Molecular evolution of bumble bee vitellogenin and vitellogenin-like genes

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Abstract
Vitellogenin (Vg), a storage protein, has been significantly studied for its egg yolk precursor role in oviparous animals. Recent studies found that vitellogenin and its Vg-like homologs were fundamentally involved in many other biological processes in social insects such as female caste differences and oxidative stress resilience. In this study, we conducted the first large-scale molecular evolutionary analyses of vitellogenin coding genes (Vg) and Vg-like genes of bumble bees, a primitively eusocial insect belonging to the genus Bombus. We obtained sequences for each of the four genes (Vg, Vg-like-A, Vg-like-B, and Vg-like-C) from 27 bumble bee genomes (nine were newly sequenced in this study), and sequences from the two closest clades of Bombus, including five Apis species and five Tetragonula species. Our molecular evolutionary analyses show that in bumble bee, the conventional Vg experienced strong positive selection, while the Vg-like genes showed overall relaxation of purifying selection. In Apis and Tetragonula; however, all four genes were found under purifying selection. Furthermore, the conventional Vg showed signs of strong positive selection in most subgenera in Bombus, apart from the obligate parasitic subgenus Psithyrus which has no caste differentiation. Together, these results indicate that the conventional Vg, a key pleiotropic gene in social insects, is the most rapidly evolving copy, potentially due to its multiple known social functions for both worker and queen castes. This study shows that concerted evolution and purifying selection shaped the evolution of the Vg gene family following their ancient gene duplication and may be the leading forces behind the evolution of new potential protein function enabling functional social pleiotropy.

Keywords
vitellogenin, Bombus, positive selection, functional pleiotropy
Vitellogenin (Vg) is a phospholipoglycoprotein, and the precursor protein of vitellin, a protein required for egg yolk formation by most oviparous species (Spitth et al., 1991). In insects, vitellogenin is synthesized in the fat body, released into the hemolymph, and finally taken up by developing oocytes to be consumed throughout embryogenesis (Hagedorn & Kunkel, 1979; Pan et al., 1969; Raikhel & Dhadialla, 1992; Tufail & Takeda, 2008). Despite its main egg yolk function, Vg is not female-specific and can also be found in males of some species although in smaller amount (Piulachs et al., 2003; Trenzek & Engels, 1986; Tufail & Takeda, 2008). Moreover, Vg has been extensively studied for its multifunctional effects in social insect life histories. In honey bee (Apis mellifera), in addition to its central involvement in the division of labor between queens and workers (Tufail & Takeda, 2008; Weil et al., 2009), Vg is known to be involved in regulation of nonreproductive features of colonies, such as aging and queen longevity (Corona et al., 2007; Excels, 1974), temporal worker division of labor (Bloch & Grozinger, 2011; Guidugli et al., 2005; Münch & Amdam, 2010; Nelson et al., 2007), and royal jelly production (Amdam et al., 2003).

In honey bee, only one conventional Vg gene can be found and has been extensively studied for its multiple phenotypic effects on both queen and worker traits (Amdam et al., 2003). Pleiotropic genes are expected to be evolutionarily constrained since mutations that increase fitness for one trait might decrease overall fitness via antagonistic effects on other traits (Otto, 2004). Interestingly however, many previous studies have not found support for a negative relationship between the pleiotropy of a given gene and its selection pressure, measured as the ratio of nonsynonymous to synonymous substitutions (dN/dS; e.g., Razeto-Barry et al., 2011; Twyman et al., 2018; Vedanayagam & Garrigan, 2015). Vg displayed high rates of adaptive evolution, and positive selection signs of this gene were repeatedly detected in eusocial hymenopteran species (Kent et al., 2011; Morandin et al., 2014; Salmela et al., 2016).

Multiple roles for a single protein are also projected to lead to a gene duplication event and to favor the multiple acquired roles. Besides the conventional Vg, three homologs called Vg-like proteins were recently discovered in ants (Morandin et al., 2014). These Vg homologs have arisen from an ancient gene duplication event. Two of these homologs, Vg-like-A and Vg-like-B, can be found in all insect species studied, while Vg-like-C was so far only found in Hymenoptera (Kohlmieier et al., 2018; Morandin et al., 2014). These homologs exhibit differences in their conserved protein domains and have undergone rapid evolution after duplications (Morandin et al., 2014). Their role is currently unknown, but their structural variation suggests variable functions. In honey bee, Vg-like-A displays the closest structural and functional similarities to Vg and responded strongly to inflammatory and oxidative conditions, thus is likely associated with the aging process (Salmela et al., 2016). Vg-like-A also showed a strong temporal expression variation and may be involved in wintering worker longevity (Ricigliano et al., 2018). Furthermore, Vg-like-A is linked to the regulation of nursing behaviors in the ant Temnothorax longispinosus (Kohlmieier et al., 2018). During duplication, Vg-like-B lost several Vg structural elements, which may suggest that Vg-like-B may perform only few of the Vg original functions, such as coping with oxidative stress (Morandin et al., 2014). Four protein domains (N-sheet, a-helical, vWFD, and polyserine linker) were found in Vg, while only the N-sheet was detected in Vg-like-C, potentially implying specialization, and could possibly be involved in neurobiological functions (Salmela et al., 2016).

Bumble bees are a group of insects belonging to the genus Bombus (Hymenoptera: Apidae). Bumble bees, honey bees, and stingless bees (Tetragonula) are phylogenetically close relatives (Peters et al., 2017). There are about 250 known bumble bee species belonging to 15 subgenera, mainly distributed in the northern hemisphere (Cameron et al., 2007; Williams et al., 2008). Bumble bees are often described as primitively eusocial because their social organization is simpler than that of the honeybee. Unlike honey bees or stingless bees, most bumble bee species have an annual cycle, with queens single-handedly founding nests (Goulson, 2010). Bumble bees pass through several distinct phases during their annual life cycle, including solitary and eusocial phases. At the final stage of their colony cycle, termed the competition phase, the queen and workers will compete intensely over the production of males (Amsalem et al., 2015).

The fascinating life history and high levels of biological and ecological heterogeneity make bumble bees an outstanding model system for the study of molecular evolution. First of all, the biological and ecological characteristics of bumble bees can largely differ among the different subgenera (Williams et al., 2008). For instance, the tongue lengths of bumble bees are very diversified among different subgenera. Some subgenera such as Orientalisombus, Subterraneombus, and Sibiricombus which favor deep flowers have very long tongues, while others have relatively short tongues (Williams et al., 2008). Bumble bees are also extremely diversified in their habitats: for example, Mecodiacombus and Alpinobombus species prefer alpine/arctic, while Orientalisombus generally use forest habitats. Most strikingly, there are obligate parasitic species in the subgenus Psithyrus which enslaves the species from other subgenera (Amsalem et al., 2015). To our knowledge, such extreme biological and ecological diversifications within a single genus have not been previously reported in honey bees or stingless bees. Also, honey bees and stingless bees are primarily tropical insects, with relatively stable environments, while bumble bees mainly occur in cool climates in general with more variable environments. Furthermore, bumble bees biological and ecological characteristics may also deviate among different species within a single subgenus. For example, in at least seven subgenera, the distribution elevation significantly varies among different species (An et al., 2014), leading to distinct genomic evolution rates among species (Lin et al., 2019). Consequently, we hypothesize that Vg and Vg-like genes should be under distinct selection forces in bumble bees compared to honey bees or stingless bees.

To date, for most multi-species Vg analysis, bumble bee Vg sequences were compared with species from other genera (Du
et al., 2019; Li et al., 2010; Salmela et al., 2016), probably due to the lack of sequence data from multiple bumble bee species. In this study, based on 27 bumble bee genomes, and combined with genomes of their phylogenetically close relatives (honey bees and stingless bees), we conducted the first large-scale molecular evolutionary analyses of bumble bee Vg and Vg-like genes to understand the selective patterns of Vg gene family in bumble bees.

2 | MATERIALS AND METHODS

2.1 | DNA extraction and libraries construction

Adult workers from nine species of Bombus (B. lantschouensis, B. sichelii, B. impetuosus, B. laesus, B. skorikovi, B. bohemicus, B. trifasciatus, B. waltoni, and B. convexus) were live-trapped using sweep nets from the field. Samples of B. trifasciatus were collected in Ji’an, Jiangxi Province, China, while the other eight species were collected in Qinghai Province, China, between 2017 and 2019. After collection, samples were immediately stored at −20°C.

DNA extractions, libraries construction, and sequencing were done by the company Novogene. In brief, DNA was extracted from whole-body of each individual (one worker per species) using a DNeasy Blood and Tissue Kit (Qiagen). Concentration and quality of extracted DNA was examined with an Agilent 2100 Bioanalyzer (Agilent Technologies). Libraries were sequenced paired-end for 150 cycles (1 × 150 bp) on an Illumina Hiseq 2000 system. Read quality was inspected with FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Clean reads were used for de novo assembly using IDBA-UD (Peng et al., 2012) with default settings, and contigs shorter than 200 bp were discarded.

2.2 | Discovery of Vg and Vg-like sequences

In addition to our nine newly sequenced Bombus species, previously published bumble bee genomes were added to our dataset (see Jackson et al., 2020; Kent et al., 2018; Lin et al., 2019; Sadd et al., 2015; Tian et al., 2019). As a result, a total of 27 bumble bee species belonging to 10 subgenera (2 to 5 species for each genus) were used for this study (Figure 1; Table S1). The nucleotide sequences of Vg, Vg-like-A, Vg-like-B, and Vg-like-C of B. impatiens were downloaded from GenBank (Vg, XP_003492277.1; Vg-like-A, XP_003494500.3; Vg-like-B, XP_012245829.1; and Vg-like-C, XP_003489777.1). We

![Figure 1](image-url)
retrieved the corresponding coding sequences from the 26 other bumble bee genomes using the software Exonerate v2.4.0 (Slater & Birney, 2005), with default settings and with B. impatiens Vg and Vg-like protein sequences as queries.

Additionally, we aimed to retrieve Vg and Vg-like sequences from stingless bees (Tetragonula spp.) and honey bees (Apis spp.). These two genera are phylogenetically close relatives of Bombus (Peters et al., 2017) and five species of each genus (Tetragonula carbonaria, T. clypearis, T. davenporti, T. hockingsi, and T. mellipes; Apis cerana, A. dorsata, A. florea, A. laboriosa, and A. mellifera) were available with genomic resources in GenBank (Table S2). To do so, we obtained Vg and Vg-like sequences from GenBank for Apis mellifera (Vg, NP_001011578; Vg-like-A, XP_001121939; Vg-like-B, XP_395423; Vg-like-C, XP_001122505) and used them as queries to extract the other Apis species sequences using Exonerate with default settings. In the same manner, the translated protein sequences of the four genes from B. impatiens and A. mellifera were used as queries to extract the corresponding sequences from the five Tetragonula species.

The Vg and Vg-like sequences from each species were aligned for each genus separately using ClustalW (Codons) program embedded in the software MEGA v10.1.7 (Kumar et al., 2018) with default settings and verified by visual inspection. The DNA sequence variations were calculated using DnaSP v 6.12.03 (Rozas et al., 2017).

The reference species tree of Bombus (Figure 1) was drawn by TreeGraph2 v2.15.0 (Stöver & Müller, 2010) according to previous studies (Cameron et al., 2007; Williams et al., 2012). The reference tree of Tetragonula (Figure S1) was drawn according to Raffiudin and Crozier (2007; Figure S1).

2.3 Molecular evolutionary analyses among different genera

We used the CODEML program in the PAML package v4.9j (Yang, 2007) to study the selection pressures affecting the different genes and to test for patterns of molecular evolution. Our aim was to estimate synonymous and nonsynonymous substitutions using the branch tests and site tests (Bielawski & Yang, 2005).

We firstly tested whether the overall selection of Vg and Vg-like genes of bumble bees deviated from honey bees and stingless bees. The M0 (one ratio) was used to estimate the overall selection (dN/dS, ratio of nonsynonymous/synonymous substitution rates) across all sites, and the alignment of each gene for each genus (Bombus, Apis, and Tetragonula) was separately calculated to compute the dN/dS ratio of each genus. Next, we calculated the pairwise dN/dS ratios (Yang & Nielsen, 2000) across the species within each genus using the YN00 program from the PAML package. The values of pairwise dN/dS ratios of each gene were compared between Bombus and each of the other two genera using a Mann–Whitney rank test in SPSS v25.0. The relationships between dN and dS and between dN/dS and dS were tested using a linear regression analysis in SPSS.

2.4 Molecular evolutionary analyses within Bombus

We first tested whether differences of selection pressures exist among the ten bumble bee subgenera. The "several dN/dS ratios" branch model (BM) was used to estimate the dN/dS ratios of each of the ten subgenera independently. The ten external branches corresponding to the ten subgenera were viewed as different foregrounds, whereas all the internal branches were viewed as a common background (Figure 1). The likelihood ratio tests (LRTs) between M0 (null model) and BM models were conducted by comparing twice the difference in log-likelihood values (2ΔlnL) against a chi-square distribution (df = 2). The obtained ten dN/dS ratios of the bumble bee subgenera were compared across the four different genes using one-way ANOVA and paired samples t tests in SPSS. Moreover, we also used the branch-site model called Model A to test for positive selections in each subgenera. The null model for Model A is Model A1, which is a modify on Model A, but with ω= 1 fixed (Yang et al., 2005; Zhang et al., 2005). In each run, one target subgenus was marked as the foreground branch while the remaining nine subgenera were viewed as backgrounds. Again, 2ΔlnL values between Model A and Model A1 were used to conduct LRTs for robustness with χ2 test (df = 1).

Lastly, we studied the extent of selection for each Vg and Vg-like gene set of Bombus individually by dividing the data into four data sets (one for each orthologous gene) and comparing the neutral model (M1a) with a model allowing positive selection (M2a). The 2ΔlnL values between the M1a and M2a models were used to test for robustness using LRTs with χ2 test (df = 2), and positively selected sites were identified with the Bayes Empirical Bayes (BEB; Yang et al., 2005).

It should be mentioned that, in order to reduce false discovery rate, a Benjamini–Hochberg correction in R program ("p.adjust" command) was used where necessary (see below).

3 RESULTS

3.1 Overall genetic variation of Vg and Vg-like genes

A total of 2,095 million DNA reads were obtained from the nine bumble bee species (B. lantschouensis, B. sichelii, B. impetuosus, B. laeisus, B. skorikovi, B. bohemicus, B. trifasciatus, B. watoni, and B. convexus), with a total size of 314 Gb (SRA accession No. PRJNA667279). Coding sequences of the four Vg genes were successfully obtained for the 27 bumble bee species (File S1). The lengths of the aligned sequences (stop codons not considered) were 5,337, 4,569, 4,260, and 960 base pairs, respectively. Vg was the most variable in terms of nucleotide sequence identity among the four genes, with 2,389 (42.98%) variable nucleotide sites, including 39 indel codons. In contrast, Vg-like-B was the most conservative, with 11.56% variable nucleotide sites, and no indel was detected. Vg-like-A and Vg-like-C...
TABLE 1 Genetic variations of Vg and Vg-like genes of the bumble bees (Bombus), stingless bees (Tetragonula), and honey bees (Apis; stop codons were not considered)

| Sequence          | Index | Vg   | Vg-like-A | Vg-like-B | Vg-like-C |
|-------------------|-------|------|-----------|-----------|-----------|
| Bombus (N = 27)   | Total sites | 5,337 | 4,569     | 4,260     | 960       |
|                   | Variable sites | 2,294 | 903       | 476       | 193       |
|                   | Variable percent | 42.98% | 19.76%    | 11.17%    | 20.10%    |
| Tetragonula (N = 5) | Total sites | 5,331 | 4,503     | 4,245     | 948       |
|                   | Variable sites | 140   | 73        | 54        | 39        |
|                   | Variable percent | 2.63%  | 1.62%     | 1.27%     | 4.11%     |
| Apis (N = 5)      | Total sites | 5,319 | 4,524     | 4,260     | 978       |
|                   | Variable sites | 839   | 514       | 194       | 213       |
|                   | Variable percent | 15.77% | 11.36%    | 4.55%     | 21.78%    |

showed similar levels of genetic variation and Vg-like-C sequence included three indel codons (Table 1).

The sequences of the four genes from five stingless bee species and five honey bee species are also provided in File S1. Unlike bumble bees, stingless bees and honey bees Vg-like-C sequence was the most variable in terms of nucleotide sequence identity among the four genes, follows by Vg and Vg-like-A (Table 1). Consistent with the bumble bee sequences, Vg-like-B was the most conserved gene for both stingless and honey bees (Table 1). The overall sequence variation information can be found in Table 1.

3.2 Molecular evolution between Bombus and the other two genera

We first characterized the extent of positive and purifying selection in each of the orthologous copies separately (Vg, Vg-like-A, Vg-like-B, and Vg-like-C). The dN/dS ratios based on the M0 model showed that Bombus conventional Vg was under strong positive selection (dN/dS = 1.311), whereas the Vg-like genes were under purifying selection (dN/dS = 0.349, 0.077 and 0.196, respectively, Table 2). In Apis and Tetragonula, all four genes were found under purifying selection. Moreover, in Tetragonula, Vg-like-A, B, and C are the most conserved copies (dN/dS = 0.295, 0.072 and 0.107, respectively, Table 2), whereas the conventional Vg evolve more rapidly (dN/dS = 0.302). In Apis, however, Vg-like-C was evolving more rapidly (dN/dS = 0.301) than the conventional Vg (dN/dS = 0.260), Vg-like-A (dN/dS = 0.211), and Vg-like-B (dN/dS = 0.076). In all three genera, Vg-like-B was the most conserved gene (Table 2).

Pairwise analyses of Vg selection showed that, for any of the three genera, dN significantly increased with increasing dS (Figure 2). Linear regression and Benjamini–Hochberg correction (n = 12) showed that the relationship of dN and dS of Bombus species follow a function of dN = 0.022 + 0.924*dS, with a strong correlation (R² = 0.882, p < .001). Similar patterns were found among Tetragonula species (dN = −6.26E-4 + 0.385 * dS, R² = 0.985, p < .001) and Apis species (dN = 0.016 + 0.212 * dS, R² = 0.788, p < .001). For all Vg orthologous copies, dN and dS were also positively correlated, at the exception of Vg-like-C sequence in Apis (R² = 0.281, p =.115, Table 3). The linear regression information is shown in Table 3 and Figure S2.

Mann–Whitney rank test and Benjamini–Hochberg correction (n = 8) showed that pairwise dN/dS values of bumble bees Vg were significantly larger than those of stingless bees and honey bees (Z = −5.393, p < .001). Pairwise dN/dS values of bumble bees Vg-like-A were significantly larger than those of honey bees (Z = −5.128, p < .001), but not significantly deviated from stingless bees (Z = −1.023, p = .350). No significant differences of pairwise dN/dS values for Vg-like-B could be detected between bumblebees and stingless bees (Z = −1.540, p =.165) nor honey bees (Z = −0.255, p = .799). In contrast, pairwise dN/dS values of bumble bees Vg-like-C were significantly smaller than those of stingless bees (Z = −3.306, p =.002) and honey bees (Z = −4.491, p < .001).

3.3 Molecular evolution within Bombus

Similar as the M0 model, the branch model showed that Bombus conventional Vg was under positive selection with dN/dS = 1.331 ± 0.302 (mean ± SD), whereas the Vg-like genes were under purifying selection (dN/dS = 0.344 ± 0.064, 0.074 ± 0.024 and 0.159 ± 0.080, respectively [Table 4; Figure 3]). One-Way ANOVA based on the branch model results showed that the dN/dS values significantly deviated among the four genes (F = 131.588, df = 3, p < .001). Paired sample t test and Benjamini–Hochberg correction (n = 6) confirmed this finding (df = 9, p < .006) and showed a rank order of Vg > Vg-like-A > Vg-like-C > Vg-like-B (Figure 3). According to the likelihood ratio test, the branch model significantly deviated from the M0 (df = 10, p < .001) for Vg, but not...
for the Vg-like genes (df = 10, p > .151; Table 4). For Bombus Vg, nine out of ten subgenera showed a dN/dS ratio over 1 (average = 1.40, range: 1.12–1.71, Table 4), with the exception of the subgenus Psithyrus (Figure 3) for which the dN/dS ratio was only 0.713.

The M2a model showed that 32.1% of the Bombus Vg codons were under purifying selection (dN/dS < 1), 46.2% under neutral selection (dN/dS = 1), and 21.6% under positive selection (ω2 = 4.111).
TABLE 5 Statistical results of site models in Vg and Vg-like genes of 27 bumble bee species

| Gene               | M1a (np = 54)                             | M2a (np = 56)                             | LRT (df = 2)       |
|--------------------|-------------------------------------------|-------------------------------------------|--------------------|
| Vg                 | p0 = 0.436, ω0 = 0.083; p1 = 0.564, ω1 = 1.000; lnL = -31,891.635 | p0 = 0.321, ω0 = 0.070; p1 = 0.462, ω1 = 1.000; lnL = -31,416.198 | 2ΔlnL = 950.874, p < .001 |
| Vg-like-A          | p0 = 0.716, ω0 = 0.064; p1 = 0.284, ω1 = 1.000; lnL = -13,541.057 | p0 = 0.746, ω0 = 0.091; p1 = 0.231, ω1 = 1.000; lnL = -13,526.160 | 2ΔlnL = 29.794, p < .001 |
| Vg-like-B          | p0 = 0.968, ω0 = 0.050; p1 = 0.032, ω1 = 1.000; lnL = -9,062.035 | p0 = 0.971, ω0 = 0.052; p1 = 0.028, ω1 = 1.000; lnL = -9,060.276 | 2ΔlnL = 3.581, p = .167 |
| Vg-like-C          | p0 = 0.877, ω0 = 0.075; p1 = 0.123, ω1 = 1.000; lnL = -2,892.983 | p0 = 0.886, ω0 = 0.078; p1 = 0.000, ω1 = 1.000; lnL = -2,892.958 | 2ΔlnL = 0.050, p = .975 |

TABLE 6 Branch-site model results for each of the 10 bumble bee subgenera (N0.95, number of sites which were positively selected with posterior probability >0.5; N0.995, number of sites which were positively selected with posterior probability >0.95)

| Foreground subgenus | LRT (df = 1)     | N0.95 | N0.995 |
|---------------------|------------------|-------|--------|
| Pyrobombus          | 2ΔlnL = 94.471, p < .001 | 52    | 10     |
| Bombus s. str.      | 2ΔlnL = 6.709, p < .012   | 3     | 0      |
| Sibiricobombus      | 2ΔlnL = 12.667, p < .001  | 26    | 1      |
| Melanobombus        | 2ΔlnL = 57.192, p < .001  | 80    | 5      |
| Alpigenobombus      | 2ΔlnL = 19.542, p < .001  | 41    | 1      |
| Thoracobombus       | 2ΔlnL = 27.831, p < .001  | 107   | 0      |
| Psithyrus           | 2ΔlnL = 0.000, p = 1.000   | 1     | 0      |
| Megabombus          | 2ΔlnL = 10.140, p < .002   | 15    | 0      |
| Subterraneobombus   | 2ΔlnL = 6.037, p = .016    | 6     | 0      |
| Mendacibombus       | 2ΔlnL = 41.450, p < .001   | 44    | 0      |

For the Vg-like genes, most codons were under either purifying selection or neutral selection (Table 5). In Bombus, Bayes Empirical Bayes analysis showed that 150 codons (8.43%) in the conventional Vg and 3 codons (0.20%) in Vg-like-A were detected as positively selected sites (probability >.95). Yet, we could not detect any positively selected sites for Vg-like-B and Vg-like-C. The M2a models were robust for Vg and Vg-like-A, but not for Vg-like-B or Vg-like-C (Table S3).

The branch-site models as well as Benjamini–Hochberg correction (n = 10) showed that for nine of the ten Bombus subgenera (except for B. Psithyrus), three or more sites were under positive selection with posterior probability >0.5. And in four subgenera (Pyrobombus, Sibiricobombus, Melanobombus, and Alpigenobombus), at least one site was under positive selection with posterior probability >.95. For the same nine subgenera, the log-likelihood values of Model A significantly deviated from its counterpart Model A1 (ΔlnL = 0.016), indicating the likelihood of this result. For Psithyrus, only one site showed signs of positive selection. However, because there were not any deviations between Model A and Model A1 (2ΔlnL = 0.000, p = 1.000), this result could not be supported. The details of the branch-site model results are listed in Table 6.

4 | DISCUSSION

Vitellogenin is a multifunctional hemolymph protein that is characterized by its central role in social insect's division of labor and affects multiple aspects of social insect life histories (Amdam et al., 2003). Bumble bees are primitively eusocial insects which represents an intermediate stage in the evolution between solitary and eusociality (Goulson, 2010). Unlike the “true” eusocial bees such as honey bees or stingless bees, most bumble bee species pass through both solitary and eusocial phases during their life cycle. Moreover, bumble bees are extremely diversified in their biological and ecological characteristics among different subgenera. Here we show that in the bumble bees, Vg has experienced elevated rate of evolution and is under strong positive selection. Its homologous genes, Vg-like genes that have diverged both from Vg and from each other, do not display such patterns. Additionally, signs of positive selection were also absent from its sister clades, Tetragonula and Apis' Vg.

A unique advantage of bumble bees is the fact that sequences from the two closest clades are publicly available, so we can investigate the forces driving vitellogenin evolution across the phylogeny tree and ask whether Vg and Vg-like genes in distinctive genus differ in their molecular evolutionary rates. Our study determines that the conventional Vg is the most rapidly evolving copy in bumblebees. Interestingly, we observed that unlike in bumble bees, no sign of positive selection could be found for the conventional Vg of both Tetragonula and Apis. The pairwise analyses showed that all the three genera had increased dN with increasing dS. However, the slope of the linear regression of Bombus was much higher than that of Tetragonula and Apis, indicating a higher overall dN/dS ratio in Bombus. Although there were considerable overlaps of dS between Bombus and the other two genera, the pairwise dN/dS values among Bombus genera were larger than any of the counterparts of Tetragonula and Apis (Figure 2). In Tetragonula and Apis, the purifying
selection pressure on Vg might be slightly lower because Vg-related traits underlying adaptive evolution may differ between the genera. Thus, our results suggest that duplication, positive evolution, and purifying selection may be major evolutionary forces driving Vg gene evolution across divergent taxa.

Vg is best known for its primary role in the formation of egg yolk in egg-laying animals (Tufail & Takeda, 2008); however, in social insects, Vg has probably acquired additional functions (Guidugli et al., 2005) and fulfills roles related to behavior and survival (Havukainen et al., 2011; Kent et al., 2011; Nelson et al., 2007; Seehuus et al., 2006). Functional pleiotropy plays an important role in molecular evolution (Paaby & Rockman, 2013). Although increased purifying selection against pleiotropic mutations (McGuigan et al., 2014) and slow rates of evolution of pleiotropic genes (Salathe et al., 2006) have been observed in some cases, more recent studies have demonstrated that gene pleiotropy may increase evolutionary rate (Razeto-Barry et al., 2011; Twyman et al., 2018; Vedanayagam & Garrigan, 2015). While viewed as a primi-tively eusocial genus, the single conventional Vg protein in bumble bees showed inclines of functional pleiotropy of bumble bee’s Vg (Razeto-Barry et al., 2011). This pattern is consistent with the fact that the positive selection that we detected may drive the evolution of novel protein function and thus enables functional pleiotropy of bumble bee’s Vg (Razeto-Barry et al., 2011). Similar patterns have been observed in the Vg genes of other eusocial hymenopteran species (Kent et al., 2011; Morandin et al., 2014; Salmela et al., 2016). Thus, our results suggest that Vg functional pleiotropy may have arisen due to strong positive selection acting on it, and may further indicate the appearance of a novel, unknown Vg function in bumble bees.

Functional pleiotropy of a gene is also predicted to lead to a duplication event, such as the duplication of the ancestral gene leading to Vg and Vg-like genes (Morandin et al., 2014). Gene duplication is an important source of new genetic material for selection to act upon (Force et al., 1999; Lynch & Force, 2000; Ohno, 1970; Zhang, 2003). After duplication, the duplicated gene copy can acquire functions different from those of the ancestral gene (Gu et al., 2002; Khaladkar & Hannenhalli, 2012; Morandin et al., 2014; Wagner, 2000). Unlike Vg, the three Vg-like genes showed signs of purifying selection in all three genera. Multiple factors could be affecting the rate of sequence evolution such as the number of pleiotropic interactions, the gene expression levels, or their tissue-specific expression patterns, that could not be detected in this study. Further studies on evolutionary patterns of Vg-like genes across social insect species and on their functions are needed to fully understand their roles in social insects and the selection pressures they experience.

An exception to the otherwise overall positive selection on vitellogenin in our set of bumble bee species is the subgenus Psithyrus. We found that the subgenus-based branch model analyses showed that almost all subgenera had a dN/dS ratio over 1. Incredibly however, the dN/dS ratio of the subgenus Psithyrus was much lower than the other subgenera (only 0.713). Moreover, the subgenus-based branch-site model analyses indicated that all subgenera but Psithyrus had significant deviations between Model A (alternative hypothesis) and Model A1 (null hypothesis). These results repeatedly indicate the distinctive evolution of Psithyrus Vg. Bumble bees in the subgenus Psithyrus have annual life cycles similar to those of typical bumble bee species, except that instead of founding their own nest and rearing workers, they steal a nest from “true” bumble bees (Goulson, 2010). Initially, these bumble bees were formerly described as a separate genus Psithyrus, but it is now widely accepted that they belong to a subgenus within Bombus, with the subgenus Thoracobombus as the sister group (Cameron et al., 2007; Williams et al., 2008). These bumblebees also exhibit social parasitism, with the absence of a worker caste, or the need to forage for nectar and pollen to provision developing larvae (Lhomme & Hines, 2019). Although it is not clear whether the lower positive selection level of Psithyrus was a secondary evolutionary event, it is probable that their fundamentally different life history has influenced the evolution of Vg even within just one genus. In fact, due to the absence of caste differentiation in Psithyrus, it is conceivable that its simplified lifestyle reduced the functional pleiotropy demand on Vg and thus caused a lower dN/dS ratio.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTION
Fang Zhao: Investigation (equal); Methodology (equal). Claire Morandin: Investigation (equal); Methodology (equal). Kai Jiang: Methodology (supporting). Tianjuan Su: Methodology (supporting). Bo He: Methodology (supporting). Gonghua Lin: Conceptualization (lead); Funding acquisition (equal); Investigation (lead); Methodology (equal). Zuhao Huang: Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (lead).

DATA AVAILABILITY STATEMENT
The genome data (BioProject ID: PRJNA667279) are available from the Sequence Read Archive of the National Centre for Biotechnology Information (NCBI). The other data generated or analyzed during this study are included in this published article and its supplementary files.

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