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RESEARCH ARTICLE

Marked interspecific differences in the neuroanatomy of the male olfactory system of honey bees (genus *Apis*)

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Abstract

All honey bee species (genus *Apis*) display a striking mating behavior with the formation of male (drone) congregations, in which virgin queens mate with many drones. Bees' mating behavior relies on olfactory communication involving queen—but also drone pheromones. To explore the evolution of olfactory communication in *Apis*, we analyzed the neuroanatomical organization of the antennal lobe (primary olfactory center) in the drones of five species from the three main lineages (open-air nesting species: dwarf honey bees *Apis florea* and giant honey bees *Apis dorsata*; cavity-nesting species: *Apis mellifera*, *Apis kochevnikovi*, and *Apis cerana*) and from three populations of *A. cerana* (Borneo, Thailand, and Japan). In addition to differences in the overall number of morphological units, the glomeruli, our data reveal marked differences in the number and position of macroglomeruli, enlarged units putatively dedicated to sex pheromone processing. Dwarf and giant honey bee species possess two macroglomeruli while cavity-nesting bees present three or four macroglomeruli, suggesting an increase in the complexity of sex communication during evolution in the genus *Apis*. The three *A. cerana* populations showed differing absolute numbers of glomeruli but the same three macroglomeruli. Overall, we identified six different macroglomeruli in the genus *Apis*. One of these (called MGb), which is dedicated to the detection of the major queen compound 9-ODA in *A. mellifera*, was conserved in all species. We discuss the implications of these results for our understanding of sex communication in honey bees and propose a putative scenario of antennal lobe evolution in the *Apis* genus.

KEYWORDS

antennal lobe, honey bee, macroglomerulus, premating isolation, reproduction, RRID: SCR_000450, RRID: SCR_003070, RRID: SCR_007353, RRID: SCR_013672, RRID: SCR_014213

1 | INTRODUCTION

In a wide range of animal species, interpopulational diversification of courtship traits and associated preferences provide a basis for premating isolation and speciation (Ritchie, 2007). In insects, reproduction generally involves the search for mating partners through the detection of sexual pheromonal blends (Ayasse, Paxton, & Tengö, 2001). Co-evolution of sexual pheromones and of the olfactory system are then critical for the emergence of barriers to genetic exchange among populations (Bacquet et al., 2015; Gabirot, Lopez, & Martín, 2012; Smadja & Butlin, 2009; Wyatt, 2010). In many cases, the females of closely related species produce the same major compound and the relative proportions of secondary components endow the pheromonal blend with its species-specificity (moths: Groot et al.,

Abbreviations: AL, antennal lobe; AMMC, antennal mechanosensory and motor centre; 9-HDA, 9-hydroxy-(E)-2-decenoic acid; 10-HDA, 10-hydroxy-(E)-2-decenoic acid; 9-ODA, 9-oxo-2-decenoic acid; HOB, methyl p-hydroxybenzoate; HVA, 4-hydroxy-3-methoxyphenylethanol; LCA, last common ancestor; LH, lateral horn; LN, local interneuron; MB, mushroom bodies; MG, macroglomerulus; OR, olfactory receptor; OSN, olfactory sensory neuron; PN, projection neurons; QMP, queen mandibular pheromone; SEZ, subesophageal zone

2006; Saveer et al., 2014; Groot, Dekker, & Heckel, 2016; honey bees: Plettner et al., 1997). During speciation, the rapid adaptation of males' olfactory system for detecting and processing females' secondary products and using them as honest sexual signals may be instrumental for maximizing the detection of the proper mating partner (Berg, Almaas, Bjaalie, & Mustaparta, 1998; Engsontia, Sangket, Chotigeat, & Satasook, 2014; Lee, Vickers, & Baker, 2006; Namiki, Daimon, Iwatsuki, Shimada, & Kanzaki, 2014; Todd, Anton, Hansson, & Baker, 1995). The comparative study of the olfactory systems of closely related species might thus help identify pivotal adaptations in the evolution of new species and the selective pressures involved.

In insect, olfactory sensory neurons (OSNs), which detect odorants, are housed in olfactory sensilla on the antennae (Zacharuk, 1985). Each OSN typically expresses a single functional olfactory receptor (OR) protein and individual OSNs hence generally respond only to a limited number of chemical compounds (de Fouchier et al., 2017; Grabe et al., 2016; Hallem & Carlson, 2006; Hallem, Ho, & Carlson, 2004; Pask et al., 2017; Slone et al., 2017). OSN axons project to the first olfactory processing center of the insect brain, the antennal lobe (AL), which contains multiple morphological and functional units, the glomeruli (Baumann, Oland, & Tolbert, 1996; Shepherd, 1974). All OSNs expressing a particular OR gene converge on the same glomerulus (Couto, Alenius, & Dickson, 2005; Vosshall, Wong, & Axel, 2000). Consequently, each glomerulus responds to a relatively small number of odorants and natural odors generally evoke an odor-specific combination of glomerular activity in the AL (Galizia & Szyszka, 2008; Sandoz, 2011). AL glomeruli are interconnected by a dense network of local, mostly inhibitory interneurons (LN) which process olfactory information, before it is conveyed by projection neurons (PN) to higher order processing centers, the mushroom bodies (MB) and the lateral horn (LH) (Abel, Rybak, & Menzel, 2001; Fonta, Sun, & Masson, 1993; Mobbs, 1982).

Within a given species, the AL displays a highly stable organization, defined by a similar number, volume and spatial arrangement of the glomeruli across individuals of the same sex. However, it usually presents clear differences between species (Anton & Homberg, 1999; Hansson & Anton, 2000). The peculiar anatomical compartmentation of the AL in well-defined morphological units (the glomeruli) provides an ideal tool for investigating anatomical and functional adaptations of the sensory system (Hansson & Stensmyr, 2011). A larger number of AL glomeruli is usually associated with an insects' sensitivity to a great diversity of semiochemicals and to enhanced olfactory discrimination abilities, whereas larger glomerular volumes suggest a larger number of OSNs feeding into this glomerulus, thereby enhancing the insects' sensitivity to certain odorants. This last situation is especially observed in the males of numerous insect species, which evolved a sexually dimorphic olfactory system tuned to the detection of female sexual signals. They are characterized in the AL by the presence of hypertrophied glomeruli, called macroglomeruli, which are usually dedicated to the detection of characteristic compounds of their conspecific pheromone (Arnold, Masson, & Budharugsa, 1985; Hansson, Christensen, & Hildebrand, 1991; Nishikawa et al., 2008; Nishino, Iwasaki, Kamimura, & Mizunami, 2012; Sandoz, 2006).

The honey bees (tribe Apini, genus *Apis*) are a group of eusocial bee species (Hymenoptera: Apidae) characterized by a set of remarkable behaviors, among which the construction of perennial, colonial nests made from wax, extreme multiple mating by queens and the use by workers of a complex communication system known as the dance language (Koeniger, Koeniger, & Tingek, 2010; Oldroyd & Wongsiri, 2006). The genus consists of nine recognized species, classically divided in three groups: the dwarf honey bees (among which *Apis florea*), the giant honey bees (among which *Apis dorsata*), and the cavity-nesting honey bees (among which *Apis mellifera*, *Apis cerana*, and *Apis koschevnikovi*) (Hepburn & Radloff, 2011; Koeniger, Koeniger, & Tiesler, 2014; Oldroyd & Wongsiri, 2006; Ruttner, 1988). These species can be differentiated by characters such as size, nest construction, complexity of the waggle dance, division of labor, and foraging behavior among others (Arias & Sheppard, 2005; Koeniger et al., 2010; Raffiudin & Crozier, 2007; Ruttner, 1988). However, all species display a very similar and particularly striking mating behavior (Baer, 2005; Hepburn & Radloff, 2011; Koeniger et al., 2014; Koeniger & Koeniger, 2000). During the mating season, when climatic conditions are adequate, sexually mature males gather in the air and form so-called drone congregations (Koeniger & Koeniger, 2000; Koeniger & Koeniger, 2004; Loper, Wolf, & Taylor, 1987; Loper, Wolf, & Taylor, 1992). When a virgin queen joins the congregation, a flock of drones attracted by olfactory signals (pheromones) rushes towards the female (Gries & Koeniger, 1996). Then, the queen mates with multiple drones which generally die directly after copulation (see Table 1; Baudry et al., 1998; Palmer & Oldroyd, 2000; Oldroyd & Wongsiri, 2006; Hepburn & Radloff, 2011).

In South-East Asia, several *Apis* species live in sympatry and reproductive isolation is maintained by a number of pre- and postzygotic barriers, as for instance different daily mating periods and locations, different genitalia shapes, incompatibilities for sperm storage and fertilization, etc. (Koeniger et al., 2010; Koeniger & Koeniger, 2000; Oldroyd & Wongsiri, 2006). The relative role that differences in olfactory sex communication play in this reproductive isolation is still unclear. One compound produced by the queen mandibular glands, 9-keto-2 (E)-decenoic acid (9-ODA), is known to attract drones from all *Apis* species tested (Butler, Calam, & Callow, 1967; Gary, 1962; Koeniger & Koeniger, 2000; Nagaraja & Brockmann, 2009; Sannasi, Rajulu, & Sundara, 1971; Shearer, Boch, Morse, & Laigo, 1970). However, queens' mandibular glands produce multiple compounds, and the ratios of individual components in the queen pheromonal blend clearly differ among species (Keeling, Otis, Hadisoesilo, & Slessor, 2001; Keeling, Slessor, Koeniger, Koeniger, & Punchihewa, 2000; Plettner et al., 1997; Slessor, Kaminski, King, Borden, & Winston, 1988). Given such interspecific differences in queen-emitted chemical signals, the question naturally arises whether this variation is reflected in the olfactory system of honey bee males.

Because the Western honey bee, *A. mellifera*, has been a traditional scientific model in the study of olfactory communication (Free, 1987; Slessor, Winston, & Le Conte, 2005; Trhlin & Rajchard, 2011) and neural processing (Galizia & Menzel, 2001; Rössler & Brill, 2013; Sandoz, 2011; Sandoz, Deisig, de Brito Sanchez, & Giurfa, 2007; Sinakevitch, Bjorklund, Newbern, Gerkin, & Smith, 2018), substantial information is available about the sensory system involved in sex communication in this species. The drones show a sexually dimorphic olfactory system including longer antennae than females, and seven

times as many olfactory placode sensilla (Brockmann & Brückner, 2001b, 2003; Esslen & Kaissling, 1976). OSNs within these placode sensilla respond to 9-ODA (Kaissling & Renner, 1968; Vareschi, 1971). Transcriptomic studies on drones' antennae have now identified four overexpressed OR genes, one of which is involved in the detection of 9-ODA (Wanner et al., 2007). Pursuant to this antennal sensory adaptation, the antennal lobe of *A. mellifera* drones shows four sexually-dimorphic macroglomeruli (Arnold et al., 1985; Brockmann & Brückner, 1999; Brockmann, Galizia, & Brandt, 2001; Nishino, Nishikawa, Mizunami, & Yokohari, 2009), one of which (MG2) specifically responds to 9-ODA (Sandoz, 2006). To this day, only one study has attempted to describe the drone AL in another *Apis* species. It showed that in the most basal extant species, the dwarf honey bee *A. florea*, drones possess only two macroglomeruli, one of which presents similarities with MG2 in *A. mellifera* (Brockmann & Brückner, 2001a). This observation suggests that the AL of honey bee drones may indeed have undergone substantial adaptations for the detection of species-specific pheromonal blends. However, to retrace the evolution of these traits, AL organization needs to be investigated in a more complete set of species.

In the present study, using anatomical staining and confocal microscopy, we analyzed AL organization in the males of five honey bee species from the three main lineages (dwarf honey bees: *A. florea*, giant honeybees: *A. dorsata*, cavity-nesting honey bees: *A. mellifera*, *A. kochevnikovi*, and *A. cerana*). To compare inter- and intraspecific variations, we also assessed structural variability in the ALs of three different populations of one of our species *A. cerana* (populations from Borneo, Thailand, and Japan). By measuring the numbers of glomeruli, their locations, shapes, and volumes, we assessed the presence of macroglomeruli in each species/population and identified putative homologies. These data provide new clues for understanding adaptive brain changes involved in premating isolation and speciation in the genus *Apis*.

2 | MATERIALS AND METHODS

2.1 | Insects

We investigated the ALs of males from five honey bee species belonging to the *Apis* genus (Hymenoptera, Apidae): two open-air-nesting species, *A. florea* and *A. dorsata*, along with three cavity-nesting species, *A. mellifera*, *A. koschevnikovi*, and *A. cerana* (Supporting Information Table S1). Drones of the dwarf honey bee, *A. florea*, were collected in Thailand (Ratchaburi province) whereas drones of the western honey bee *A. mellifera* were caught in Gif-sur-Yvette (France). Three sympatric species, the giant honey bee *A. dorsata*, the eastern hive bee *A. cerana* and the red honeybee *A. kochevnikovi* were captured in Borneo, Malaysia (Tenom). We also assessed possible differences between three *A. cerana* populations: *A. cerana* from Borneo (Tenom), Thailand (Chumpon province), and Japan (Itoshima city, Fukuoka prefecture). Despite clear genetic and morphometric differences among these populations, they are not yet consensually recognized as subspecies (Hepburn & Radloff, 2011; Oldroyd & Wongsiri, 2006; Radloff et al., 2010; Smith, Villafuerte, Otis, & Palmer, 2000). In this study, we thus named *A. cerana* populations by their geographical origin, for example, *A. cerana* Borneo, *A. cerana* Thailand, and *A. cerana* Japan. All specimens were kept at least 3 months in preservation solutions before the dissection (see Supporting Information Table S1 for details).

2.2 | Brain preparation

The drones were decapitated, and the heads were placed in 4% PFA and kept for at least 5 days at 4 °C to fix the brains homogeneously. The heads were then washed three times in phosphate-buffered saline (PBS) solution and the brains were dissected out. Neuropils were stained in 10% neutral red solution for 3 h (Neutral Red Solution, Buffered; Sigma-Aldrich, Egham, UK). They were then washed in PBS solution (3 × 10 min) and dehydrated in series of ascending ethanol concentrations (50%, 70%, 90%, 95% and 3 × 100%, 10 min each). Finally, the brains were clarified in methylsalicylate (Sigma-Aldrich, Steinheim, Germany) for at least 3 days at 4 °C.

2.3 | Confocal microscopy

For observation of the drones' ALs, clarified brains were mounted on aluminum slides with a central hole filled with methylsalicylate, and subsequently covered by thin coverslips. Each AL was scanned with a laser scanning confocal microscope (LSM700; Carl Zeiss, Jena, Germany). Neutral red staining was visualized using 555 nm excitation produced by a solid-state laser. Using a water immersion objective (20× Plan-Apochromat, 1.0 DIC), AL optical sections were acquired at a resolution of 1,024 × 1,024 pixels (0.313 μm/pixels; *x*, *y*) with 1 μm intervals (*z*). Serial optical sections were then saved as LSM Files using the ZEN software (version 8.1; Carl Zeiss; RRID: SCR_013672).

3 | 3D RECONSTRUCTIONS

To reconstruct AL glomeruli, confocal image stacks were first opened with ImageJ software (ImageJ; RRID: SCR_003070) and the Bio-formats library plugin (RRID: SCR_OOO450). After adjusting brightness and contrast, image stacks were saved as TIFF files and imported in three-dimensional (3D) analysis software (Amira 5.4.3, FEI, Berlin, Germany; RRID: SCR_007353). AL glomeruli were individually reconstructed by manual labeling in three planes of the image stacks (*xy*, *xz*, and *yz*) before applying the *Wrap* function, hence obtaining a 3D model. Fit of the 3D model was assessed visually by the experimenter. In the case of non-spherical structures which cannot be reliably reconstructed using 3D wrapping, a different approach was used. The glomerulus was outlined on several frontal sections (*xy*) along the ventrodorsal axis (*z*) and the *Interpolate* function was then used to build the 3D models. This choice only aimed at producing the best fitting model for each glomerulus.

3.1 | Neuroanatomical analysis and macroglomerulus naming system

Spatial directions given in all figures are based on the neuraxis (see Figure 1h in Strausfeld, 2002). The "frontal" surface of the brain

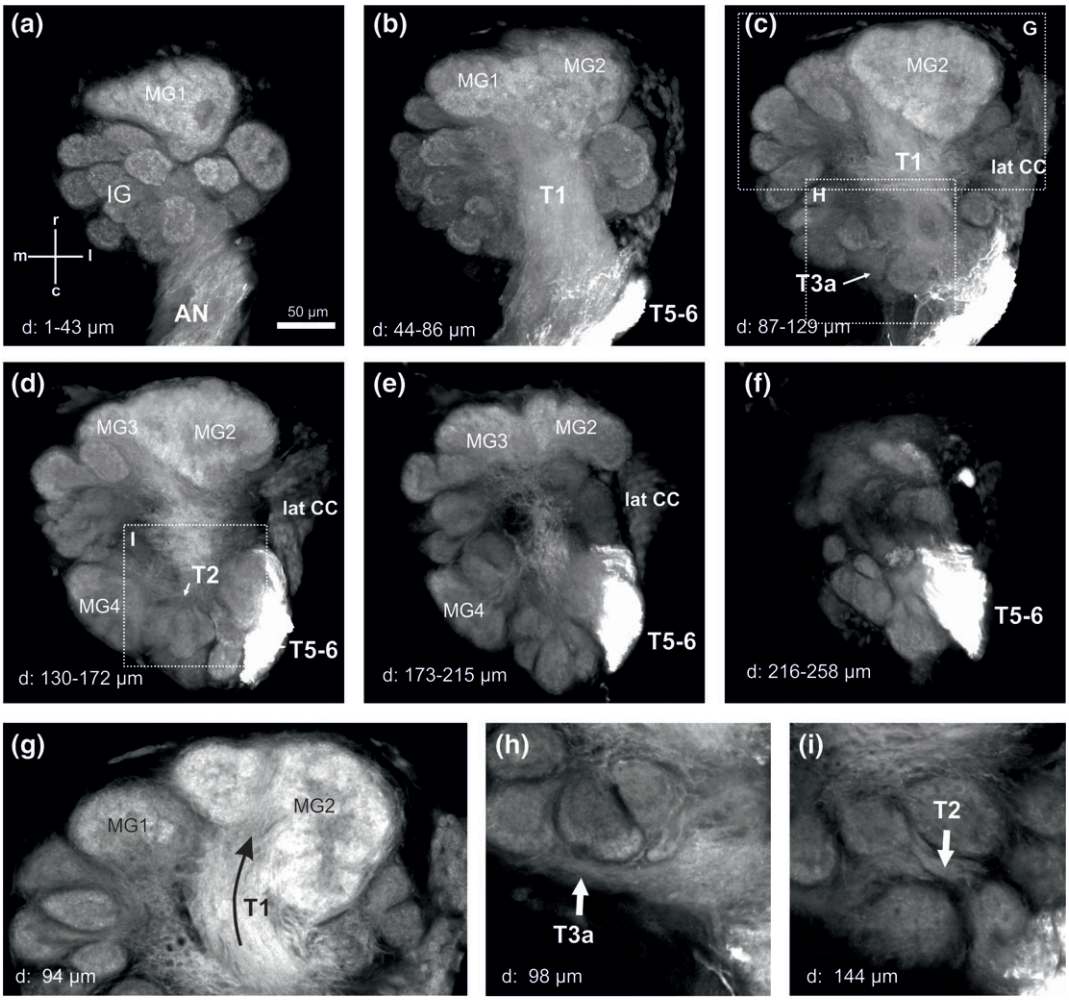

FIGURE 1 Landmarks in the antennal lobe of *Apis* drones. (a–f) Serial Z projections (~40 µm thickness) through the antennal lobe of a drone *Apis mellifera*, after antennal backfill with microruby. (g–i) Details from single optical section outlined in (c,d). (g) Massive innervation of MG2 by T1; (h) Outer innervation of isomorphic glomeruli by T3a; (i) Detail of small T2 tract on its way to innervate MG4. M: medial; l: lateral; r: rostral; c: caudal; MG1–4: macroglomeruli 1 to 4; T1, T2, T3a, T5–6: sensory neuron subtracts from the antennal nerve; IG: isomorphic glomeruli; lat CC: lateral cell cluster; d: depth

corresponds to its most ventral region in the neuraxis. When referring to the AL, caudal points toward the bees' mouthparts, while *rostral* points toward the ocelli. Using the antennal nerve orientation as a landmark, serial optical sections of each AL were carefully aligned on the caudorostral axis to facilitate inter- and intraspecific comparisons. The location, shape, and innervation pattern of the macroglomeruli were used to assess their putative homology. Traditionally, within species, macroglomeruli are named with numbers, depending on their position on a ventrodorsal axis (Arnold et al., 1985; Brockmann & Brückner, 2001a; Nishino et al., 2009; Wanner et al., 2007). Such a numbering system does not work well for interspecific comparisons. In this study, we thus introduced a different naming system useful across species and using letters, from MGa to MGf. To optimize its compatibility with previous descriptions of the model species *A. mellifera*, macroglomeruli of *A. mellifera* drones termed MG1-MG4 (Brockmann & Brückner, 2001a; Sandoz, 2006; Wanner et al., 2007) or GC1-GC4 (Arnold et al., 1985; Nishino et al., 2009) are identified as MGa to MGd in this study. In the same way, their putative homologs in the other *Apis* species are named as MGa-MGd and two additional macroglomeruli which were observed in some other species but without any correspondence in *A. mellifera* were termed MGe and MGf.

3.2 | Data analysis and statistics

To compare the neuroanatomical organization of the AL across different honey bee species, at least five ALs per species and population were 3D reconstructed. These reconstructions provided the number of glomeruli in each AL and the volume of each glomerulus within this AL.

To compare the numbers of AL glomeruli among species and populations, a Kruskal-Wallis test was used. It was followed by pairwise comparisons using Dunn's post hoc test, which includes a correction for multiple comparisons. These tests were performed with Statistica 8.0 (StatSoft, Tulsa, OK; STATISTICA, RRID: SCR_014213).

The volume of each glomerulus was measured as the added volume of all labeled voxels using Amira statistical tools. The total volume of an AL was measured by adding up the volumes of all its glomeruli. As we were not interested in absolute differences in volume across species and individuals, the volume of each glomerulus was

normalized according to the whole AL volume. For each glomerulus, this value thus indicated the percentage of volume occupied by this glomerulus in the AL. Absolute volumes are provided for each species/population in Supporting Information.

To define the macroglomeruli within the ALs of each species/population, we used a standard statistical measure applied in previous studies on hymenopteran ALs (Couto, Lapeyre, Thiéry, & Sandoz, 2016; Kuebler, Kelber, & Kleineidam, 2010; Roselino, Hrncir, da Cruz, Giurfa, & Sandoz, 2015; Streinzer, Kelber, Pfabigan, Kleineidam, & Spaethe, 2013). The average volume of each of the largest glomeruli was compared with the overall distribution of glomerular volumes within each species. A quantitative threshold that defines extreme outliers within a distribution (similar to the "outer fence," Zar, 2010) was used as follows:

$$V_{outlier} > V_U + k\,(V_U\text{-}V_L),$$

With V_U the upper percentile (90%) and V_L the lower percentile (10%) of glomerular volume distribution. We used $k = 3$ as a conservative value that successfully categorized macroglomeruli in several hymenopteran species (leaf-cutting ants: Kuebler et al., 2010; eucerine bees: Streinzer et al., 2013; stingless bees: Roselino et al., 2015; hornets: Couto et al., 2016). Glomeruli whose volume was above this threshold were considered as macroglomeruli. An example calculation is given in Supporting Information Figure S1.

4 | RESULTS

4.1 | Antennal lobe morphology in the *Apis* genus

To investigate the organization of the male honey bee AL, the brains of different *Apis* species and populations were stained with neutral red and the antennal lobes were scanned under confocal microscopy, allowing the delimitation of the AL's individual units, the glomeruli. We analyzed the male ALs of five species, *A. florea* ($N = 5$), *A. dorsata* ($N = 10$), *A. mellifera* ($N = 6$), *A. koschevnikovi* ($N = 7$), and *A. cerana*, in which three populations were compared: Borneo ($N = 5$), Thailand ($N = 5$), and Japan ($N = 5$) (Supporting Information Table S1). Visual observation of the ALs in the different species/populations showed the same general organization, with four main tracts of OSNs entering the AL from the antennal nerve, as previously described in details in *A. mellifera* drones (Figure 1; Arnold et al., 1985; Nishino et al., 2009). In all species, part of the antennal nerve (described as tracts T5–6 in *A. mellifera*, Suzuki, 1975; Abel et al., 2001; Figure 1) bypasses the AL on its caudolateral side towards the AMMC (antennal mechanosensory and motor centre) and SEZ (subesophageal zone). In addition to these tracts, similar landmarks were found in the different species, like for instance a conspicuous cluster of local/projection neuron somata on the lateral AL side (lat CC in Figure 1), with a thick bundle of neurites entering the AL. Drone ALs mostly contained isomorphic glomeruli, but also in all species and populations a limited number of highly enlarged glomeruli.

We first analyzed glomeruli numbers and found that they differed significantly among the different species/populations (Kruskal-Wallis test, $H = 38.74$, 6 *df*, $p < .001$; Figure 2). *Apis* drones generally possess more glomeruli than previous inferred from counts in *Apis mellifera*

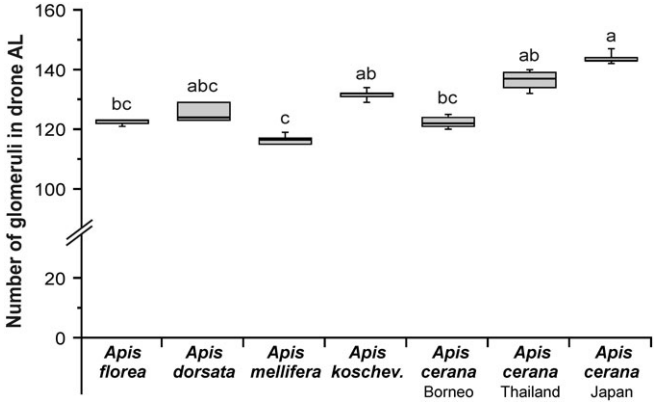

FIGURE 2 Number of glomeruli in the different species and populations of *Apis*. Box plots show the median (bold line) and interquartile range (25–75%) of the number of AL glomeruli and the whiskers indicate 10% and 90% of the distribution. The number of glomeruli was heterogeneous among the seven species and populations of *Apis* (Kruskal-Wallis test, 6 *df*, $H = 38.7$, $p < .001$), ranging from 116.5 glomeruli in *Apis mellifera* to 143.8 in *Apis cerana* Japan. Letters indicate significant differences in pairwise multiple comparisons (Dunn's test) after a Kruskal-Wallis test

(~103 in Arnold et al., 1985; 107–109 in Kropf, Kelber, Bieringer, & Rössler, 2014; 116 in Nishino et al., 2009). Remarkably, the AL of this species contained the lowest number of glomeruli in our sample, with 116.5 ± 0.6 (mean ± *SD*) glomeruli. It was significantly less than in the other cavity nesting species, *A. koschevnikovi* (131.7 ± 0.6) and *A. cerana*, at least for its populations from Thailand (136.4 ± 1.5) and Japan (143.8 ± 0.9) (posthoc Dunn tests, $p < .01$). Interestingly, drones from the three *A. cerana* populations showed heterogeneous numbers of glomeruli, the Borneo population presenting significantly less glomeruli (122.4 ± 0.9) than the Japan population (Dunn tests, $p < .05$). The two open-air-nesting species showed no significant difference in glomeruli numbers, with 122.4 ± 0.4 glomeruli in *A. florea* and 125.5 ± 0.85 in *A. dorsata* (Dunn tests, $p = 1.00$). We conclude that differences in the numbers of glomeruli do not scale with evolutionary divergence: while evolutionary distant species like *A. florea* and *A. mellifera* displayed similar glomeruli numbers, some closely related populations of the same species, like *A. cerana* Borneo and *A. cerana* Japan, did.

4.2 | Macroglomeruli

Based on volumetric measures, we aimed to identify the macroglomeruli within the ALs of the different species and populations. Figure 3 presents the distribution of glomerular volumes in relative units with respect to the total volume of each AL (see box plots). To assess whether the largest glomeruli are voluminous enough to be considered as macroglomeruli, we used a standard statistical threshold that defines extreme outliers in a volume distribution (dotted lines in Figure 3, see example calculation in Supporting Information Figure S1). Between two and four outliers were found in drone ALs depending on the species (colored dots in Figure 3). Drone ALs of the open-air-nesting species, *A. florea* and *A. dorsata*, contained two glomeruli above the macroglomerular volume threshold. As previously described, the AL of *A. mellifera* drones showed four strongly enlarged

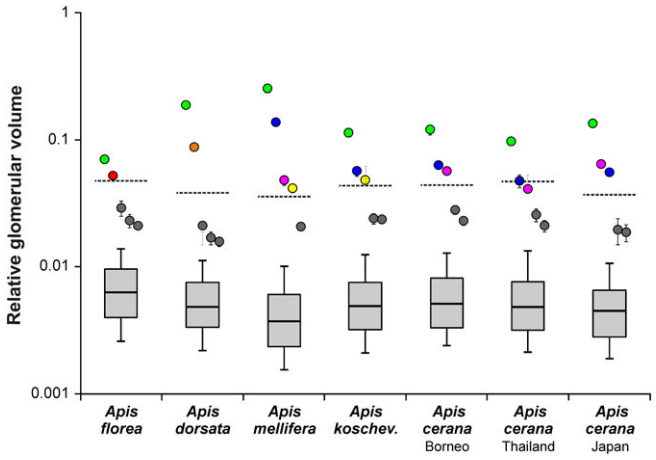

FIGURE 3 Distribution of the relative glomerular volume and position of the five largest glomeruli. Box plots show for each species and population the distribution of the glomerular volumes relative to the total AL volume. The boxes encompass the interquartile range (25–75%) and the medians are indicated by bold lines, whereas whiskers show the 10th and 90th percentiles of relative volume distribution. The dotted lines indicate the statistical macroglomerulus threshold and glomeruli with relative volumes above the threshold are shown with colored circles (macroglomeruli), whereas glomeruli with relative volumes below the threshold are represented by gray circles. All species possess at least two hypertrophied glomeruli with volumes exceeding the statistical threshold. One glomerulus below the threshold was colored in *A. cerana* Thailand (magenta) due to its strong similarity with a macroglomerulus at the same location in the two other *A. cerana* populations. The relative volumes are represented along a logarithmic scale (*Y*-axis) for easier comparison across species and populations [Color figure can be viewed at wileyonlinelibrary.com]

glomeruli, which all passed the threshold. In the other cavity-nesting species, *A. koschevnikovi* and *A. cerana*, three hypertrophied glomeruli were above the volume threshold. In the Thailand population of *A. cerana*, only two of the three enlarged glomeruli were above the threshold. The third large glomerulus was however still 8.5 times larger than the median glomerular volume, and its anatomical characteristics did not allow differentiating it from the same glomerulus in the two other populations. We thus chose to consider it a macroglomerulus in the next steps of this work.

To understand potential homologies existing among these hypertrophied glomeruli we compared their ranking in the volume distributions, their spatial arrangement with regards to local landmarks and their shape in all species and populations (Figure 4). Overall, we identified six macroglomeruli over all species and populations, which we termed MGa to MGf (see summary in Table 1). They are presented in 3D reconstructions of the drone ALs of the different species/populations in Figure 5. Their possible below-threshold homologous glomeruli in the different species/populations are shown in Supporting Information Figure S2. The six *Apis* macroglomeruli are described below from the most ventral to the most dorsal:

- In the most ventral part of the AL of all cavity-nesting species, *A. mellifera*, *A. koschevnikovi* and *A. cerana*, a macroglomerulus termed MGa is located in the rostrolateral region (Figures 4g,j,m, p,s and 5). It generally classifies as the second largest glomerulus in each species' volume distribution (Table 1). It was previously identified as MG1 or GC1 in *A. mellifera* (Arnold et al., 1985; Brockmann et al., 2001; Nishino et al., 2009) and has a similar shape and position in all cavity-nesting species, although its size is greatly expanded in *A. mellifera*. It is innervated by the T1 tract of OSNs, which conspicuously traverses the AL from the antennal nerve (caudal) to the rostrolateral AL side (Arnold et al., 1985; Brockmann et al., 2001; Nishino et al., 2009).
- The largest glomerulus in all species is MGb, which is located in the rostrolateral part of the AL, at mid-depth on the ventrodorsal axis (Figures 4b,e,h,k,n,q,t and 5). In all species, it is nested rostrally against the lateral cell cluster. Its shape varies from an egg-shape (open nesting species) to a bean shape (cavity nesting species). This macroglomerulus, previously described as MG2 or GC2 in *A. mellifera*, is directly innervated by the T1 tract.
- Besides MGb, males of *A. mellifera* and *A. koschevnikovi* present another macroglomerulus, termed MGc (in Figures 4h,k and 5). This macroglomerulus called MG3 or GC3 in *A. mellifera* (Arnold et al., 1985; Brockmann et al., 2001; Nishino et al., 2009) occupies the medial flank of MGb and is closely opposed to it. The other cavity-nesting species, *A. cerana* and one of the open-nesting species *A. dorsata* do show a large glomerulus at the same location (third and fourth glomerulus in terms of volume respectively for *A. dorsata* and *A. cerana*) but it is below the statistical threshold for being considered a macroglomerulus (Table 1).
- In both *A. mellifera* and *A. cerana*, a hypertrophied glomerulus termed MGd occupies the medial side of the AL (Figures 4h,n,q,t and 5). This macroglomerulus described as MG4 or GC4 in *A. mellifera*, is innervated by a fine but remarkable tractus, T2, and is located near the landmark glomerulus B02 (Arnold et al., 1985; Brockmann et al., 2001; Nishino et al., 2009). MGd was clearly found in all three *A. cerana* populations, but in one population (Thailand) its volume was just below the statistical macroglomerulus threshold (Figure 3). The fact that it generally had the third largest volume of all glomeruli (Table 1), along with its T2 innervation, shape, and location, support its homology in both species.
- In each of the open-nesting species, *A. florea* and *A. dorsata*, a macroglomerulus was found in the most dorsal layers of the ALs close to the so-called "necklace" glomeruli (T4 innervated, Flanagan & Mercer, 1989; Nishino et al., 2009). This macroglomerulus was the second largest glomerulus in both open-nesting species, after MGb (Table 1; Figure 5). Despite the dorsal location in both species, close examination of its relative position with regards to common landmarks in both species suggests that it does not correspond to homologous macroglomeruli. Indeed, while the *A. dorsata* macroglomerulus is located on the medio-rostral side, close to the T5–6 tract on the way to the AMMC, the *A. florea* macroglomerulus is found more caudally, separated from the T5–6 tract by ordinary-sized glomeruli. For this reason, the macroglomerulus were termed MGe in *A. florea* (Figures 4cand 5) and MGf in *A. dorsata* (Figures 4f and 5). Glomeruli at these locations in cavity-nesting species were clearly below the statistical threshold, although in *A. koschevnikovi* and *A. cerana* from Borneo and Thailand a glomerulus at the MGf location showed the sixth largest volume (Table 1).

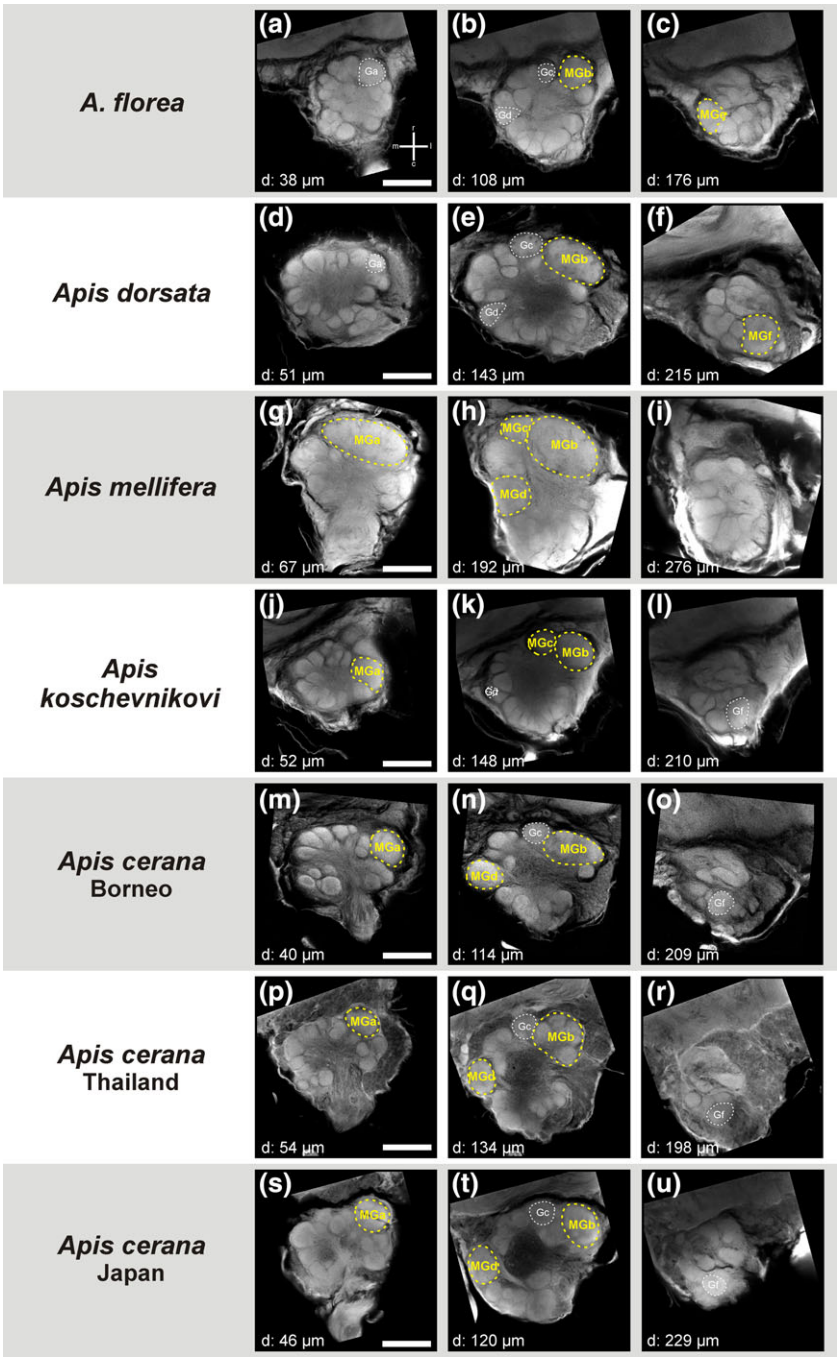

FIGURE 4 Macroglomeruli in the male antennal lobe of different *Apis* species and populations. Confocal sections through the left AL are presented from the most ventral (left) to the most dorsal (right). Identifiable macroglomeruli (MG) are encircled with dotted lines and labeled MGa to MGe from the most ventral to the most dorsal (see text). Species/populations are *Apis florea* (a–c), *A. dorsata* (d–f), *A. mellifera* (g–i), *A. koschevnikovi* (j–l), *A. cerana* Borneo (m–o), *A. cerana* Thailand (p–r), *A. cerana* Japan (s–u). A similar organization plan of the drone AL is observed across the genus *Apis*, yet macroglomerular organization varies among species, but not among populations. For each confocal image, values in the bottom left corner indicate the depth (d) of the optical section from the ventral surface of the AL. The scale bars indicate 100 µm. R, rostral; c, caudal; m, medial; l, lateral [Color figure can be viewed at wileyonlinelibrary.com]

In conclusion, we found clearly differing numbers and arrangements of macroglomeruli in the different *Apis* species, but a conserved macroglomerular equipment in the different populations of *A. cerana*. The four macroglomeruli that were previously described in *A. mellifera* (MGa-MGd) find a correspondence in at least one of the other *Apis* species. Two additional macroglomeruli (MGe and MGf) which are absent in *A. mellifera*, were identified in the open-air-nesting species, *A. florea* and *A. dorsata*.

4.3 | Relative investment in macroglomerular structures

To assess the relative investment of the different honeybee species and populations in macroglomerular structures, and possibly in sex communication, we compared the proportion of their AL occupied by macroglomeruli. The absolute volume of macroglomerular structures and whole AL are given for each species and population in supplementary material (Supporting Information Figure S3). The relative volume

TABLE 1 Putatively homologous macroglomeruli

macroglomerular units	*A. florea*	*A. dorsata*	*A. mellifera*	*A. koschevnikovi*	*A. cerana* Borneo	*A. cerana* Thailand	*A. cerana* Japan
a	**Ga** 2.10 ± 0.07% # 5	**Ga** 1.68 ± 0.20% # 4	**MGa** 13.74 ± 0.52% # 2	**MGa** 5.69 ± 0.48% # 2	**MGa** 6.31 ± 0.14% # 2	**MGa** 4.75 ± 0.55% # 2	**MGa** 5.55 ± 0.22% # 3
b	**MGb** 7.02 ± 0.17% # 1	**MGb** 18.93 ± 1.03% # 1	**MGb** 25.40 ± 1.32% # 1	**MGb** 11.37 ± 0.52% # 1	**MGb** 12.05 ± 1.06% # 1	**MGb** 9.72 ± 0.41% # 1	**MGb** 13.46 ± 0.59% # 1
c	**Gc** 0.69 ± 0.12% # normal vol	**Gc** 2.09 ± 0.15% # 3	**MGc** 4.15 ± 0.30% # 4	**MGc** 4.82 ± 0.15% # 3	**Gc** 2.80 ± 0.20% # 4	**Gc** 2.57 ± 0.30% # 4	**Gc** 1.96 ± 0.46% # 4
d	**Gd** 1.39 ± 0.20% # 8	**Gd** 1.57 ± 0.13% # 5	**MGd** 4.81 ± 0.40% # 3	**Gd** 1.04 ± 0.11% # normal vol	**MGd** 5.67 ± 0.36% # 3	**MGd*** 4.10 ± 0.27% # 3	**MGd** 6.43 ± 0.19% # 2
e	**MGe** 5.21 ± 0.37% # 2	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*
f	*N/A*	**MGf** 8.72 ± 0.52% # 2	*N/A*	**Gf** 2.21 ± 0.17% # 6	**Gf** 2.06 ± 0.14% # 6	**Gf** 1.99 ± 0.23% # 6	**Gf** 1.18 ± 0.07% # 13

For each species/population (columns), the features of identified macroglomeruli or their respective isomorphic glomeruli are shown. Relative volume ± *SD* (in % of whole AL volume) is given between brackets. The rank (#) of this glomerulus among all AL glomeruli of each species/population is also indicated. "# normal vol" refers to putatively homologous isomorphic glomeruli which can be identified but whose volume does not rank in the upper 10% of this species' distribution. "N/a," nonavailable, refers to cases where a homologous isomorphic glomeruli cannot be identified with confidence, usually due to the lack of a clear neuroanatomical features.

occupied by macroglomeruli differed among *Apis* species and populations (Kruskal-Wallis test, $H = 36.27$, 6 *df*, $p < .001$; Figure 6). The Western honeybee *A. mellifera* displayed the highest investment in macroglomerular structures with four macroglomeruli accounting in total for 48.1 ± 1.3% of the AL volume. This species thus invests significantly more in macroglomeruli than *A. florea* (12.2 ± 0.5%), *A. koschevnikovi* (21.9 ± 0.6%), and *A. cerana* Thailand (18.6 ± 1.0%) (Dunn tests, $p < .01$). The two open-air-nesting species showed a discrepancy in the AL volume invested in macroglomeruli (Figure 6) with 27.6 ± 1.1% in *A. dorsata* but only 12.2 ± 0.5% in *A. florea* (Dunn test, $p < .01$). No significant difference in macroglomerular investment was observed among the three populations of *A.cerana* (Borneo: 24.0 ± 1.3%, Thailand: 18.6 ± 1.1%, and Japan 25.4 ± 0.8%, Dunn tests, NS).

Lastly, we compared the investment of each species in the putatively homologous macroglomeruli. In Figure 7, significant differences for a given macroglomerulus are represented by different letters.

- MGa, which is present in all cavity-nesting species, accounts for 13.7 ± 0.5% of total AL volume in *A. mellifera*. It occupies a significant higher portion of the AL in this species compared to other cavity nesting species, with 5.7 ± 0.5% in *A. kochevnikovi*, 4.7 ± 0.5% in *A. cerana* Thailand and 6.4 ± 0.2% in *A. cerana* Japan (Kruskal-Wallis test, $H = 17.37$, 4 *df*, $p = .002$; Dunn tests, $p < .029$). The difference with *A. cerana* Borneo was, however, not significant (6.3 ± 0.1%; Dunn test, NS).
- MGb is the most voluminous glomerulus in all studied species and populations (Supporting Information Figure S4), but the relative volume it occupies varies widely (Kruskal-Wallis test, $H = 37.81$, 6 *df*, $p < .001$; Figure 7). Thus, MGb represents from 7.0 ± 0.2% of total AL volume in *A. florea* to 25.4 ± 1.3% in *A. mellifera*. Among open-nesting bees, its relative volume is higher in *A. dorsata* than *A. florea* (Dunn test, $p < .001$). Among cavity-nesting bees, it is higher in *A. mellifera* than in *A. koschevnikovi* and the Thailand population of *A. cerana* (Dunn tests, $p < .011$).
- MGc occupies 4.2 ± 0.3% of total AL volume in *A. mellifera* which is slightly, but significantly, less than the 4.8 ± 0.2% found in *A. koschevnikovi* (Mann–Whitney *U* test: $Z = -2.21$, $p = .022$).
- MGd which is present in *A. mellifera* and all populations of *A. cerana* also shows a significant heterogeneity (Kruskal-Wallis test, $H = 12.69$, 3 df, $p = .005$), with a higher relative volume in *A. cerana* Japan (6.4 ± 0.2%) than in *A. cerana* Thailand (4.7 ± 0.5%; Dunn test, $p = .005$).

Overall, each macroglomerulus occupies at least 4% of total AL volume. MGb, which is consistently the most voluminous macroglomeruli in all species and population, generally accounts for more than half of the total macroglomerular investment. The second largest macroglomerulus, MGe/MGf in open-air-nesting species or MGa in cavity nesting species, is usually twice smaller than the MGb. The two sister species, *A. koschevnikovi* and *A. cerana*, show comparable macroglomerular investment especially with regard to MGa and MGb. The main difference between these two cavity-nesting species lies in the presence of MGc in *A. koschevnikovi* or MGd in *A. cerana*. However, both MGc and MGd are present in the third cavity-species, *A. mellifera*. At the population level, we observed a single difference between *A. cerana* Japan and *A. cerana*

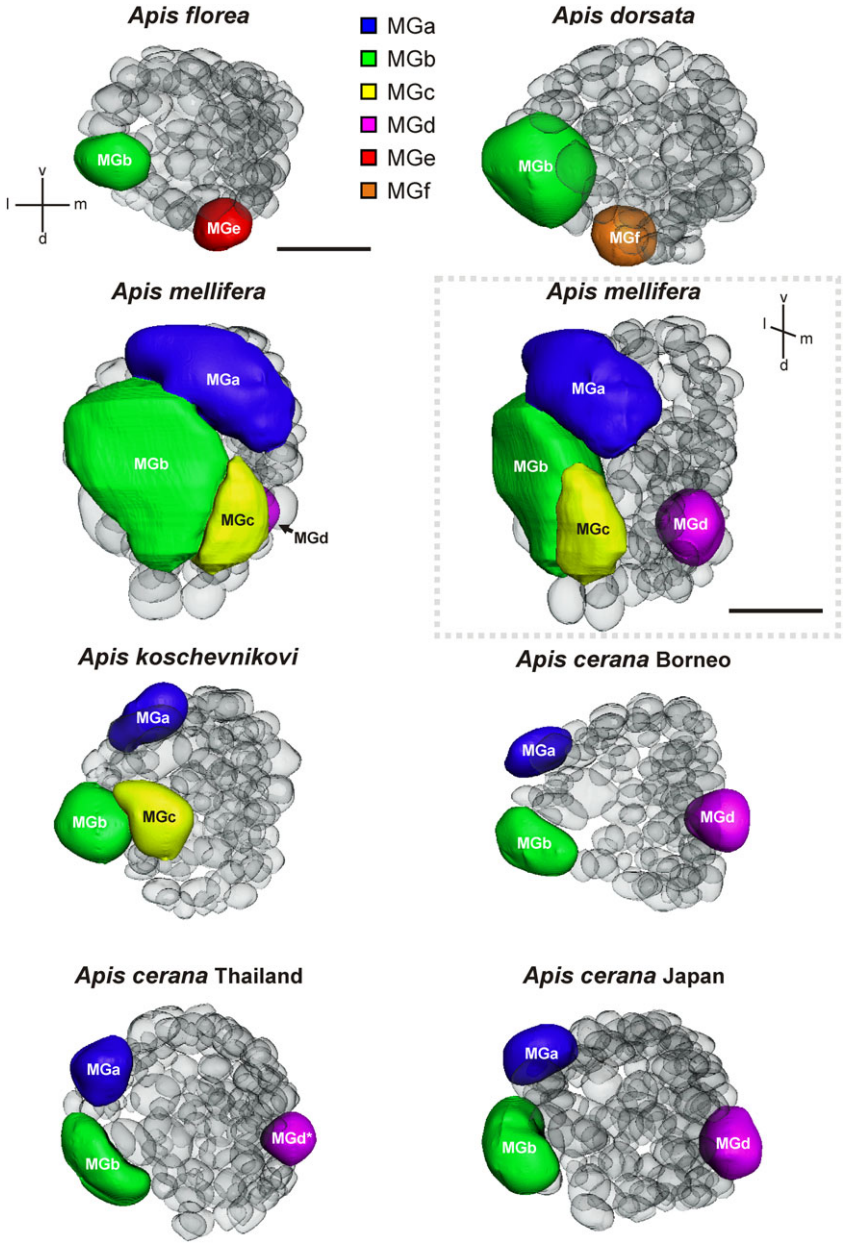

FIGURE 5 Three-dimensional (3D) models of the antennal lobes of different *Apis* species and populations with their respective macroglomeruli. 3D models of left antennal lobes are represented in a rostral view as this view best represents relative positions in all *Apis* species and populations. The antennal nerve faces behind each lobe. In a box (gray dotted line), the *Apis mellifera* AL is presented with a light rotation along the mediolateral axis for better visibility of MGd and easier comparison across species. Putatively homologous macroglomeruli are presented with the same color across species/populations. Scale bars indicate 100 μm. V, ventral; d, dorsal; m, medial; l, lateral [Color figure can be viewed at wileyonlinelibrary.com]

Thailand, with a higher relative investment for MGd in *A. cerana* Japan.

5 | DISCUSSION

We investigated the organization of the drone AL in five *Apis* species and assessed its interpopulational variability. Our results show that the number and volume of the glomeruli in male ALs vary between species, and to a lesser extent, between *A. cerana* populations. All male ALs of a given species present macroglomeruli ranging from two in open-air-nesting species to three and four in cavity-nesting species. Overall, taking into account possible homologies, we identified six different macroglomeruli in the *Apis* genus.

5.1 | Glomeruli numbers in *Apis* drones

Glomeruli are generally less numerous in males than in females of eusocial Hymenoptera (Couto et al., 2016; Kuebler et al., 2010; Mysore et al., 2009; Nishikawa et al., 2008; Stieb, Kelber, Wehner, & Rössler, 2011; Streinzer et al., 2013; Zube & Rössler, 2008). This lower number of glomeruli in males is often attributed to a "limited" behavioral repertoire of males (Arnold et al., 1985; Kuebler et al., 2010; Streinzer et al., 2013). Honey bee drones do not forage and are

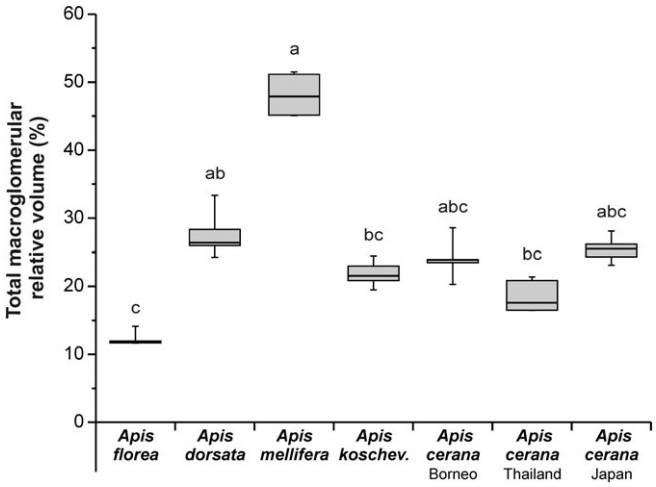

FIGURE 6 Macroglomerular investment across *Apis* species and populations. The box plots represent the median (bold line) and interquartile range (25–75%) of the relative volume occupied by all macroglomeruli in each species/population. Wiskers indicate the 10th and 90th percentiles. The whole macroglomerular volume is very different among species, from 12.2% in *Apis florea* to 48.1% in *Apis mellifera* (Kruskal-Wallis test, 6 df, $H = 36.3$, $p < .001$)

indeed mostly known for their role in the insemination of virgin queens during reproduction (Free, 1957; Koeniger et al., 2014; Winston, 1987). Accordingly, a large part of their AL is occupied by macroglomeruli, thought to be involved in sex communication (Arnold et al., 1985; Brockmann & Brückner, 2001a; Sandoz, 2006). They spend, however, most of their life within the darkness of the hive and need to use a significant range of olfactory cues to orientate and interact with other colony members. Later, when flying out of the colony, they may use olfactory cues together with visual cues to find their way to and from the congregations. It should be noted that drones' glomeruli numbers, as found in our study, are still much higher than total numbers found in many insect species displaying an extensive range of olfactory-mediated behaviors (for instance ~50 in Drosophila, Laissue et al., 1999; Kondoh, Kaneshiro, Kimura, & Yamamoto, 2003; and ~65 in moths; Rospars, 1983; Anton & Hansson, 1994; Skiri, Rø, Berg, & Mustaparta, 2005). Thus, despite lower number of olfactory glomeruli compared to their conspecific females, honey bee males may still have a good capacity for discriminating a wide range of nonsexually related odorants.

Usually, glomeruli numbers are well conserved within a given phylogenetic group (Schachtner, Schmidt, & Homberg, 2005). Our results show that glomeruli numbers in the drone AL are quite heterogeneous across *Apis* species. These disparities are not strictly lineage-dependent because closely related species sometimes showed larger differences than more-distant species collected at the same location. This supports the idea that glomeruli numbers are also the result of each species/population's adaptation to its local olfactory environment. This is particularly striking when comparing different populations of *A. cerana*, like those from Japan (~144 glomeruli) and Borneo (~122 glomeruli). Gene flow between Borneo and continental populations is thought to have ceased ~16–18,000 years ago (Smith et al., 2000). Since macroglomerular structures appear stable among *A. cerana* populations, our results suggest stabilizing selection on the sexual communication system. On the contrary, selective pressures for adapting to these different environments may have driven the emergence/disappearance of ordinary glomeruli in the different populations. Changes in the numbers of glomeruli are thought to follow, via a yet unknown process, the birth and death of new olfactory receptor proteins, through gene duplication and pseudogenization/deletion, respectively (Andersson, Löfstedt, & Newcomb, 2015; Ramdya & Benton, 2010). Although our comparisons were made on the drone AL, the number of glomeruli is usually considered a worker-related trait in bees, as it underlies its capacity to efficiently discriminate numerous olfactory cues, a crucial ability during foraging. We thus predict that the pattern we described here on drones will also be found in the workers of these species.

5.2 | Conservation and divergence of macroglomerular structures

We observed a remarkable diversity of AL macroglomeruli in the different *Apis* species, with both highly conserved and species-unique units. The most striking example of a conserved unit is MGb, which was consistently present at the same location, showing the highest

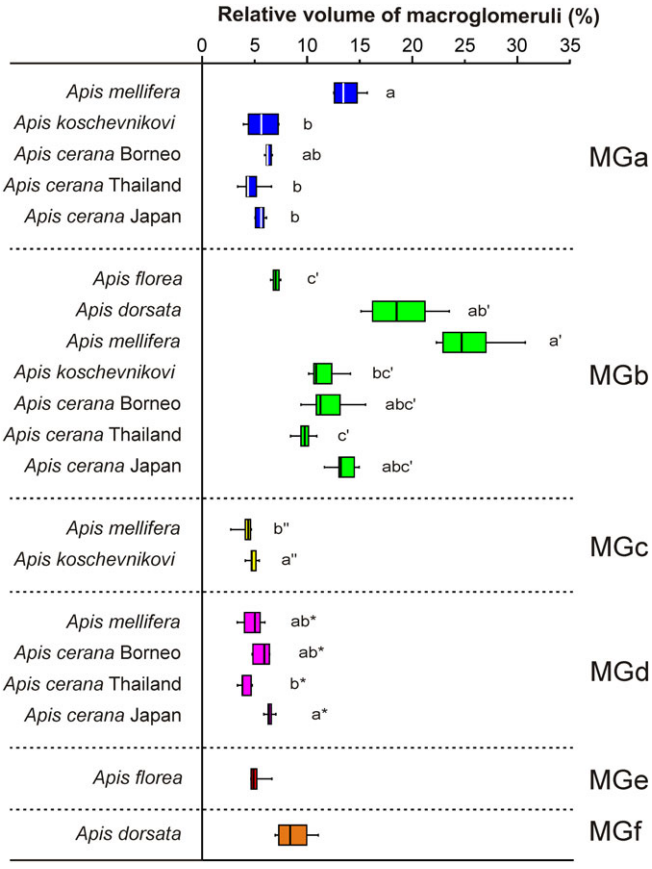

FIGURE 7 Comparison of the relative volume of putatively homologous macroglomeruli across species/populations. Significant differences in the relative volume of each macroglomerulus is observed among species and populations. The strongest heterogeneity is observed for macroglomerulus MGb. Different letters indicate significant differences in Mann–Whitney *U* tests or multiple comparison tests (Dunn's post hoc test) after significant Kruskal-Wallis tests [Color figure can be viewed at wileyonlinelibrary.com]

volume and exhibiting the same dense innervation by the T1 olfactory tract in all investigated species. This observation suggests that MGb may have been present in the last common ancestor of these bees, which lived about 10 million years ago (Arias & Sheppard, 2005; Garnery, Vautrin, Cornuet, & Solignac, 1991; Raffiudin & Crozier, 2007). The fact that it was conserved throughout *Apis* evolution, supports the idea that it played an enduring and crucial role in honey bee reproduction. Nonetheless, the relative investment in MGb varied greatly across species (from 7 to 25%) suggesting that selection on the number of OSNs targeting this macroglomerulus might have occurred. In *A. mellifera* the extraordinary investment in MGb is mirrored by the strongly increased number of placode sensilla at the periphery compared to other species (Brockmann & Brückner, 2003; Esslen & Kaissling, 1976) and the high sensitivity of the drone antenna to 9-ODA (Brockmann, Brückner, & Crewe, 1998). This extraordinary investment in MGb may have provided drones in this species with the ability to detect the queen pheromone (here 9-ODA) from longer distances than other species (Brockmann & Brückner, 2003). The five other macroglomeruli were found in only some of the species. Generally, these macroglomeruli seem to have appeared from the enlargement of preexisting glomeruli. For all the additional macroglomeruli found in cavity-nesting species (MGa, MGc, and MGd), relatively large, but subtreshold, glomeruli could be found at the same location in the ALs of *A. dorsata* and/or *A. florea* (Figure 4, Supporting Information Figure S2 and Table 1). A remarkable case is MGa, whose isomorphic counterpart in *A. florea* was qualitatively considered as a macroglomerulus in a previous study lacking a statistical macroglomerulus definition (MG1; Brockmann & Brückner, 2001a). We must remark here that our work attempted to provide a common volumetric, statistically grounded, definition of macroglomeruli, pinpointing extraordinary investments in particular units. It must, however, not be understood as suggesting that subthreshold units do not play a significant biological role in each species' biology. Only functional data, like electrophysiology or in vivo imaging, may provide information on their functions. In any case, the fact that these macroglomeruli have undergone remarkable size increases through evolution suggests that they may have represented a major transition in the reproductive communication system of honey bees and the rise of the extant group of cavity-nesting species. Although our analyses especially highlighted the size increase of specific glomeruli, the opposite phenomenon might also have occurred. Within extant cavity-nesting species, the sister species *A. koschevnikovi* and *A. cerana* show similar relative volumes of MGa and MGb, but only MGc was classified as a macroglomerulus in *A. koschevnikovi* and only MGd in *A. cerana*. Although we cannot exclude the possibility of an independent volume increase of the same glomerulus in *A. mellifera* and in one or the other of the cavity-nesting species, due to the presence of large isomorphic glomeruli at these locations, the most probable scenario involves the gradual atrophy of one macroglomerulus in each of the two sister species (red stars in Figure 8).

5.3 | A model of macroglomerular evolution in honey bees

These different observations are summarized in a putative scenario of honey bee drone AL evolution (Figure 8), placing possible evolutionary events on a consensus phylogenetic tree of *Apis* species established previously using molecular and morphological data (Arias & Sheppard, 2005; Raffiudin & Crozier, 2007). According to this tree, dwarf bees (e.g. *A. florea*) would have diverged first, then giant bees (e.g. *A. dorsata*) and finally the group of extant cavity-nesting species would have diversified. Note that there is an ongoing controversy about the life history traits of the last common ancestor (LCA) of extant *Apis* species, that is, if it was an open-nesting or a cavity nesting species (Koeniger, Koeniger, & Phiancharoen, 2011; Oldroyd & Wongsiri, 2006). Irrespective of this question, our model concentrates on drone AL evolution proposing a scenario which would minimize the number of events (five appearances, blue stars; two disappearances—red stars). In this scenario, only MGb was present in the LCA, probably involved in the detection of virgin queens'

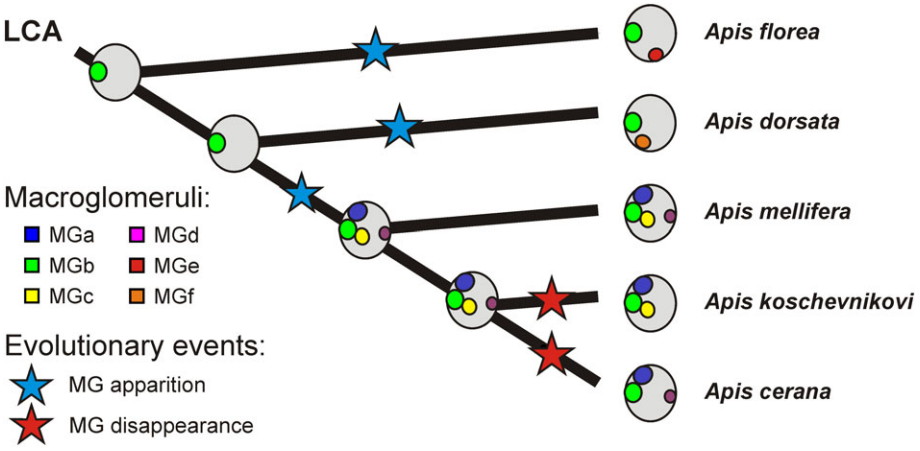

FIGURE 8 Putative model of drone antennal lobe evolution in the *Apis* genus. This model posits that the last common ancestor (LCA) of honey bees carried one macroglomerulus (MGb) dedicated to the detection of a major queen pheromone component. Evolution of honey bees was accompanied with a diversification of macroglomeruli, with the appearance (blue stars) of, respectively, 1, 1, and 3 macroglomeruli in lineages of dwarf bees (*Apis florea*), giant bees (*Apis dorsata*), and extant cavity nesting bees (*A. mellifera*, *A. koschevnikovi*, *A. cerana*). Secondary losses (red stars) of two glomeruli would have happened later, during the separation of *A. cerana* and *A. koschevnikovi* [Color figure can be viewed at wileyonlinelibrary.com]

pheromone (9-ODA in extant species). Divergence and the apparition of new species would have been accompanied by a diversification of sex pheromone compounds and accordingly the advent of new macroglomeruli, like MGe in dwarf bees, MGf in giant bees and MGa, MGc and MGd in cavity-nesting bees. Whereas *A. mellifera* would have kept all four of these macroglomeruli, secondary losses/reductions may have occurred in *A. koschevnikovi* (MGd) and *A. cerana* (MGc). Other models, which would involve convergent appearance of some macroglomeruli are possible (see above), but in our view less likely. It will be important now to precise this model by describing the drone ALs of the remaining known species in the *Apis* genus (the dwarf bee *A. andreniformis*, the giant bee *A. laboriosa*, and the cavity-nesting *A. nuluensis* and *A. nigrocincta*).

Previous neuroanatomical studies in moths have provided prominent examples of a stable macroglomerular complex at the AL entrance of males with only slight morphological reorganisations in the course of evolution (Berg, Galizia, Brandt, & Mustaparta, 2002; Hildebrand, 1996; Kazawa et al., 2009; Namiki et al., 2014; Rospars, 1983; Skiri et al., 2005). In this "functional model" of macroglomerulus evolution, these hypertrophied glomeruli are stable structures across species and adaptations mainly occur at a functional level through mutations of OR genes, shifting their receptive range to detect new compounds. Such system may be advantageous because it allows conserving all downstream neural circuits involved in the elicitation of sexual behaviors. In honey bees, however, drone macroglomeruli are diverse and appear at different locations in the different species. This corresponds more to a "structural model" of macroglomerulus evolution, in which adaptations of males' olfactory system would occur through increases in the numbers of OSNs targeting an existing, ordinary sized, glomerulus, progressively increasing its volume and its sensitivity to specific compounds. Under this model, ORs' receptive range would evolve only slowly. As more and more honey bee genomes are sequenced and their ORs annotated (Karpe, Jain, Brockmann, & Sowdhamini, 2016), we should be able to verify this point soon in this group.

5.4 | Biological function of macroglomeruli

To date, the functional implication of only one macroglomerulus (MGb), in only one *Apis* species (*A. mellifera*), is known. Using optophysiological AL recordings, specific responses to the major component of the queen pheromone, 9-ODA, were recorded in MGb (termed MG2 in Sandoz, 2006). A transcriptomic study identified an olfactory receptor gene, AmOR11, which is overexpressed in *A. mellifera* drones and specifically responds to 9-ODA when expressed in *Xenopus* oocytes (Wanner et al., 2007). Interestingly, an ortholog of AmOR11 was also found to be overexpressed in *A. florea* males (AfOR11, Karpe et al., 2016). Taking into consideration that males of all honey bee species are attracted to 9-ODA (Butler et al., 1967; Gary, 1962; Koeniger & Koeniger, 2000; Nagaraja & Brockmann, 2009; Sannasi et al., 1971; Shearer et al., 1970), and that all investigated species possess MGb, we expect to find an ortholog to AmOR11 overexpressed in the males of all these species. We propose that this structurally homologous MGb would form a labeled line across the *Apis* genus with an unbroken functional history in the detection and processing of 9-ODA (Sandoz, 2006; Sandoz et al., 2007; Wanner et al., 2007). Since functional data on the other macroglomeruli are lacking, we can only speculate about their biological functions. In insects, male macroglomeruli are traditionally considered to be adaptations for the processing of female-emitted mating signals. In *Apis*, because drone (and subsequently queen) flying times of different sympatric species are segregated during the day, the sexuals of different species will very rarely meet in the wild (Koeniger et al., 2014). Consequently, it does not seem necessary for honey bee drones to invest a substantial part of their olfactory system (macroglomeruli) in the recognition of queen-emitted compounds beyond 9-ODA. One cannot exclude, however, that secondary components of the queen pheromonal blend may have played a role during speciation in these species, together with other reproductive isolation processes. Additionally, more and more data in *A. mellifera* point to the use by drones of drone-produced pheromones in the formation of the congregations (Bastin, Cholé, Lafon, & Sandoz, 2017b; Brandstaetter, Bastin, & Sandoz, 2014; Gerig, 1972; Lensky, Cassier, Notkin, Delorme-Joulie, & Levinsohn, 1985; Villar, Wolfson, Hefetz, & Grozinger, 2017). These cues may also be used by virgin queens to find the congregations (Bastin, Savarit, Lafon, & Sandoz, 2017a). Although no data are yet available in other *Apis* species, an interesting hypothesis is that some of the described drone macroglomeruli may process male-produced pheromonal compounds. This being said, we must remain open to the possibility that some drone macroglomeruli may relate to nonsexual traits, representing adaptations to each species' lifestyle. For instance, drones spend most of their lives within/on the nest. In cavity-nesting species, finding food/nurse bees in the complex nest structure may require better olfactory detection/processing capacities than in air-nesting species with a single vertical comb. To progress on these questions, new behavioral data on the use of queen- and drone-olfactory cues by Asian honey bee species are needed. Current efforts to find the odor ligands of ORs that are overexpressed in the drones of the different species compared to females may also provide a helpful strategy toward this goal.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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