

Asymmetric genetic differentiation: evidence for hybridization between two sympatric termite species in nature

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May 26, 2020

Abstract

Hybridization between species is likely to be associated with a new ecological impact. However, in termites, reports of hybridization mostly focus on hybrid zones caused by species invasion or the development of initial-stage colonies. In this study, we combined microsatellite genotyping with mitochondrial DNA sequencing to investigate the hybridization and adaptive introgression between two sympatric, long-differentiated related termite species, *Reticulitermes flaviceps* and *R. chinensis*, in nature. Similar levels of mitochondrial and nuclear genetic diversity were found in *R. flaviceps* and *R. chinensis*. Asymmetric interspecific genetic differentiation was observed between mitochondrial and nuclear genes, with high genetic divergence found in mitochondrial DNA but low genetic divergence in nuclear genes. Our results indicated a lack of mitochondrial gene exchange in *R. flaviceps* and *R. chinensis* but unconstrained nuclear introgression between them. This asymmetric genetic differentiation between nuclear and cytoplasmic material strongly suggests that there is interspecific hybridization between *R. flaviceps* and *R. chinensis* in nature, which provides new insight into the dynamics of hybridization and its potential consequences for speciation in termites.

Introductions

Hybridization may cause significant impacts on the types and levels of genetic divergence between species (Abbott *et al.* 2013; Payseur& Rieseberg 2016; Zhang *et al.* 2016). This divergence includes neutral divergence, adaptive introgression and coevolution, which may accumulate in different ways. Some species may acquire novel characteristics or phenotypes through interactions with hybrids, including advantages derived from genetic recombination and disadvantages resulting from allelic or environmental incompatibilities (Abbott *et al.* 2013; Meier *et al.* 2017). In addition to its genetic consequences, hybridization can influence the species composition of communities. When two species with different populations exist in the same habitat, individuals from smaller populations may mate more frequently with members of larger populations, resulting in the dilution of the smaller populations. Some endangered species may become extinct as a result of hybridization (Levin *et al.* 1996).

For the past several decades, hybridization was considered to be relatively rare, especially in animals (Abbott *et al.* 2013; Heliconius Genome 2012). However, recent studies have suggested that hybridization in animals is more common than previously thought, providing primary material for both speciation and evolution (Abbott *et al.* 2013; Kulmuni& Pamilo 2014; Song *et al.* 2011). Approximately 10-30% of animal and plant species hybridize regularly. High rates of hybridization have been reported in many groups, including birds, insects and fishes (Abbott *et al.* 2013; Capblancq *et al.* 2015; Pennisi 2016). Incomplete barriers between species may lead to adaptive introgression through the transmission of beneficial alleles between species via backcrosses, which may occur between closely related species.

Two closely related termite species, *Reticulitermes flaviceps* Oshima and *Reticuliterme chinensis* Snyder,

are common forest and structural insect pests in China (Liet *et al.* 2014). They are consistently recognized as distinct species on the basis of multiple criteria, including morphology, biology (colony life history) and mitochondrial genome levels (Bourguignon *et al.* 2016). In terms of morphological characteristics, the colour of the *R. flaviceps* alate pronotum is yellow, but that of *R. chinensis* is black. The eclosion and swarming of *R. chinensis* occur in the same year at intervals of approximately half a month, but in *R. flaviceps*, eclosion occurs in the first year, and swarming occurs in the second year, with intervals of approximately 4 months. Despite the differences in swarming times, some overlap of dispersal periods and the reproductive season persists in the two species. More importantly, *R. flaviceps* and *R. chinensis* share stable habitats, including nesting and foraging sites. Thus, reproductive individuals of the two termite species may encounter each other in nature while searching for mates and nest sites. In preliminary laboratory experiments, we found that a preference for conspecific partners was absent when these species encountered each other during the reproductive season. Pairs established in the laboratory can produce surviving hybrid larvae (Wu *et al.* 2020), suggesting that hybridization and adaptive introgression occur in the context of incomplete barriers between *R. flaviceps* and *R. chinensis*. However, whether hybridization and adaptive introgression occur in nature is still unclear.

Here, we combined microsatellite (simple sequence repeat SSR) genotyping with mitochondrial DNA sequencing to analyse the genetic structure of the sympatric species *R. flaviceps* and *R. chinensis* and to answer the following three questions: (1) whether hybridization and adaptive introgression occur between the two termite species in nature; (2) whether similar genetic differentiation exists in their mitochondrial DNA (mtDNA) and SSR (nDNA); and (3) the effect of hybridization and introgression on the genetic structure and evolution of the termites.

Materials and methods

Termite collection

Termite samples were collected from Wuhan, Nanjing and Chengdu cities in China. Congeneric *R. flaviceps* and *R. chinensis* share common habitat in these regions, which provides ideal conditions for studying the interactions between the two species in hybrid zones. The sampling of *R. flaviceps* and *R. chinensis* populations was performed from October 2017 to May 2018 in the three areas indicated above. Alates of the two species were collected during this period, which aided in species identification. In total, we sampled 67 colonies, including 10 *R. flaviceps* and 15 *R. chinensis* colonies from Chengdu, 10 *R. flaviceps* and 15 *R. chinensis* colonies from Wuhan, and 10 *R. flaviceps* and 15 *R. chinensis* colonies from Nanjing (colonies were assigned to species based on the morphology of alates; see Fig. 1). The distance between the colonies was at least 1000 m to avoid repeated sampling of the same colony. More than 30 individuals (including workers, soldiers and alates) were collected from each colony. All the individuals from the same colony were placed in absolute ethanol and stored at 4°C for genetic analysis.

Species identification, COII sequencing and microsatellite genotyping

Species were identified by combined analysis of the mitochondrial COII gene and morphology. Whole-genomic DNA was extracted from each termite sample using a TIANamp Genomic DNA Kit (Tian Gen Biotech Co., Ltd) according to the recommendations of the manufacturer. The extracted DNA was stored at 4°C until analysis. Subsequently, we obtained sequence data for a mitochondrial gene (COII) and nuclear markers (microsatellite loci) from each individual sample. For the mitochondrial COII gene, PCR was performed using the primers A-tLeu, 5'-CAGATAAGTGCATTGGATTT-3', and B-tLys, 5'-GTTTAAGAGACCATTACTTA-3' (Simon *et al.* 1994), with an annealing temperature of 56°C (Miura *et al.* 2000). For nuclear DNA, PCR was performed using ten pairs of microsatellite primers: Rs03, Rs78, Rs76, Ra50, Ra79, Ra95, Ra128, Ra141 and Ra144 (see supplementary materials, Table S1). The conditions for each PCR assay were based on those described by Wu *et al.* (2020). GeneMapper v4.0 (Applied Biosystems) and Peak Scanner v1.0 (Applied Biosystems) were used for the analysis of the results.

We only used sequences for which the two-direction chromatograms were well matched. Sequences were spliced using DNASTAR Lasergene v7.1 (<http://www.dnastar.com/t-allproducts.aspx>). After splicing was

completed, all sequences were saved in Fasta format and imported into MEGA7 for comparison. The sequences that were not neat at both ends were deleted so that all sequences of the same length were retained. Haplotypes were estimated from the mitochondrial data using DnaSP v5 (Librado& Rozas 2009). The maximum likelihood phylogenetic tree, with 100 bootstrap replicates, and the estimated best substitution model (GTRGAMMA) were obtained using MEGA v. 7.

Genetic diversity is an important measure for evaluating the genetic structure and reproductive strategies of populations. We characterized the nuclear genetic diversity of *R. flaviceps* and *R. chinensis* using the following parameters: mean number of effective alleles per locus (N_e), observed heterozygosity (H_o) and expected heterozygosity (H_e). These analyses were performed using GenAIEx v. 6.5 and GENEPOP v. 1.2. We also investigated genetic diversity based on mitochondrial data using DnaSP v5, including haplotype diversity and nucleotide diversity (h and π). We tested the differences in genetic diversity (N_e , H_e and H_o) between the species via T-test analysis.

Genetic differentiation and estimated gene flow

A female gamete contributes both nuclear and cytoplasmic material, whereas a male gamete does not carry cytoplasmic material contributing to the formation of a zygote. When hybridization occurs between species, the nuclear genes of the offspring are a combined product of the different parental species, whereas the cytoplasmic genes of the offspring are contributed by their mother and are not recombinant (Greiner *et al.* 2015; Hagstrom *et al.* 2014). Thus, interspecies hybridization may lead to asymmetric genetic differentiation between nuclear and cytoplasmic material. There will be high genetic differentiation of nuclear genes but low genetic differentiation of cytoplasmic genes. Thus, we calculated the genetic differentiation of the termite species at the mitochondrial gene (COII) and nuclear gene (SSR) levels. F_{ST} , a standardized measure of the genetic variance among populations, was calculated using GenAIEx v. 6.5, DnaSP v5 and FSTAT v. 2.9.3.2. Gene flow was indirectly measured using genetic frequency data.

To determine whether there was species-related genetic clustering of the populations of *R. flaviceps*, *R. chinensis* and their hybrid populations, we employed a Bayesian approach for inferring population structure using STRUCTURE 2.3. We ran the admixture model with correlated frequencies, with 10 independent runs for each K (from 1 to 10) performed with 10,000 MCMC repetitions and a burn-in of 10,000. We used the mean maximum estimated logarithm of the posterior probability of the data for each K (LnP(D)) to identify the most likely number of clusters, and we used DK values combined with species identification to obtain the optimal K.

Results

Species identification and haplotypes

Sixty-seven sympatric colonies of *Reticulitermes* termites were identified using a series of morphological and mitochondrial COII gene analyses (Fig. 2). There were 38 colonies belonging to *R. flaviceps* and 29 colonies belonging to *R. chinensis*. Both species were distributed across Chengdu, Nanjing and Wuhan. For the mitochondrial markers, 661 bp of COII was resolved in 67 individuals. In all samples, 31 unique haplotypes were identified based on the 92 variable sites (14 singleton variable sites and 78 parsimony-informative sites) derived from the 67 aligned individuals based on the mitochondrial COII gene sequences. Among the different sample plots, nine haplotypes were found in Nanjing, 12 haplotypes in Wuhan and 18 haplotypes in Chengdu. The phylogenetic tree constructed via the ML method showed two major clades within the collected sampled species (Fig. 2). The first clade comprised samples showing haplotypes similar to *R. flaviceps*, and the second clade comprised samples showing haplotypes similar to *R. chinensis*.

Mitochondrial and nuclear genetic diversity

Similar levels of overall genetic diversity were found in *R. flaviceps* and *R. chinensis* in the mtDNA sequences. The index values of haplotype diversity and nucleotide diversity for *R. flaviceps* ($h = 0.81$, $\pi = 0.036$, Table 1) were similar to those for *R. chinensis* ($h = 0.854$, $\pi = 0.034$, Table 1). Haplotype diversity (h) in different plots ranged from 0.657 to 0.848, with a mean of 0.734, in *R. flaviceps* and from 0.4 to 0.978, with a mean of

0.674, in *R. chinensis*. Nucleotide diversity (π) in *R. flaviceps* and *R. chinensis* ranged from 0.008 to 0.02, with a mean of 0.014, and from 0.002 to 0.028, with a mean of 0.013, respectively.

For nuclear genetic diversity, there was no significant difference in the number of effective alleles between the populations of *R. flaviceps* and *R. chinensis* (N_e : Fig. 3A; $t = 0.44$, $p = 0.66$). The number of effective alleles ranged from 1.25 to 8.54, with a mean of 4.39, in *R. flaviceps* and from 1.06 to 10.01, with a mean of 4.15, in *R. chinensis*. Similarly, there were no significant differences in the observed heterozygosity (H_o : Fig. 3B; $t = 1.17$, $p = 0.25$) or expected heterozygosity (H_e : Fig. 3C; $t = 0.78$, $p = 0.44$) between the populations of *R. flaviceps* and *R. chinensis*. The observed heterozygosity at different loci ranged from 0.22 to 0.80, with a mean of 0.50, in *R. flaviceps* and from 0.22 to 0.85, with a mean of 0.54, in *R. chinensis*. The expected heterozygosity ranged from 0.20 to 0.88, with means of 0.72, in *R. flaviceps* and from 0.05 to 0.90, with a mean of 0.69, in *R. chinensis*.

Genetic differentiation and gene flow

Based on the mitochondrial and nuclear genetic results, the genetic differentiation between the two species was calculated using F-statistics (Table 2). We found that *R. flaviceps* and *R. chinensis* displayed asymmetric genetic differentiation between their mitochondrial and nuclear genes ($t = 10.72$, $p < 0.001$). The mtDNA COII gene analysis showed high genetic divergence and low gene flow between the species ($F_{st} > 0.62$, $N_m < 0.15$, Table 2), but the nuclear genes (SSRs) showed low genetic divergence and high gene flow between the species ($F_{st} < 0.12$, $N_m > 2.63$, Table 2). The asymmetric genetic differentiation between the nuclear and cytoplasmic material indicated that there was interspecific hybridization between *R. flaviceps* and *R. chinensis*.

Nuclear introgression from either *R. flaviceps* into *R. chinensis* or *R. chinensis* into *R. flaviceps* was unconstrained in the sympatric area. Among 38 colonies possessing *R. flaviceps* mtDNA haplotypes, an *R. chinensis*-specific nuclear haplotype was observed at proportions ranging from 1.5% (Wuhan) to 23.7% (Chengdu), with a mean of 10% (Table 3). Similarly, in 29 colonies possessing *R. chinensis* mtDNA haplotypes, a *R. flaviceps*-specific nuclear haplotype was found at proportions ranging from 2.2% (Wuhan) to 14.9% (Chengdu), with a mean of 6% in the colonies on a genetic basis. In other words, approximately 10% of female individuals of *R. flaviceps* mated with males of *R. chinensis*, and approximately 6% of female individuals of *R. chinensis* mated with males of

R. flaviceps.

Genetic clustering

The assignment test analysis using STRUCTURE also showed evidence of nuclear introgression in three plots (Fig. 4). As the species were identified, the most appropriate K was $K = 2$ for all plots, indicating that the colonies of both Nanjing and Wuhan were appropriately divided into two species-specific genotypic clusters, although a few gene introgressions were observed. In contrast, the populations of *R. flaviceps* and *R. chinensis* in Chengdu were much more mixed and showed little evidence of species differentiation.

Discussion

Hybridization results in variation with new combinations of alleles through the same mode of sexual reproduction in species, which may provide a basis for adaptive evolution (Abbott *et al.* 2013). However, sexual reproduction can result only in nuclear gene recombination, whereas cytoplasmic genes are maternally inherited (Birky 2001; Greiner *et al.* 2015). Thus, asymmetric gene flow between cytoplasmic and nuclear loci is considered to provide evidence of interspecific hybridization (Setoguchi & Watanabe 2000). Our results showed that *R. flaviceps* and *R. chinensis* exhibited asymmetric genetic differentiation between their mitochondrial and nuclear genes. There was low gene flow and high genetic divergence of mitochondrial genes, while there was high gene flow and low genetic divergence of nuclear genes between the species. Additionally, the identified *R. flaviceps*/*R. chinensis* colonies possessed *R. chinensis*/*R. flaviceps* nuclear genes. These results suggested that interspecies hybridization and gene exchange occurred in the two termite species in nature.

What are the possible factors causing hybridization? In termites, the overlap of the distribution and swarming of related species is common, and the sex-pairing pheromone is similar in sibling species (Aldrich& Kambhampati 2009; Capblancq *et al.*2015; Hartke& Rosengaus 2011). More importantly, neither males nor females possess external sclerotized genitalia (Hartke& Baer 2011). Thus, the indicated prezygotic reproductive barrier may be largely absent in some sibling termite species. Second, the initial colony is usually established under monogamy (Korb& Hartfelder 2008; Thorne *et al.*1999). Many monogamous individuals may fail to mate with conspecific partners due to the sex ratio, predators and/or other factors (Matsuura *et al.* 2002). In this case, their mating with heterospecific partners and nest building favour improved fitness when they encounter each other. Additionally, in some subterranean termites, workers can develop into secondary reproductives (Haverty& Howard 1981; Su *et al.* 2014), and dealates that fail to mate can also live for a long time; they may find a homosexual partner and co-establish a stable nest (Mizumoto *et al.* 2016). Hence, it is possible that foraging workers fuse to form a heterospecific virgin colony and then mate with heterosexual dealates, which provides a possible mechanism for hybridization between dealates and secondary reproductives developing from workers.

Adaptive introgression of genetic material between incompletely isolated species may cause various impacts on genetic diversity. It can contribute to genetic diversity or facilitate genetic assimilation depending on the size and construction of populations (Seehausen 2004). For social termites, many colonies are established by founders from the same natal colony (DeHeer& Vargo 2005; Vargo& Husseneder 2009). One closed colony can be composed of kin, which may lead to inevitable inbreeding. In fact, high genetic diversity is still maintained in natural populations (Huang *et al.* 2013; Vargo& Husseneder 2009). Fortunately, hybridization and introgression between incompletely isolated species may explain why genetic diversity can be maintained in natural populations of termites with respect to inbreeding. We previously noted that *R. flaviceps* and *R. chinensis* possess similar behaviours and lack conspecific preferences in the process of reproduction (Wu *et al.* . 2019). At the same time, we found that artificial hybrid colonies can produce living offspring under laboratory conditions. Combined with the results regarding genetic exchange between the species in nature in this study, we further confirmed the speculation that the maintenance of high genetic diversity in termites is related to adaptive introgression and hybridization.

In general, incomplete reproductive barriers occur in young species because they remain in the initial stage of differentiation. For example, a host shift led to prezygotic reproductive isolation between the original species and host races in *Rhagoletis pomonella* , but the postzygotic reproductive barriers were invalid due to the short time of differentiation (Dambroski& Feder 2007). However, hybridization between long-diverged species often negatively affects the viability and fertility of hybrid offspring, preventing gene flow between species (Anderson *et al.* 2010; Brideau *et al.* 2006; Brucker& Bordenstein 2013; Fitzpatrick 2008; Moyle& Nakazato 2009). Inconsistent with that prediction, the differentiation time between *R. flaviceps* and *R. chinensis* is 2-3 million years (Bourguignon *et al.* 2016). Our results indicated that they exhibit incomplete reproductive barriers and that there is gene flow between the two species. Similar patterns of gene exchange are also reported in the related tapeworm species *Schistocephalus solidus* and *S. pungitii* despite their differentiation 20-25 million years ago (Henrich& Kalbe 2016). These results suggested that interspecific gene exchange shows not relationship with the time of species differentiation.

During the process of evolution and speciation, differentiated species are often re-fused due to diffusion or environmental changes, which can result in adaptive introgression or speciation via hybridization. The phylogenetic relationship of this evolutionary process cannot be described through a binary bifurcation structure but by a network, namely, reticulate evolution. Therefore, the existing phylogenetic tree based on a binary bifurcation structure cannot truly describe the evolution of species (Pennisi 2016). Additionally, hybridization can reduce differences between taxa or even cause a genetic homogenizing effect (Bernal *et al.* 2017; Schierenbeck 2011). Alternatively, hybridization results in the introgression of genetic material and accelerates speciation (Abbott *et al.* 2013; Bendesky *et al.*2017; Payseur& Rieseberg 2016). However, hybridization and gene introgression existed in the termite *Reticulitermes* (Dedeine *et al.* 2016). Neither the above-described reductions in genetic differences between species nor speciation are observed in the termite *Reticulitermes* . If this were the case, there would clearly be strong selection against hybridization

to maintain species integrity in nature. Further studies on the maintenance mechanisms of species will still be required in the future.

Acknowledgments:

We thank Profs. Min Liu, Chun Chen and Dr. Yanan Dong for valuable suggestions on an earlier draft of this manuscript. We also thank Zhen Cheng, Xingying Zhao, Hao Wang and Yongyong Gao for their helps in termite collections. This research work was supported by the National Natural Science Foundation of China [31772516].

Author Contributions

Conceived and designed the model: J.W. and Q.H. Performed the research: J.W. H.X and Q.H. Wrote the paper: J.W. A. H. and Q.H. All authors revised the manuscript and approved the final version.

Competing interests The authors declare no competing interests

Availability of data and material

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

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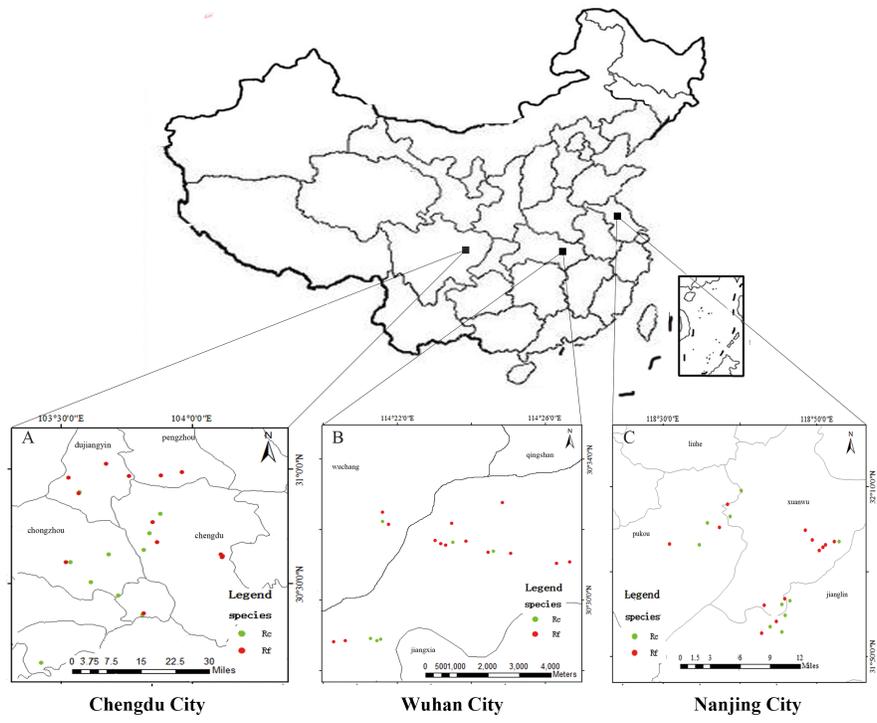
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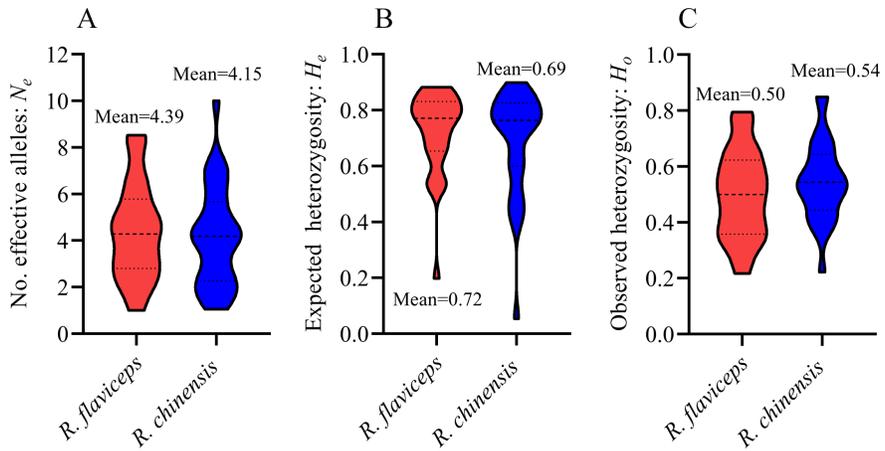
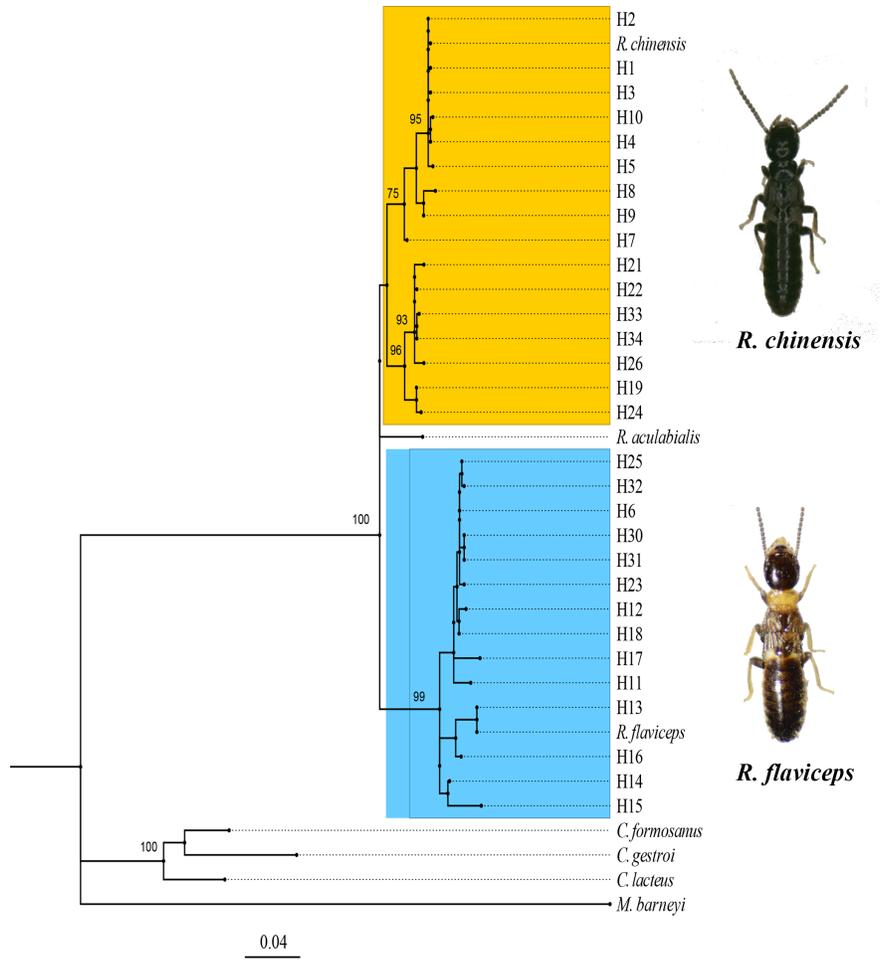
Fig. 1 The sampling map for *R. chinensis* and *R. flaviceps* populations in three plots of China.

Fig. 2. Maximum likelihood (ML) tree and morphology of termites. The numbers on the branches indicate the bootstrap percentages. Thirty-one unique haplotypes were divided into two clusters and combined with the haplotypes of *R. chinensis* and *R. flaviceps* .

Fig. 3. The violin plot and the genetic diversity index of *R. flaviceps* and *R. chinensis*.

Fig. 4. Genetic clusters of termite samples assigned by STRUCTURE. The colonies are arranged by their areas. Green and red represent the assigning probability to the two major clades.

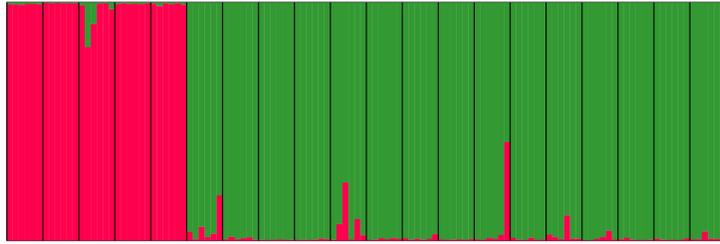




A Nanjing K=2



B Wuhan K=2



C Chengdu K=2

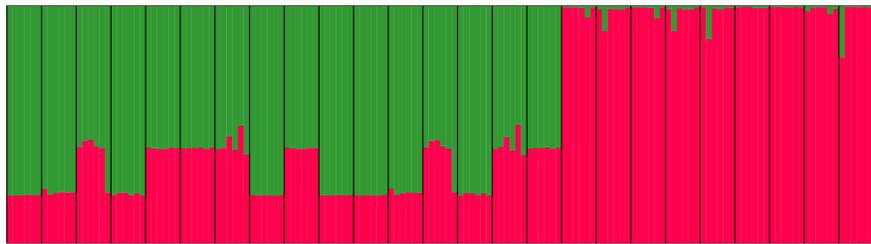


Table 1. Genetic diversity indices for CO II in *R. chinensis* and *R. flaviceps*.

Populations	<i>N</i>	Number of Haplotypes (<i>N_h</i>)	Haplotype diversity (<i>h</i>)	Nucleotide diversity (π)
<i>R. chinensis</i>	29	13	0.81±0.079	0.0359±0.00222
Nanjing	5	4	0.644±0.152	0.02848±0.00794
Wuhan	14	2	0.400±0.237	0.00221±0.00131
Chengdu	10	9	0.978±0.054	0.01083±0.0026
<i>R. flaviceps</i>	38	18	0.854±0.041	0.03415±0.00249
Nanjing	17	4	0.679±0.112	0.00871±0.00499
Wuhan	6	7	0.657±0.138	0.01329±0.00626
Chengdu	15	9	0.848±0.088	0.02013±0.00371

Table 2. F -Statistics and estimates of gene flow between *R. flaviceps* and *R. chinensis*.

Sampling plots	Mitochondrial DNA (mtDNA)		Nuclear gene (nDNA, SSR)	
	F_{st}	N_m	F_{st}	N_m
Nanjing	0.6912	0.11	0.117±0.022	2.639±0.517
Chengdu	0.7435	0.09	0.039±0.016	13.804±4.209
Wuhan	0.6206	0.15	0.087±0.013	3.294±0.591

Table 3. Percent of sampled colonies assigned to genotypes based on ten SSR markers.

Populations	Number colonies	Percent of colonies assigned to genotypes	
		<i>R. chinensis</i>	<i>R. flaviceps</i>
<i>R. chinensis</i> (possessing mtDNA haplotypes of <i>R. chinensis</i>)			
Chengdu	10	0.851	0.149
Nanjing	5	0.974	0.026
Wuhan	14	0.978	0.022
<i>R. flaviceps</i> (possessing mtDNA haplotypes of <i>R. flaviceps</i>)			
Chengdu	15	0.237	0.763
Nanjing	17	0.048	0.952
Wuhan	6	0.015	0.985