



# Harden up: metal acquisition in the weaponized ovipositors of aculeate hymenoptera

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## Abstract

The use of metal ions to harden the tips and edges of ovipositors is known to occur in many hymenopteran species. However, species using the ovipositor for delivery of venom, which occurs in the aculeate hymenoptera (stinging wasps, ants, and bees) remains uninvestigated. In this study, scanning electron microscopy coupled with energy-dispersive X-ray analysis was used to investigate the morphology and metal compositional differences among aculeate aculei. We show that aculeate aculei have a wide diversity of morphological adaptations relating to their lifestyle. We also demonstrate that metals are present in the aculei of all families of aculeate studied. The presence of metals is non-uniform and concentrated in the distal region of the stinger, especially along the longitudinal edges. This study is the first comparative investigation to document metal accumulation in aculeate aculei.

**Keywords** Scanning electron microscopy · Energy-dispersive X-ray spectroscopy · EDS · Aculeata · Aculeus · Cuticle · Metal accumulation

## Introduction

Aculeata (ants, bees, and stinging wasps) are the most conspicuous of the hymenopteran insects, and are known predominantly for the capacity to inflict a painful sting (Piek 1986). Different groups inflict varying amount of pain,

with the most severe responses (as perceived by humans) delivered by taxa including bullet ants (*Paraponera*), tarantula hawk wasps (*Pepsis*), and armadillo wasps (*Synoecca*) (Schmidt 2016). Uniquely among venomous animals, the venom apparatus of aculeates is evolutionarily derived from the female's ovipositor (Robertson 1968). The weaponisation of the ovipositor is associated with the evolution of stinging aculeates diverging from parasitic wasps and may have helped drive the enormous radiation of aculeates (Branstetter et al. 2017).

The ovipositors of parasitic hymenopterans are subject to considerable abrasion and need to be both hard and wear resistant, especially in groups that must repeatedly drill through wood or other dense substrates concealing their hosts. Many parasitoids can also flex their ovipositors as they navigate this structure through the substrate to locate their hidden hosts (Quicke 2014). Research has shown that parasitic ovipositors are enriched with transition metals such as zinc and manganese, which are hypothesised to affect the mechanical properties of the cuticle (Kundanati and Gundiah 2014; Quicke 1998). In contrast, aculeate aculei are not required to drill through a hard substrate and thus do not have these same requirements for abrasion and wear-resistance. This dichotomy between the use of

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the ovipositor in parasitic hymenopterans and the aculeus in aculeates suggests that a metal compositional difference may not be unexpected.

The aculeate aculeus is primarily used for the injection of venom. Aculeates can be divided into social and solitary groups and the functions of venoms in each group are different. Solitary aculeates envenomate their prey to paralyse and preserve it, whereas social aculeates often use the aculeus to defend their colonies from vertebrate predators (Starr 1985, 1989), while at the same time retaining some offensive capabilities (Fisher 1993). Although the importance of the aculeus in driving social evolution within aculeates has been questioned by some (e.g., Kukuk et al. 1989), this difference in function between solitary and social groups does suggest that the aculeus morphology and metal content may also differ in these taxa in a phylogenetic context. Hence, documenting these patterns using comparative methods can provide the phylogenetic context for assessing the role of the aculeus in insect social evolution.

The primary aim of our study was to investigate the morphological adaptations of aculeate aculei and secondly to determine if there are any metals present in the aculei of aculeates. Here, using a comparative approach, we used a scanning electron microscope (SEM) with an energy-dispersive X-ray (EDS) detector to characterize the morphology and composition of the aculeate aculeus for the first time. This research is the first step to understand the evolution of these understudied insect venom systems and how they impact the evolution and ecology of aculeates.

## Materials and methods

### Sample preparation

One specimen of each species in Table 1 was investigated. In species with negative findings regarding metal composition, further specimens were analysed to provide confirmation. The studied species were selected to provide detailed sampling within several major groups of Aculeata including ants (Formicidae), apid bees (Apidae), vespids wasps (Vespidae), and several other aculeate lineages. Species were selected to match those used in ongoing study on venom composition within aculeates to allow comparisons between ovipositor size and venom profiles. All specimens are vouchered at the Smithsonian National Museum of Museum of Natural History, Washington DC; voucher codes are provided in Table 1. The aculei were excised from the abdomen and washed in 80% ethanol. Samples were allowed to air dry and then adhered on a conductive double-sided (adhesive) carbon tape and carbon coated.

### Energy dispersive X-ray analysis (EDS)

Scanning electron microscopy was performed using the Hitachi S3700-N scanning electron microscope (SEM) in high vacuum mode. Energy-dispersive X-ray spectrometry (EDS) was performed using a Bruker XFlash 4010 silicon drift detector using Esprit v1.9.4 software by Bruker. Samples were imaged and analysed using a beam energy of 20 keV at approximately 10 mm working distance. Areas of interest were first imaged, and then regions of interest were selected for EDS analysis. No suitable metal-bearing carbon-rich standard is currently available to match the material being assessed, hence, an interactive standardless peak-background (P/B) ZAF matrix correction was used to estimate compositions within the Esprit software. The P/B ZAF correction routine is well suited to this study given its ability to measure the composition of non-flat specimen geometry.

Hyperspectral X-ray mapping was applied to investigate the distribution of the transition metals present. Acquisition conditions for elemental imaging were 15 keV, 10 mm working distance, and 25° tilt towards the EDS detector to increase the take-off angle from 35 to 60°. Specimens tilted in this fashion suffer a reduced nitrogen absorption by carbon, for example, owing to a shortened path length within the aculeus. Specimen tilting toward the detector additionally reduces shadowing effects from the three-dimensional aculeus (Goldstein et al. 1992). X-ray images extracted from the hyperspectral datasets are presented as net count maps, with background counts and adjacent peak contributions removed.

### Phylogenetic comparative analyses

A phylogeny was assembled using inferred evolutionary relationships within various taxa from previous studies (Arévalo et al. 2004; Ascher et al. 2001; Borowiec et al. 2017; Brady et al. 2006; Branstetter et al. 2017; Cameron et al. 2007; Cardinal et al. 2011; Hasegawa and Crozier 2006; Lopez-Osorio et al. 2014; Perrard et al. 2013; Santos et al. 2015; Schmidt 2013; Schmitz and Moritz 1998; Willis et al. 1992; Wilson et al. 2012) and was used for all further analyses conducted in R v3.2.5 (R-Core-Team 2011) using the ape package (Paradis et al. 2004) for general handling of phylogenetic and trait data. Ancestral states were estimated and reconstructed over the tree to investigate the evolutionary history of the traits and consequently their relation to one another over time. To provide a comparative estimate of the barbs present on the aculeus, the ratio of the height of the barb relative to the base of the barb was calculated. This calculation corrected for any

**Table 1** Presence of metals in the aculei

Sociality	Family	Species	Voucher code	Fe	Cu	Mn	Zn	Ti
Social	Apidae	<i>Apis mellifera</i>	USNMENT 01111826	1	1	1	–	–
	Apidae	<i>Apis cerana</i>	USNMENT 01110537	1	–	–	–	–
	Apidae	<i>Apis dorsata</i>	USNMENT 01080706	2	–	1	–	–
	Apidae	<i>Apis florea</i>	USNMENT 01111300	2	–	–	–	1
	Apidae	<i>Bombus impatiens</i>	USNMENT 01000423	–	–	1	–	1
	Apidae	<i>Bombus huntii</i>	USNMENT 01003891	–	–	1	–	–
	Apidae	<i>Bombus sonorus</i>	USNMENT 01033975	3	–	2	–	1
Solitary	Apidae	<i>Centris rhodipus</i>	USNMENT 01248229	–	–	1	–	1
	Apidae	<i>Diadasia rinconis</i>	USNMENT 01248238	2	–	1	–	1
	Apidae	<i>Peponapis pruinosa</i>	USNMENT 01248231	1	–	1	–	–
	Apidae	<i>Xylocopa rufa</i>	USNMENT 01248232	1	–	–	–	1
Social	Formicidae	<i>Ectatomma tuberculatum</i>	USNMENT 01125478	1	–	1	–	–
	Formicidae	<i>Gnamptogenys mordax</i>	USNMENT 01125485	–	–	1	–	–
	Formicidae	<i>Rhytidoponera metallica</i>	USNMENT 01125467	1	–	–	1	1
	Formicidae	<i>Pogonomyrmex maricopa</i>	USNMENT 01125460	1	–	–	3	1
	Formicidae	<i>Pogonomyrmex occidentalis</i>	USNMENT 01125468	1	–	–	–	–
	Formicidae	<i>Pogonomyrmex rugosus</i>	USNMENT 01125487	–	–	–	1	–
	Formicidae	<i>Myrmecia gulosa</i>	USNMENT 01125476	1	–	–	–	–
	Formicidae	<i>Myrmecia nigripes</i>	USNMENT 01125463	1	–	–	–	1
	Formicidae	<i>Myrmecia pilosula</i>	USNMENT 01125934	1	–	–	–	–
	Formicidae	<i>Myrmecia rufinodis</i>	USNMENT 01125465	1	–	–	1	1
	Formicidae	<i>Myrmecia simillima</i>	USNMENT 01125470	–	–	–	–	–
	Formicidae	<i>Myrmecia tarsata</i>	USNMENT 01125473	1	–	–	–	–
	Formicidae	<i>Daceton armigerum</i>	USNMENT 01125479	–	–	–	2	1
	Formicidae	<i>Paraponera clavata</i>	USNMENT 01125475	–	–	2	–	1
	Formicidae	<i>Pachycondyla crassinoda</i>	USNMENT 01125477	1	–	–	–	–
	Formicidae	<i>Dinoponera gigantea</i>	USNMENT 01125471	1	–	–	1	–
	Formicidae	<i>Leptogenys elongata</i>	USNMENT 01125488	1	–	–	–	–
	Formicidae	<i>Odontomachus bauri</i>	USNMENT 01125482	–	–	–	1	–
	Formicidae	<i>Diacamma</i> sp.	USNMENT 01125480	–	–	–	1	–
	Formicidae	<i>Platythyrea lamellosa</i>	USNMENT 01125486	1	–	–	1	–
Formicidae	<i>Odontoponera</i> sp.	USNMENT 01125483	–	–	–	–	–	
Formicidae	<i>Streblognathus aethiopicus</i>	USNMENT 01125474	–	–	–	–	–	
Formicidae	<i>Tetraponera aethiopus</i>	USNMENT 01125484	1	–	–	–	–	

Table 1 (continued)

Sociality	Family	Species	Voucher code	Fe	Cu	Mn	Zn	Ti
Social	Vespidae	<i>Agelaia myrmecophila</i>	USNMENT 01248201	1	–	–	–	1
	Vespidae	<i>Brachygastra mellifica</i>	USNMENT 01125241	–	–	–	–	–
	Vespidae	<i>Mischocyttarus flavitarsus</i>	USNMENT 01248210	3	–	–	–	3
	Vespidae	<i>Parachartergus fraternus</i>	USNMENT 01125240	–	–	–	–	–
	Vespidae	<i>Polistes canadensis</i>	USNMENT 01248205	1	1	–	–	1
	Vespidae	<i>Polistes comanchus navajoe</i>	USNMENT 01248212	1	–	–	–	–
	Vespidae	<i>Polistes dorsalis</i>	USNMENT 01125242	1	1	–	3	–
	Vespidae	<i>Polistes flavus</i>	USNMENT 01248206	1	1	–	1	1
	Vespidae	<i>Polistes major castaneocolor</i>	USNMENT 01248214	2	–	–	–	1
	Vespidae	<i>Polybia occidentalis</i>	USNMENT 01125244	1	–	–	1	1
	Vespidae	<i>Polybia rejecta</i>	USNMENT 01248215	1	–	–	–	–
	Vespidae	<i>Polybia sericea</i>	USNMENT 01248216	1	–	–	–	1
	Vespidae	<i>Polybia simillima</i>	USNMENT 01125243	1	–	–	2	1
	Vespidae	<i>Ropalidia stigma</i>	USNMENT 01248247	–	–	–	–	–
	Vespidae	<i>Dolichovespula maculata</i>	USNMENT 01248220	1	–	–	–	–
	Vespidae	<i>Dolichovespula arenaria</i>	USNMENT 01248219	1	–	1	–	–
	Vespidae	<i>Vespa luctuosa</i>	USNMENT 01248249	1	–	–	–	–
	Vespidae	<i>Vespa mandarina</i>	USNMENT 01248221	3	–	–	–	–
	Vespidae	<i>Vespa simillima</i>	USNMENT 01248222	1	–	–	–	–
	Vespidae	<i>Vespa tropica</i>	USNMENT 01248253	1	–	–	–	–
	Vespidae	<i>Dolichovespula arctica</i>	USNMENT 01238524	1	–	1	–	–
	Vespidae	<i>Vespula pensylvanica</i>	USNMENT 01248224	1	–	–	–	–
	Vespidae	<i>Vespula vulgaris</i>	USNMENT 01248225	2	–	–	–	–
	Vespidae	<i>Synoeca septentrionalis</i>	USNMENT 01248218	1	–	1	–	–
	Vespidae	<i>Belonogaster juncea colonialis</i>	USNMENT 01248246	–	–	–	–	–
	Vespidae	<i>Provespa anomala</i>	USNMENT 01248248	–	–	–	–	–
Solitary	Mutillidae	<i>Dasymutilla chiron</i>	USNMENT 01248237	1	–	–	–	–
	Mutillidae	<i>Dasymutilla gloriosa</i>	USNMENT 01248228	1	–	–	–	–
	Mutillidae	<i>Dasymutilla klugii</i>	USNMENT 01248204	3	–	–	1	1
	Scoliidae	<i>Scoliidae</i> sp.	USNMENT 01248235	–	–	–	–	–
	Crabronidae	<i>Stictia carolina</i>	USNMENT 01248245	1	–	–	–	–

Scale: 1 (<0.3%), 2 (0.31–0.6 wt%), 3 (0.61–1.5 wt%) or – (no metal detected)

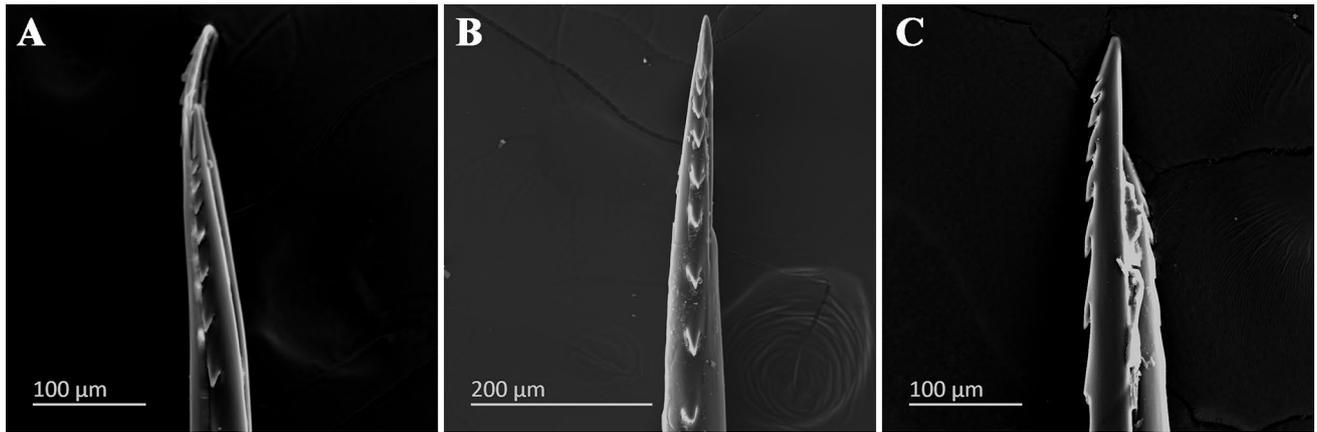
size bias between species giving an independent measure degree that the aculeus was barbed. The degree the aculeus was barbed and metal concentrations (Zn, Fe, Mn, Cu, and Ti) were reconstructed by maximum likelihood in the contMap function in phytools (Revell 2012). We then fit PGLS models (Symonds and Blomberg 2014) in caper (Orme et al. 2015) to test for relationships between metal content and degree of barbs.

## Results

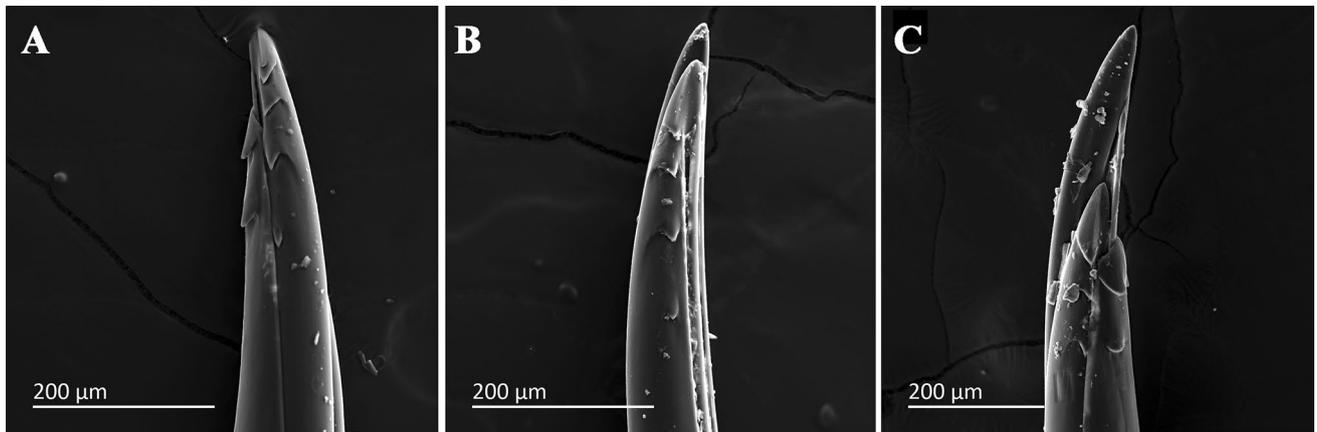
### Morphology

Scanning electron micrographs showed striking differences in the aculeus morphology of Apoidea and other Aculeata (Figs. 1, 2, 3, 4 and 5). The tip of the aculeus

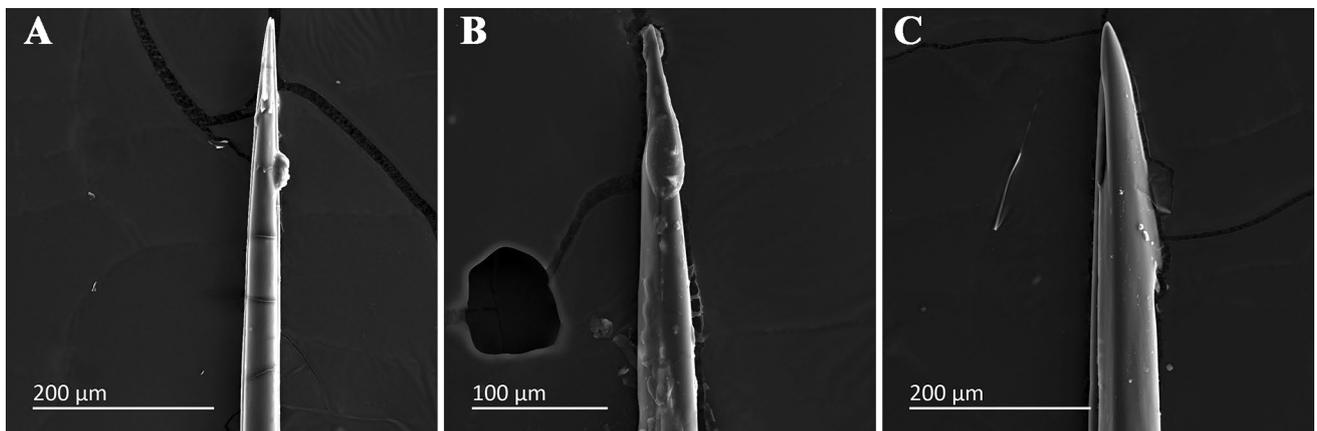
of *Apis* species is armed with backward sloping barbs (Fig. 1). *Bombus aculei* have fewer barbs at the tip and the barbs are much smaller (Fig. 2). Barbs on the aculeus shaft of *Xylocopa* species are undeveloped or absent (Fig. 3). *Centris* and *Diadasia* bees have a short bump-like protrusion on the end of the aculeus (Fig. 3). Barbs on the aculei of Vespidae and Formicidae show a greater degree of morphological variation than found in Apoidea (Figs. 4, 5, 6). Ancestral reconstruction showed that barbs present on the aculeus either had a single common ancestor and has convergently lost multiple occasions or has evolved convergently on multiple, perhaps as many as seven, occasions (Fig. 9). Notably, *Agelaia* and *Mischocyttarus* both lack serrations despite being nested within a group of highly serrated species, rendering serrated social wasp species non-monophyletic.



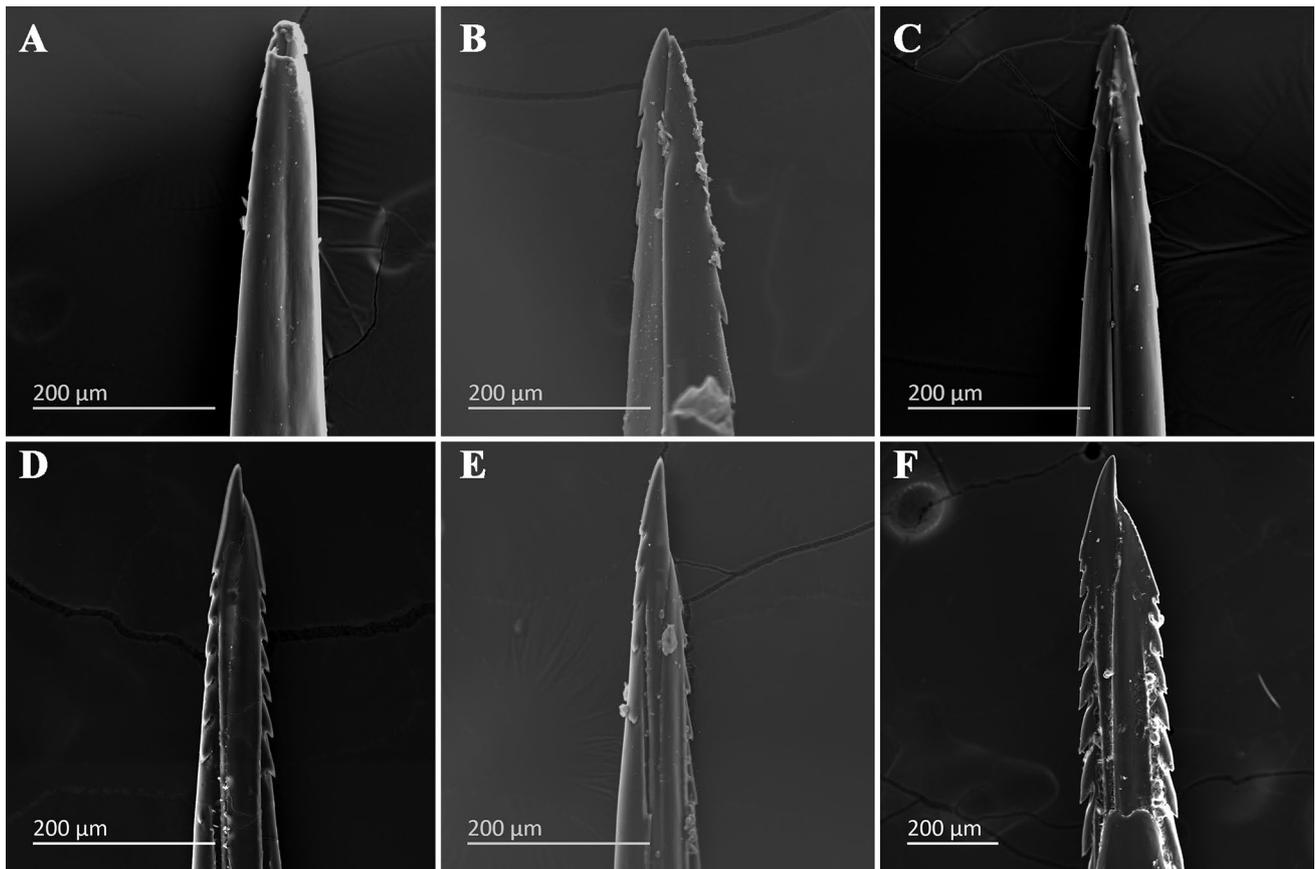
**Fig. 1** Scanning electron microscope images of the *Apis* genus representatives: **a** *Apis cerana* lateral view; **b** *Apis dorsata* lateral view; and **c** *Apis mellifera* ventral view



**Fig. 2** Scanning electron microscope images of the *Bombus* genus representatives: **a** *Bombus impatiens* lateral view; **b** *Bombus huntii* lateral view; and **c** *Bombus sonorus* lateral view

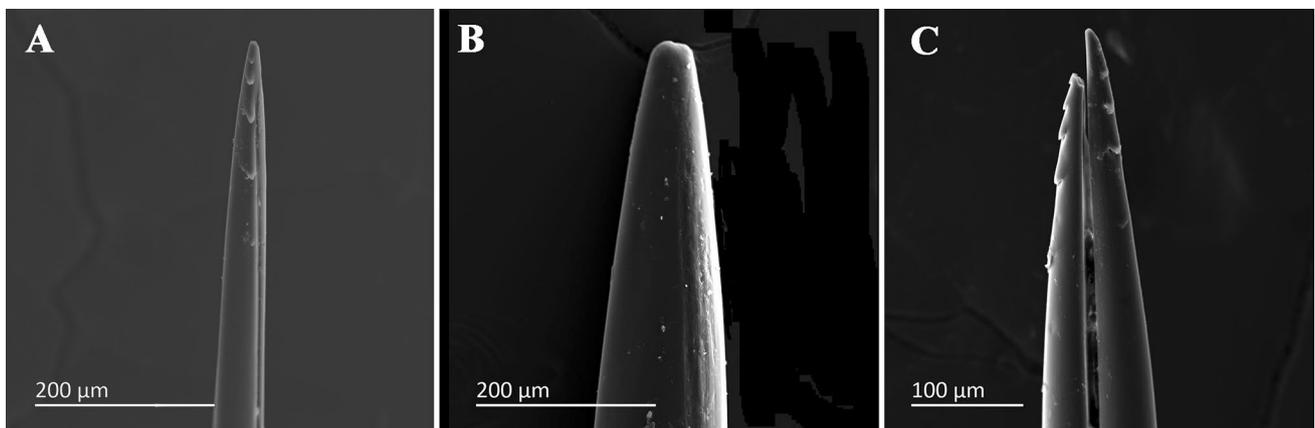


**Fig. 3** Scanning electron microscope images of the solitary bee representatives: **a** *Centris rhodipus* dorsal view; **b** *Diadasia rinconis* dorsal view; and **c** *Xylocopa rufa* lateral view

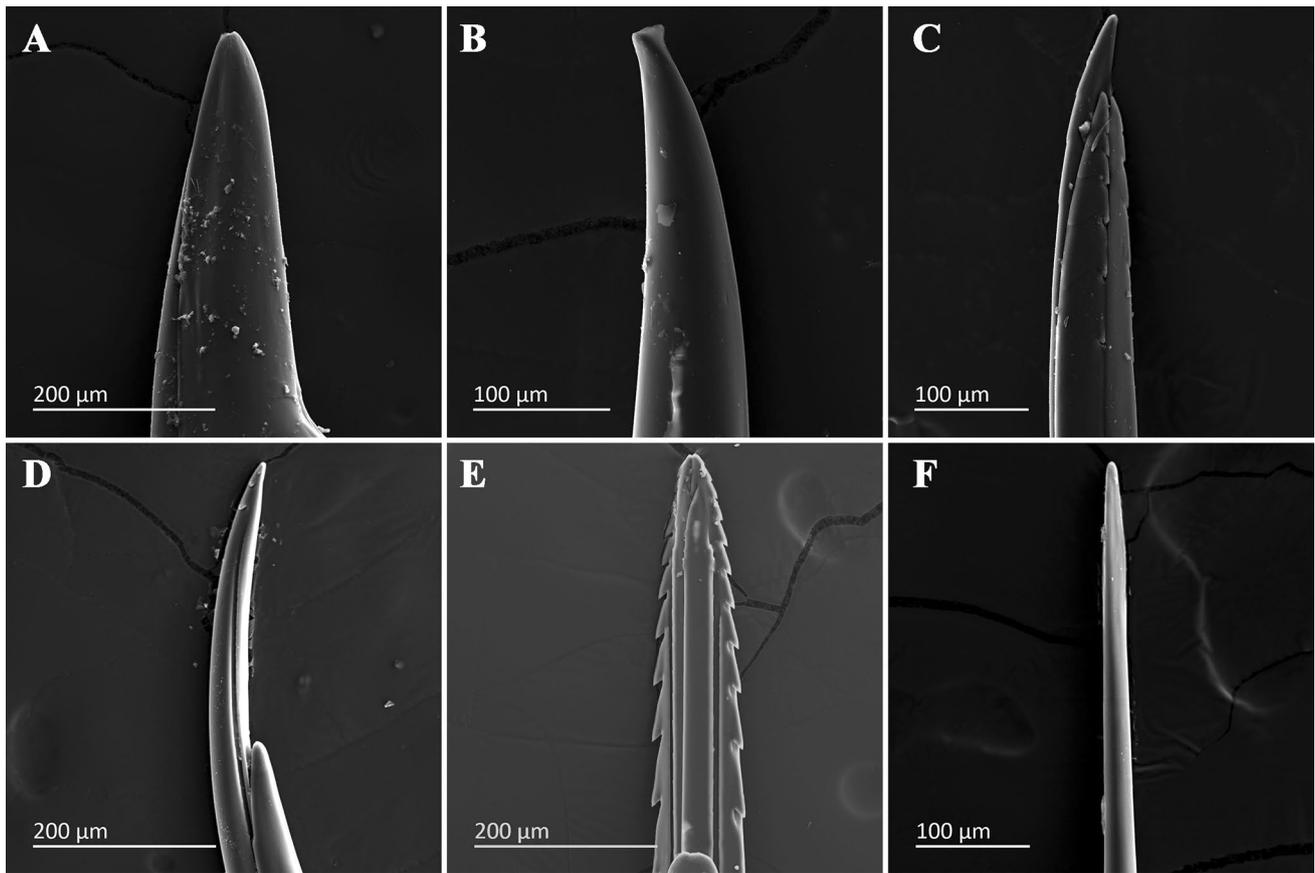


**Fig. 4** Scanning electron microscope images of the social wasp representatives: **a** *Agelaiia myrmecophila* lateral view; **b** *Dolichovespula maculata* lateral view; **c** *Polistes dorsalis* lateral view; **d** *Provespa*

*anomala* lateral view; **e** *Synoeca septentrionalis* lateral view; and **f** *Vespa mandarinia* lateral view



**Fig. 5** Scanning electron microscope images of the solitary wasp representatives: **a** *Dasyutilla glorisa* lateral view; **b** *Scolidae* sp. dorsal view; and **c** *Stictia carolina* ventral view



**Fig. 6** Scanning electron microscope images of Formicidae representatives: **a** *Ectatomma tuberculum* dorsal view; **b** *Gnamplogenys mordax* dorsal view; **c** *Myrmecia gluosa* lateral view; **d** *Odontoponera*

spp. ventral view; **e** *Paraponera clavata* ventral view; and **f** *Pogonomyrmex occidentalis* dorsal view

### Elemental composition and distribution

Iron (Fe), zinc (Zn), manganese (Mn), titanium (Ti) and copper (Cu) were found to be present in the aculei and almost exclusively in the distal region. The metals were found in minor concentrations between 0.02 and 1.5 mass percent (Table 1). Metals were detected in all aculeate families studied (Figs. 7, 8, 9, 10). Zinc was detected in the families Formicidae, Mutillidae and Vespidae. Among all metals for which high concentrations were detected, zinc content showed the highest taxonomic diversity, suggesting multiple convergent increased accumulations of this metal content. Copper was identified primarily in *Polistes* species, particularly *Polistes dorsalis*, but also in *Apis mellifera*. Manganese was found in most families with the major exception of Formicidae, but concentrations above 0.3% were only in two species: *Bombus sonoroides* and *Paraponera clavata*. High concentrations of iron were found only in the aculeus of the giant vespid species *Vespa mandarinia*, while a high concentration of titanium was

restricted to *Leptogenys elongata*. Representative spectra of metal accumulation in aculeate aculei are presented in Fig. 7. Figure 8 shows the compositional imaging performed on *Paraponera clavata* with metals concentrated in the distal region of the aculeus, non-uniformly distributed. Potassium, chloride and phosphorous are scattered throughout the end of the aculeus, whereas zinc is restricted to the distal end of the aculeus.

### Phylogenetic generalised least squares regression

We found that barbs present on the aculeus were not related to sociality (PGLS:  $t = 1.28$ ,  $df = 1$ ,  $p = 0.20$ ) or the presence of Ti (PGLS:  $t = -0.13$ ,  $df = 1$ ,  $p = 0.89$ ), Fe (PGLS:  $t = 0.89$ ,  $df = 1$ ,  $p = 0.37$ ), Mn (PGLS:  $t = -0.27$ ,  $df = 1$ ,  $p = 0.79$ ), and Cu (PGLS:  $t = 1.25$ ,  $df = 1$ ,  $p = 0.21$ ). However, higher levels of Zn (PGLS:  $t = 1.91$ ,  $df = 1$ ,  $p = 0.06$ ) in the aculeus were marginally associated with barbs in the aculeus.

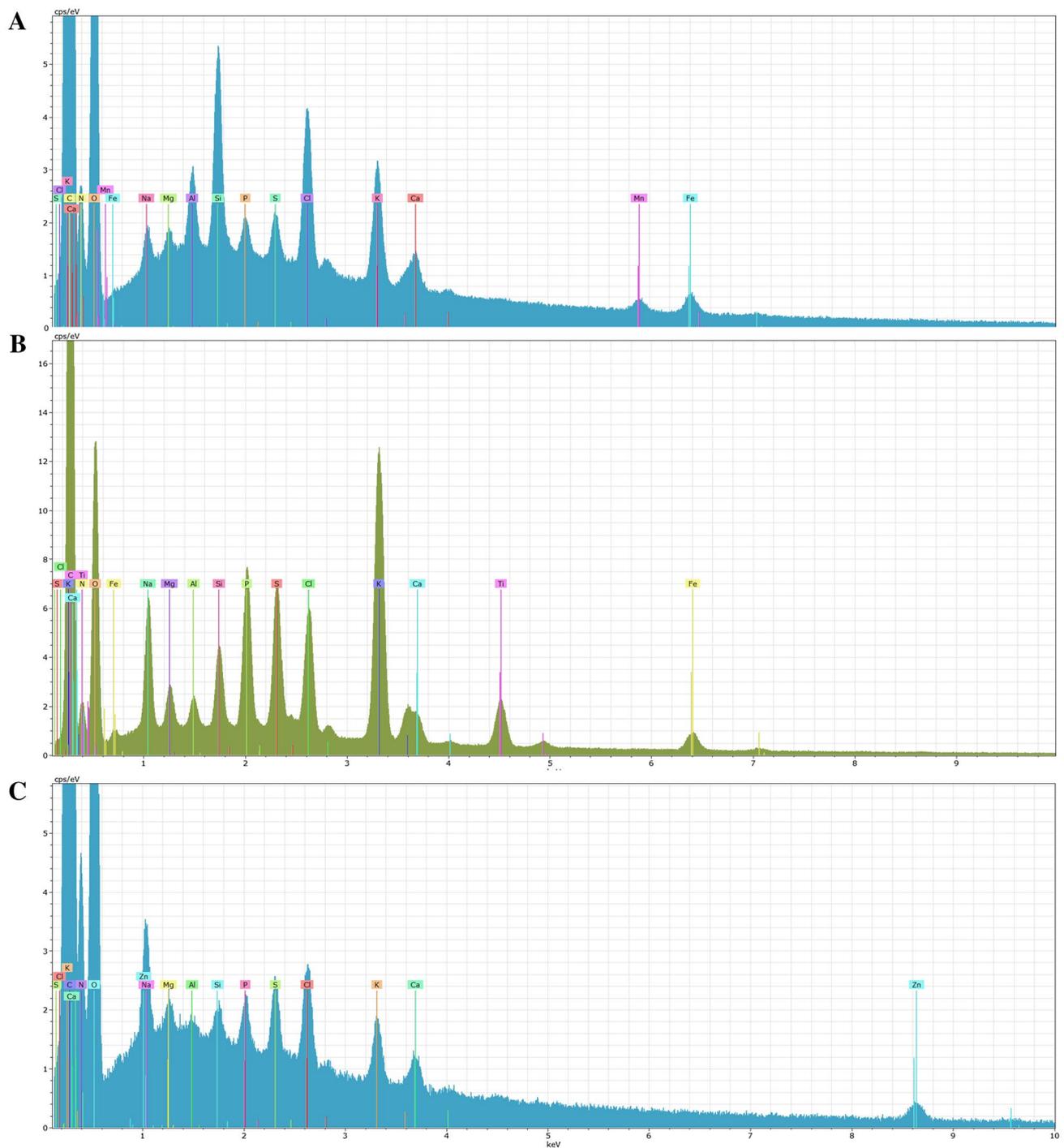


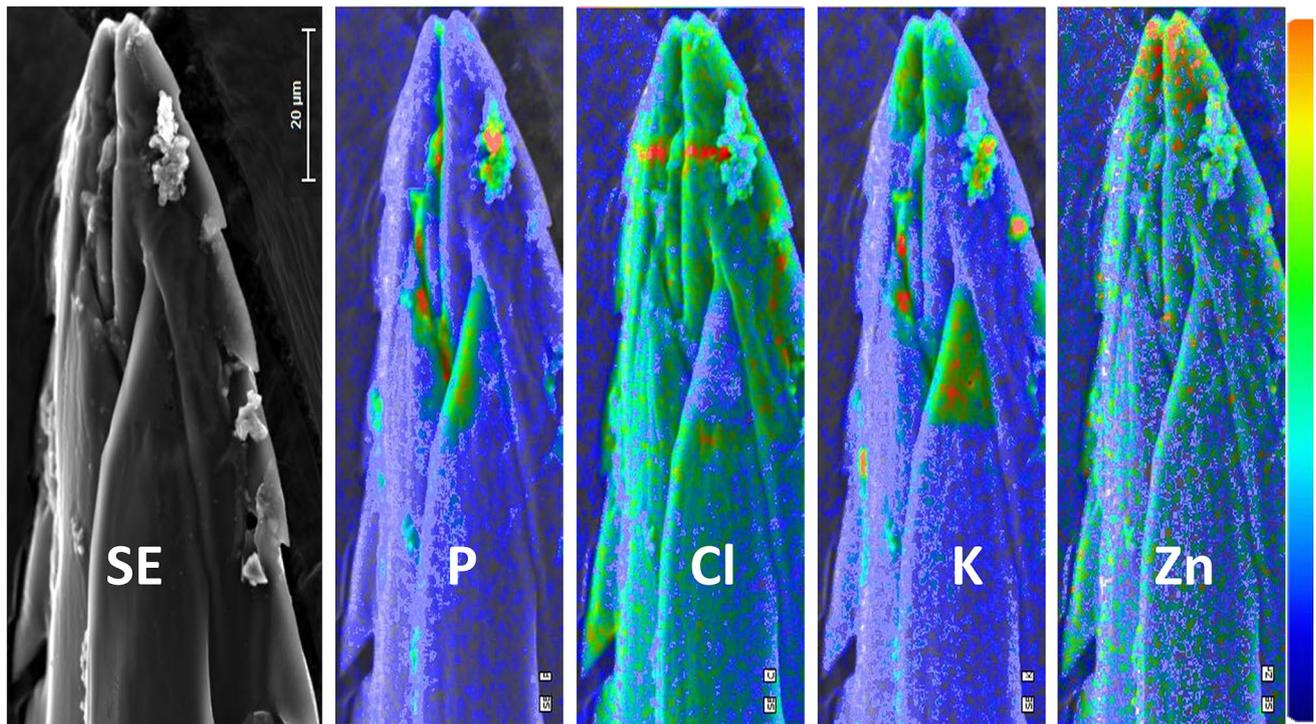
Fig. 7 Representative energy-dispersive X-ray spectra: **a** *Bombus sonorus*; **b** *Miscocyttarus avitarsus*; **c** *Pogonomyrmex maricopa*

## Discussion

### Morphology

Scanning electron micrographs showed striking differences in the morphologies of aculei across aculeates. The aculei in the genus *Apis* are all armed with backward sloping barbs

(Fig. 1). These barbs are one of the reasons aculeus autotomy (the self-amputation of the aculeus) occurs in honeybees (Hermann 1971). It has also been established that the muscles surrounding the aculeus in *Apis* species are significantly reduced, which contributes to aculeus autotomy (Maschwitz and Kloft 1981). The distance between each successive barb increases proximally in *Apis*. This arrangement has been



**Fig. 8** Compositional imaging of *Paraponera clavata* stinger with 25° tilt and TOA 60°

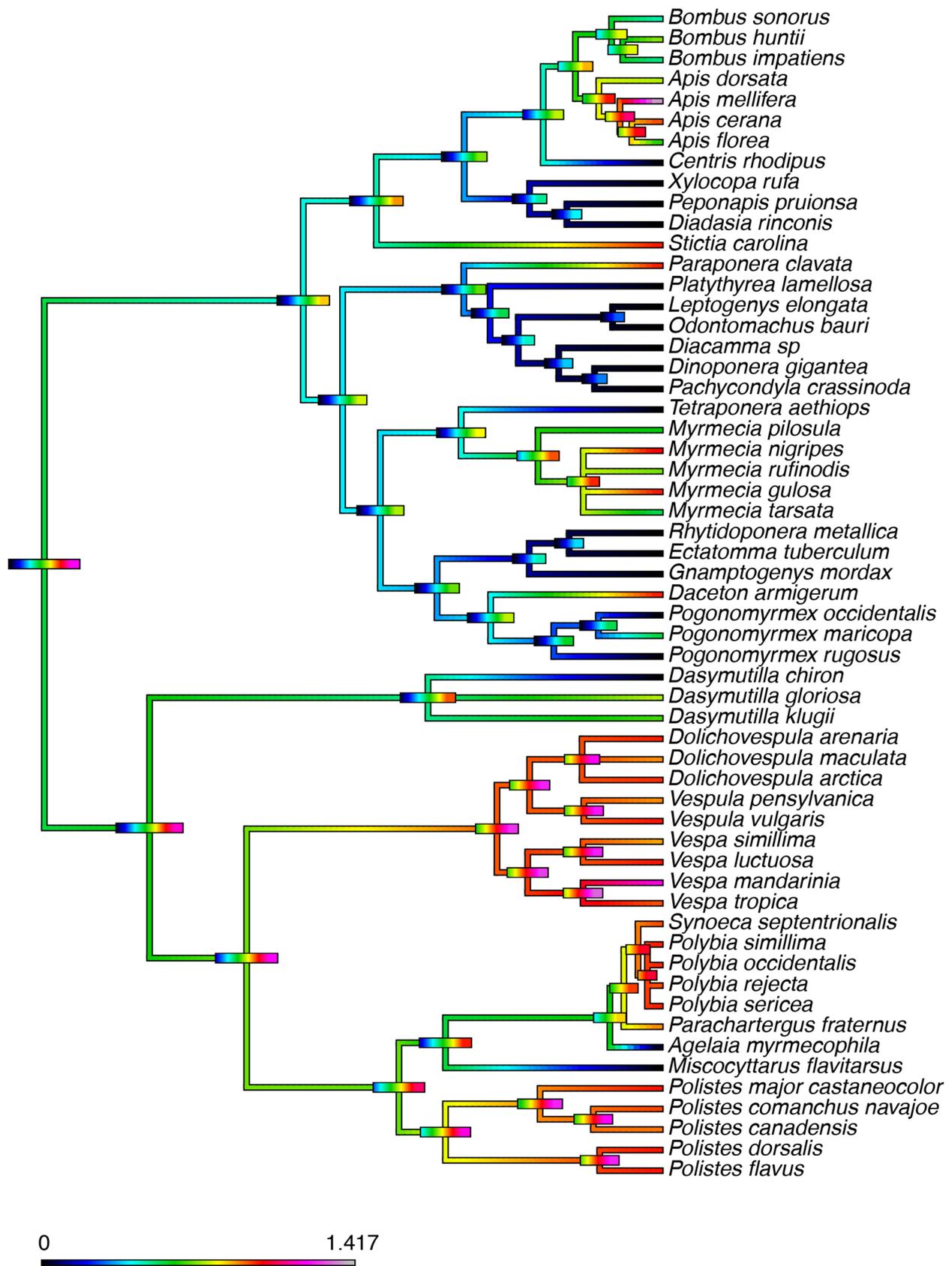
postulated to assist in the firm penetration of each acute barb into the victim's body, thus ensuring that the aculeus cannot be retracted (Ramya and Rajagopal 2008). In contrast, species in the genus *Bombus* are able to retract their aculeus, which is reflected in the morphology of the barbs on the aculeus (Fig. 2). No solitary bee species show aculeus autotomy, which is consistent with the evidence provided herein by the lack of barbs (Fig. 3), although *Centris rhodipus* and *Diadasia rinconis* do have a short bump-like protrusion on the end of the stinger (Fig. 3a, b).

The aculeus of species examined from the family Vespidae showed a range of morphological variations from small barbs to large barbs (Fig. 4). Aculeus autotomy has also been found in genera of Vespidae (Shorter and Rueppell 2012). The aculei from the solitary wasp species (Fig. 5) were similar to the aculei of solitary bee species (Fig. 3) in lacking any substantial barbs. The aculei from Formicidae predominately lacked barbs (Fig. 6).

Aculeus autotomy is only known to occur in social Hymenoptera and has evolved independently on at least three separate occasions; bees of the genus *Apis*, ants of the genus *Pogonomyrmex*, and several species in the tropical wasp tribes Epiponini, Polistini, and Rhopalidiini (Hermann 1971, 1984; Sledge et al. 1999). Aculeus autotomy has previously been attributed to barb size (Hermann 1984). The species found with barbs on the

aculeus all belonged to groups that are known to present with aculeus autonomy with the exception of *Polistes* and *Mischocyttarus*, which have not been found to autotomise their aculeus. This suggests that barb presence and size may facilitate aculeus autotomy but are most likely not a requirement for this trait.

Barbs on the aculeus may be the ancestral trait in aculeates that has subsequently been secondarily lost in certain groups of aculeates, or they may have evolved separately on numerous occasions. If barbed aculei are the ancestral state this would follow that barbs have been found in hymenopterans outside aculeates; evidenced by the superfamilies Ichneumonoidea and Chalcidoidea (Kundanati and Gundiah 2014; Quicke et al. 1999). There have been no studies done on the morphology of the aculeus in Chrysidoidea. However, research focusing on aculeus morphology Chrysidoidea and Symphyta may further elucidate the evolutionary trajectory of this trait and whether it is the result of convergent evolution. The barbs have become more prominent in certain lineages of aculeates, most notably Apidae (Figs. 1, 2). However, as seen in certain Formicidae species (Fig. 6), barbs have been lost in some aculeate lineages. This loss of barbs on the aculeus in some lineages of aculeates is not significantly dependent on sociality but rather some other undetermined selection pressure.



**Fig. 9** Ancestral state reconstructions over branches for serrations where warmer colours represent a greater degree of serration in the aculeus

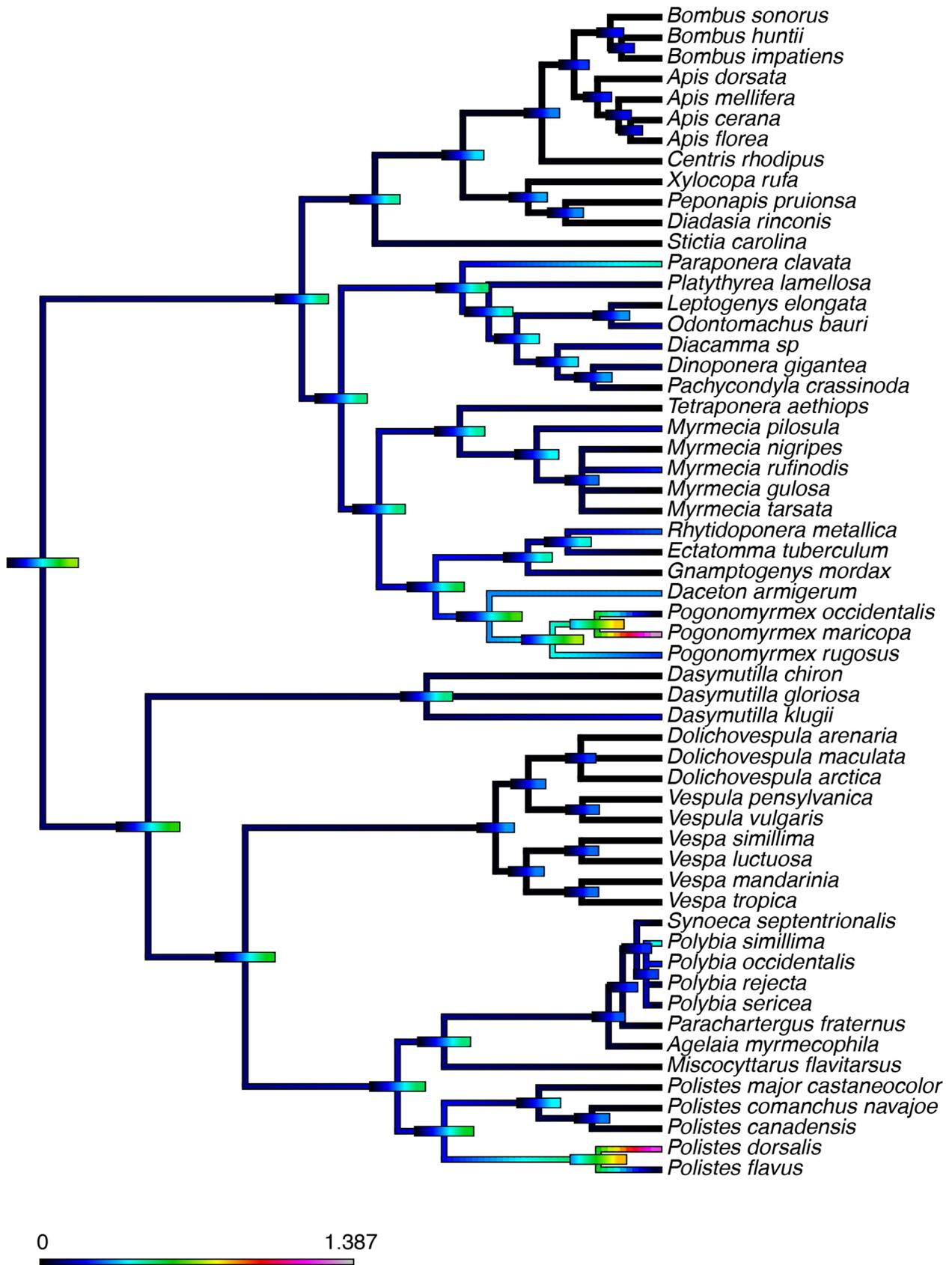


Fig. 10 Ancestral state reconstructions over branches for zinc where warmer colours represent a higher concentration of zinc in the aculeus

## Elemental composition and distribution

We also examined the presence of metals in the aculei. Transition metals were found in all families of aculeates studied; however, there were species within most of these families that do not show the presence of any detectable metals in the aculeus (Table 1). As shown previously in parasitoids, there is considerable variation between taxa in whether or not they have high concentrations of metals associated with the aculeus (Quicke 1998). This variation may be informative of the life history of the species, but further studies would be needed to confirm this.

Metals identified in the cuticle of the aculeus were iron, zinc, manganese, titanium and copper. Phylogenetic analyses found no strong relationship between the presence of iron, copper, manganese, or titanium and barbs on the aculeus, indicating a lack of direct evolutionary selection pressure between barbs present on the aculeus and concentrations of these metals (Figs. 11, 12, 13, 14). However, there was a marginally positive relationship between barb presence and zinc (Fig. 10). Earlier research has shown that zinc enrichment in insects plays a functional role in the enhancing mechanical properties such as hardness, which may be why it has been linked to barb presence in this study (Edwards et al. 1993; Hillerton and Vincent 1982; Schofield et al. 2002). Manganese-enriched cuticles have been linked to increases in cuticle density and/or resistance to fracture (Morgan et al. 2003). Thus, indicating a role in species of this study, which is not co-selected for in relation to degree of barbing, but guided by unknown selection pressures. Iron and copper, while also quite common in the species sampled, are less studied and the particular effect these metals have on cuticle mechanical properties remains unclear.

Iron, zinc, manganese, and copper were found at 0.02–1.5 weight mass percent of the cuticle (Table 1). This is a relatively minor fraction of the bulk composition and it has been postulated that these metals are present at levels too low to affect changes in mechanical properties (Schofield et al. 1997). It seems likely that the low percentage of metals compared to those found in parasitoid species may be a result of the aculei not being required to be especially hardened or abrasion resistant, in comparison to wood-boring in gall-wasps or fig-wasps, for example. However, the cuticle is a complex matrix and still not well understood regarding its mechanical properties. The hardest insect material found was in the jaws of a jewel beetle larva that lacked the presence of metals and fed on wood. In seeming contradiction, the similarly dark-coloured adult beetle mandibles contain the transition metal manganese, but were significantly softer (Cribb et al. 2010). This suggests that even though there are only low concentrations of metals present in the aculeus, it is not necessarily a softer material.

SEM-EDS point analyses reveal that metals occur almost exclusively around the tips and edges of the aculeus but rarely in the surrounding areas of the cuticle. Thus, while the overall metal content is low, the selective placement suggests an adaptive functional role. Consistent with this, enhanced amounts of zinc have also been found concentrated mainly in the ovipositor tips of several wood-boring wasps (Quicke 1998), several gall-parasitoid wasps (Polidori et al. 2013), and the parasitic fig wasp *Apocrypta* (Kundanati and Gundiah 2014). To investigate the compositional differences further, compositional imaging was undertaken on the bullet ant *Paraponera clavata*. Again, the elemental distribution of the metals was non-uniform and concentrated in the distal region of the aculeus where the mechanical impact is expected to be highest. The tip of the *P. clavata* aculeus was enriched in chlorine, phosphorus, potassium, and zinc by factors of 6–100 times relative to bulk chitin and protein. This compositional imaging highlights that the presence of metals is concentrated in the tips of the aculei, which is to be expected with the tip bearing the brunt of any mechanical damage.

The forms of metal selectivity and sequestration is of interest to understand the mechanisms of metal binding in the organism; however, these processes are not well understood. There is limited evidence to support habitat, diet or phylogeny in determining why certain metals are selected (Cribb et al. 2008; Fontaine et al. 1991; Hillerton et al. 1984; Hillerton and Vincent 1982; Schofield 2005; Schofield et al. 2002). Recently, it has been postulated that genetic and cellular regulation may have the greatest control over which metals are utilised, rather than environmental metal availability (Degtyar et al. 2014). The mechanisms for the biological sequestration of metals have not been well studied either. However, two potential binding mechanisms have been proposed: binding to amino acid side chains of cuticular proteins, or binding to catecholate ligands, which accumulate during cuticle sclerotization (Schofield 2005). Nevertheless, our results do not allow us to distinguish these potential mechanisms and this is, therefore, an open question for future research. The link found between the presence of zinc and barb presence on the aculeus is interesting and could be useful as a model system to study the roles that these different metals play in the mechanical properties of the insect cuticle.

## Conclusion

In conclusion, we used scanning electron imaging coupled with energy-dispersive X-ray analysis to investigate the structure and metal composition of the aculeate aculeus. Through such methods, we show unique morphological features in the aculeus of aculeates, which are not associated

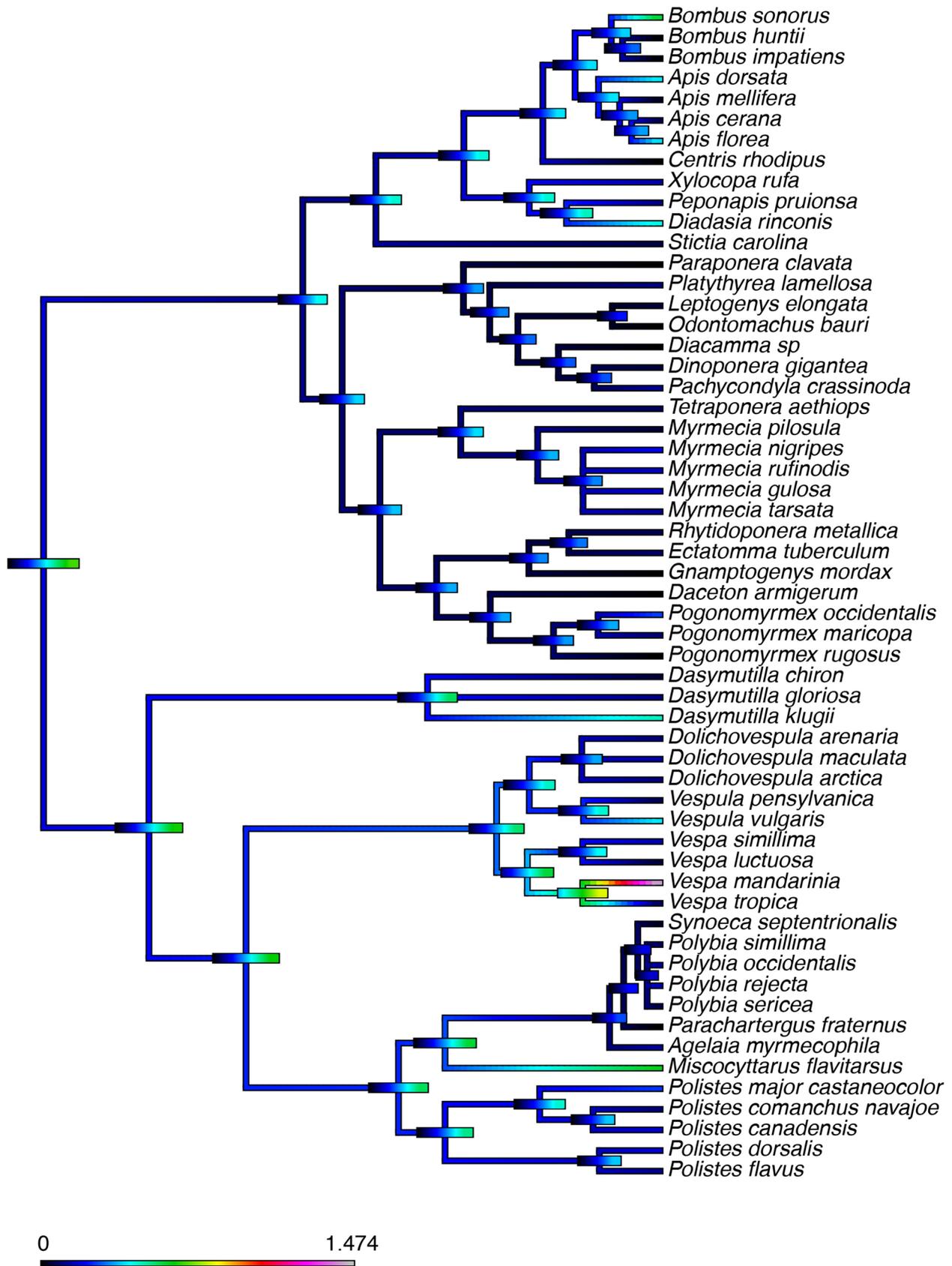


Fig. 11 Ancestral state reconstructions over branches for iron where warmer colours represent a higher concentration of iron in the aculeus

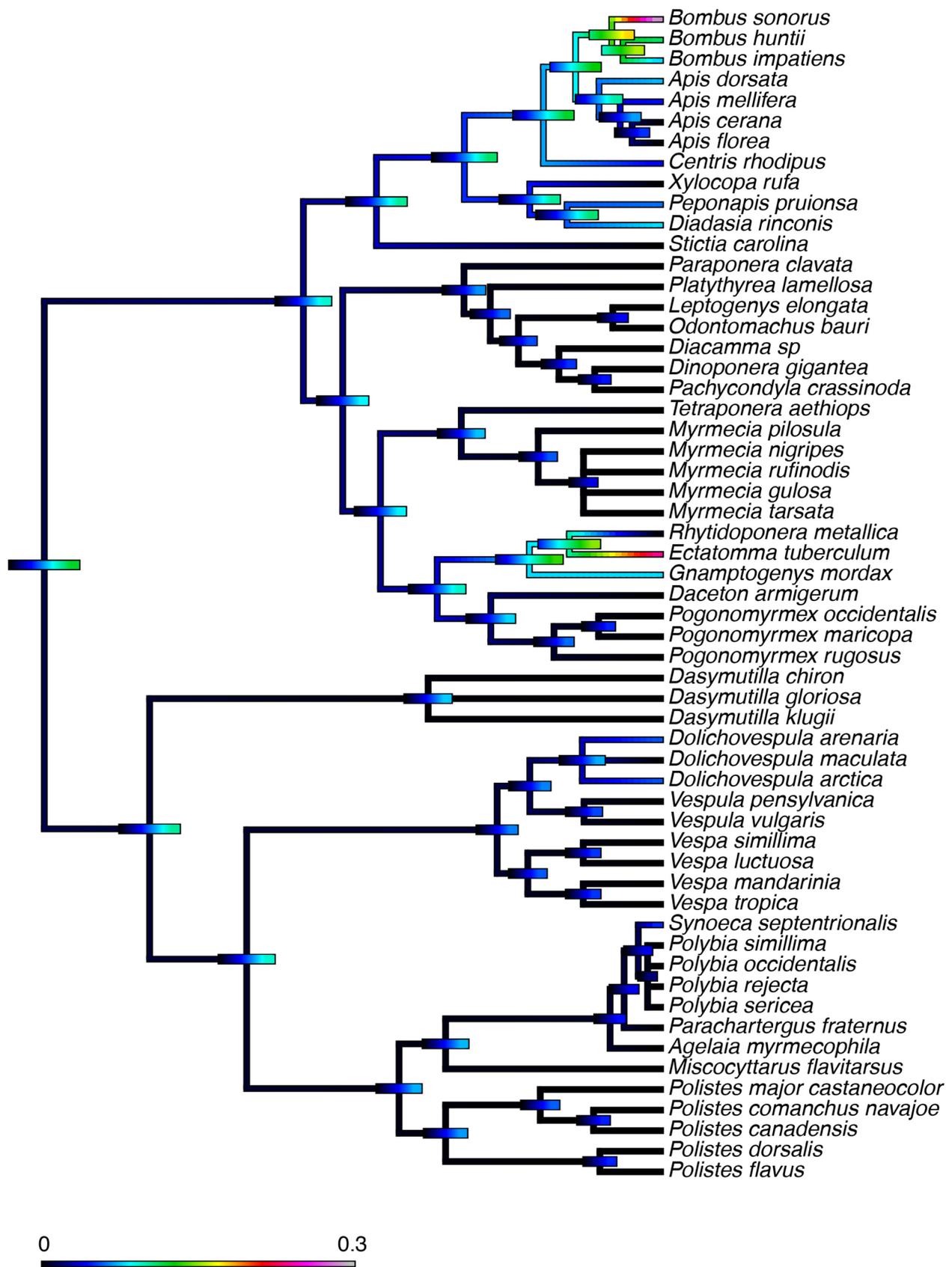


Fig. 12 Ancestral state reconstructions over branches for manganese where warmer colours represent a higher concentration of manganese in the aculeus

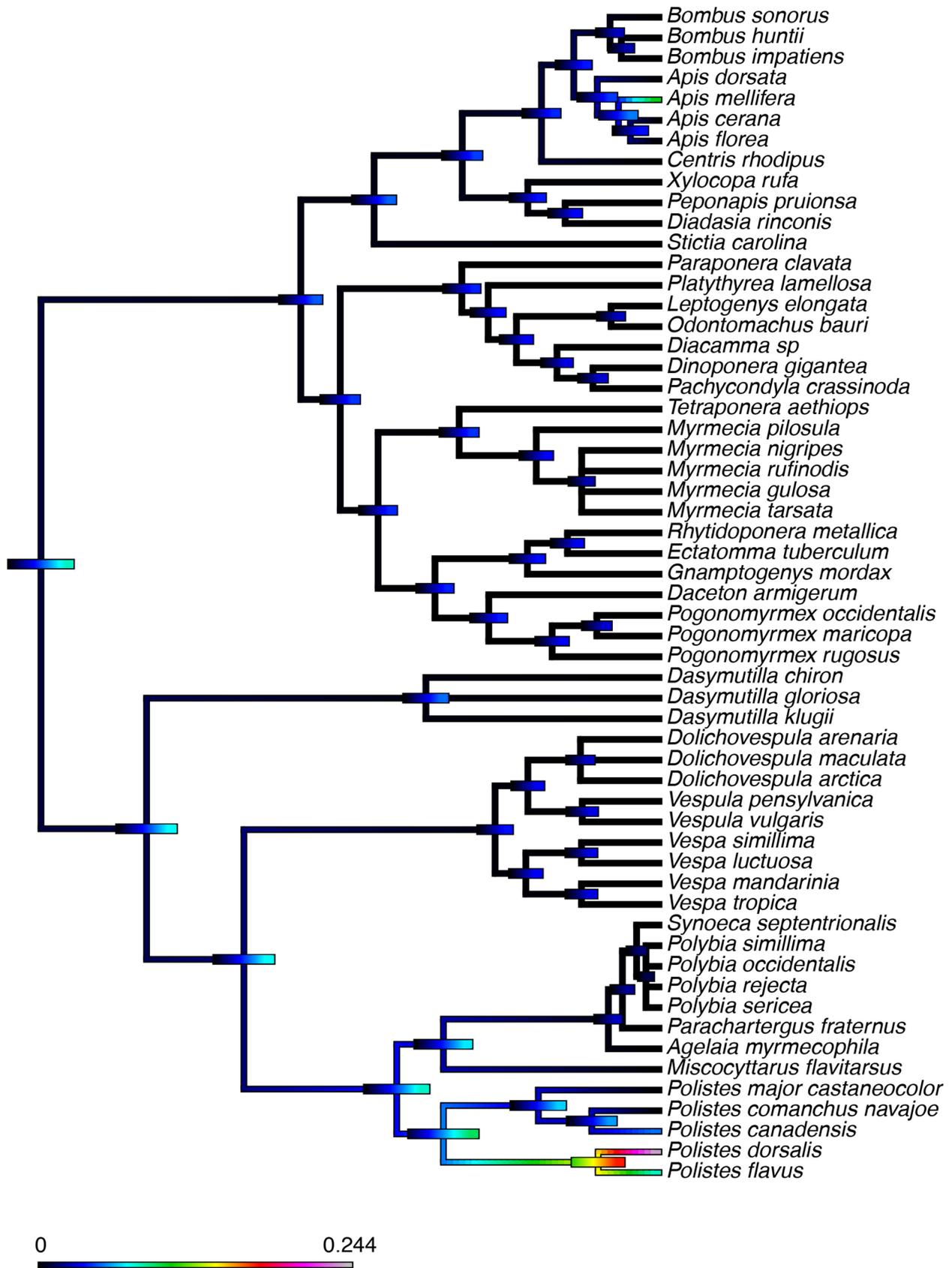


Fig. 13 Ancestral state reconstructions over branches for copper where warmer colours represent a higher concentration of copper in the aculeus

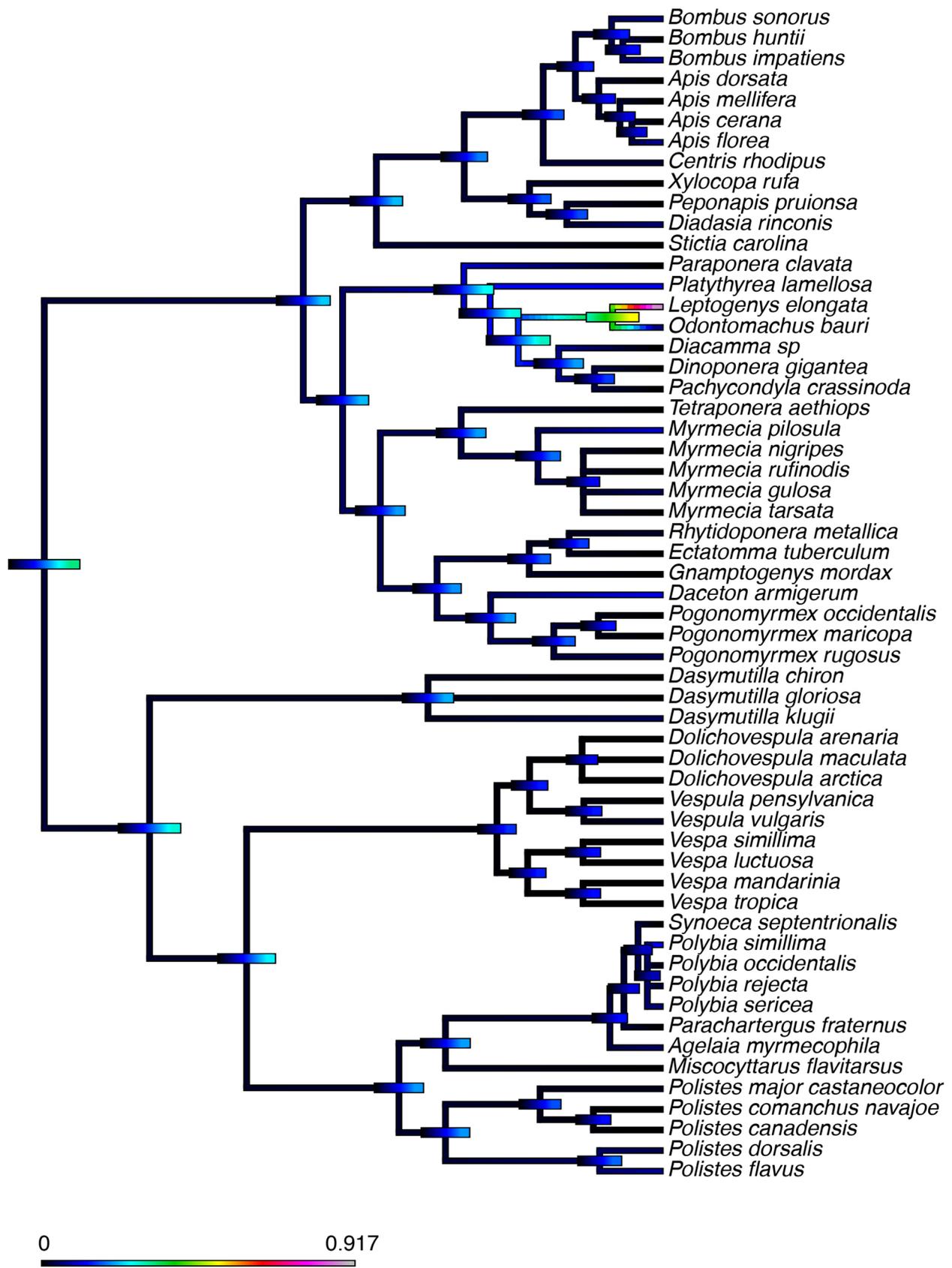


Fig. 14 Ancestral state reconstructions over branches for titanium where warmer colours represent a higher concentration of titanium in the aculeus

with sociality. Our findings are also the first to show metal accumulation in ovipositors that are used exclusively for the delivery of venom. This research aids in understanding the evolution of these understudied insect venom systems and will have wide-reaching effects pertaining to the evolution and ecology of stinging wasps, bees, and ants.

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