Genomic Analyses Reveal Demographic History and Temperate Adaptation of the Newly Discovered Honey Bee Subspecies *Apis mellifera sinisxinyuan* n. ssp

Chao Chen,[†,1,2] Zhiguang Liu,[†,1,2] Qi Pan,[†,3] Xiao Chen,[1] Huihua Wang,[1] Haikun Guo,[1] Shidong Liu,[4] Hongfeng Lu,[3] Shilin Tian,[3] Ruiqiang Li,[3,5] and Wei Shi[*,1,2]

[1]Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing, China

[2]Key Laboratory of Pollinating Insect Biology, Ministry of Agriculture, Beijing, China

[3]Novogene Bioinformatics Institute, Beijing, China

[4]Apiculture Management Center of Xinjiang Uygur Autonomous Region, Urumqi, Xinjiang, China

[5]Peking-Tsinghua Center for Life Sciences, Biodynamic Optical Imaging Center (BIOPIC) and School of Life Sciences, Peking University, Beijing, China

[†]These authors contributed equally to this work.

***Corresponding author:** E-mail: shiweibri@126.com.

Associate editor: Michael Rosenberg

Abstract

Studying the genetic signatures of climate-driven selection can produce insights into local adaptation and the potential impacts of climate change on populations. The honey bee (*Apis mellifera*) is an interesting species to study local adaptation because it originated in tropical/subtropical climatic regions and subsequently spread into temperate regions. However, little is known about the genetic basis of its adaptation to temperate climates. Here, we resequenced the whole genomes of ten individual bees from a newly discovered population in temperate China and downloaded resequenced data from 35 individuals from other populations. We found that the new population is an undescribed subspecies in the M-lineage of *A. mellifera* (*Apis mellifera sinisxinyuan*). Analyses of population history show that long-term global temperature has strongly influenced the demographic history of *A. m. sinisxinyuan* and its divergence from other subspecies. Further analyses comparing temperate and tropical populations identified several candidate genes related to fat body and the Hippo signaling pathway that are potentially involved in adaptation to temperate climates. Our results provide insights into the demographic history of the newly discovered *A. m. sinisxinyuan*, as well as the genetic basis of adaptation of *A. mellifera* to temperate climates at the genomic level. These findings will facilitate the selective breeding of *A. mellifera* to improve the survival of overwintering colonies.

***Key words:* *Apis mellifera sinisxinyuan*, new subspecies, temperate climates, population history, adaptation.**

Article

Introduction

Identifying adaptive variation driven by different climate factors is a major focus in evolutionary biology. Both phenotypic and genetic variation among populations can be influenced by climatic factors, as has been demonstrated in a variety of species such as *Arabidopsis*, *Drosophila*, sheep, and human (González et al. 2010; Hancock, Brachi, et al. 2011; Hancock, Witonsky, et al. 2011; Lv et al. 2014). Detecting climate-mediated selective signatures is important for understanding not only the genetic basis of adaptation to local environment but also the potential effects of climate change on populations (Balanyà et al. 2009). In addition, identifying adaptive polymorphisms can shed light on functionally important variants (Nielsen et al. 2007).

The western honey bee (*Apis mellifera*) is an interesting species for studying climate-driven adaptations. *Apis mellifera* is widely distributed in Africa, western Asia, and Europe, with at least 24 subspecies that can be divided into four lineages based on morphometric and molecular studies: A-lineage in Africa, M-lineage in western and northern Europe, C-lineage in eastern Europe, and O-lineage in the Middle East and western Asia (Ruttner 1988; Garnery et al. 1992; Arias and Sheppard 1996; Whitfield et al. 2006; Han et al. 2012; Wallberg et al. 2014). *Apis mellifera* originated in tropical/subtropical regions and subsequently radiated into temperate zones, and adaptation to temperate climates is a key step in its evolutionary history (Ruttner 1988; Han et al. 2012). Temperate and tropical populations of *A. mellifera* show fundamental differences in a variety of traits (Winston 1987), most of which involve coping with long, cold winters (Southwick and Heldmaier 1987; Ruttner 1988; Amdam et al. 2005). However, few molecular studies have been conducted to investigate the genetic responses to temperate climates in *A. mellifera*.

At present, studies on the adaptation of *A. mellifera* to temperate climates mainly focus on European honey bees, in

particular *A. m. mellifera* (Maurizio and Hodges 1950; Fluri et al. 1977; Seeley 1985, and references therein; Southwick and Heldmaier 1987; Ruttner 1988; Amdam et al. 2005; Meixner et al. 2007; Seehuus et al. 2013). *Apis mellifera mellifera* is distributed along the northwest boundary of the range of *A. mellifera* in Europe, and is well known for its ability to survive harsh winters. However, to enhance our understanding of the species adaptation to temperate climates, it is preferable to also study populations in the northeast region of its range in northwest China, Mongolia, Kazakstan, or southern Russia. We found a new, winter-tolerant population of *A. mellifera* in Xinyuan prefecture, located in Xinjiang Uygur Autonomous Region, China. The natural environment in Xinyuan is characterized by long, cold winters due to a combination of high latitude and altitude, with a frost season of 185–205 days. However, the genetic diversity and evolutionary history of this group had not been extensively studied.

In this study, we investigated the Xinyuan honey bee population's demographic history and genome-level adaptation to temperate climates. We collected ten feral colonies at different locations across the Xinyuan prefecture and resequenced the whole genomes of a total of ten individuals, each from a separate colony (supplementary table S1, Supplementary Material online). We also downloaded resequenced data from representative populations in each of the four previously delineated lineages of *A. mellifera*, including *A. m. mellifera*, *A. m. carnica*, *A. m. ligustica*, *A. m. anatoliaca*, and *A. m. scutellata*. Using a population genomic approach, we estimated the relationships and identified genomic polymorphisms in these honey bee populations and reconstructed the demographic history to elucidate how temperate climates shape the divergence of Xinyuan bees and their close relatives. We also identified rapidly evolving genes in honey bees from Xinyuan to gain insight into genetic basis of adaptation to temperate climates.

Results

We selected 45 individuals from seven populations (the Xinyuan bees, *A. m. mellifera*, *A. m. carnica*, *A. m. ligustica*, *A. m. anatoliaca*, *A. m. scutellata*, and *A. cerana* as outgroup) (fig. 1A and supplementary table S2, Supplementary Material online). Resequencing of the ten Xinyuan bees generated a total of 178.9 million paired-end reads of 100 bp read length (17.9 Gb of sequence, supplementary table S1, Supplementary Material online). Genome alignment of the Xinyuan bee sequences indicated an average depth of 8.08-fold and coverage of 89.86% relative to the *A. mellifera* reference genome (234.087 Mb; supplementary table S3, Supplementary Material online) (The Honeybee Genome Sequencing Consortium 2006). After stringent quality filtering, we identified a total of 2,123,243 high-quality population single nucleotide polymorphisms (SNPs) in the 45 individuals (supplementary table S4, Supplementary Material online), and 988,217 small indels (shorter than 50 bp) (supplementary table S5, Supplementary Material online).

Evolutionary and Demographic History

Population Structure and Divergence

To infer the phylogenetic relationship of the Xinyuan bees and other populations, we constructed a phylogenetic tree using the neighbor-joining algorithm based on the pairwise genetic distances between populations using polymorphic SNPs in whole genome. The tree showed that honey bees from Xinyuan and *A. m. mellifera* were clustered into a subclade, and that they potentially originated from a common ancestor (fig. 1B). We also used the SNPs in coding and noncoding regions to construct neighbor-joining trees and obtained the same topological structures (supplementary fig. S1, Supplementary Material online). Principle component analysis using EIGENSOFT3.0 (Patterson et al. 2006) and population structure analyses using *frappe* (Tang et al. 2005) corroborated these patterns. In particular, the first principal component (variance explained = 16.90%, Tracy–Widom $P = 1.12E\text{-}06$) separated the outgroup *A. cerana* from the other populations. The second principal component (variance explained = 14.89%, Tracy–Widom $P = 9.51E\text{-}14$) indicated that Xinyuan honey bees and *A. m. mellifera* were separated from the other subspecies (fig. 1C, supplementary fig. S2 and table S6, Supplementary Material online). Clustering analyses with *frappe*, grouped honey bees from Xinyuan and *A. m. mellifera* into the same cluster if K was less than 5, but with K equal to 5 they were divided into two groups (fig. 1D). Our resequenced data support the placement of the Xinyuan honey bees in the M-lineage (same as *A. m. mellifera*), which is consistent with the results of morphometric and mitochondrial analysis (supplementary fig. S3, Supplementary Material online).

To further characterize the relationship between the Xinyuan bees and *A. m. mellifera*, we used MCMCtree method in package PAML 4.5 (Yang 2007) to estimate the divergence times between the populations. This analysis revealed that the divergence time between *A. m. mellifera* and the honey bees from Xinyuan is about 132 ka (fig. 2A). In comparison, divergence time between *A. m. carnica* and *A. m. ligustica*, two well-characterized subspecies from the C-lineage, is only about 30 ky before the present, an estimate comparable to that in a previous study (Wallberg et al. 2014). Identity score (IS) analysis also indicated that Xinyuan bees and *A. m. mellifera* have less similarity than that of *A. m. carnica* and *A. m. ligustica* (supplementary fig. S4, Supplementary Material online). Therefore, we propose that the Xinyuan honey bees is a new subspecies of *A. mellifera* (*A. m. sinisxinyuan* n. ssp.).

Description of *A. mellifera sinisxinyuan* n. ssp

Holotype Worker bee; China, Xinjiang Uygur Autonomous Region, Xinyuan prefecture, 43°17′51″N, 83°42′48″E, elevation 1,709 m. Institute of Apicultural Research honey bee collection, sample number 0999-0096.

Paratypes Several worker bees, same data as holotype.

Etymology The name refers to Xinyuan prefecture in China as the location where this subspecies is first discovered.

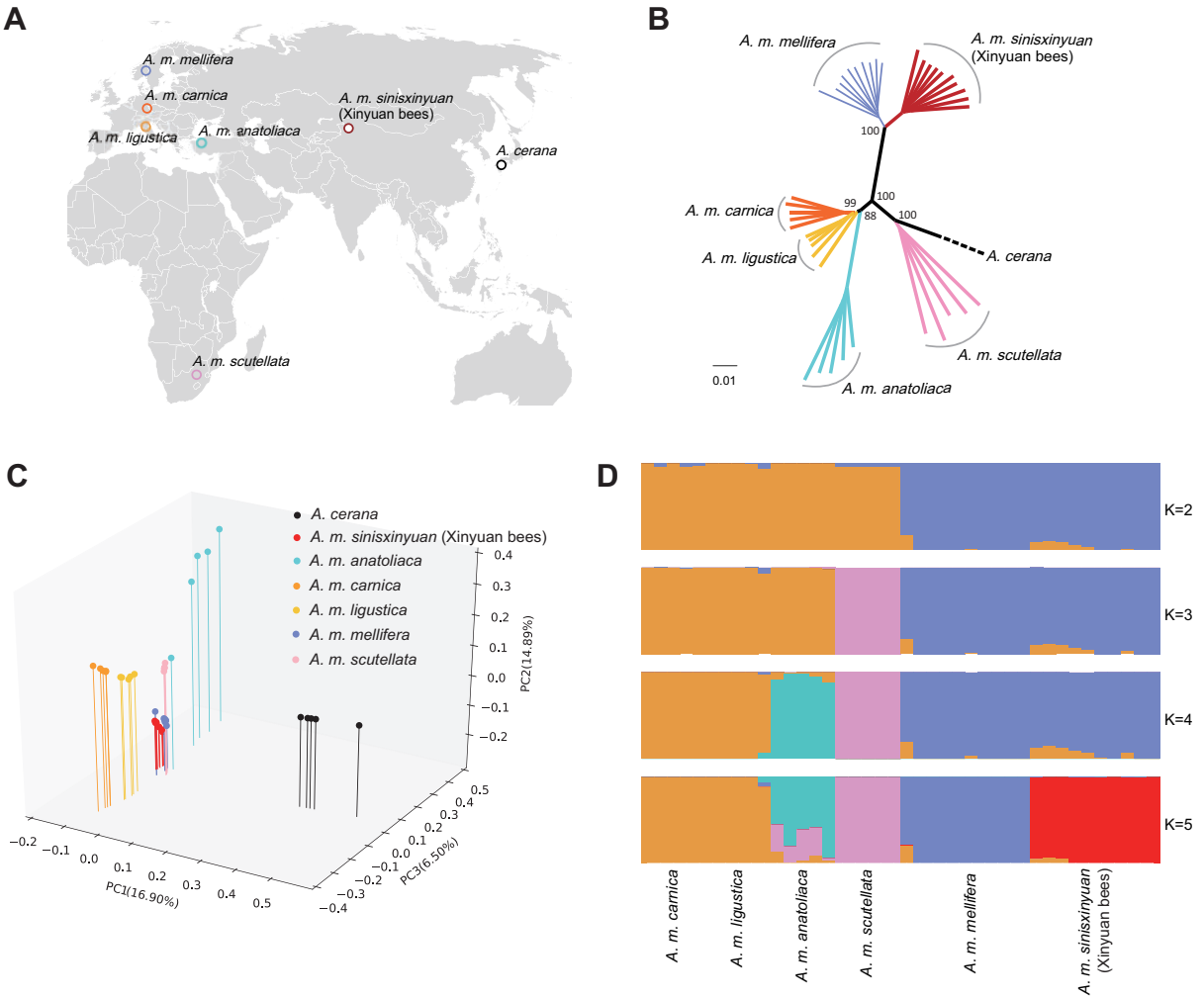

Fig. 1. Population genetics. (*A*) Geographic locations of the studied honey bees. (*B*) Neighbor-joining phylogenetic tree of honey bee populations derived from 100 bootstrap replicates. The scale bar represents the evolutionary distances measured by p-distance. (*C*) Three-way PCA plots of honey bee populations. (*D*) Genetic structure of the honey bees. Analyses with *frappe* show the clustering of sample into 2–5 groups. The proportion of the individual's genome from each ancestral population is shown by the length of each colored segment.

Distribution The subspecies is distributed in the Yili River Valley in western China.

Apis mellifera sinisxinyuan is a dark bee with black abdomens and gray hair. Worker bees have very long abdominal hair, longer than the European dark bee, *A. m. mellifera*, and almost twice as long as *A. m. ligustica* and *A. m. pomonella*. The body is slender and small, considerably smaller than its relative *A. m. mellifera*. The cubital index, sternite 6 index, and tarsus index of *A. m. sinisxinyuan* are comparable to that of *A. m. mellifera*. In addition, proboscis of Xinyuan honey bees is short. Mean values and standard deviation of selected morphometric characters are presented in supplementary table S7, Supplementary Material online.

Workers bees of *A. m. sinisxinyuan* are very aggressive. Preliminary observations found that it has excellent overwintering ability, and the temperature threshold for worker bees to take cleaning flights and to collect honey is low. It is also featured by fast spring build up and high honey production (Liuz et al., unpublished data).

Demographic History

To explore the demographic history of *A. m. sinisxinyuan*, we first estimated changes in effective population size using a Pairwise Sequentially Markovian Coalescent (PSMC) method (Li and Durbin 2011). The result shows that the effective population size of *A. m. sinisxinyuan* fluctuates over time with earth climate (fig. 2*B*), with high effective population size during cold periods. The most dramatic decline in effective population size occurs during Marine Isotope Stage 5 (80–130 ka) (Lisiecki and Raymo 2005; Jouzel et al. 2007), the last major interglacial period in history, after which the effective population size began to increase and peaked during the Last Glacial Maximum (20–26.5 ka) (Clark et al. 2009). Effective population size declined after the Last Glacial Maximum, when the global temperature increased. This pattern strongly suggests a strong effect of global temperature on the effective population size of *A. m. sinisxinyuan*. Notably, the divergence between *A. m. sinisxinyuan* and *A. m. mellifera* (132 ka) occurred at the beginning stage of the effective population size decline, suggesting population decline in the

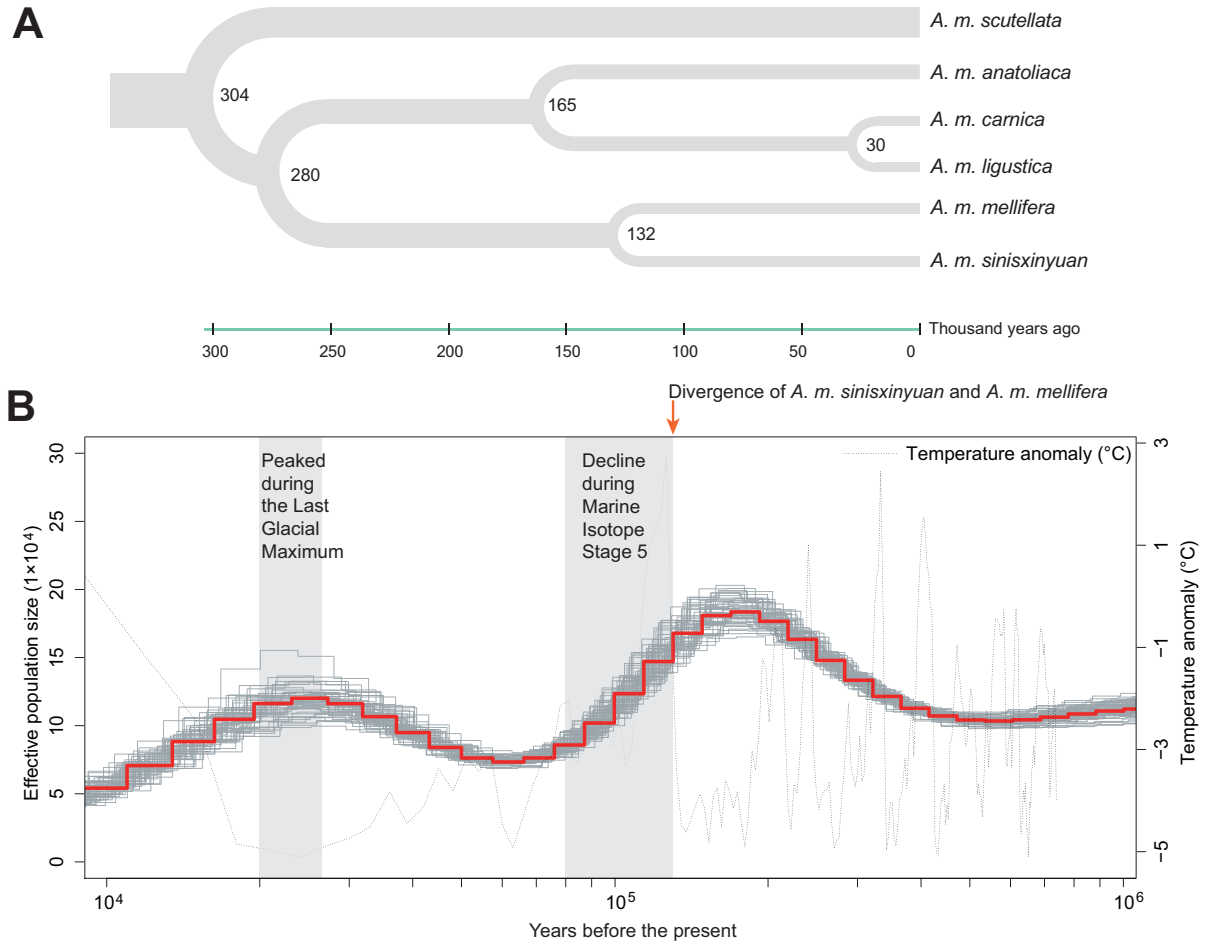

FIG. 2. Population genetics and demographic history. (*A*) Time of divergence between populations. Number at each node represents the time of divergence in thousands of years. (*B*) Demographic history of Xinyuan bees (*Apis mellifera sinisxinyuan*). The red line indicates the effective population size measured by the scaled mutation rate per generation (0.53×10^{-8}), the gray solid lines illustrate the PSMC estimates for 50 rounds of bootstrap. The generation time is 1 year. Shaded areas represent the Last Glacial Maximum (left) and Marine Isotope Stage 5 (right).

common ancestor of *A. m. sinisxinyuan* and *A. m. mellifera* may result in the geographical separation.

Genes under Selective Sweep with Temperate Climates

Although *A. m. mellifera* and *A. m. sinisxinyuan* are well adapted to temperate climates, the tropical *A. m. scutellata* do not show the characteristics suitable for a life in temperate regions (Terada et al. 1975; Southwick and Heldmaier 1987). To identify potential mechanisms for the adaptation, we pooled *A. m. sinisxinyuan* and *A. m. mellifera* samples and compared with the tropical *A. m. scutellata*. Genes under selective sweep were classified into Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000) and Gene Ontology (GO) (Ashburner et al. 2000) functional categories based on orthologs in the fruit fly (*Drosophila melanogaster*). In total, we identified 6.16 Mb of selective sweep regions, encompassing 454 genes with both high F_{ST} and high θ_π-ratio (fig. 3A). Several highly enriched functional categories were identified, including "membrane depolarization" (four genes, $P = 9.64E\text{-}05$), "dopamine metabolic process" (four genes, $P = 1.131E\text{-}03$), "regulation of lipid storage" (five genes, $P = 1.981E\text{-}03$), "flight behavior" (five genes, $P = 2.020E\text{-}03$) (supplementary table S8, Supplementary Material online), and the Hippo signaling pathway (five genes, $P = 3.995289E\text{-}02$; supplementary table S9, Supplementary Material online).

Genes Associated with Regulation of Lipid Storage

The enrichment of genes for lipid storage reflects the crucial role of fat body in *A. mellifera*'s adaptation to temperate climates. We found a selected gene *Indy* (I'm not dead yet), which is a cotransporter related to adult longevity and is abundantly expressed in fat body (Rogina et al. 2000; Seehuus et al. 2013). Another selected gene *Foxo* (forkhead box O; fig. 3*B*–*D*) is a transcription factor involved in the insulin signaling pathway, and regulates cell number and lipid metabolism (Jünger et al. 2003; Vihervaara and Puig 2008). In *A. mellifera*, *Foxo* is hypothesized to play a key role in the longevity of both winter bees and queens, through the regulation of the glycolipoprotein Vitellogenin content in fat body (Corona et al. 2007; Aurori et al. 2013). Other selected genes include *δCOP* and *ζCOP*, both are members of the vesicle-mediated Coat Protein Complex I (COPI), a regulator of lipid

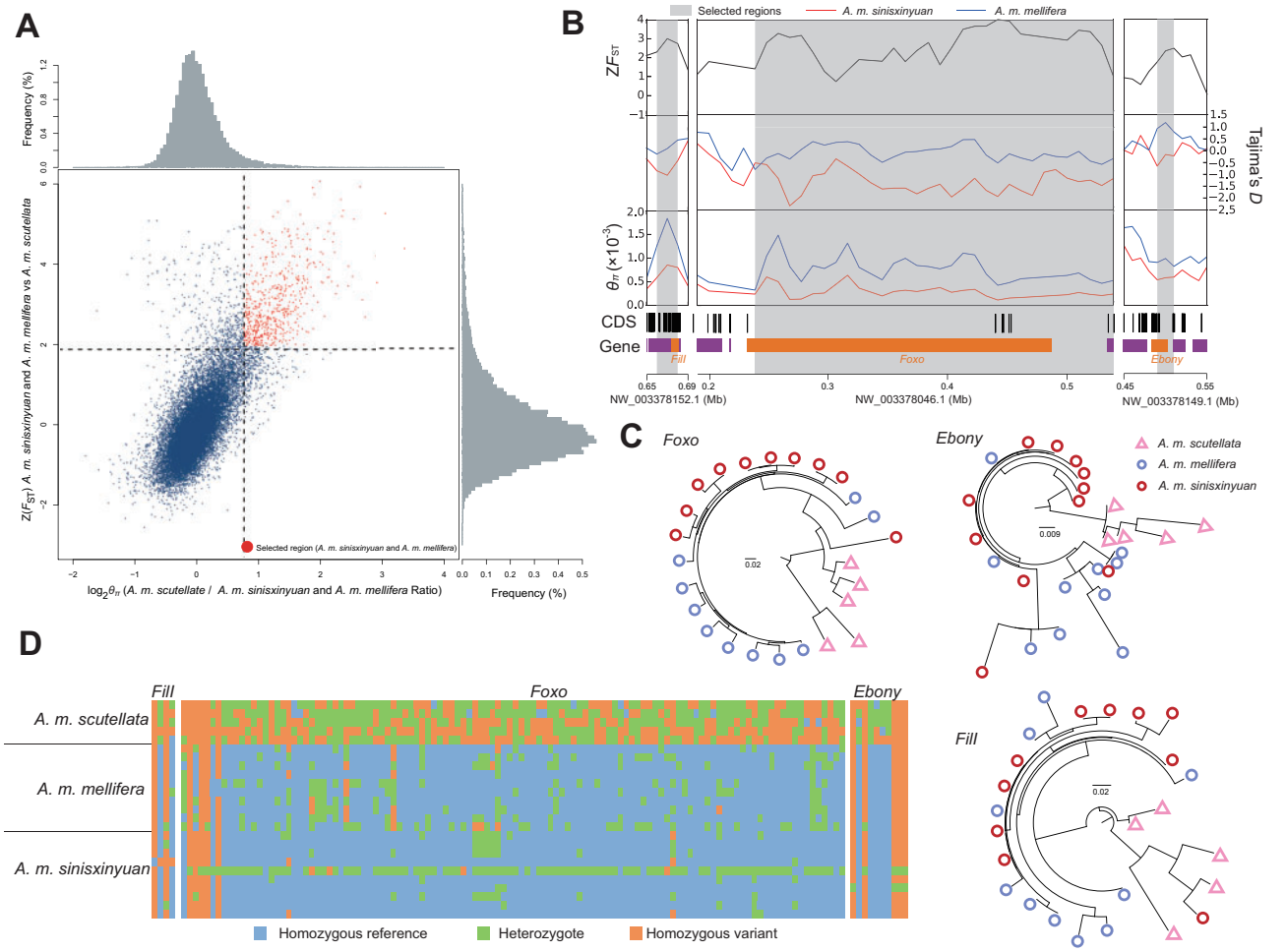

Fig. 3. Genomic regions with strong selective sweeps in *Apis mellifera sinisxinyuan* and *A. m. mellifera*. (*A*) Distribution of θ_π ratios ($\theta_{\pi,\ A.\ m.\ sinisxinyuan\ \text{and}\ A.\ m.\ mellifera}/\theta_{\pi,\ A.\ m.\ scutellata}$) and F_{ST} values, calculated in 20-kb windows sliding in 10-kb steps. Data points on the right of the vertical dashed line (corresponding to the 5% left tail of the empirical θ_π ratio distribution), and above the horizontal dashed line (5% right tail of the empirical F_{ST} distribution) were identified as selected regions for *A. m. sinisxinyuan* and *A. m. mellifera* (red points). (*B*) Examples of genes with strong selective sweep signals in *A. m. sinisxinyuan* and *A. m. mellifera*. F_{ST}, θ_π, and Tajima's *D* values are plotted using a 10-kb sliding window. Shaded genomic regions were the regions with strong selective signals for *A. m. sinisxinyuan* and *A. m. mellifera*. Genome annotations are shown at the bottom (black bar, coding sequences; purple bar, genes). The boundaries of *Fill*, *Foxo*, and *Ebony* are marked in orange. (*C*) The gene trees for *Fill*, *Foxo*, and *Ebony* for *A. m. sinisxinyuan*, *A. m. mellifera*, and *A. m. scutellata*. (*D*) Genotypes of SNPs in putative selective sweeps containing *Fill*, *Foxo*, and *Ebony* in *A. m. sinisxinyuan*, *A. m. mellifera*, and *A. m. scutellata*.

homeostasis (Beller et al. 2008). Taken together, our results suggest the importance of fat body in overwintering honey bees.

Genes Associated with the Control of Flight Muscle

Two of the overrepresented GO categories were "flight behavior" and "membrane depolarization," which are related to the control of flight muscle. Gene *Fill* (flightless I) shows clear signals of a selective sweep in the temperate population, and is involved in the structural organization of indirect flight muscle (fig. 3*B*–*D*) (de Couet et al. 1995), the muscle that generates heat (Tautz 2008). We also found that four out of the five genes in the "membrane depolarization" category were selected. Membrane depolarization is a crucial step in the initiation of action potential in muscle fibers, whereas action potential determines muscle contraction. In honey bee, low temperate reduces the frequency of action potential and hence the wing beat frequency (Bastian and Esch 1970), selected genes in membrane depolarization in the temperate populations may enhance their ability to fly under low temperature. Alternatively, they may also be related to efficient generation of heat.

Genes Related to the Neural System

A number of selected genes in top five GO terms in our analysis are related to the neural system. We found that four genes (*Tan*, *Ebony*, *Manf*, and *Dysbindin-A-like*) were selected in "dopamine metabolic process." Dopamine is one of the main neurotransmitters in *A. mellifera* and has profound effects on behaviors (Scheiner et al. 2002; Beggs et al. 2007; Beggs and Mercer 2009; Agarwal et al. 2011). Among these selected genes, *Tan* and *Ebony* (fig. 3*B*–*D*) code for a pair of N-β-alanyldopamine (NBAD) hydrolase and NBAD-synthase, which function together to regulate the level of dopamine

(Borycz et al. 2002; Pérez et al. 2010, 2011). Moreover, three selected genes (*Tan*, *Tbh*, and *Iav*) in "flight behavior" were also related to neurotransmitters, among which *Tbh* is a tyramine beta-hydroxylase converting tyramine into octopamine (Livingstone and Tempel 1983), and *Iav* (inactive) is a gene involved in octopamine biosynthesis (Roshina and Pasyukova 2007). One major characteristic setting *A. mellifera* apart from most other insects is their social behavior, which is closely linked to the neural system (Scheiner et al. 2002; Zayed and Robinson 2012). Selected genes in this category may regulate the behavior under an environment with seasonal climate change.

The Hippo Signaling Pathway

Selected genes in the temperate versus tropical comparison are enriched in the Hippo signaling pathway. Interestingly, we also found enrichment of selected genes in this pathway in an *A. m. sinisxinyuan* versus *A. m. mellifera* comparison (three genes, $P = 3.8240E\text{-}02$; supplementary table S10, Supplementary Material online). However, only one of the selected genes is common in both comparisons (fig. 4*A*). Enrichment of selected genes in this highly conserved pathway indicates its importance in the process of adaptation. One of the selected genes is *Ds* (Dachsous; fig. 4*B*), an upstream regulator of the Hippo signaling pathway (Cho and Irvine 2004; Willecke et al. 2008). There were also three selected genes identified in the downstream of the Hippo signaling pathway, namely *Yki* (Yorkie), *Sd* (Scalloped), and *14-3-3*. Yki is a transcription coactivator which acts as a key regulator of apoptosis and proliferation (Huang et al. 2005); Sd, a partner of Yki, forms a complex with Yki that binds to DNA and regulates the activity of target genes (Goulev et al. 2008); 14-3-3 is an inhibitor of Yki that binds to Yki and prevents it from entering the nucleus to activate corresponding genes (Ren et al. 2010). Yki, Sd, and 14-3-3 together regulate the expression of downstream effector genes in the Hippo signaling pathway (fig. 4*A*). The Hippo signaling pathway is responsible for the control of organ size during development through cell proliferation and apoptosis (Halder and Johnson 2011; Zhao et al. 2011), and may underlie the differences in size of fat body between winter fat bees and regular bees.

Discussion

Our discovery of *A. m. sinisxinyuan* further extends the eastern boundary of *A. mellifera*'s distribution into western China. This is the first discovery of an *A. mellifera* subspecies in China. *Apis mellifera sinisxinyuan* is also the only subspecies in the M-lineage found outside of Europe. Molecular dating shows that *A. m. sinisxinyuan* diverged from *A. m. mellifera* about 132 ka. Analyses of the population history of the cold-tolerant *A. m. sinisxinyuan* suggest that long-term global temperature has had a substantial influence on its effective population size, with high effective population size under low temperature. A genome-wide scan for signatures of selective sweeps identified a set of candidate genes targeted by natural selection during adaptation to temperate climates. Notably, the Hippo signaling pathway appears to play an important role in the evolution of temperate populations.

Previously, phylogenetic trees constructed from subspecies in the four lineages largely mirrored their geographic range, in that subspecies within the same lineage showed contiguous distributions (Wallberg et al. 2014). Specifically, the ranges of subspecies from the M-lineage are northern and western Europe (Ruttner 1988). However, the discovery of *A. m. sinisxinyuan* in Asia disrupts this pattern, suggesting a more complex evolutional history. Moreover, although it has been puzzling that the closely related *A. mellifera* and *A. cerana* showed allopatric distributions, the area between the distributions of these two species is not well studied (Sheppard and Meixner 2003). Our discovery of *A. m. sinisxinyuan* in this area further narrows the gap between the distribution of *A. mellifera* and *A. cerana*, and further research into this area may provide additional insight into the evolution of both species.

The discovery of *A. m. sinisxinyuan* raises questions about the origin and colonization of the M-lineage. The existence of *A. m. sinisxinyuan* in the eastern Tian Shan mountains suggests a northern migration route of the M-lineage from Asia into Europe as proposed by Garnery et al. (1992). *Apis mellifera sinisxinyuan* may represent an eastern branch during the migration, whereas *A. m. mellifera* represents the western branch. Alternatively, it is also possible that the origin of the expansion was in the Tien Shan Mountains with *A. m. mellifera* representing westward expansion of the M-lineage. Similar questions were raised when the O-lineage *A. m. pomonella* was discovered in the western Tian Shan Mountains, suggesting that the Tian Shan Mountains are also possibly the origin of the O-lineage (Sheppard and Meixner 2003). Further exploration in the Tian Shan Mountains and Xinjiang area may be important in understanding the evolutionary history of *A. mellifera*.

Population genomics of *A. m. sinisxinyuan* further reveals the history of the newly discovered subspecies. Based on the historical pattern of effective population size of *A. m. sinisxinyuan* and the divergence time between *A. m. sinisxinyuan* and *A. m. mellifera* (fig. 2), we propose a scenario of population history of *A. m. sinisxinyuan*, in which global temperature plays an important role: 1) Before Marine Isotope Stage 5, when *A. m. sinisxinyuan* and *A. m. mellifera* had not separated, the common ancestor of *A. m. sinisxinyuan* and *A. m. mellifera* had a monolithic distribution throughout Eurasia (fig. 5*A*). 2) During Marine Isotope Stage 5, elevated global temperature caused habitat reduction of the common ancestor, resulting in two geographically separated populations: The ancestor of *A. m. sinisxinyuan* in Asia and the ancestor of *A. m. mellifera* in Europe (figs. 2*B* and 5*B*). 3) After Marine Isotope Stage 5, the ancestor of the *A. m. sinisxinyuan* expanded its range as the weather became colder, peaking during the Last Glacial Maximum (figs. 2B and 5C). 4) On the one hand, in the post Last Glacial Maximum period, rising global temperature reduced suitable habitat for *A. m. sinisxinyuan*, whereas on the other hand, migration to new habitat was probably not an option due to the surrounding barriers formed by Tian Shan Mountains, deserts, and the Tibetan

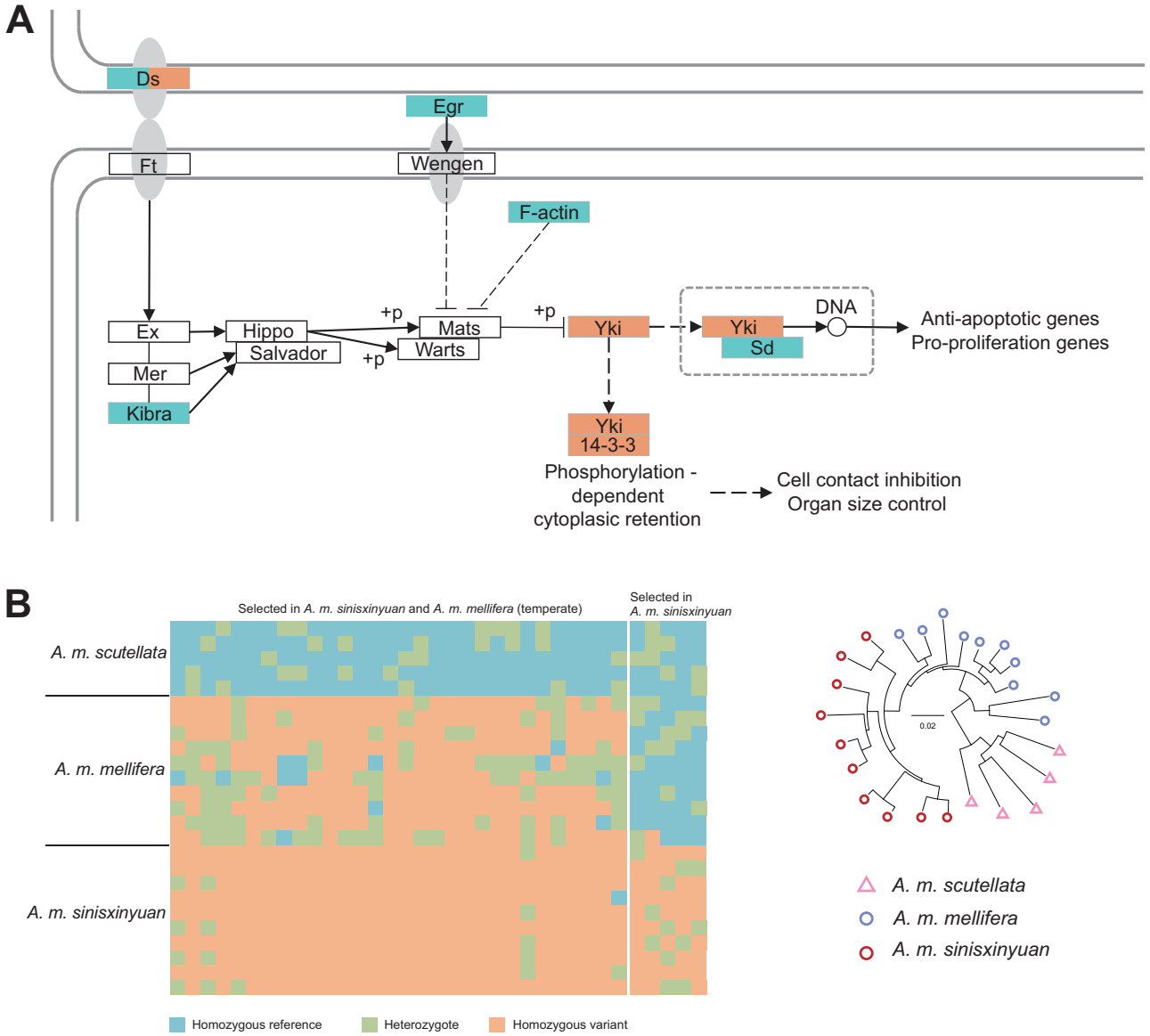

Fig. 4. (*A*) Selection in the Hippo signaling pathway. Selected genes in temperate versus tropical comparison and *Apis mellifera sinisxinyuan* versus *A. m. mellifera* comparison are in blue and orange, respectively. Annotations, abbreviations, and connections are in accordance with KEGG standards, with solid lines and dashed lines representing direct and indirect relationships, respectively. (*B*) Genotypes of SNPs and the gene tree of *Ds* (Dachsous) in *A. m. sinisxinyuan*, *A. m. mellifera*, and *A. m. scutellata*.

Plateau. As a result, *A. m. sinisxinyuan* experienced another decline in effective population size (fig. 5*D*).

The drop in effective population size of *A. m. sinisxinyuan* under elevated global temperature raises concerns for its protection. *Apis mellifera sinisxinyuan* is currently found only in very limited areas, which have been largely protected from human activities due to mountain barriers. However, global warming, together with increased human activity in this area (including beekeeping), poses a serious threat to the already limited population. In addition, another subspecies from the O-lineage, *A. m. pomonella* (Sheppard and Meixner 2003), may also exist in this area (Meixner MD, unpublished data). Furthermore, the two subspecies may be separated by a hybrid zone, given that there is no physical barrier between them, and the existence of *A. m. pomonella* in Xinyuan has the potential to impair the genetic integrity of *A. m. sinisxinyuan* through hybridization and introgression. Measures should be taken immediately for effective protection of *A. m. sinisxinyuan*.

Genetic variation in the genome of temperate populations of *A. m. sinisxinyuan* and *A. m. mellifera* can be characterized by adaptations to the extended cold winter, particularly rapidly evolving genes associated with the fat body. Several selected genes are related to lipid metabolism, which has been shown to be important in many organisms at high altitudes and/or latitudes (Swanson and Liknes 2006; Cheviron et al. 2012; Qiu et al. 2012; Qu et al. 2013; Liu et al. 2014). In addition, several selected genes related to fat body are also known to influence longevity. Abundant fat body and prolonged life span are characteristics of specialized winter bees, which are observed in temperate populations but not tropical populations (Winston 1980; Winston and Katz 1981). The fat winter

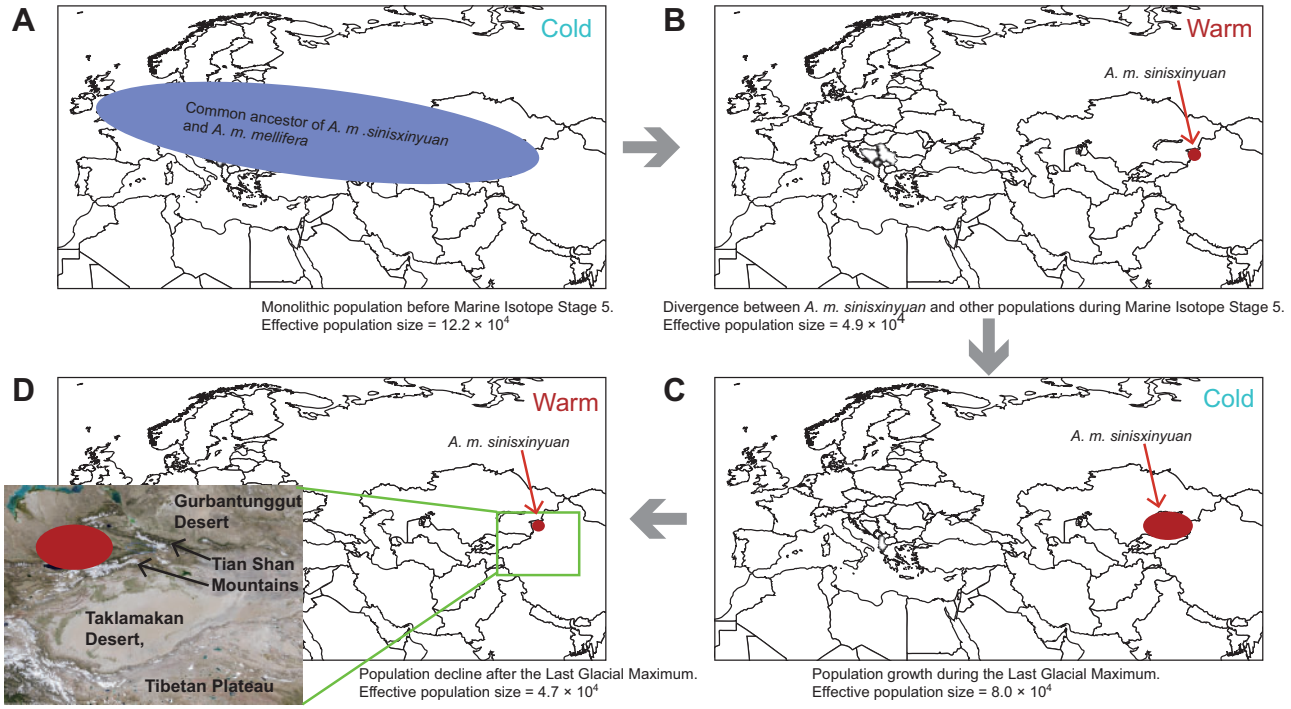

Fig. 5. Geography and possible population dynamics of *Apis mellifera sinisxinyuan* population in history. (*A*) Monolithic population of the ancestor of *A. m. sinisxinyuan* and *A. m. mellifera* spanning Europe and Asia. (*B*) Divergence of *A. m. sinisxinyuan* from other populations. (*C*) Increased population size of *A. m. sinisxinyuan* during the Last Glacial Maximum. (*D*) Population decline of *A. m. sinisxinyuan* after the Last Glacial Maximum.

bees can live for 6–8 months as opposed to 25–35 days in summer bees (Maurizio and Hodges 1950; Free and Spencer-Booth 1959). Previous studies have shown that a major stored component in fat body, Vitellogenin, is associated with longevity (Amdam et al. 2005). Taken together, fat body may play a central role in promoting longevity in overwintering bees.

The development of fat body may be greatly influenced by the Hippo signaling pathway, another important target of selection. Three lines of evidence support this hypothesis: First, the Hippo signaling pathway has a profound effect on the regulation of liver size in mammals (Halder and Johnson 2011; Hong et al. 2015), and the mammalian liver is analogous to fat body in insects in that it is essential for detoxification, energy storage, and utilization (Liu et al. 2009; Arrese and Soulages 2010). Second, the abundance of the regulatory protein 14-3-3 in the Hippo signaling pathway is influenced by the insulin pathway (Chan et al. 2011), which has a strong effect on fat body (Corona et al. 2007; Nilsen et al. 2011; Ihle et al. 2014). Third, the Hippo signaling is also connected to the insulin signaling pathway (Gotoh et al. 2015).

Another characteristic of overwintering honey bees is the ability to maintain stable nest temperature during winter. Unlike many other insects, the eusocial *A. mellifera* species do not survive cold winters in a dormant state (Salt 1969; Sinclair 2015). Instead, they have developed a unique socially cooperative mechanism to maintain high nest temperature during winter: Individual bees form a tight winter cluster to minimize heat loss, and within the winter cluster, heater bees generate heat to maintain a temperature of approximately 20 °C. Heat is generated in heater bees by decoupling the flight muscle from wings allowing flight muscles to vibrate without wing movement, generating heat. In addition, foragers warm themselves in the morning by shivering with flight muscles (Tautz 2008). Selection on the selected genes related to flight in temperate populations may involve in heat generation. For example, the *Fill* gene affect the structure of indirect flight muscle (de Couet et al. 1995), and selection on this gene may result in a muscle structure that can generate heat rapidly and/or efficiently. Selected genes related to flight may also influence the ability of honey bees to fly under low temperature. Cold flight muscles are unable to generate high enough wing beat frequencies for bees to fly (Josephson 1981), probably due to reduced frequency of membrane action potentials under low temperature (Bastian and Esch 1970). Because membrane depolarization is required to generate action potentials, the selected genes in membrane depolarization may enhance the ability of temperate honey bees to fly under cold condition.

Conclusion

Our study benefits from the recent discovery of *A. m. sinisxinyuan*, and provides insights into the history and genetic basis of adaptation of *A. mellifera* to temperate climates at the genomic level. Our integrative analyses highlight the role of historical global temperature in the long-term population dynamics of *A. m. sinisxinyuan*, and shed light on the underlying mechanisms of adaptation to temperate climates in *A. mellifera*. In addition, our result may facilitate the genetic

breeding of *A. mellifera* to reduce overwintering losses, which have been a severe problem in many main beekeeping countries (van der Zee et al. 2012, 2014; vanEngelsdorp et al. 2012; Spleen et al. 2013; Pirk et al. 2014; Steinhauer et al. 2014; Lee et al. 2015).

Materials and Methods

Sampling

Xinyuan honey bees were collected in Yili River Valley, located in the northwest of Xinjiang Uygur, China, in August, 2011. Workers were collected from 20 colonies either from wild nests or human-made hives populated with wild-caught swarms, in four sites across Xinyuan prefecture. Geographic coordinates for the sample sites are 43°17′ 39″N, 83°35′ 23″E, 43°18′ 7″N, 83°39′ 48″E, 43°17′ 51″N, 83°42′ 48″E, and 43°18′ 5″N, 83°39′ 59″E, with elevations of 1,484, 1,566, 1,709, and 1,566 m, respectively. Bees from each colony were preserved in 90% ethanol. Individuals were chosen randomly from each colony and subjected to further analysis.

Sequencing

Ten Xinyuan honey bees, each from a different colony, were sequenced on the Illumina sequencing platform (HiSeq 2500) using standard procedures. The 500-bp paired-end libraries were prepared to generate approximately 8× raw coverage. A total of 17.9 Gb of clean sequence data were generated (supplementary tables S1 and S3, Supplementary Material online).

To analyze the evolutionary history of Xinyuan and other populations, we also downloaded the publicly available sequence data of 35 individuals from other representative populations (Supplementary table S2, Supplementary Material online) (Wallberg et al. 2014). The downloaded data were generated using the AB SOLiD technology with 4–6× coverage per sample.

Reads Mapping and Quality Control

All reads were preprocessed for quality control and filtered using our in-house script in Perl before mapping. Paired reads were discarded if the number of N's in either of the paired reads exceeded 10%. Also, the number of low quality ($Q \leq 5$) bases in a single read was restricted to less than 50%. 76.6 Gb of high-quality, clean reads were kept and mapped to the latest version of the honey bee apiMel4.5 reference genome using BWA-MEM with default parameters except for the "-t -k 32 -M -R" option (Li and Durbin 2009) (supplementary table S3, Supplementary Material online). Alignment bam files were sorted using samtools and duplicated reads were removed. Sequencing coverage and depth for each sample were calculated (supplementary table S3, Supplementary Material online).

The mapping rates of reads between the 35 individuals in the downloaded data (50–60%) and the 10 Xinyuan individuals (96%–97%) show significant discrepancy due to differences in sequencing platforms. Compared with the mapping technology of LifeScope (Wallberg et al. 2014), BWA set up a set of stricter parameters to judge alignment. To increase the accuracy of SNP calling and population variation analyses, we chose BWA as our read alignment software. We tested the difference of mapping rate with the two methods using data from a sample. The result revealed that the mapping rate of BWA was less than LifeScope by 20%.

SNP Calling and Genotyping

SNPs were detected using SAMtools' mpileup (Li et al. 2009). The reference genome was subdivided into segments and analyzed in parallel. Population-based genotypes for the 45 individuals were created by SAMtools. About 4.7×10^6 raw SNPs were detected. The output was further filtered meeting the following criteria:

(1) Quality score more than 20.
(2) SNPs within 5 bp around a gap to be filtered.
(3) The sum of read depths across all populations was [4, 1,000].
(4) Ignoring multinucleotide polymorphisms.
(5) The average Phred scaled base quality of the variant allele was $\geq$20.
(6) The unobserved variant allele constituted $\leq$50%.

A total of 2.1×10^6 SNPs were retained for downstream analyses after applying the filters above.

Principal Component Analysis

The software EIGENSOFT3.0 (Patterson et al. 2006) was used for principal component analysis with biallelic SNPs of the 45 individuals. We plotted the first three significant components (supplementary fig. S2, Supplementary Material online). To some extent, the discrete points reflect the real structure of population. A Tracy–Widom test was used to determine the significant level of the principal components.

Population Structure

To estimate individual admixture assuming different numbers of clusters, the population structure was investigated using the program *frappe* (Tang et al. 2005), with a maximum-likelihood method. We increased the coancestry clusters spanning from 2 to 5 and ran analysis with 10,000 iterations.

Phylogenetic Tree

We constructed a neighbor-joining tree using pairwise genetic distances matrix data of all individuals, calculated by TreeBest (http://treesoft.sourceforge.net/treebest.shtml). The bootstrap was set to 1,000 times to assess the branch reliability. With *A. cerana* as outgroup, the result was in clear separation among all the individual samples. The Xinyuan honey bees and *A. m. mellifera* derived from the same branch.

Divergence Time Estimation

We used the putative sequence of coding region based on the allele frequency of each population, which was concatenated to one supergene for each species. Divergence times were estimated by MCMCtree (Yang 2007) package in PAML 4.5, using soft fossil constraints under various molecular clock models. The time unit is 100 My. We added three calibrations to edit the tree, one for the most recent common ancestor of

A. cerana and *A. mellifera*, [0.06, 0.09] (Arias and Sheppard 1996), one within *A. mellifera*, [0.0015, 0.0035], and the last between *A. m. ligustica* and *A. m. carnica* [0.0002, 0.00035] (Wallberg et al. 2014). The clock variable was set to 2. The process was ran to sample 10,000 times using HKY85 as the substitution model, with the sample frequency set to 50 after a burn-in of 50,000 iterations.

Linkage-Diseqrilibrium Analysis

Linkage disequilibrium was calculated on the correlation coefficient (r^2) statistics of two loci using Haploview software (Barrett et al. 2005), with minor allele frequency (MAF) greater than 0.05. Five individuals were randomly chosen from each population, and SNPs in each population were extracted to perform the analysis. Parameters were set as follows: "-maxdistance 4 -n -dprime -minMAF 0.05." The longer the physical distance, the lower the value of r^2, so the maxdistance was set to 4 K.

Effective Population Size

We used the PSMC model (Li and Durbin 2011) to obtain detailed changes in effective ancestral population sizes of Xinyuan population. The sequencing data of the Xinyuan populations were about 10×. The high sequencing depth was to ensure the quality of consensus sequence. The genotypes of individuals were determined based on the alignment BAM files using SAMTOOLS (Li et al. 2009). Bases of low sequencing depth (a third of the average depth) or high depth (twice of the average) were masked. The utility fq2psmcfa (provided with the PSMC software) was used to convert this diploid consensus sequence to the required input format. The parameters were set as follows: -N30 -t15 -r5 -p 45*2. The generation time (g) was set as an estimate of 1 year. We used a mutation rate (μ) of 5.3×10^{-9} per bp per generation.

Gene Annotation

We annotated *A. mellifera* gene set within functional categories based on the corresponding *D. melanogaster* orthologs. The genes were annotated by a homology-based method. *Apis mellifera* coding sequence was blasted to the *D. melanogaster* peptide sequences downloaded from Ensembl database (Release 74), with the parameter "-p blastx -m8 -e 1e-5 -F F" and the minimum alignment peptides more than 50. The correspondence relationship of *A. mellifera* gene and ontology category was decided by the best hit score of alignment, and the GO information was obtained from BioMart, with the *D. melanogaster* reference gene set (BDGP6).

Selection Analyses

The allele frequencies of variable sites were used to identify regions potentially affected by long-term selection using two complementary approaches. We calculated population fixation statistics (F_{ST}) and nucleotide diversity (θ_π) for each sliding window (in 20-kb windows with 10-kb step size). We Z-transformed the distribution of F_{ST} and calculated the log value of θ_π ratios. The putative selection targets were extracted based on being in the top 5% of log-odds ratios for both θ_π and F_{ST}. Our analyses compared *A. m. sinisxinyuan* (Xinyuan honey bees) and *A. m. mellifera* with *A. m. scutellata*, and *A. m. sinisxinyuan* with *A. m. mellifera*.

Genomic Similarity

The similarity of the sequenced genomes in pairwise comparisons was calculated by identical score for SNPs. The identity score (IS) (Rubin et al. 2010; Ai et al. 2015) between two samples was measured by the distance score Ds*i* of allele to the reference allele at a given SNP site *i*:

$$\mathrm{Ds}i = \begin{cases} 0/0, & 0 \\ 1/1, & 1 \\ 0/1, & 0.5 \end{cases} \quad (1)$$

The identical scores was the total accumulated score within a 20-kb window divided by the total number of SNPs. The formula of IS was as below:

$$\mathrm{IS} = \frac{\sum_{i=1}^{N} (1 - |\mathrm{Ds}_{1i} - \mathrm{Ds}_{2i}|)}{\text{number of SNPs in the window}} \quad (2)$$

N is the total number of SNPs within a 20-kb window. The identical scores of a single site were calculated as 1 minus the difference of Ds between the two samples (supplementary fig. S4, Supplementary Material online).

Data Accessibility

All short-read sequence data of this study have been deposited in the NCBI Short Read Archive (SRP065991), under the BioProject PRJNA301648.

Supplementary Material

Supplementary note, figures S1–S4, and tables S1–S10 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

This work was supported by the Chinese Academy of Agricultural Sciences (The Agricultural Science and Technology Innovation Program, grant No. CAAS-ASTIP-2015-IAR) and the earmarked fund for Modern Agro-industry Technology Research System (grant No. CARDS-45-KXJ1). The authors thank Tianqi Liu and Liqing Zhang for their help in field sampling; Yong Jin and Qian Wang for their assistance in preparing the figures; and Mark Camara for helpful comments and proofreading of the manuscript. They also thank the Oberursel Institute, Germany, for morphological reference data.

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