *Stenoheriades* encompasses Morocco, southern Spain and Sicily, southeastern Europe to easternmost Turkey and southern Turkey to the Levant and the Arabian peninsula.

Heriades integra Benoist, 1934, is treated as a member of the genus *Stenoheriades* by Griswold (1985, 1994) and Ungricht *et al.* (2008). Unfortunately, the male holotype originating from northernmost Morocco could not be located at the Muséum national d'Histoire naturelle Paris. However, the original description lists several characters, such as spined axilla, lack of a transverse carina between vertical and horizontal part of tergum 1 and rounded tergum 7, which exclude its membership in *Stenoheriades*. Instead, these characters solely apply to *Osmia (Hoplosmia) scutellaris* Morawitz, 1868, rendering the name *Heriades integra* syn. nov. a junior synonym of *Osmia scutellaris*.

No morphological characters are known so far to differentiate between the males of *S. asiatica, S. coelostoma* and *S. levantica*, raising the question whether the male types of *S. asiatica* might be conspecific with *S. coelostoma* or *S. levantica*. As females of a *Stenoheriades* species have been collected near the type locality of *S. asiatica* (Nur mountains, Hatay province, Turkey; Friese, 1921), which neither belong to *S. coelostoma* nor *S. levantica*, the *Stenoheriades* species with mandibles of normal shape, a shallow impression at the clypeal margin and a basal tubercle on the labrum (see identification key) is most probably *S. asiatica*. This assumption is in line with the currently known distribution range of *S. coelostoma* and *S. levantica*, which is—compared with *S. asiatica*—more western and more southern, respectively (see species accounts). The clarification of the species identity of *S. asiatica* renders the information about the geographic range of *S. asiatica* given by Ungricht *et al.* (2008) erroneous: *S. asiatica* neither occurs in Europe nor in western Turkey but ranges from central to easternmost Turkey. The *Stenoheriades* species that is distributed in southeastern Europe and western Turkey is *S. coelostoma*, which has been erroneously synonymized with *S. asiatica* by Griswold (1994).

Species accounts

eingeddica species group

The species of this group, which contains two southern African taxa beside *S. eingeddica* (Griswold, 1985, 1994), are characterized by the distinctly carinate preoccipital ridge, the carinate omaulus, a dorsal projection on the metapleuron, distinct interantennal carinae in the female (Fig. 8) and the denticulate transverse preapical ridge of male tergum 6 (Fig. 9).

Stenoheriades eingeddica Griswold, 1994

Stenoheriades eingeddicus Griswold, 1994: 18. Type material: Holotype ♂, "Ejn Geddi Palestine" (Israel), Ministry of Agriculture and Natural Resources Nicosia.

New records. ISRAEL AND PALESTINE, *Arava valley:* Iddan, 26.–27.4.1996, 8.5.1996, 9.4.2011; En Yahav, 15.4.–4.5.2010; 2km N En Yahav, 28.4.2010; Hazeva, 16.4.2011; Wadi Shahak, 11.4.2012; *Judean Desert:* En Zeelim, 5km N Masada, 7.5.1996; *Negev:* 50km SE Beer Sheva, Wadi En Aqrabbim, 8.5.1996. JORDAN: Zarga Main, 7.5.1995; Dead Sea env., 16.4.1996. YEMEN: Wadi Aniz SSW Sana, 7.10.2005; Hawf NE Albhaydah, 14.10.2005.

Literature records. OMAN: Dhofar, Ayun Pools. 10.10.1977 (Griswold, 1994).

Distribution. Desert areas of the Levant (Jordan, Israel and Palestine) and Arabian Peninsula (Oman, Yemen).

Pollen hosts. Probably oligolectic on Asteroideae (Asteraceae) (based on 10 pollen samples from 5 different localities); all pollen loads examined consisted exclusively of pollen grains of the *Aster* type. Flower records: *Atractylis carduus* (Griswold, 1994); *Anvillea garcinii*, *Pulicaria undulata* (label records).

Nesting biology. Several individuals were observed flying around dead *Acacia* wood in the Negev desert (C. Praz and C. Sedivy, personal communication), suggesting that insect burrows in dead wood are used as nesting site.

coelostoma species group

The species of this Palaearctic group are characterized by the presence of a subapical tuft of long bristles on the female labrum and the bidentate transverse preapical ridge of male tergum 6 (Fig. 15).

Stenoheriades asiatica (Friese, 1921)

Heriades asiaticus Friese, 1921: 175. Type material: Syntypes ♂, "Airan" (Turkey).

New records. SYRIA: N of Latakia, Slinfeh env., 7.6.1999. TURKEY, *Adiyaman:* Nemrut Dagi, Karadut, 2.7.1993; Karadut env., 50km N Adiyaman, 1.6.2001; *Hakkari:* Uludere, 5.6.1977; 22km S Beytüssebap, 2.6.1980; 10km W Uludere, 4.6.1980; 19km S Beytüssebap, 26.6.1985; *Hatay:* Bademli, 6km W Hassa, 13.5.2002; Akcali, 35km S Hakkari, 21.6.2010; *Sanliurfa:* Halfeti (Birecik), 31.5.1998; *Siirt:* 5km E Eruh, 26.5.1983.

Distribution. Central to easternmost Turkey and southwards to northern Syria; westernmost records: Bademli (Hatay province, Turkey) and Latakia (Syria).

Pollen hosts. Probably oligolectic on Asteroideae (Asteraceae) (based on 10 pollen loads from 6 different localities); all pollen loads examined consisted exclusively of pollen grains of the *Anthemis* type except for one load that additionally contained a small percentage of pollen grains of the *Taraxacum* type, suggesting that Cichorioideae might occasionally be exploited as pollen hosts as well.

Nesting biology. Unknown.

Stenoheriades coelostoma (Benoist, 1935)

Heriades coelostoma Benoist, 1935: 277. Type material: Lectotype ♀, by designation of Zanden (unpublished), "Asie mineure: Taurus" (Turkey), Muséum national d'Histoire naturelle Paris. The synonymization with *Stenoheriades asiatica* (Friese, 1921) in Griswold (1994) is erroneous (see above).

Pseudoheriades (Stenoheriades) hofferi Tkalců, 1984: 2. Type material: ♂, "Bulgaria mer.-occ. Peju Javorov (im Struma-Tal)", Tkalců Collection Praha. Type species of *Stenoheriades* Tkalců. New synonymy based on type material and distribution.

New records. BULGARIA: Sandanski, 13.7.1966, 26.5.–31.7.1967, 1.–30.6.1969; Pirin, 5.1967; Rodopi, Parvenec, 12.6.1996; Rodopi, Hrabrino, 15.6.1997; Trakia, Proslav, 10.7.1997. CROATIA: Makaraska river, Drvenik, 16.6.2000; Melnik, 25.6.2007. GREECE, *Attica:* Kifissia N Athen, 30.4.1977; Athen, Daphni, 10.5.1986; Fokida Chriso, 3km W Delfi; 5.5.1998; *Epirus:* Smolikas, 8.1980; *Lesvos:* 4.6km N Plomari, 12.6.2004; *Peloponnes:* Olympia (Alfios valley), 13.5.2000; Achaia, Zachlorou, N Kalavrita, 31.5.–1.6.2002; Loutra, 30km SEE Olympia, 2.5.2005; Kato Loussi, 20.6.2008; *Thasos:* Agios Georgios, 22.5.2012; Melissourgos, 19.6.2012; *Thessaly:* Mt. Ossa, 28.5.1988. TURKEY, *Adana:* Pozanti, 28.5.1979, 6.7.1983; Sihli, 3km NW Tekir, 12.8.1983; Candirlar, 11.7.1996; *Amasya:* Amasya, 5.7.1977; *Ankara:* Beynam, 15km S Ankara, 23.7.1987; Ankara, 15.6.2006; Camlidere env., 17.6.2006; *Antalya:* Taskesigi, 100km E Antalya, 25.5.1990; Köstebek, 24.6.1991; N Akseki, 19.6.1998; Side-Kumköy, 60km E Antalya, 4.–17.4.2004; Elmali env., 6.7.2006; between Korkuteli and Tefenni, 6.8.2006; Konyaalti, 25.5.2009; *Aydin:* between Akcaköy and Ödemis, 28.5.2006; *Bolu:* 17km S Seben, 17.6.1998; *Eskisehir:* Inönü, 1.8.1991; *Izmir:* Boz Dag, 6.1990; Dikili env., W Izmir, 19.6.1998; Izmir env., 19.–20.6.1998; 10km NE Ödemis, 3.7.2006; *Konya:* Seydisehir, Teke Gec, 1.8.1904; 10km S Karaman, 19.6.1985; Mt. Aydos/Eregli, 7.8.1991; 30km S Aksehir, 24.6.1998; 10km S Aksehir, 25.6.1998; *Kütahya:* 28km SSE Kütahya, 12.7.2006; 20km NEE Kütahya, 13.7.2006; *Manisa:* 35km SEE Salihli, 30.6–2.7.2006; *Mersin:* Namrun, 3.6.1983; Kiskalesi, Silifke, 9.–13.5.1988; Uzuncaburc, 30km N Silifke, 28.5.1996; Cornelek, 40km E Mut, 29.5.1996, 18.6.1997; 30km NW Erdemli, 20.6.1996; Taurus mountains, 20km NW Erdemli, 5.7.1996; Eksiler, 20km W Silifke, 17.6.1997; Kirobasi, 60km E Mut, 19.6.1997; Kuzucubelen, 28.5.1998, 15.6.2000; Aslanli, 30km N Erdemli, 17.6.1998; 30km N Kuzucubelen, 15.6.2000; Erdemli, 28.6.2000; Sahmurlu, 23.5.2005; *Mugla:* Seki, 70km NE Fethiye, 7.7.1981; Gökbel, Dalyan, 26.5.2005; *Nevsehir:* Capadoica, Ürgüp, 13.6.1998; *Nigde:* W Ciftehan, 20.6.1981; *Yozgat:* Saraykent env., 70km E Yozgat, 12.6.2001.

Distribution. Southeastern Europe (Bulgaria, Croatia, Greece) to central Turkey; easternmost record: Candirlar (Adana province, Turkey).

Pollen hosts. Oligolectic on Asteroideae (Asteraceae) (based on 35 pollen loads from 15 different localities); all pollen loads examined consisted exclusively of pollen grains of the *Anthemis* type. Flower records: *Anthemis arvensis* (label record).

Nesting biology. Unknown.

Stenoheriades levantica Müller, spec. nov.

Holotype: ISRAEL AND PALESTINE: Mt. Carmel, Mitla, 300m, 32°44'11N 34°59'91E, 17.4.2000, ♀ (leg. S.P.M. Roberts). Deposited in the Entomological Collection of ETH Zurich.

Paratypes: ISRAEL AND PALESTINE: Banyas, 26.5.1991, 2♀ (leg. K. Warncke); Tel Dan, 26.5.1991, ♀ (leg. K. Warncke); Mt. Meron, 30.5.1991, ♂ (leg. K. Warncke); Mt. Tabor, 580m, 28.5.1991, 8♀, ♂ (leg. K. Warncke); Mt. Carmel, Mitla, 17.4.2000, ♂ (leg. S.P.M. Roberts); Judean Foothills, Park Britannia, 24.4.2011, ♀ (leg. T. Koznichki); Ramat haNadiv, 24.4.2012, ♀ (leg. T. Shapira); Nahal Ar'ar, 4.5.2012, ♂ (leg. G. Pisanty). JORDAN: North Shuna env., 29.–30.4.1996, 7♀ (leg. M. Halada); NW of Ailun, 850m, 20.5.2007, ♀ (leg. Z. Kejval). SYRIA: Ganawat, 16.5.1995, 3♀, 7♂ (leg. K. Denes). Deposited in the Entomological Collection of ETH Zurich, the Oberösterreichische Landesmuseum Linz and the private collection of M. Schwarz (Ansfelden).

Additional records. ISRAEL AND PALESTINE: Park Britannia, 12.3.2013, ♀ (leg. Y. Berner); Ya'ar Adulam, 3.4.2013, ♀ (leg. Y. Berner).

Literature records. LEBANON: Djezzine, 2.6.1953 (Mavromoustakis, 1955, as *S. coelostoma*).

Diagnosis. The female of *S. levantica* is characterized by a median impression at the clypeal margin as are the females of the closely related *S. asiatica* and *S. coelostoma*. It differs from *S. coelostoma* by the two-teethed mandible with a straight upper margin (Fig. 12), and from both *S. asiatica* and *S. coelostoma* by the deep, well limited and regularly semicircular clypeal impression (Fig. 14) and the lack of a distinct triangular tubercle at the labral base (Fig. 14). The male of *S. levantica* is morphologically identical with the males of *S. asiatica* and *S. coelostoma*.

Description: Second segment of labial palpus about three times as long as first segment. Preoccipital ridge carinate. Parapsidal line distinctly longer than half length of tegula. Basal zone of propodeum very short, medially about half as long as metanotum, longitudinally ridged and with distinct transverse carina along its posterior margin. Posterior surface of propodeum polished, without punctures except for lateral parts, which are rather densely punctured. Tibial spurs of hind leg yellowish to yellowish-white, nearly straight and regularly tapering towards acute apex. Declivous part of tergum 1 polished and almost devoid of punctures, distinctly separated from horizontal part by sharp and raised carina.

FEMALE: Body length 5–7mm. Mandible with two apical teeth and straight, uninterrupted upper margin (Fig. 12); its base with roundish to oval impression, well developed tubercle at its lower and weakly developed tubercle at its upper margin; basal half of mandibular inside without projections below cutting edge. Labrum extending beyond closed mandibles, basally flat or rarely with minute roundish tubercle (Fig. 14). Clypeus with medioapical impression, which is rather deep, well limited and of regular semicircular shape (Fig. 14); clypeal impression medially with longitudinal carina, which is continuous to developed only basally. Maximal width of genal area about as wide as compound eye. Apical margin of terga 1–4 with uninterrupted whitish hair bands; apical hair band on tergum 5 only weakly developed. Scopa yellowish-white.

MALE: Body length 5–7mm. Antennal segments 3–4 shorter than wide. Base of mandible with roundish impression. Labrum basally with distinct tubercle of oval to triangular shape. Clypeus medioapically slightly emarginated. Maximal width of genal area about 0.6x as wide as compound eye. Hypostomal area covered with dense whitish pilosity, which increases in length towards the occiput. Apical margins of terga 1–5 each with sparse whitish hair band. Tergum 6 with distinct lateral flaps; its transverse preapical ridge with two median teeth separated from each other by roundish incision (Fig. 15). Tergum 7 bifid (Fig. 15). Sternum 1 strongly bulged. Hair fringe at apical margin of sternum 4 more than half as long as hair fringe at apical margin of sternum 3. Sternum 5 with triangular incision; its lateral lobes apically with fringe of short bristles at inner edge. Sternum 6 medially with deep depression of oval shape, which bears two short rows of minute and stiff bristles at its posterior end. Apex of gonoforceps slightly bent inwards.

Distribution. Levant (southern Syria, Lebanon, Jordan, Israel and Palestine).

Pollen hosts. Oligolectic on Asteroideae (Asteraceae) (based on 24 pollen loads from 12 different localities); all pollen loads examined consisted exclusively of pollen grains of the *Anthemis* type except for one load that contained pollen of the *Aster* type. Flower records: yellow Asteraceae flowers (Mavromoustakis, 1955).

Nesting biology. Unknown.

Etymology. *levanticus* = referring to the Levant, the geographic area encompassing the eastern Mediterranean region that ranges from southernmost Turkey to the Sinai peninsula.

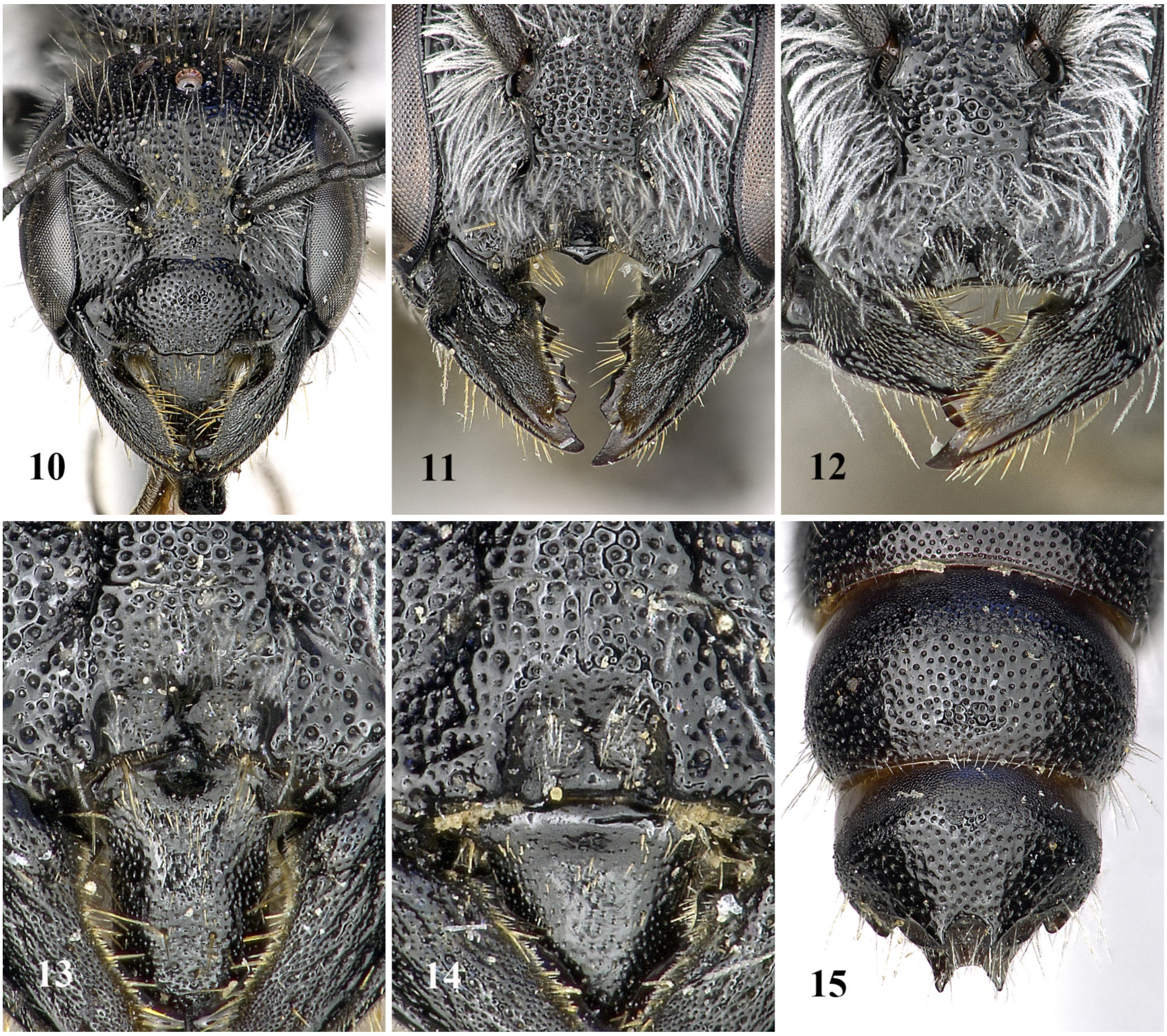

FIGURE 10–15. 10: *Stenoheriades maroccana,* head of female. 11: *Stenoheriades coelostoma,* mandibles of female. 12: *Stenoheriades levantica*, mandibles of female. 13: *Stenoheriades asiatica,* clypeus and labral base of female. 14: *Stenoheriades levantica,* clypeus and labral base of female. Figure 15: *Stenoheriades levantica*, terga 5–7 of male.

Stenoheriades maroccana (Benoist, 1928)

Heriades maroccana Benoist, 1928: 212. Type material: Lectotype ♀, by designation of Zanden (unpublished), "Aïn Leuh" (Morocco), Muséum national d'Histoire naturelle Paris.

New records. ITALY, *Sicily:* Etna south, 21.6.2012. MOROCCO: Meknes, 20.3.1992. SPAIN, *Andalucia:* Granada, Cerro del Chupa, 1.6.1986; Sierra Cazorla, Palomas, 17.6.2003.

Distribution. Southernmost Europe (southern Spain, Sicily) and northern Africa (Morocco).

Pollen hosts. The only three pollen loads available so far (from three localities) contained pollen of Cichorioideae (Asteraceae). Flower records: *Sonchus, Calendula stellata* (label records).

Nesting biology. Several females and males were observed at a wooden phone post in Morocco (Benoist, 1928b), suggesting that insect burrows in dead wood are used as nesting site.

Key to the Palaearctic species of *Stenoheriades*

Females

1 Apical margin of clypeus straight or medially projecting (Figs. 8, 10). 2

1* Apical zone of clypeus with median impression (Figs. 13, 14). 3

2 Interantennal area with two raised carinae (Fig. 8). Preoccipital ridge raised to a carina. Paraocular area, pronotal lobe and base of tegula covered with dense, rather short and appressed white pilosity (Fig. 8). Apical margin of clypeus straight, medially with three more or less distinct short teeth (Fig. 8). Clypeus weakly and evenly rounded. Mandible short and broad with three distinct apical teeth (Fig. 8). Labrum short, projecting for less than half of its length beyond closed mandibles, its apical zone not edged longitudinally and more than half as wide as basal width of labrum. Punctation of head and thorax coarse except for clypeus, where minute punctures are intermixed with large punctures (Fig. 8); largest punctures on supraclypeal area larger than largest punctures on clypeus. Body length 7–8mm. . . . . ***Stenoheriades eingeddica***

2* Interantennal area without raised carinae (Fig. 10). Preoccipital ridge edged, but not raised to a carina. Paraocular area, pronotal lobe and base of tegula covered with sparse, long and erect whitish pilosity (Fig. 10). Apical margin of clypeus medially with short and narrowly rectangular projection (Fig. 10), which is apically straight or bears up to three weak protuberances. Clypeus distinctly bulged in its basal half. Mandible long and slender with two distinct apical teeth and small tooth-like projection along upper margin (Fig. 10). Labrum long, projecting for almost half of its length beyond closed mandibles, its apical fourth sharply edged longitudinally and less than half as wide as basal width of labrum. Punctation of head and thorax rather fine; largest punctures on supraclypeal area smaller than largest punctures on clypeus (Fig. 10). Body length 6–7.5mm.. . . . . ***Stenoheriades maroccana***

3 Mandible with three teeth (Fig. 11); the two preapical teeth are separated by a shallow emargination and moved upwards, so that the upper margin of the mandible is nowhere straight. Mandible basally distinctly swollen, with strong roundish tubercle at its upper margin and with two small, more or less distinct roundish projections positioned on the mandibular inside slightly below the upper margin (Fig. 11). Body length 6–7mm. . . . . ***Stenoheriades coelostoma***

3* Mandible with two teeth (Fig. 12), upper margin straight above preapical tooth. Mandible basally not distinctly swollen, with weakly developed tubercle at its upper margin and without projections on the mandibular inside below the cutting edge (Fig. 12).. 4

4 Median impression at apical margin of clypeus shallow, indistinctly limited and not regularly semicircular (Fig. 13). Base of labrum with distinct, more or less triangular tubercle (Fig. 13). Body length 5–7mm. . . . . ***Stenoheriades asiatica***

4* Median impression at apical margin of clypeus deep, clearly limited and regularly semicircular (Fig. 14). Base of labrum flat, rarely with very small roundish tubercle (Fig. 14). Body length 5–7mm. . . . . ***Stenoheriades levantica***

Males

1 Transverse preapical ridge of tergum 6 with several acute teeth (Fig. 9). Paraocular area, clypeus, pronotal lobe and base of tegula covered with dense, rather short and appressed white pilosity. Base of terga 2–4 distinctly constricted. Punctation of head and thorax coarse; punctures on supraclypeal area larger than punctures on clypeus. Body length 6–7mm. . . . . ***Stenoheriades eingeddica***

1* Transverse preapical ridge of tergum 6 with two median teeth separated from each other by a roundish or triangular incision (Fig. 15). Paraocular area, clypeus, pronotal lobe and base of tegula covered with sparse, long and erect whitish pilosity. Base of tergum 2 weakly, of terga 3–4 barely perceivably constricted. Punctation of head and thorax rather fine; punctures on supraclypeal area and clypeus of about the same size. 2

2 Medioapical part of clypeus slightly protruding and raised, with three, rarely more short teeth. Antennal segments 3–4 quadrate to slightly longer than wide. Hypostomal area sparsely covered with long whitish hairs. Base of mandible without roundish impression. Sternum 1 slightly bulged. Hair fringe at apical margin of sternum 4 about half as long as hair fringe at apical margin of sternum 3. Sternum 5 with large and roundish incision; lateral lobes of sternum 5 with narrow fringe of short bristles along apical margin. Apex of gonoforceps bent inwards at almost right angles. Body length 5.5–7mm. . . . . ***Stenoheriades maroccana***

2* Medioapical part of clypeus neither protruding nor raised, without teeth or sometimes with median protuberance. Antennal segments 3–4 shorter than wide. Hypostomal area covered with dense whitish pilosity. Base of mandible with roundish impression. Sternum 1 strongly bulged. Hair fringe at apical margin of sternum 4 more than half as long as hair fringe at apical margin of sternum 3. Sternum 5 with rather small and triangular incision; lateral lobes of sternum 5 apically with fringe of short bristles only at their inner edge. Apex of gonoforceps slightly bent inwards. Body length 5–7mm. No morphological characters are known to distinguish the following three species in the male sex. To the present knowledge, the species have an allopatric distribution:
. . . . ***Stenoheriades coelostoma***: southeastern Europe to central Turkey
. . . . ***Stenoheriades asiatica***: central to easternmost Turkey, northern Syria
. . . . ***Stenoheriades levantica***: southern Syria, Lebanon, Israel and Palestine, Jordan

Acknowledgments

M. Schwarz (Ansfelden), F. Gusenleitner (Oberösterreichisches Landesmuseum Linz) and W. Arens (Bad Hersfeld) loaned a large amount of material of *Hofferia* and *Stenoheriades* for study. C. Praz (Université de Neuchâtel) and C. Villemant (Muséum national d'Histoire naturelle Paris) helped locating type material. D. Bénon provided photos on habitat and nesting site of *Hofferia schmiedeknechti*. H. Baur (Natural History Museum Bern) kindly provided access to a digital imaging system for taking photomicrographs. C. Rasmussen, M. Rightmyer Gee and an anonymous reviewer made valuable comments on the manuscript.

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Appendix 2:

Rozen, J. G., Jr, Pisanty, G., Trunz, V., Bénon, D., & Dorchin, A. (2015). Nesting biology, flower preferences, and larval morphology of the little-known Old World bee *Ochreriades fasciatus* (Apoidea: Megachilidae: Megachilinae). *American Museum Novitates*, *3830*, 18.

AMERICAN MUSEUM NOVITATES

Number 3830, 18 pp. April 21, 2015

Nesting Biology, Flower Preferences, and Larval Morphology of the Little-Known Old World Bee *Ochreriades fasciatus* (Apoidea: Megachilidae: Megachilinae)

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ABSTRACT

Herein we present information on the nesting behavior of *Ochreriades fasciatus* (Friese) found occupying beetle galleries in dead trunks and branches of certain trees and shrubs in Israel. We also describe the pre- and postdefecating larvae thereby making known the mature larva for this uncommon Old World genus. Females of *O. fasciatus* build linear nests in existing burrows in dead wood; depending on the length of the burrow, 1-5 cells are placed in one nest. The cell partitions are made of hardened mud, while the nest plug consists of pebbles fixed together with mud. *Ochreriades fasciatus* is oligolectic on Lamiaceae and probably strongly associated with the two related genera *Ballota* and *Moluccella*. It is hoped that information concerning its nesting biology, host-plant relationships, as well as larval development and anatomy will eventually prove valuable in determining the phylogenetic position of this genus relative to other megachiline bees.

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ISSN 0003-0082

INTRODUCTION

Ochreriades Mavromoustakis, 1956, is a rare, Old World genus of megachilid bees that has a restricted and disjunct distribution. It contains only two described species: *O. fasciatus* (Friese, 1899), known from very few locations in the Middle East (Jordan, Syria, and Israel; Müller, 2014) and *O. rozeni* Griswold, 1994, known from the single holotype female from Namibia (Griswold, 1994). In adult morphology, *Ochreriades* is unusually distinctive, as follows: (1) elongate adult body shape, more so than any other megachilid, with pronotum elevated and surrounding scutum anteriorly; (2) yellow integumental markings, unique within the osmiine and suggesting tribe Anthidiini; and (3) very long mouthparts (fig. 6), with proboscis nearly reaching tip of metasoma. The genus was originally suggested to be allied to *Chelostoma*, at that time considered closely related to *Heriades* (Mavromoustakis, 1956). Griswold (1994), however, showed that both *Chelostoma* and *Ochreriades* did not have the distinctive features of members of the *Heriades* group of osmiine genera (for details, see Michener, 2007: 448–449) and suggested that both genera may be closer to some members of the *Osmia* group of genera such as *Hoplitis (Alcidamea)*. The phylogenetic position of *Ochreriades* has been examined in few studies and remains unsettled. A cladistic analysis of morphological characters suggested a sister relationship between *Ochreriades* and *Chelostoma*, although with weak bootstrap-support values (Gonzalez et al., 2012). Two molecular studies have assessed the position of *Ochreriades* within osmiine and megachilid bees (Praz et al., 2008; Litman et al., 2011). In both cases, *Ochreriades* was not closely related to *Chelostoma*, but its position varied within Megachilinae, as sister to all other Osmiini (*Chelostoma* included), sister to Anthidiini + Osmiini + Megachilini, or sister to Megachilini + Osmiini. In all cases, support for the position of *Ochreriades* was weak.

In the present paper, we describe the nesting biology of *O. fasciatus*, examine its host-plant relationships and pollen-collecting behavior, and provide a description of the mature larvae.

In mid-July 2013 C.J.P. contacted J.G.R. to ask whether he would like to examine the larva of the rare bee *O. fasciatus*, which had been discovered by a group of Israeli and Swiss students (V.T., D.B., and A.D.) on the Golan plateau, northern Israel. G.P. visited the site in June 2014 and collected many nest-bearing branches. From this material G.P. sent some larvae to J.G.R. and also sent nest-bearing branches to Neuchatel University, Switzerland, where further studies were pursued by J.G.R. and C.J.P. with assistance by V.T. in late September/early October 2014. Preserved larvae were sent both from Israel and Switzerland to the AMNH to be examined by J.G.R.

METHODS

For examination, larvae and cocoons were prepared following the procedures outlined by Rozen and Hall (2011). To examine the floral preferences of *O. fasciatus*, D.B. analyzed the pollen provisions of six nests from the Golan site using light microscopy. Small quantities of the provisions were embedded in glycerol gelatin on a microscope slide. The pollen was identified to family under 400× magnification using a reference collection and the literature cited in Müller (1996a).

NESTING BIOLOGY

V.T., D.B., and A.D. originally discovered nests of *O. fasciatus* in a semiarid, open shrubland dominated by the deciduous shrub *Ziziphus lotus* (L.) Lam. (Rhamnaceae) near Kibbutz Lehavot ha-Bashan, on the slopes descending from the Golan Heights into the Hula Valley (N 33º08′32″ E35º39′12″, 138 m elev.; hereafter "Golan site") (figs. 1, 2). Nests were located in dead, erect cypress trees (*Cupressus sempervirens* L. (Cupressaceae)) planted along a dirt road and surrounded by several bushes of *Ballota undulata* (Fresen.) Bentham (Lamiaceae), one of the main host plants of *O. fasciatus*. Nests were distributed across all parts of the cypress trees from bottom to top, including the main trunk and side branches. Although the cypress trees were dead, the wood was very hard, and contained many beetle emergence holes (see below), in which the nests were located. Approximately 50 nests were discovered in a single dead tree in May 2013 after observing many females entering or sealing the burrows. In addition, approximately 20 nests were found in dead branches of an unknown species of deciduous tree. Around 80 more nests were discovered by G.P. in two dead trees when he visited the site in June 2014 when bee activity had almost ceased; nests were identified by the pebble-containing nest-closure plug assumed to be characteristic of the species.

In June 2014 G.P. discovered a second site in a Mediterranean shrubland located in the Judean Foothills, 1.2 km west of Kibbutz Bet Guvrin (N 31°36′51″ E 34°52′50″, 260 m elev.; hereafter "Judean Foothills site") (fig. 3), approximately 180 km SSW from the first site. The vegetation was dominated by multitrunk buckthorn (*Rhamnus lycioides* L. (Rhamnaceae)) and mastic (*Pistacia lentiscus* L. (Anacardiaceae)) shrubs about 2 m tall; *B. undulata* bushes grew mostly at the periphery of the shrubs, half-shaded. Each buckthorn and mastic shrub possessed dozens of thin trunks (diameter ca. 2-4 cm) growing sideways, some of them alive and bearing leaves and others dead. In four dead trunks of one of the buckthorns, close to the ground (5-50 cm above ground), he found 10 nests of *O. fasciatus*, three still active and the rest sealed. As in the previous site, the wood containing the nests was very hard.

All the nests examined in both sites were located in existing burrows in firm wood, strongly suggesting that females do not excavate burrows. Instead, they exploit the burrows premade by other insects, as in many other megachilid bees such as *Heriades* and *Chelostoma* (Westrich, 1989; Müller et al., 1997). In the nests described here, most burrows were excavated by metallic wood-boring (jewel) beetles (Buprestidae), which can be identified by the distinctly oval shape of the burrows in cross section. Four buprestid larvae in total were found inside the wood, two at each site. However, D.B. and V.T.'s discovery of adult *O. fasciatus* nesting in very small (diameter ca. 1.9–2.0 mm), perfectly round holes in another kind of wood from the same area supports the conclusion that this bee will use burrows made by other insects in other kinds of wood.

Nests of *O. fasciatus* consist of a single burrow leading from a hole in the wood surface to the cell or linear series of cells inside (figs. 11, 13). As already indicated, most nests seen were built in the more oval burrows of buprestids. The entrances are approximately 3-7 mm wide (range 2.5-9 mm; $n = 8$), and burrow diameters are consistent with those of their entrances. In most nests examined, the first 1-3 cm of the burrow are oriented at an angle to the wood surface, whereas the more distal part of the burrow runs more or less parallel to the wood grain.

FIGURES 1–3. Habitat photographs of observation/collection sites of *Ochreriades fasciatus*. **1.** Golan site from a distance showing cypress trees along roadway (picture by D.B. and V.T.). **2.** Close-up of Golan site with dead cypress tree containing nests of *O. fasciatus* at left (picture by D.B. and V.T.). **3.** Judean Foothills site showing G.P. collecting at nesting site (picture by A. Gotlieb).

Behind the entrance plug there is an open space of variable length before the first cell (i.e., the last cell that was built) is reached (fig. 11). The cells are generally located in the distal, straight portion of the burrow. Most cells were 8–11 mm long and their diameters in cross section were the same as that of the burrow, i.e., cells were also oval in cross section.

Cells are arranged in a single, continuous linear series, front to rear, along the burrow, so that their long axes are more or less aligned with the wood grain (figs. 11, 13). The cell front is always the end closer to the nest entrance. In one three-celled nest, there was a 15 mm open space between the most proximal cell and the two distal ones. It is important to point out that the arrangement of cells running with the wood grain is dictated by the feeding habits of the buprestid larva to find edible tissue; it is not determined by the female bee. This cell positioning

FIGURES 4–10. Macrophotographs of adults and nest entrances of *Ochreriades fasciatus.* **4.** Female on upper lip of flower of *Ballota undulata* tapping stamens with the undersurface of her metasoma and thereby collecting pollen on her metasomal scopa. **5.** Male feeding on nectar from flower of *B. undulata*, showing lack of contact between dorsum of small bee and stamens above and thereby not contributing to pollen transfer. **6.** Female with mouthpart extended flying toward flower of *B. undulata*. **7.** Entrance covered by single large pebble. **8.** Another covered by cluster of pebbles. **9.** Female bringing in pebble to cover nest entrance. **10.** Female with pollen-filled scopa about to enter nest. (Figs. 4, 5 by G.P., figs. 6–10 at Golan site by D.B. and V.T.)

parallels what has been reported for such megachilids as *Lithurgus chrysurus* Fonscolombe (Lithurgini) in which the behavior of the nest-making female bee determines cell orientation (Rozen, 2013).

Larvae and provisions are located in the distal part, i.e., rear, of the cell; the provisions are semiliquid, as in *Hoplitis (Hoplitis) adunca* (Panzer), and do not form a firm pollen mass. They occupy the entire rear end of the cell. Young larvae are sedentary and firmly connected to the provisions. Postdefecating larvae in cocoons have their head end at the front of the cocoon. Dissected burrows (including those from the Judean Foothills site) contained between one and five cells.

Cell partitions (figs. 12, 15) and burrow closures, i.e., nest plugs (fig. 12), are made of hardened mud and dissolve in water within a few minutes, suggesting that the mud is made with nectar and not with resin or glandular secretions. No pebbles are associated with cell partitions. The external surface of the nest plug (figs. 7, 8, 11, 12) is distinct from that of the partitions, as it incorporates relatively large pebbles (diameter 1-2 mm) that the female introduces with her mandibles (fig. 9). These pebbles are either glued together with mud, sometimes only weakly so or are inserted into moist mud. Their presence allows easy identification of completed nests even when the bees are inactive. The plug is usually located at the entrance of the burrow with pebbles extruding from the wood surface (figs. 7, 8) but can sometimes be as much as 1-2 cm inside the burrow (fig. 12), even invisible from outside. In the smallest burrows, the visible part of the plug may consist of one single pebble (fig. 7). Cell partitions are somewhat concave on both sides; their thicknesses tend to be 1.0–1.5 mm (maximally up to 2.0 mm) at the periphery but narrower toward the center.

In the Golan site, females were observed during provisioning by V.T. and D.B. Females first enter the burrow head first (fig. 10), presumably either to deliver nectar onto the provisions or to inspect the cell for parasites. After a few seconds, they come out, their metasomal scopa still filled with pollen, turn around at the nest entrance and enter the burrow, this time metasoma first. They stay in the nest slightly longer than the first time and eventually leave the nest once their scopa is empty. This observation suggests that females are not able to turn around inside the burrows (at least the narrow ones), as is also the case for many cavity- or stem-nesting bees such as *Chelostoma* and *Heriades* (this behavior may be universal in cavity-renting bees that nest in cavities whose diameters are only slightly greater than the bee using them).

Cocoon Structure and Fecal Placement: The cocoon shape of *O. fasciatus* (fig. 14) is dictated by the shape of the nest burrow and by the spacing of the partitions. Because most nests observed were in tunnels presumably made by larval Buprestidae, burrows are oval (not circular) in cross section. Furthermore, in cases where the buprestid larva is large, the cocoon may not adhere to all parts of the burrow wall.

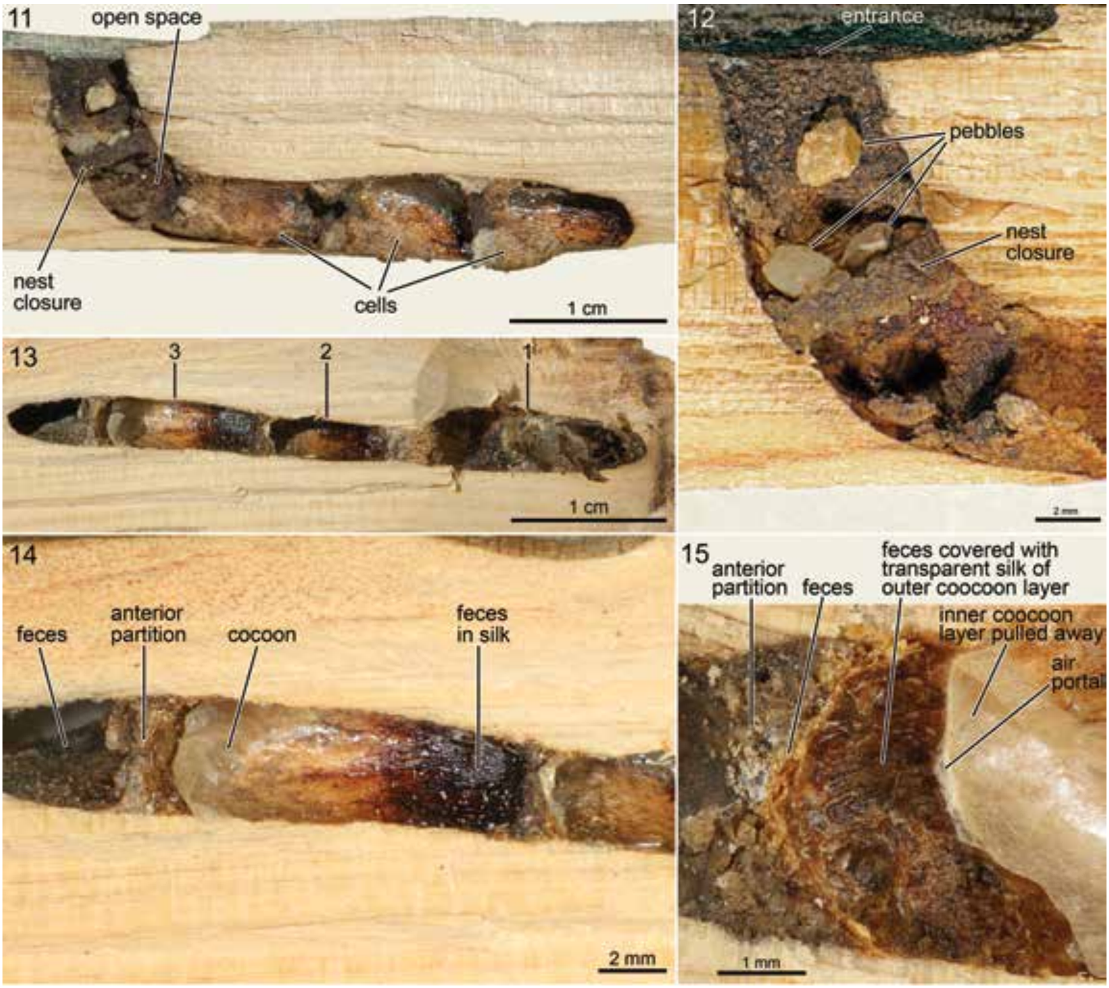
In general the cocoon is elongate, semitransparent, pale, more or less cylindrical in shape, and rounded at both ends (fig. 14). It is approximately 10 mm long and has a diameter dictated by and therefore slightly less than the burrow diameter. As a comparison, the body lengths of female bees are on average 8 mm (range 7–10 mm). About halfway to the rear, the surface gradually darkens with smears of black feces that farther toward the rear darken to a completely black, shiny, but opaque surface. The inner surface of the cocoon front is white, smooth, evenly curved, rather opaque, and composed of a fine webbed silk. In cross section the fabric at the front consists of an inner layer separate from but lying immediately next to the outer layer.

By examining completed cocoons of *O. fasciatus*, we recognized that cocoon spinning and defecation are interrelated, overlapping activities of the last larval instar. Before silk production starts, the larva deposits light brown feces with a faintly greenish cast against the anterior cell partition (figs. 14, 15). The pellets tend to be moist and blend together to form a mottled brown band immediately behind the darker grayish-brown partition of soil made by the female (fig. 15). Although the thickness of the two bands at their peripheries is sometimes similar, the fecal mass thins in some cases toward its center, creating in these cases a concave posterior surface to the fecal mass, at times allowing small pebbles of the soil partition in front to be exposed. Other times, as in figure 15, the fecal layer is far less concave.

The larva then spreads a very thin transparent sheet of silk over the inner surface of the fecal layer and along the wooden surface of the anterior cell wall. Thus is formed the outer layer of the front of the cocoon. The silk adhering to the mottled feces (figs. 15, 16) is so transparent that it was only first detected along a torn edge. However, widely scattered fine silk fibers attach it to the more substantial cocoon fabric that later will become the inner layer of the anterior part of the cocoon. Thus, the inner wall of the cocoon can rather easily be torn from the anterior part of the cocoon (figs. 14, 15). However, toward the rear of the cocoon the inner and outer cocoon layers more closely fuse to one another and incorporate the subsequent fecal deposits, accounting for the darkening of the cocoon rear. These feces are now black and smeared between layers of silk (fig. 14). Toward the cell rear, the cocoon fabric clings more tightly to the cell wall. Where the feces are the thickest the cocoon's texture becomes almost leathery.

These observations indicate that fecal production starts shortly before cocoon spinning and is completed while silk production continues. Furthermore, fecal coloration darkens as defecation continues, as has also been reported for some other Megachilidae (Rozen and Hall, 2011).

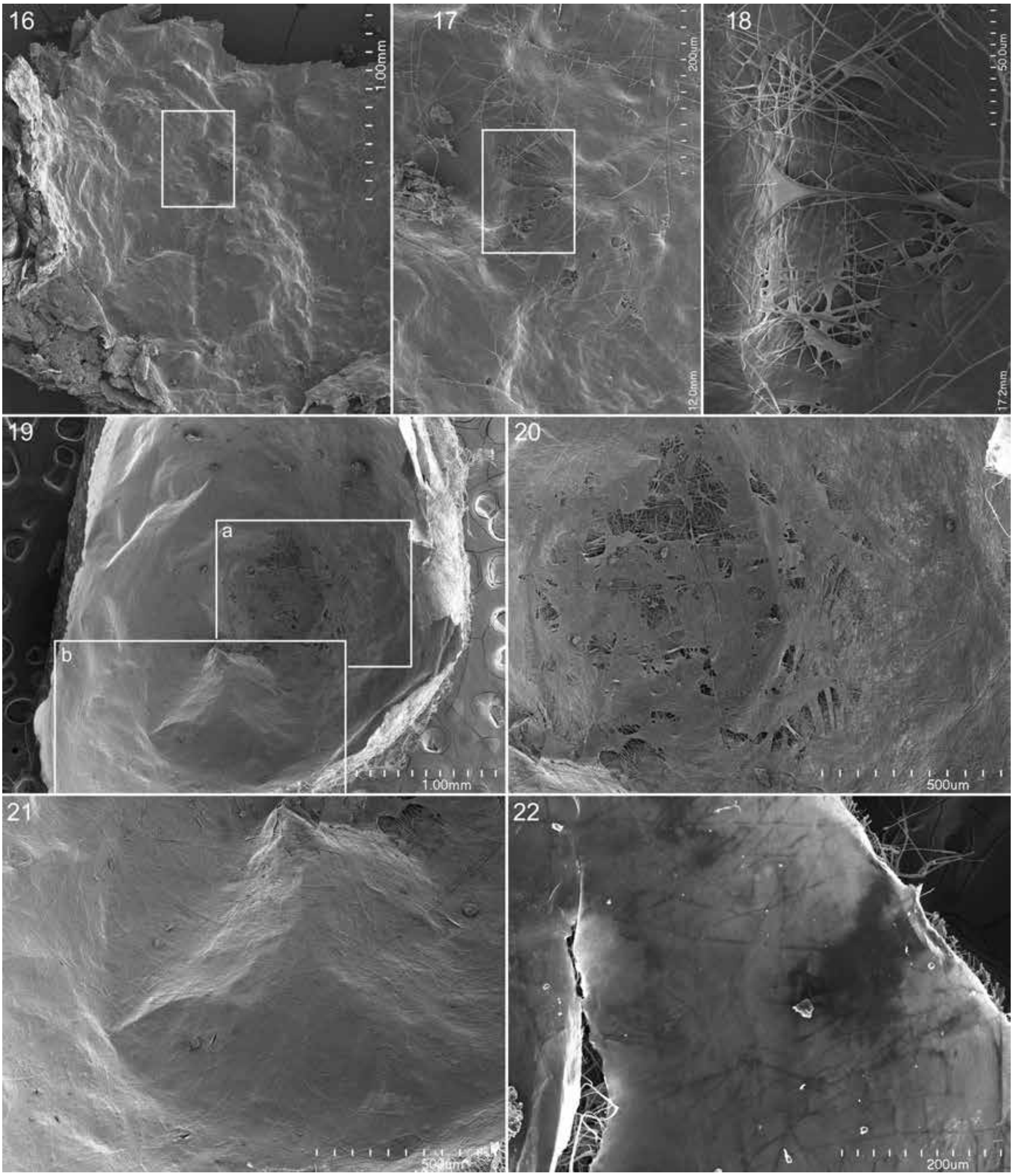
Several recent studies (Rozen and Hall, 2011; Rozen et al., 2011; Rozen and Mello, 2014) have pointed out that cocoons appear to serve several functions, among which are: exclusion of parasites and regulation of cell humidity over long periods. These studies also point out that air exchange between the interior of the cocoon and the surrounding environment is affected by a heavily screened air portal usually at the front end of the cocoon, sometimes referred to as the filter area or cocoon nipple. In the case of the cocoon of *O. fasciatus*, the air exchange portal indeed appears to be at the front end of the cocoon, identified by an irregular cluster of holes in the inner, sheetlike silk lining (figs. 19, 20), in front of which is a dense mass of fibrous white silk (fig. 15). The portal presumably functions to exclude parasites while permitting air exchange between the inside and outside atmospheres. Elsewhere, the inner surface of the inner layer of the cocoon is covered with a thin, clear, cellophanelike sheet of silk (figs. 19, 21, 22) providing a moisture-proof barrier. What is not certainly understood is the route of air exchange through the thin outer layer of silk that covers the feces deposited at the front end of the cell. Perhaps that silk is fenestrated. Alternatively air may be exchanged further back along the cell wall where the outer and inner layers meet and fuse. However, it should be noted that recent investigations on the cocoon of a larval *Hoplitis* demonstrates that the air passageway in that



FIGURES 11–15. Macrophotographs of nests of *Ochreriades fasciatus* in firm cypress wood, side view. **11.** Entire nest of three cells, which had been opened to remove contents. **12.** Close-up of entrance, showing recessed cell closure with pebbles above. **13.** Another nest with three cells, entrance to the left but not visible. **14.** Cell 3 from that nest with partial cocoon; front end of cocoon intact but partly pulled away from anterior partition; rear end of cocoon partly removed to reveal texture of inner surface with black feces imbedded in silk. **15.** Front end of cell from yet another nest, with inner layer of cocoon farther removed from outer transparent layer of cocoon appressed to mottled feces.

genus opens to the exterior by a ring of openings that circle the front of the cocoon where the outer cocoon layer attaches to the inner layer (to be fully described in a forthcoming paper).

Parasitism and Predation: No cleptoparasitic bees were associated with nests of *O. fasciatus*. However, five larvae of at least two species of predatory checkered beetles (Cleridae) were found inside the logs harvested from the Golan site. At least two of these larvae were found inside *O. fasciatus* nests, one of which was in the middle of a four-celled nest whose remaining cells on both sides contained uninjured bee larvae. Several specimens of *Leucospis dorsigera* Fabricius, 1775 (Hymenoptera: Chalcidoidea: Leucospidae), were observed flying



FIGURES 16–18. SEM micrographs of central part of anterior cell partition of *Ochreriades fasciatus* with surface covered by transparent layer of silk of front end of cocoon now made visible by reflected electrons. Note feces and part of anterior cell partition, upper left. **17, 18.** Sequential close-ups of rectangle in **16.** showing silk surface. FIGURES 19–21. SEM micrographs of inner surface of front end of cocoon showing fenestrations of central air portal. **19**. Entire front end. **20.** Close-up of central part identified by rectangle **a,** figure 19. **21.** Close-up of central part identified by rectangle **b,** figure 19. FIGURE 22. SEM micrograph of piece of inner surface of cocoon wall with bits of debris on glassy smooth, transparent inner surface of silk that had bee applied over fibrous silk.

around the dead tree containing the nests of *O. fasciatus* in the Golan site. Three dead adults of the same species were found in occupied nests of *O. fasciatus*.

FLORAL PREFERENCES AND FORAGING BEHAVIOR

Ochreriades fasciatus appears to be strictly oligolectic on Lamiaceae. Females were observed collecting pollen from *Ballota undulata* at both nesting sites, as well as at several other locations in Israel and Jordan (Golan Heights, 2 km NW Hamat Gader, 3 May 2010, leg. C. Sedivy and C. Praz; Golan Heights, 7 km N Ein Gev, 2 May 2010, leg. C. Sedivy and C. Praz; Jordan, Wadi Shuʿayb, 20 km W Amman, 22 April 2007, leg. C. Sedivy and C. Praz. In addition, females were also observed collecting pollen from *Moluccella laevis* L. (Israel, Judean Foothills, Nahshon, leg. G. Pisanty; 1 km SE Beit Nir, leg. G. Pisanty). The analysis of the pollen provision from the nests of *O. fasciatus* reveals that all six nests sampled contained only tricolpate Lamiaceae pollen, probably belonging to *Ballota undulata* (although pollen identification was possible only to the family level). In addition, Andreas Müller kindly made available analyses of the pollen contained in the scopa of 14 museum specimens of *O. fasciatus*. The pollen loads from these females originating from five localities in Syria (8 females), Jordan (1 female) and Israel (5 females) consisted entirely of tricolpate Lamiaceae pollen (Müller, 2014), further suggesting that *Ochreriades* is oligolectic on Lamiaceae, probably with a strong or exclusive preference for *Ballota* and *Moluccella*. Males also actively patrol the host plants, often hovering at 10-20 cm from the host plant in search of females. Flowers from other families are occasionally visited but probably for nectar only, including *Citrullus lanatus* (Thunb.) Matsum. and Nakai (Cucurbitaceae; 1 male), *Heliotropium* spp. (Boraginaceae; 1 male), and *Lavatera punctata* All. (Malvaceae; 1 female) (G.P., personal obs.).

The method of pollen collection by *O. fasciatus* females is noteworthy. The Lamiaceae are strongly nototribic, i.e., the flower is bilaterally symmetrical, the anthers are placed in the upper corolla, and pollen is deposited onto the dorsal surface of the floral visitor when it forages for nectar. Many bees specializing on the Lamiaceae, or on other nototribic flowers, possess modified hairs on the clypeus or frons; these hairs are short, nonplumose, usually thickened basally, and often slightly bent downward or wavy apically (Müller, 1996b). They form a short comb or brush that is used for extracting the pollen from the upper lip. However, *O. fasciatus* entirely lacks modified pilosity on the clypeus. Rather, the females climb the upper lip of the flower and repeatedly tap their metasomal scopa directly against the anthers (fig. 4). The presumably unrelated bee *Protosmia (Nanosmia) minutula* (Pérez) shows similar behavior on other Lamiaceae (e.g., *Teucrium montanum* L. (Müller, 1996b; Müller et al., 1997: 321), and one unidentified species of *Protosmia (Protosmia)* was also observed to collect pollen in a similar way at the Golan site (V.T., D.B.). *Ochreriades fasciatus* females alternate these pollen-collecting visits with nectar visits, in which they land on the lower lip of the flower and insert their proboscises into the corolla. The corolla of both *Ballota* and *Moluccella* is moderately deep and adapted to large, long-tongued bees such as *Anthophora*. This suggests that the particularly long mouthparts of *O. fasciatus* (and *O. rozeni*, whose host plant is unknown) that nearly reach the tip of the metasoma are an adaptation to reach the nectar of their host plants. Müller (1996b) stated that pollination of Lamiaceae by bees was likely achieved mostly during nectar visits, as pollen-collecting females restrict their pollen visits to flowers in the male phase (many Lamiaceae are strongly protandrous; Müller, 1996b, and references therein). Interestingly, it appears that both sexes of *O. fasciatus* are too small to come in contact with the anthers during nectar visits on their host plants (fig. 5), and the overall contribution of *O. fasciatus* to pollination of their host plants may be very limited.

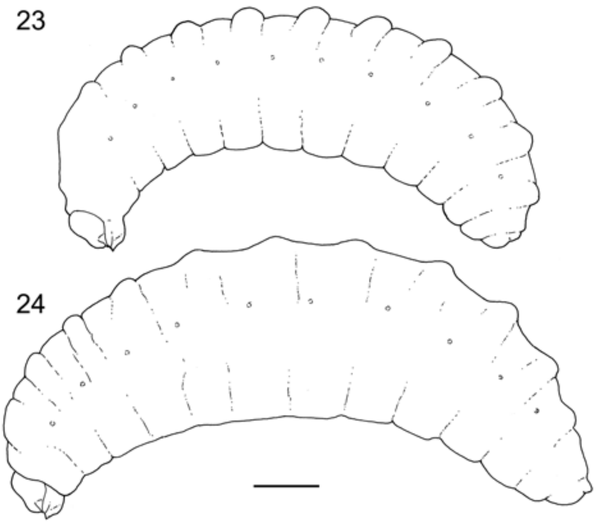
DESCRIPTION OF THE MATURE LARVAE OF *OCHRERIADES FASCIATUS*

Figures 23–37

Diagnosis: The mature larva of *O. fasciatus* (figs. 23, 34) closely resembles other known larvae of the Megachilinae. The moderate body form between robust and slender is more slender than those of Anthidiini (Michener, 1953: figs. 109, 114, 119, 120; Rozen and Hall, 2012: fig. 52; Rozen, 2015), but the apically bidentate mandible (figs. 33–36) is typical for the family (except for certain *Stelis*), even though the apically rounded teeth are less common. Body vestiture on fifth instars (figs. 27–29) is also a family feature, but is substantially reduced in *O. fasciatus* compared with many family members and seems to consist of only setae, not spicules. The dense cluster of curved setae below the anus on abdominal segment 10 seems unusual (fig. 30). The dentate atrial wall of the spiracle (fig. 37) is a common, though not unique, feature of the family; the elongate, parallel-sided subatrium may be less common. As in all megachilids, paired dorsal tubercles are absent, but many larval megachilids exhibit more or less developed, middorsal intersegmental tubercles on midbody segments (Michener, 1953: fig. 114; Rozen and Hall, 2012: figs. 18, 52) (such tubercles seem to arise from the posterior edge of the caudal annulet and involve the partly surrounding extreme anterior edge of the following cephalic annulet). In some cases such tubercles are small and obscure and therefore easily overlooked. However, in *O. fasciatus* there is no hint of these tubercles.

The following description is based on both pre- and postdefecating larvae.

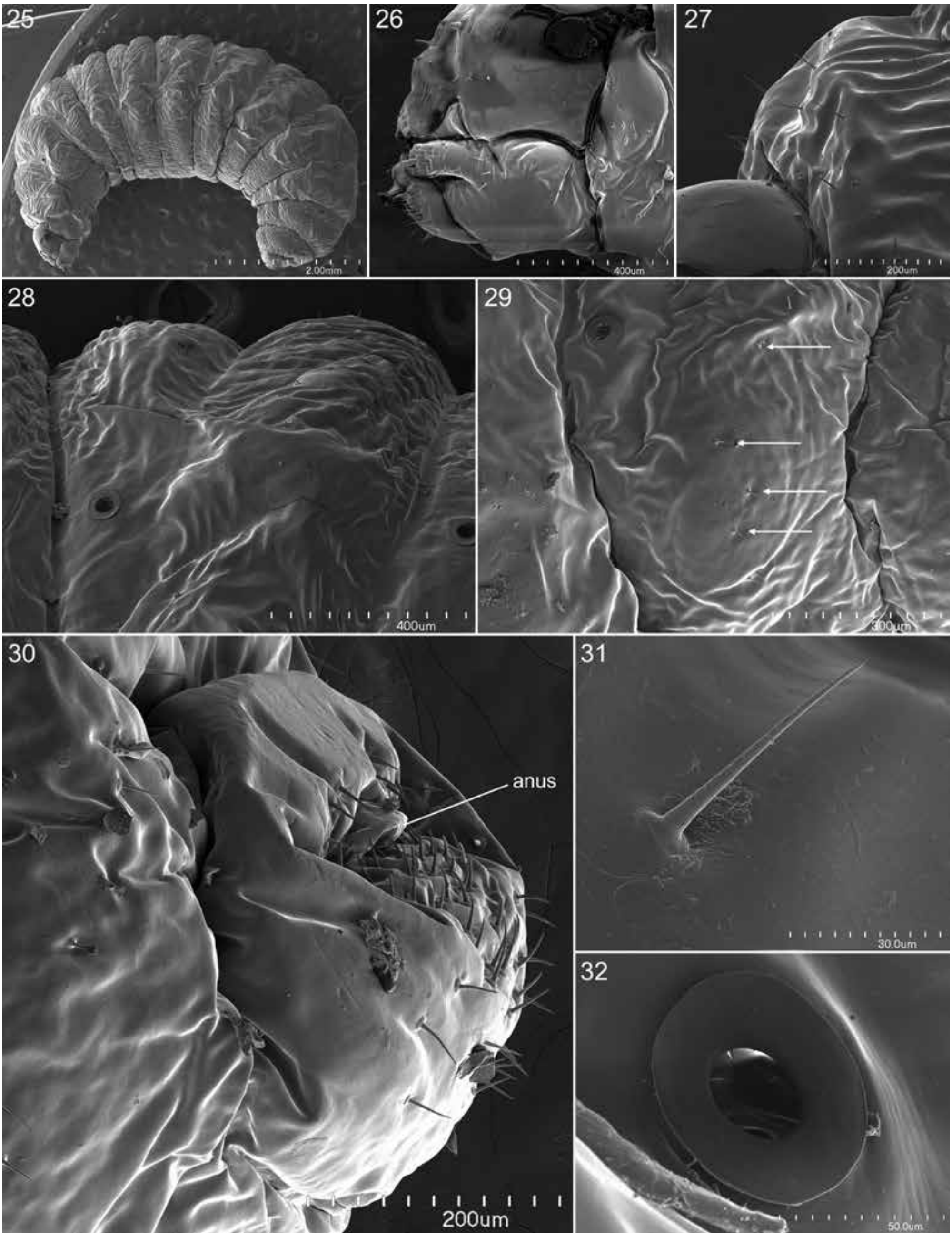
Description: **Head**: Head moderately small in relation to body size (figs. 23–25); oriented in normal, hypognathous position relative to thorax. Setae moderately long but sparse to nonexistent on upper part of head capsule; those of maxillary and labial apices large, curved, moderately abundant, and conspicuous. Head capsule unpigmented except at points of articulations with mandibles; labrum faintly pigmented except transverse labral sclerite slightly darker; mandibles moderately pigmented except mandibular apices and areas of articulation with head capsule strongly pigmented; maxillary sclerites faintly pigmented; salivary lips strongly projecting, deeply pigmented; antennal papilla, maxillary and labial palpi all uniformly moderately pigmented. Spiculation apparently absent even on hypopharynx, not on maxilla. Coronal ridge present for less than one-third distance from postoccipital ridge toward level of antennae in frontal view; postoccipital ridge well developed, bending forward somewhat toward median line on top of head; hypostomal ridge well developed, giving rise to pronounced dorsal ramus that extends posteriorly for short distance before ending abruptly in front of postoccipi-



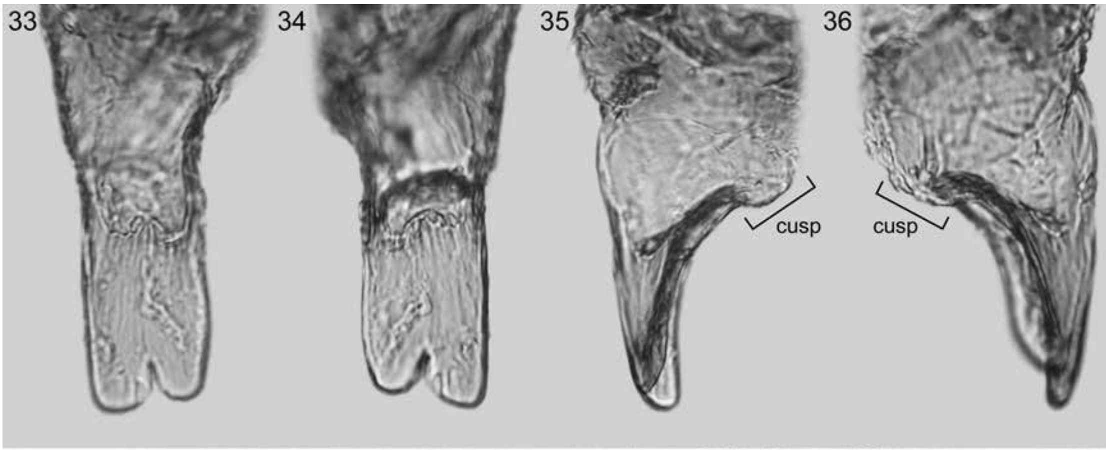
FIGURES 23, 24. Diagrams of post- and predefecating larvae of *Ochreriades fasciatus*, lateral views, to same scale, respectively. Setae not depicted.

tal ridge; both hypostomal ridge and ramus staining darkly; posterior part of ridge bending strongly mesad, forming deeply recessed posterior tentorial pit at junction with posterior tentorial bridge; posterior bridge absent in postdefecating larva because specimen preparing to molt; internal pleurostomal ridge obviously present but not well defined; epistomal ridge moderately well developed from anterior mandibular articulation to anterior tentorial pit; from pit, ridge extending vertically until fading out above level of antennal papilla (as in *Haetosmia*); hence ridge not extending across to opposite side of head. Tentorium mostly absent because of impending ecdysis. Parietal bands deeply incised. In lateral view, clypeus not projecting much beyond frons, antenna arising from faint prominence, and labrum not extending much beyond clypeus. Diameter of basal ring of antenna about two-thirds distance from closest point on ring to center of anterior tentorial pit; antennal papilla distinctly but not strongly pigmented, moderately large and elongate, longer than twice basal diameter, apically rounded, bearing perhaps three sensilla apically. Lower margin of clypeus angled upward at midline, so that at midpoint margin nearly at level of anterior tentorial pits. Labrum deeply emarginated apically; labral sclerite transverse but poorly defined, unevenly pigmented.

Mandible (figs. 33–36) moderately robust; apex darkly pigmented, bidentate with ventral tooth longer than dorsal tooth; mandibular apex approximately parallel sided in inner and outer views (figs. 33, 34); both teeth on postdefecating larva broadly rounded apically; dorsal apical edge of dorsal tooth faintly, irregularly uneven; ventral apical edge of ventral tooth also faintly uneven; apical concavity defined; cuspal area (fig. 36) developed, projecting, with surface irregularly uneven; outer mandibular surface with single conspicuous long curved seta near base. Maxillary apex strongly bent mesad in frontal view, so that maxillary palpus subapical in position; cardo distinct, posterior end directed toward posterior tentorial pit; stipes weakly sclerotized except for conspicuously long stipital rod that is darkly stained by dye, at posterior end articulat-



FIGURES 25–32. SEM micrographs of postdefecating larva of *Ochreriades fasciatus*, all lateral views or approximate lateral views. **25.** Entire larva, near lateral view. Note that SEM image accentuate texture of surfaces that have been modified by critical-point drying in contrast to outline shape defined by camera lucida illustration (fig. 23). **26.** Close-up of lower part of head and prothorax. **27.** Pronotum, showing scattered, elongate, tapering setae. **28.** Dorsal part of abdominal segment 3, showing short setae and division of cephalic and caudal annulets. **29.** Lateral lobe of abdominal segment 8, showing paucity of setae (arrows). **30.** Abdominal segment 10, close-up, showing abundant curved sharp setae below anus. **31.** Dorsal pronotal seta, close-up. **32.** Close-up of spiracle, outer view.



FIGURES 33–36. Microphotographs of cleared right mandible of postdefecating larva of *Ochreriades fasciatus*. **33.** Outer view with position of seta (removed during dissection) near base indicated by arrow. **34.** Inner view. **35.** Dorsal view. **36.** Ventral view. FIGURE 37. Microphotograph of spiracle, side view, showing elevated atrial rim, dentate atrial wall, and long, multichambered subatrium. FIGURE 38. Microphotograph of spiracle of fourth instar, side view, showing long subatrium without chambers.

ing with cardo, at anterior end broadening and branching to form weakly pigmented articulating arm of stipes; maxillary and labial palpi elongate, probably more than two times basal diameters, both pigmented like antennal papilla but slightly thinner than papilla. Labium clearly divided into prementum and postmentum; apex moderately narrow in frontal view; premental sclerite apparently absent but border between pre- and postmentum distinctly incised; prementum projecting dorsally at midline and sclerotized, pigmented on some specimens, forming dorsal bridge of premental sclerite that extends between apices of articulating arms of stipes; postmentum nonsclerotized. Salivary lips strongly projecting, transverse, with inner surface bearing parallel longitudinal grooves; width of lips slightly less than distance between bases of labial palpi. Hypopharynx distinctly separated pair of nonspiculate mounds.

Body (figs. 23–25, 27–32): Body vestiture without spicules, consisting only of slender, pale setae, tapering to fine points, arising from small but distinct alveoli; these setae inconspicuous but moderately elongate and tapering (figs. 27, 31); setae moderately abundant on elevated dorsal surfaces of thorax and widely scattered on anterior ventral surface of thorax; some setae present on dorsal surfaces of caudal annulets of abdominal segment 8, 9, and 10, on ventral surface of abdominal segments 8 and 9, and especially abundant on abdominal segment 10 below anus (fig. 30); dorsal surface elsewhere with scattered short inconspicuous setae (fig. 28); lateral lobe of abdominal segment 8 (i.e., area below level of spiracle) with approximately 2–4 setae (fig. 28). Body form of postdefecating larva moderate in lateral outline between robust and slender (figs. 23, 25); body segments gradually increasing in height with abdominal segments 4 to 6 having greatest diameters; paired body tubercles absent, but caudal annulets of most body segments projecting farther than cephalic annulets and surprisingly uniform in appearance; middorsal intersegmental tubercles totally absent; lateral lobes of most body segments uniformly moderately developed. Body form of predefecating larva in lateral outline (fig. 24) with midbody segments having greatest diameter and outline tapering forward and backward from there. On pre- and postdefecating larvae (figs. 23, 24), venter of abdominal segment 9 not produced, as is true for all known larval Megachilidae; abdominal segment 10 attached to middle of segment 9 in lateral view; anus positioned toward top of segment 10. Spiracles (figs. 32, 37) unpigmented, subequal in diameter; atrium globular with width not much greater than depth, projecting little above body wall, with rim; diameter of atrial opening about 1.5 times radial width of peritreme (as measured on SEM micrograph, fig. 32); atrial inner surface with rows of wrinkles concentric with primary tracheal opening; some wrinkles giving rise to rows of concentrically directed spicules; primary tracheal opening with collar (i.e., integument of first chamber of subatrium tending to be more robust than that of subsequent chambers); subatrium long, with about 20 or more chambers of approximately equal size except one or two next to atrium slightly larger in diameter. Sex characters unknown.

MATERIAL EXAMINED: Two postdefecating larvae: Israel: Lehavot ha-Bashan (Hula Valley), coordinates: N 33°08′32″ E 35°39′12″, 138 m elev., May 5, 2013 (V. Trunz, D. Bénon) within dead cypress wood (*Cupressus sempervirens* L.). The following were collected as nests in the field at N 33°08′28″ E 35°39′27″, 170 m elev., on June 13, 2014, (G. Pisanty) and nests were opened on dates indicated: 1 predefecating larva, VI-16-2014; 6 predefecating and 2 postdefecating larvae, VI-18-2014. From nests collected by G. Pisanty, June 13, 2014, and sent to Neuchatel: 2 postdefecating larvae IX-30-2014 (C. Praz); 2 postdefecating larvae, IX-31-2014 (C. Praz).

REMARKS: One of the larvae sent by G.P. was a young fifth instar as judged by its substantially smaller size than any other predefecating specimen. Loosely attached to the body was a bundle of its cast exoskeletons, a condition frequently encountered in the Megachilidae, probably promoted by the earlier instars' inability to move from where they had been deposited as an egg. The small fifth clearly exhibited its distinctive long body vestiture as well as well-developed salivary lips. Among the cast exoskeletons, paired mandibles and some other head parts of the third and fourth instars were clearly visible. Not surprisingly both sets of mandibles were apically bifid and body exuviae lacked setae. Although spiracles of the third instar were difficult to evaluate, those of the fourth instar (fig. 38) showed a funnel-shaped, heavily sculptured atrium and a long, parallel-sided, faintly curved subatrium not divided into chambers.

CONCLUDING REMARKS

The present paper introduces many hitherto unknown aspects of the nesting and foraging biology of the rare bee *O. fasciatus*. An important question is whether these biological aspects, as well as larval morphology and cocoon structure, may provide useful phylogenetic information to settle the hitherto unclear phylogenetic placement of *Ochreriades* within Megachilidae. With respect to larval anatomy, larval *O. fasciatus* has no middorsal intersegmental tubercles whereas they have been illustrated for a number of species of *Hoplitis* (Enslin, 1925: figs. 3, 4), but larvae of other important taxa are still uncollected and unknown. We therefore do not expand further here on the comparative anatomy of osmiine larvae. This will be the subject of a subsequent paper. Regarding the nest architecture, the nest construction in *O. fasciatus* is somehow similar to what is observed in the genus *Chelostoma*, especially in the fact that partitions are made of mud (without incorporated pebbles) while the nest plug includes both mud and pebbles (Westrich, 1989; Müller et al., 1997). The pebbles included in the nest plug are comparatively larger and the proportion of mud in the plug is lower in *O. fasciatus* than in *Chelostoma*. In spite of these differences, the inclusion of pebbles into the nest plug is a noteworthy similarity between *O. fasciatus* and *Chelostoma*. However, other osmiine lineages are known to include pebbles into the nest plug but not into the cell partitions. Bees of the genus *Heriades* use resin as nesting material (Matthews, 1963; Westrich, 1989; Müller et al., 1997). While the cell partitions are made of pure resin, the nest plug consists of resin into which small pebbles, sand grains, dirt, slivers of wood, dry plant fragments, and other miscellaneous detritus are added (Matthews, 1963; Westrich, 1989; Müller et al., 1997). Use of stones and other detritus is most probably a barrier to nest enemies such as birds, parasites, or parasitoids, a likely underestimated mortality factor in solitary bees (Elz et al., 2015). Based on these observations, one wonders whether the incorporation of pebbles into the nest plug is homologous among the various osmiine lineages discussed above or the result of convergent evolution due to high predator or parasite pressure in cavity-nesting bees. Lastly, although floral preferences may not constitute a phylogenetically reliable character, one comparison between host specialization in *Ochreriades* and *Chelostoma* is noteworthy. Sedivy et al. (2008) studied the floral preferences of *Chelostoma* in detail. They found that most species of *Chelostoma* were oligolectic on various hosts, as is *Ochreriades*, yet a striking difference with *Ochreriades* is the fact that zygomorphic (or bilateral) flowers were entirely absent from the host plants of *Chelostoma*. In conclusion, although information on the nesting biology, mature larva, and floral preferences presented herein does not currently shed light on the phylogenetic relationships of *Ochreriades* to other osmiines taxa, it does provide new information that can be compared when more complete studies of the other taxa are forthcoming.

Another consideration: Although phylogenetic information is important and interesting, it is not the only goal of natural history. Understanding and knowledge of the whole organism (all life stages plus the respective anatomy and behavior during those stages) and determining how the organism is adapted to its environment are other goals. With respect to *O. fasciatus*, the study is far from complete. Among questions yet to be answered: What is the anatomy of its egg? Where and in what position is it deposited? How many larval instars are there? It has been hypothesized that megachilid larvae do not crawl until they reach the fifth stadium (Rozen and Hall, 2011). Is that true for *O. fasciatus*? In what developmental stage does the species overwinter? Are we certain where adults mate?

ACKNOWLEDGMENTS

Stephen Thurston, Senior Scientific Assistant, AMNH, arranged all plates of illustrative material for this publication and took macrophotographs of nests. Eli S. Wyman, Curatorial Assistant, AMNH, prepared immature specimens for SEM examination, produced all SEM images, and edited the text. The “import controls” section of the Swiss federal food safety and veterinary office arranged an import permit for the larvae of *Ochreriades*. Ariella Gotlieb accompanied G.P. when visiting the Judean Foothills site. Alex Shlagman and Igal Kushnir helped with wood dissection. Ariel-Leib-Leonid Friedman (Tel Aviv University) identified beetle larvae, and Hannes Baur (Natural History Museum, Bern) kindly identified *Leucospis* specimens. V.T. and D.B.’s trip to Israel was funded by the University of Neuchatel. C.J.P. thanks Claudio Sedivy, Andreas Müller, and Sara Bangerter for making a field trip to Jordan possible. Andreas Müller kindly enabled us to present unpublished analyses of pollen loads of *Ochreriades fasciatus*.

All authors extend their appreciation to the two anonymous reviewers for their thoughtful corrections of and improvements to the manuscript.

J.G.R. extends his sincere thanks to Robert G. Goelet, Chairman Emeritus of the Board of Trustees, American Museum of Natural History, for enabling his participation in this study.

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