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A Survey of Common Pathogens of *Apis* spp. in Wild Non-cave Honeybees in Southwest China

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ABSTRACT: Honeybees are crucial pollinators with significant ecologic value. The decline of wild honeybee populations has been recognized and documented during recent decades. However, the health status of wild non-cave *Apis* spp., including giant and dwarf honeybees, remains generally unknown. We investigated eight common viruses and five bacterial or fungal pathogens in four wild non-cave honeybee species at 11 locations in Southwest China. As a result, *Melissococcus plutonius*, the pathogenic agent of European foulbrood, was detected in all the species, and the sequences were identical to the pathogen in managed cave honeybees. Only one virus, black queen cell virus (BQCV), was positive in one dwarf species, *Apis florea*, in our study. The positive BQCV infected three *A. florea* colonies in Guangxi Province, with distinct sequences from this virus reported in cave honeybees or in the same host in the nearby Yunnan Province. Although our results indicated a low pathogenic level of common diseases in the wild non-cave *Apis* spp. in Southwest China, the conservation of these wild pollinators is of importance in light of the noticeable decline in populations and the irreplaceable position of pollination.

Key words: *Apis* spp., pathogen, virus, wild pollinator.

Honeybees (Hymenoptera: Apidae; *Apis*) are major pollinators with notable ecologic benefit to ecosystem functions (Potts et al. 2010). Currently, nine *Apis* species have been identified (Zheng et al. 2018). The western honeybee, *Apis mellifera*, and the eastern honeybee, *Apis cerana*, have been widely employed for commercial crop pollination and hive products. Their population health has garnered extensive attention during the last decades (Ellis and Munn 2005; Neumann and Carreck 2010). In contrast, due to restricted distribution, limited commercial value, and inaccessible habitats, the health status of wild honeybee species is largely uncharacterized, although a sharp decline in the aerial single-combed honeybee populations, which play a primary role in the functions of natural ecosystems at specific altitudes, has been documented during recent decades (e.g., Teichroew et al. 2017). Therefore, we investigated microbial infections in the four wild non-cave honeybee species in Southwest China, *Apis laboriosa*, *Apis dorsata*, *Apis andreniformis*, and *Apis florea*.

These bees build exposed single-combed nests under inaccessible cliff faces, in deep vertical river valleys, on a tree branch, or on the eaves of buildings (Takahashi and Nakamura 2003; Hepburn et al. 2014). *Apis laboriosa* and *A. dorsata* are the two giant migratory honeybees inhabiting tropical and subtropical regions of Southeast Asia and have been reported in China, Vietnam, Nepal, Bhutan, and India (Takahashi and Nakamura 2003). Dwarf *A. andreniformis* and *A. florea* bees span the lowlands of South Asia, Southeast Asia, and the Middle East (Hepburn and Radloff 2011). Recently, *A. cerana* beekeeping has increased in scale in China due to government-led emphasis on indigenous organisms and poverty alleviation. The sympatric honeybee species may potentially lead to competition for nest sites and spillover of pests and pathogens (Geslin et al. 2017). Several common *Apis* pathogens have been reported to infect wild honeybees (Allen et al. 1990; Zhang et al. 2012; Saraithong et al. 2015); therefore, we screened for eight common honeybee viruses, acute bee paralysis virus, black queen cell virus (BQCV),

FIGURE 1. (a) Locations of *Apis laboriosa* (triangle), *Apis dorsata* (circle), *Apis andreniformis* (diamond), and *Apis florea* (square) sampled in Southwest China, 2015 to 2019. The numbers in parentheses following the localities indicate the number of collected colonies (after the comma) and the ones with positive *Melissococcus plutonius* (before the comma); this was the only pathogenic agent detected in all four non-cave *Apis* spp. in our survey. (b) Neighbor-joining phylogenetic relationships of black queen cell virus (BQCV) detected in *A. florea* in Nanning, Guangxi Province (triangle designated). Seven reference sequences were retrieved from the GenBank database with the virus's host species and locality information.

chronic bee paralysis virus, deformed wing virus, Israeli acute bee paralysis virus, Kashmir bee virus, sacbrood virus, and Varroa destructor virus-1; and five bacterial or fungal pathogens, *Paenibacillus larvae*, *Melissococcus plutonius*, *Ascosphaera apis*, *Nosema apis*, and *Nosema ceranae*, in eleven *A. laboriosa*, eight *A. dorsata*, three *A. andreniformis*, and six *A. florea* colonies in Yunnan, Tibet, Guangxi, and Hainan provinces from 2015 to 2019 (Fig. 1 and Supplementary Material Table S2). Adult honeybee workers were collected and kept on dry ice and then stored at –80 C.

From each colony, we homogenized 15–20 bees and extracted RNA and DNA simultaneously using an isolation kit (Omega Bio-Tek, Norcross, Georgia, USA) following the manufacturer's protocol. Synthesis of cDNA was conducted with RNA products following the instructions of the ReverTra Ace qPCR RT Master Mix (Tiangen, Beijing, China). We carried out PCR amplification on the obtained DNA and cDNA in a 20-µL reaction volume mixture (2×Taq PCR StarMix, Vazyme Biotech, Nanjing, China); the protocol used an initial denaturation at 94 C for 2 min, followed by 35 cycles of 94 C for 30 s, *X* C (see Supplementary Material Table S1 for annealing temperature) for 30 s, and 72 C for 30 s, with a final elongation step at 72 C for 5 min (Gong et al. 2016). Negative (water) and positive (cloned sequence) controls were included in each run of the reaction. The primer sequences used are shown in Supplementary Material Table S1. We electrophoresed PCR products (8 µL per reaction) in 2% Tris-acetate-ethylenediaminetetraacetic acid agarose gel containing 0.01% Gelview (Bio-Teke, Beijing, China) and visualized these under ultraviolet light (Peiqing, Shanghai, China). Positive PCR products were sequenced from both ends using an automated DNA capillary sequencer (Applied Biosystems 3730xl, Foster City, California, USA). The sequences obtained were confirmed using BLAST (NBCI 2020) and were submitted to GenBank (accession nos. BQCV, MN929042–MN929044).

We did not detect any viruses in the wild *A. laboriosa*, *A. dorsata*, and *A. andreniformis* colonies, similar to findings by Allen et al. (1990) in *A. laboriosa*. Coinfection of pathogens was not observed in our study (Supplementary Material Table S2). It has been widely acknowledged that *A. cerana* harbors fewer parasites and viruses than *A. mellifera* (Ellis and Munn 2005; Ai et al. 2012; Hepburn et al. 2014), which may be explained by superior hygienic behavior (Lin et al. 2016) and the disease-resistance mechanism of *A. cerana* that leaves dead drone broods sealed (Boecking 1999; Rath 1999). Similarly, *A. laboriosa* and *A. dorsata* seal diseased, mite-infested, or dead drone and worker broods (Woyke et al. 2004), which acts as a more efficient disease-resistance mechanism than removal of dead broods to limit pathogen spread within colonies. The high tendency of the eastern wild *Apis* spp. to change nest sites

in response to conditions such as food shortage, disease, or interference by natural predators (Allen et al. 1990; Woyke et al. 2004) and the migratory character of *A. laboriosa* and *A. dorsata* (Neumann et al. 2000), resulting in new nest sites, are recognized as effective means of escaping diseases.

We found BQCV infecting three *A. florea* colonies in Nanning, Guangxi Province. The evolutionarily analysis was inferred in MEGA7 using the neighbor-joining algorithm based on the maximum composite likelihood mode (Kumar et al. 2016). The phylogenetic tree shows that BQCV detected in our samples and in *A. florea* and *A. dorsata* in Sipsongpanna, Yunnan Province (Zhang et al. 2012), clustered together and separately from this virus in managed *A. cerana* and *A. mellifera* (Fig. 1b). Further studies are needed to determine whether the differentiation of BQCV strains is due to the role of geography (e.g., Mookhploy et al. 2015) or host species (e.g., Gong et al. 2016).

We detected *M. plutonius*, the causative bacteria of European foulbrood, in all four species (Fig. 1a and Supplementary Material Table S2); sequences were identical to the pathogen found in *A. mellifera* and *A. cerana* after BLAST and evolutionary analysis. There are no previous reports of *M. plutonius* in *A. dorsata*. In China, *A. cerana* is severely threatened by European foulbrood (Zhou et al. 2000), which may lay a foundation for disease transmission to the wild *Apis* spp.; there are multiple contact opportunities between *A. cerana* and wild *Apis* spp. during foraging or robbing (Fig. S1a). Although *Ascosphaera apis*, the pathogenic agent of chalkbrood, seriously impacts *A. mellifera* colonies in China (Zheng et al. 2018), we did not find this pathogen in the wild bees. The introduced *A. mellifera* cannot establish feral populations in the heavily forested areas as well as can indigenous *A. cerana*, leading to a limited chance of interaction with wild bees. This also holds true for *N. ceranae* and for *P. larvae*, which is pandemic in *A. mellifera* but was not detectable in our samples.

Although our study suggests that common *Apis* diseases do not play a central role in the survival of wild *Apis* spp. in Southwest China, a sharp population decline has been recognized and documented locally during recent decades (e.g., Teichroew et al. 2017). Since the PCR primers were developed based on the cavity-nesting honeybee species *A. mellifera*, we cannot rule out the possibilities that the wild non-cave honeybees may be hosts of pathogens despite these not being detected in our survey or that they are under threat from other emerging pathogens. Additionally, viruses are not equally detectable even when they are equally present, due to variation in virulence, seasonality, and caste associations (Ryabov et al. 2019; Abril and Jurvansuu 2020). Our study only sampled adult worker bees; further studies would be required to detect infection in drone bees, as well as the bee brood, at this location in different seasons.

Nests of wild *Apis* spp. are commonly poorly accessible by human beings; however, the high price of the honey (due to scarcity and being more natural) brings about excessive destructive exploitation by honey hunters (Fig. S1b). Furthermore, a dearth of foraging and nesting sites as a result of land-use change, overgrazing by livestock, destruction of forests, competition with managed honeybees, and inappropriate use of pesticides collectively threaten the life cycle of wild honeybees (Potts et al. 2010). The significant functional role of the non-cave giant and dwarf *Apis* spp. to wild plant communities in local ecosystems should be paid more attention in view of the altitude they inhabit, where there are few other pollinators. Creating reserves in the habitat with large numbers of wild *Apis* spp. will be necessary to limit the introduction of managed honeybees and to reduce nest competition and pathogen spillover.

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SUPPLEMENTARY MATERIAL

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