福建农林大学
FUJIAN AGRICULTURE AND FORESTRY UNIVERSITY

The 46th Apimondia in Canada

Confirmation and Application of SNPs Related to Chalkbrood Resistance in Larvae of *Apis mellifera*

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Contents

1. Background

What's chalkbrood?

A fungal disease, caused by *Ascosphaera apis*

(Bailey, 1967; Gilliam *et al*., 1978; Flores *et al*., 1996)

How to transmit?

Food sharing, nursing, drifting drones and contaminated materials etc.

(Gilliam and Vandenberg, 1997)

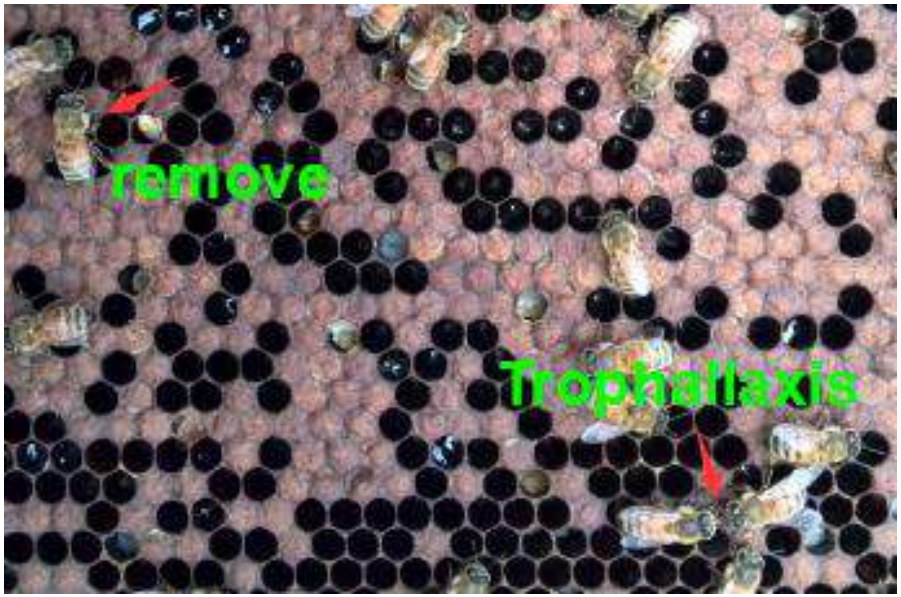
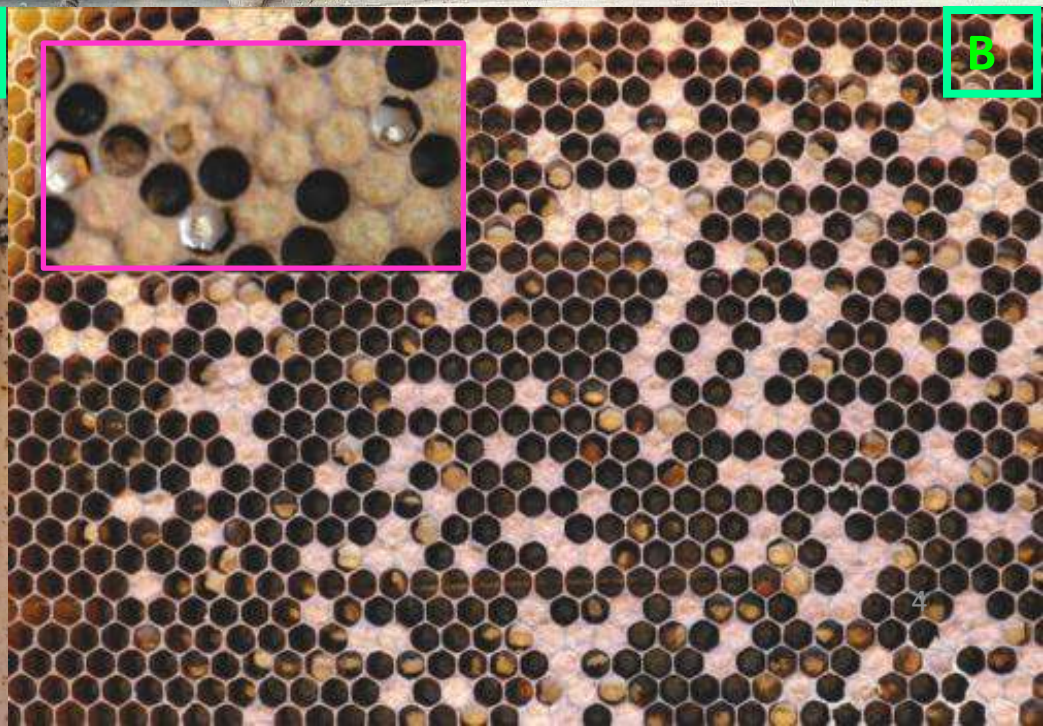


Fig. 1
A. Normal brood comb
B. Infected brood comb
C. Hive



How to fight against chalkbrood?

- Chemicals (synthetic fungicide), management and sanitation, natural compounds, antagonistic micro-organisms and breeding etc.

(Spivak & Gilliam 1998; Aronstein & Murray 2010)

- Hygienic behavior: primary mechanism of resistance to chalkbrood

(Gilliam 1998; Invernizzi *et al*., 2011)

- Breed honey bee line resistant to chalkbrood based on larvae themselves

(Holloway *et al*., 2012)

Identify genetic markers indicating chalkbrood-resistance of the colonies based on larvae

1.1 Why SNPs?

SNPs: DNA sequence variations on genome sequence (>1%; one in 1000 bases).

(Kowk 2003)

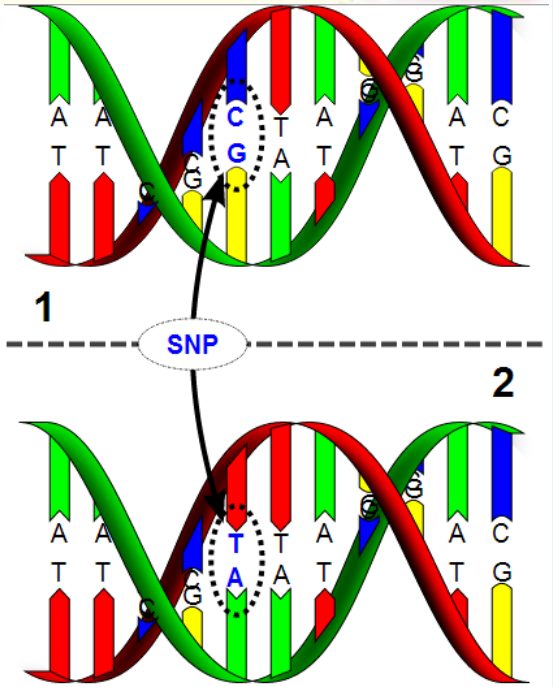


Fig 2. Diagram of SNP

(wikipedia)

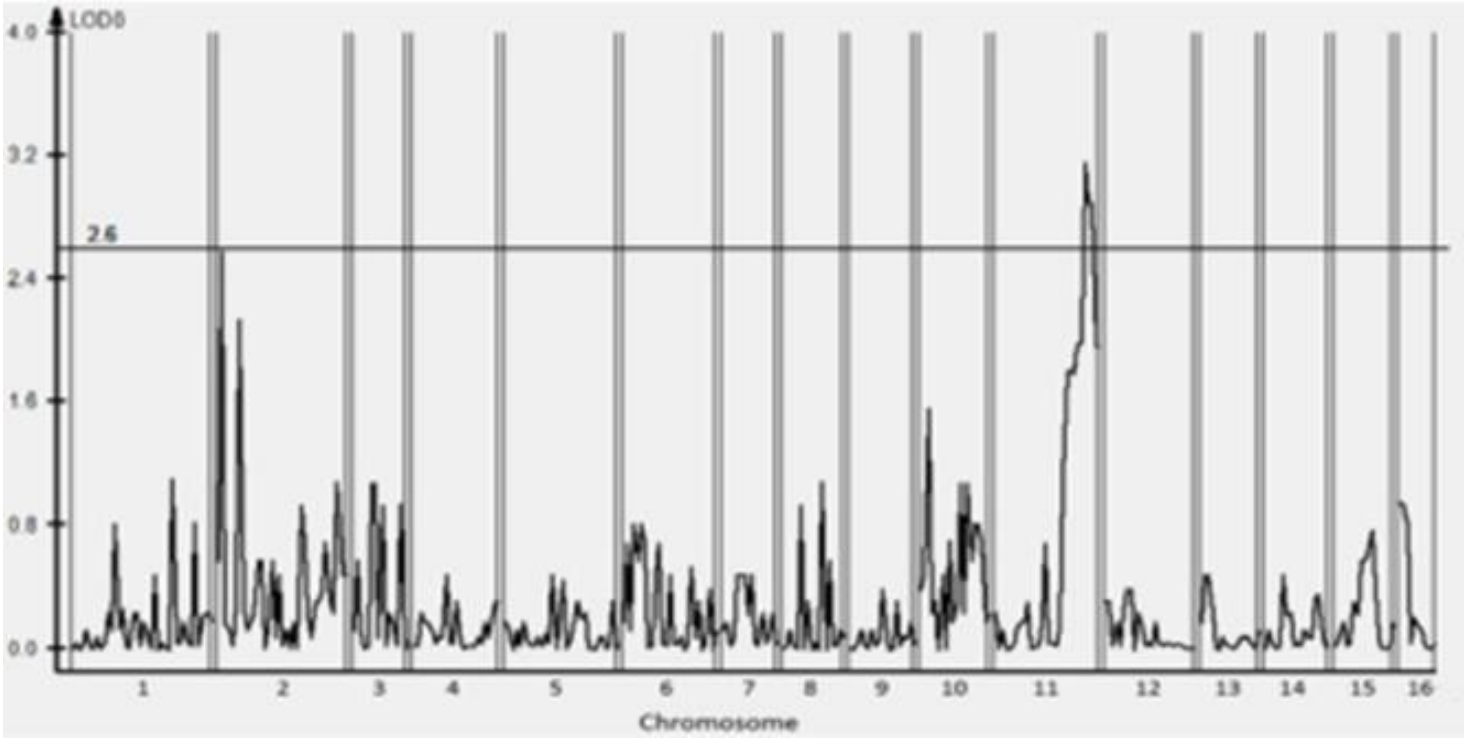
Advantages:

- ◎ Most abundant variations across the genome
- ◎ Bi-allelic, easily to be identified
- ◎ Fast and amenable to large-scale, high-throughput analyses
- ◎ Find genes or markers associated with diseases

(Vignal *et al*., 2002; Altmann *et al*., 2012)

1.2 Previous Studies

◊ QTLs found chromosome 2 and 11 associated with chalkbrood resistance. (Holloway *et al*., 2012)



Resistance correlation, larval modality VS deriving colonies

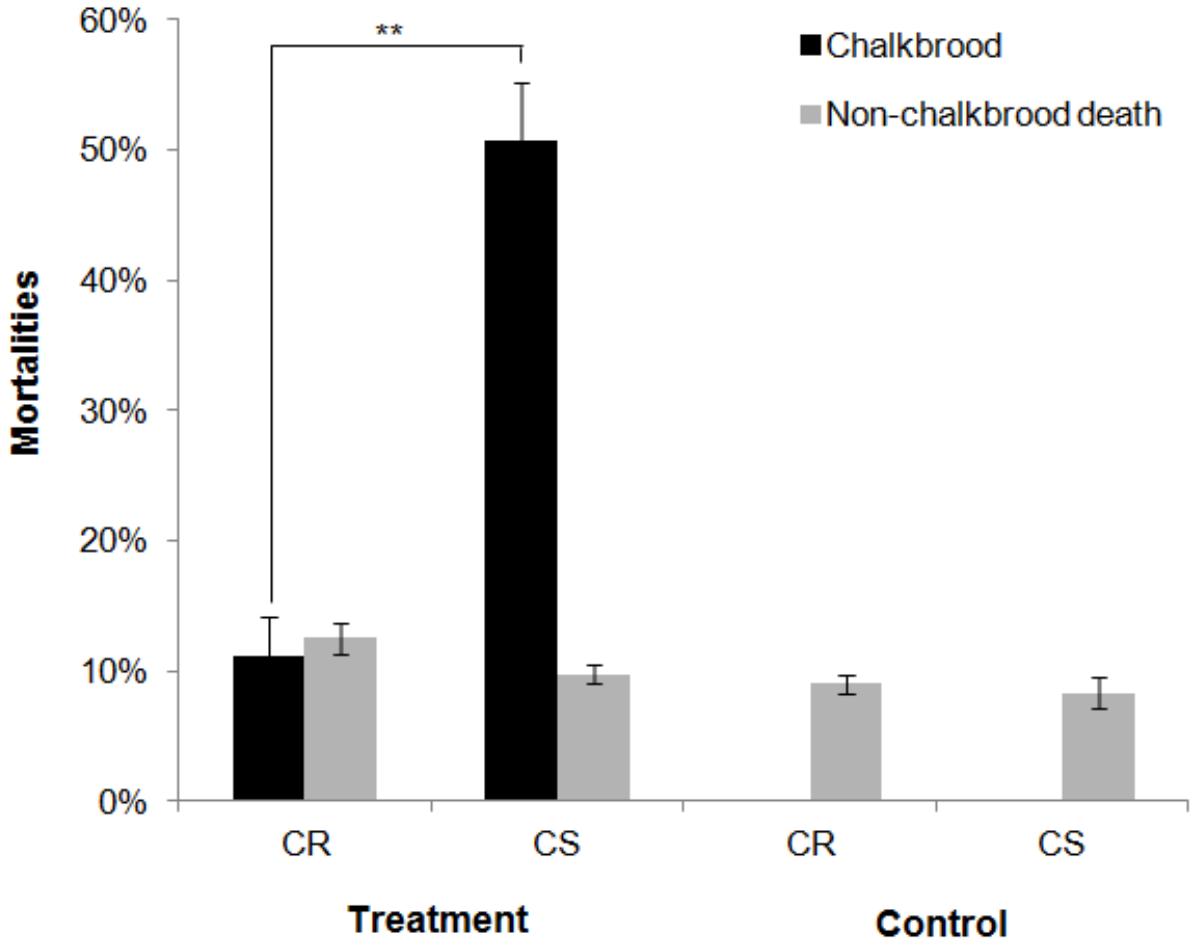


Fig 3. Comparison of larval mortality in chalkbrood-resistant and -susceptible colonies

Table 1. 71 candidate SNPs associated with chalkbrood resistance in six resistant larva samples

Number	Chromosome	Chromosome coding	Reference base	Base in resistant samples	Coordinate position in genome	symbol	Annotation
1	2	NW_003378082.1	G	A	709253	Fim	fimbrin
2	2	NW_003377976.1	C	Y	166404	LOC408715	lachesin-like
3	2	NW_003378082.1	C	T	721655	Fim	fimbrin
4	2	NW_003377991.1	G	R	716721	LOC410888	lachesin-like
5	2	NW_003377928.1	A	G	500608	LOC724736	semaphorin-1A-like
–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–
69	11	NW_003378088.1	C	T	4108245	Fng	fringe glycosyltransferase
70	11	NW_003377923.1	C	C	843167	Mrjp5	major royal jelly protein 5
71	11	NW_003377923.1	C	C	843184	Mrjp5	major royal jelly protein 5

(Limin Yan, 2012)

2. Molecular markers screening

8 colonies, 2014

1 CR and 1 CS colony

Res and Sus sample DNA

PCR

SNP genotype

Exclude false-positive SNPs

Confirmation

48 colonies, 2015

3 CR and 3 CS colonies

3rd larvae for DNA sample

Same process as the initial confirmation

Chalkbrood resistance-associated SNPs

Main methods

2.1 CR and CS olony selection

Mummies

Grind

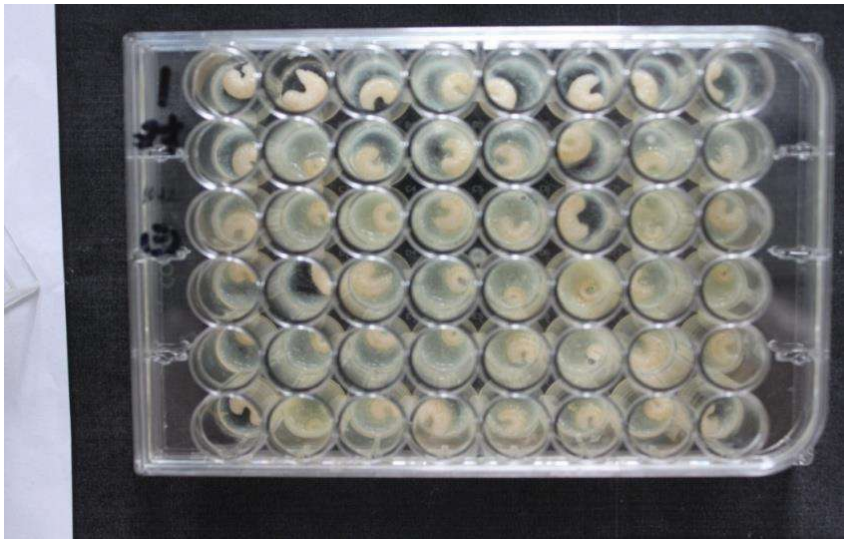
Inoculate

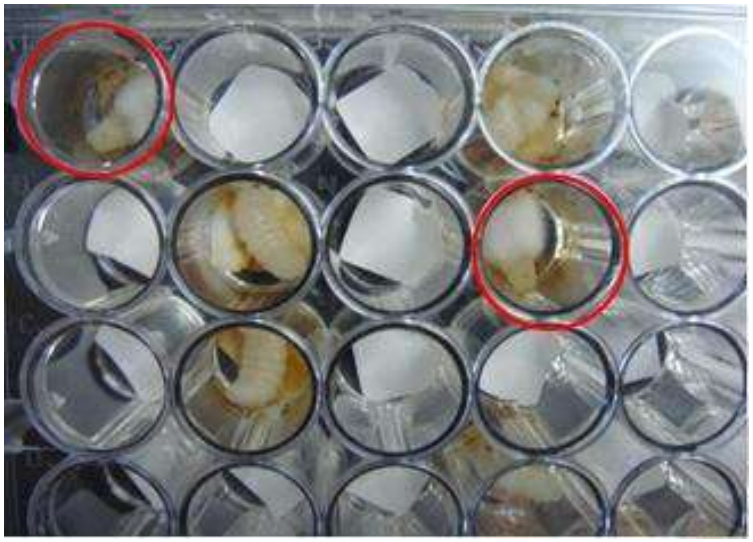
Observe

20 black mummies and 50 g pollen for each colony

Jensen *et al*. (2013)

2.2 Selecting resistant (Res) and susceptible (Sus) larvae





Larvae rearing *in vitro*

Larval diet:

50% fresh frozen royal jelly (v/v), 6% D-glucose (w/v), 6% D-frutose (w/v), 1% yeast extract (w/v) and 37% sterile deionized water

Note: The 3rd instar larvae, a dose of 5 × 10^5 spores

Jensen *et al*. (2009; 2013)

Results

Resistance assessment of colonies

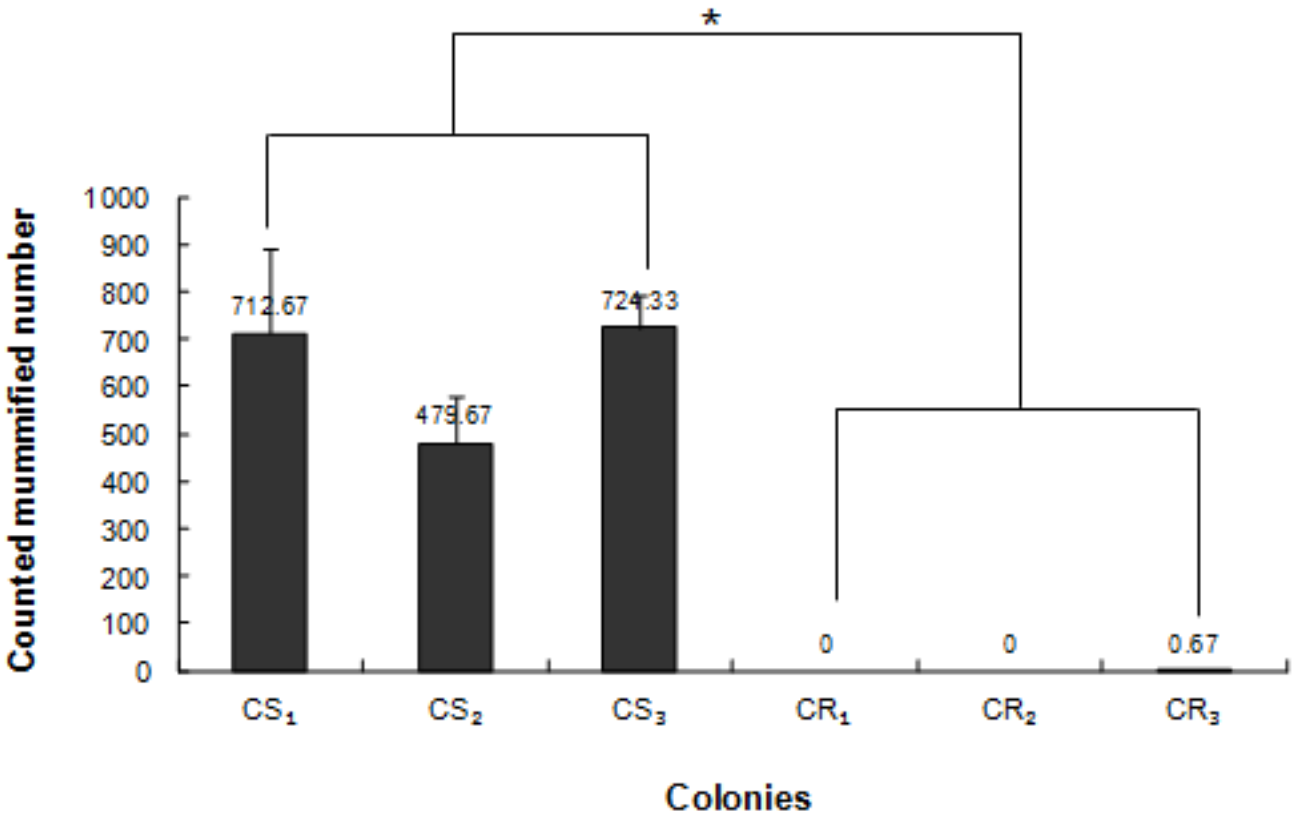


Fig 1. Comparisons of mummy number in diseased and asymptomatic colonies

Mean ± S.E. (n = 3); one-way ANOVA - LSD, * $P < 0.05$)

Initial validation

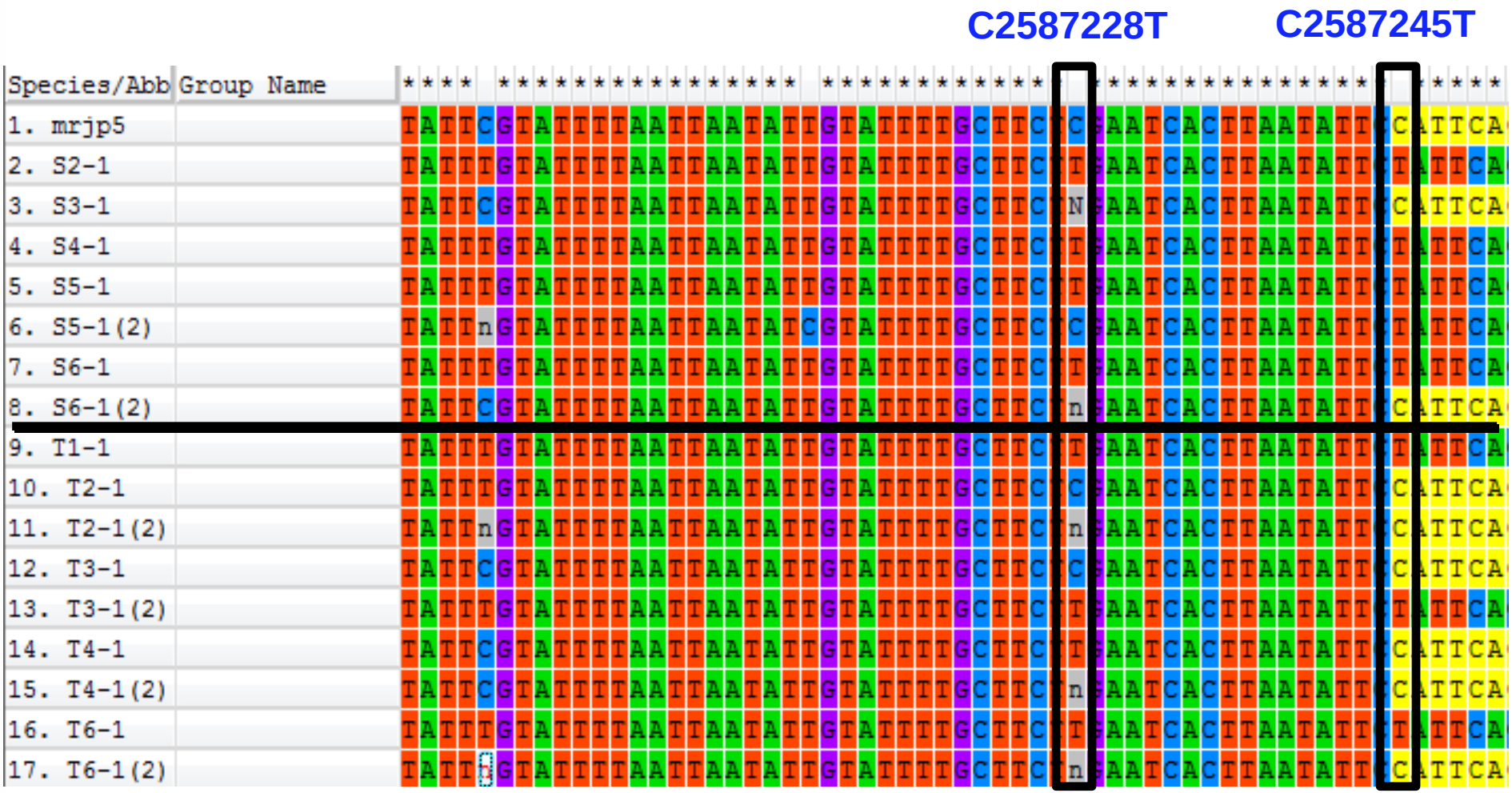


Fig 3. Alignment of sequencing result

Table 1. Eight SNP candidates after initial validation

Number	Chromosome	Chromosome coding	Reference base	Corresponding base in resistant samples	Coordinate position in genome	Symbol	Annotation
6	2	NW_003378123.1	G	A	2608140	LOC551167	multidrug resistance protein homolog 49-like
33	11	NW_003377923.1	C	C	843167	Mrjp5[a]	major royal jelly protein 5
34	11	NW_003377923.1	C	C	843184	Mrjp5[b]	major royal jelly protein 5
36	11	NW_003378088.1	T	C	1044450	LOC410318	otoferlin-like
52	11	NW_003378155.1	G	T	349321	LOC100578939	single Ig IL-1-related receptor-like
60	11	NW_003378088.1	G	R	148661	LOC100577879	carbonic anhydrase-related protein 10-like
66	11	NW_003377973.1	A	G	188523	LOC408343	potassium voltage-gated channel protein Shaker-like%
68	11	NW_003378088.1	T	W	1501725	DNAH7	Dynein, axonemal, heavy chain 7

Further validation

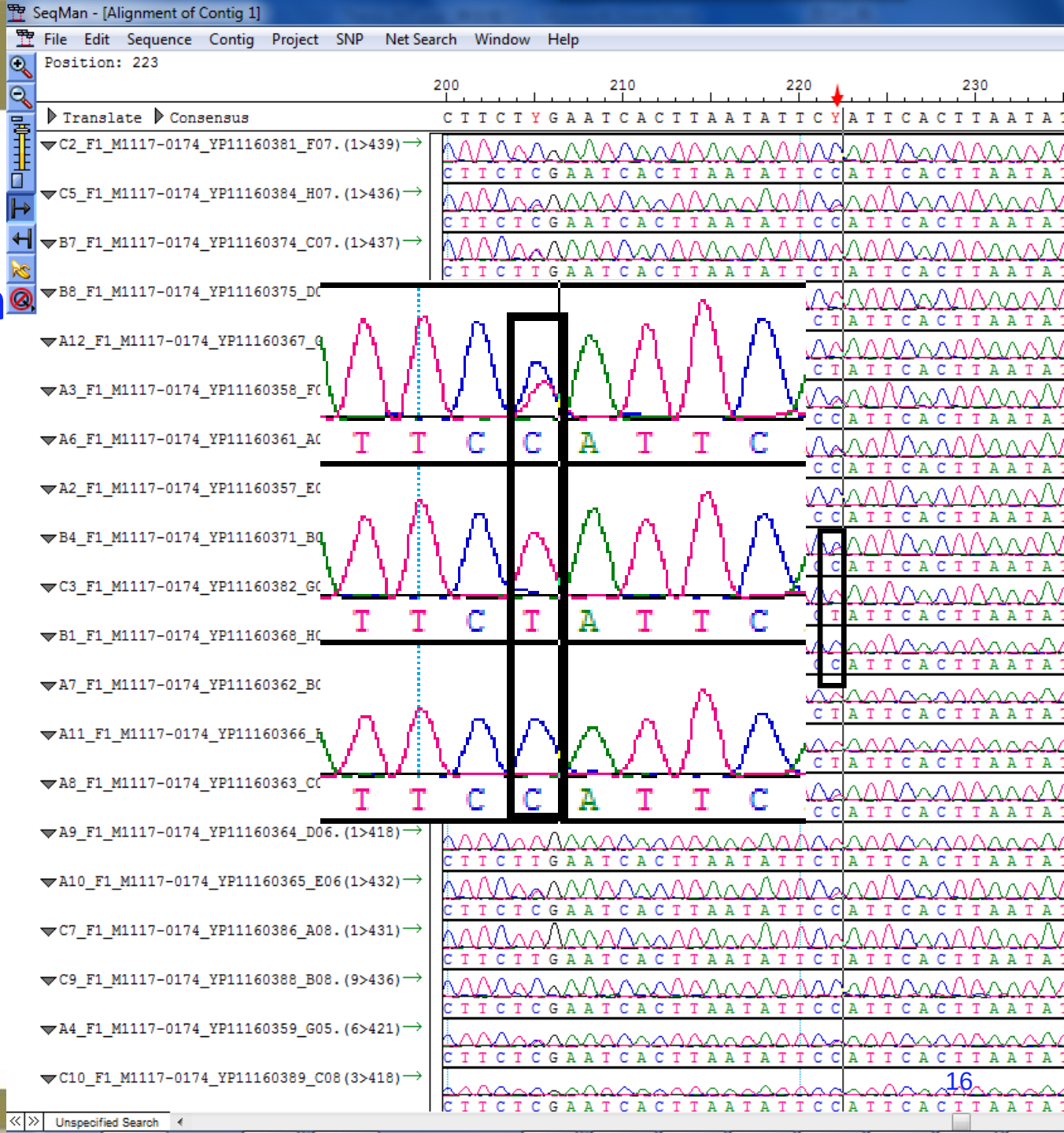


Fig 4. Manually inspect sequencing trace data using DNA star (Seqman)

Table 2. Distribution of C and T allele frequencies of SNP C2587245T

Category	Colony	Number of larvae	Frequency of genotypes			Frequency of alleles[c]					
			C/C	T/T	C/T	C	P_C[a]	Mean ± S. E.	T	P_T[b]	Mean ± S. E.
CR	CR_1	53	20	3	30	70	0.6604		36	0.3396	
	CR_2	48	9	10	29	47	0.4896		49	0.5104	
	CR_3	44	10	0	34	54	0.6136	0.5812 ± 0.0465	34	0.3864	0.4121 ± 0.0510
CS	CS_1	49	0	27	22	22	0.2245		76	0.7755	
	CS_2	47	6	20	21	33	0.3511		61	0.6489	
	CS_3	47	6	10	31	43	0.4574	0.3443 ± 0.0673	51	0.5426	0.6557 ± 0.0673

[a]significant difference in independent-samples *t*-tests ($P < 0.05$);

[b]significant difference in independent-samples *t*-tests ($P < 0.05$);

[c]significant difference in chi-square tests ($\chi^2 = 27.191$, $P < 0.001$).

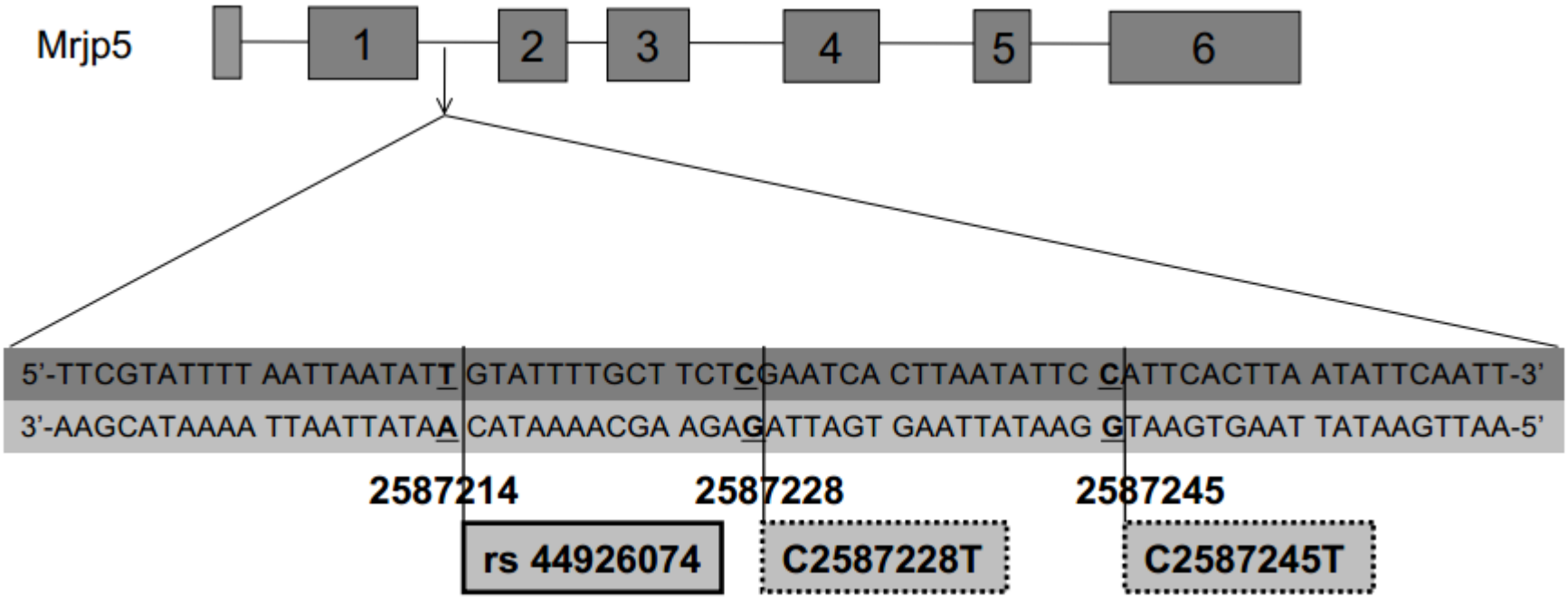


Fig 5. Diagram of SNPs distribution located at the second intron of *Mrjp 5*, chromosome 11

Table 3. Comparison of the C allele frequency in FQ and HB lines

Colony lines	Colony No.	P_C	P_T	Sample No.
FQ	1	52.08	47.92	24
	2	43.75	56.25	24
	3	56.25	43.75	24
	4	66.67	33.33	24
HB	1	34.78	65.22	23
	2	0	100	19
	3	29.55	70.45	22

$P < 0.05$

Insect Molecular Biology

Insect Molecular Biology (2016) **25**(3), 239–250 doi: 10.1111/imb.12216

Larva-mediated chalkbrood resistance-associated single nucleotide polymorphism markers in the honey bee *Apis mellifera*

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Abstract

Chalkbrood is a disease affecting honey bees that seriously impairs brood growth and productivity of diseased colonies. Although honey bees can develop chalkbrood resistance naturally, the details underlying the mechanisms of resistance are not fully understood, and no easy method is currently available for selecting and breeding resistant bees. Finding the genes involved in the development of resistance and identifying single nucleotide polymorphisms (SNPs) that can be used as molecular markers of resistance minimize the negative effects of chalkbrood on managed honey bees.

Keywords: chalkbrood, disease resistance, *Apis mellifera*, breeding, SNP, genome resequencing.

Introduction

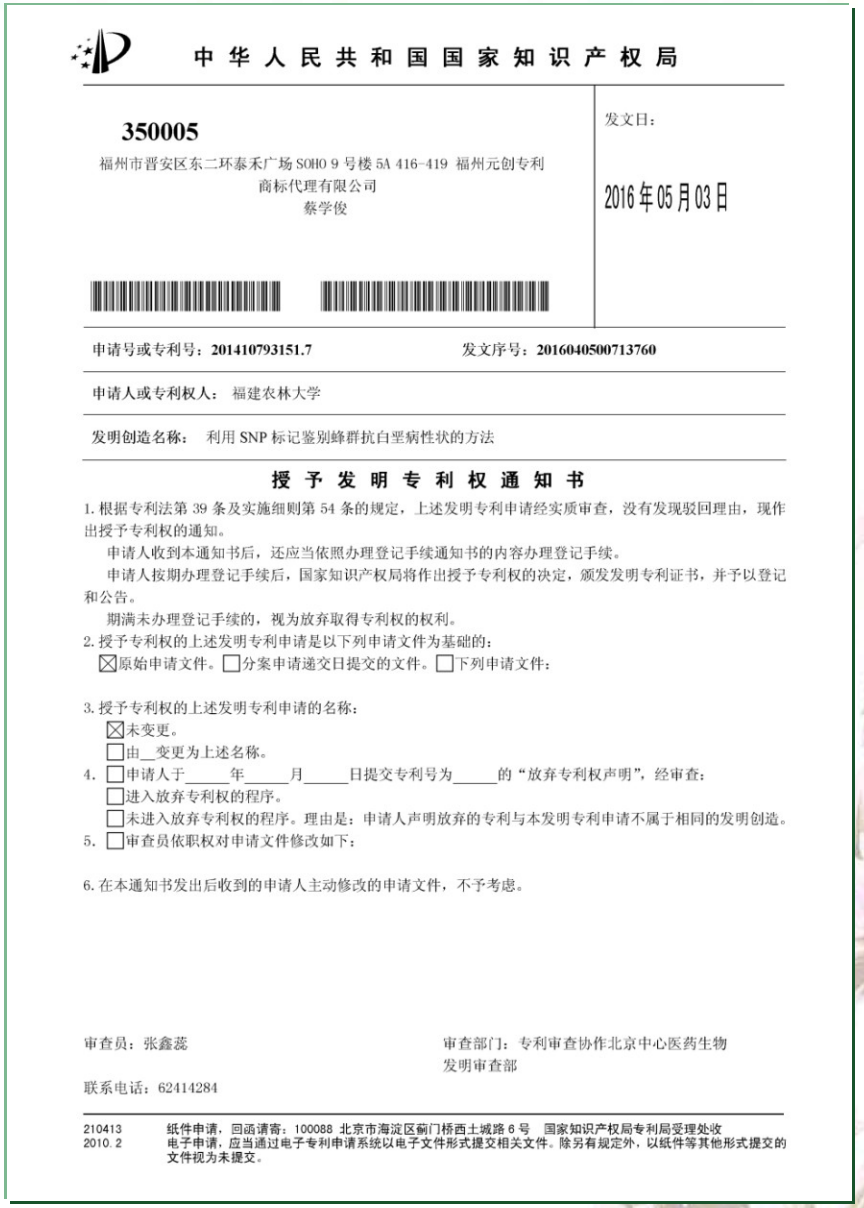
Chalkbrood is a disease of the honey bee *Apis mellifera* caused by the fungus *Ascosphaera apis*. The fungus infects honey bee larvae and causes significant harm to population growth and colony productivity. The disease is typically common during the spring months in most regions around the world (Aronstein & Murray, 2010; Jensen *et al.*, 2013).

The pattern of chalkbrood disease distribution can be affected by complex interactions between environmental factors and host genetics. High humidity and low temperature have been found to increase the prevalence of chalkbrood disease (Puerta *et al.*, 1994; Flores *et al.*,

Got Patent in China

利用SNP标记鉴别蜂群抗白垩病性状的方法 获国家发明专利授权

(专利号: ZL 201410793151.7)



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发文日：
2016 年 05 月 03 日

申请号或专利号：201410793151.7　　发文序号：2016040500713760

申请人或专利权人：福建农林大学

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3. Molecular assistant breeding

Fengqian No.1 Honeybee (FQ No.1) was selected from high royal jelly yield breed (11 lines from Zhejiang province) with honeybee closed population breed method and molecular assistant breeding method.

Honeybee breed (Fengqian No.1 Italian Bee) with chalkbrood resistance and high RJ yield

蜂王浆优质高产抗白垩病蜂种培育
From 2010--- now

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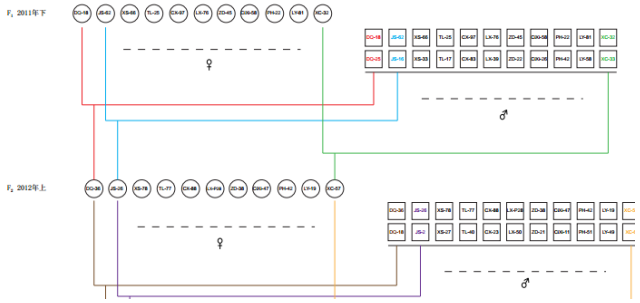
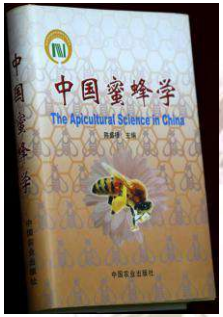
FQ No.1 Breeding technology and Part family tree

“蜂强1号”意蜂培育技术与部分系谱图

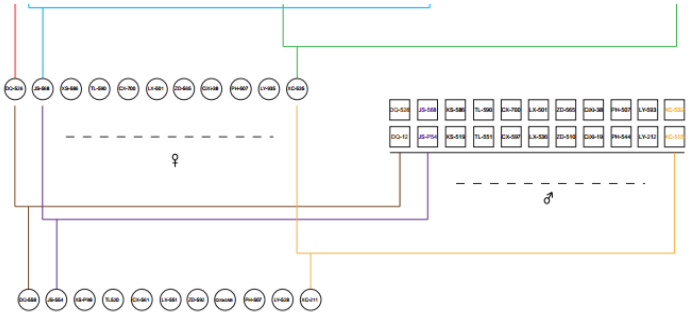
2010~2017年，采用蜜蜂闭锁群体育种技术培育13代—–“蜂强1号”意蜂

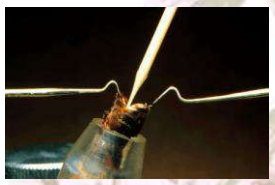


Isolated mating and artficial insemination

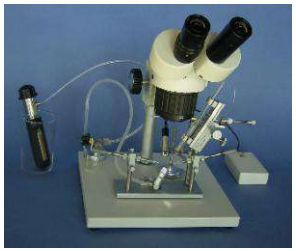


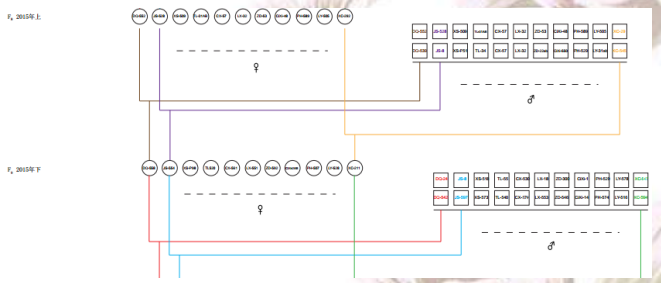
SNP assistant breeding





蜜蜂
人工授精





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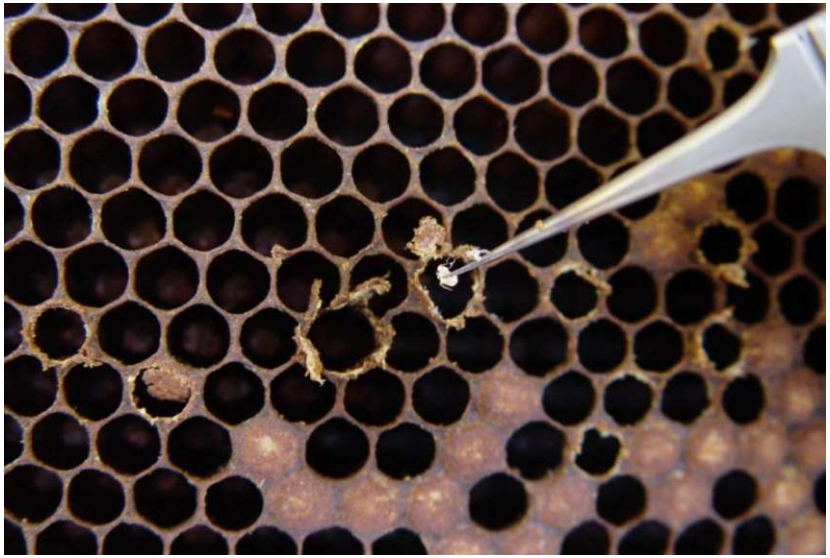

(Su SK, et al., 2007)

证书号第425980号

发明专利证书

发明名称：无伤害提取活体蜜蜂DNA的方法

发明人：苏松坤；蔡芳；杜宏沪；陈盛禄

专利号：ZL 2006 1 0052873.2

专利申请日：2006年8月10日

专利权人：浙江大学

授权公告日：2008年9月3日

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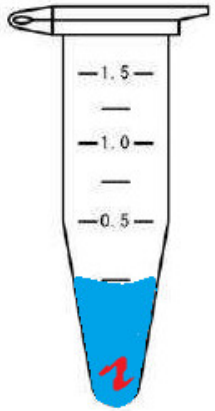
2008年9月3日

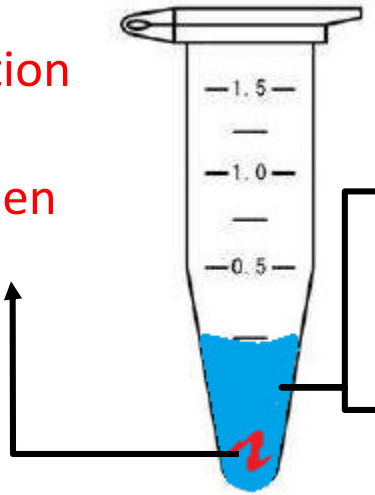
第1页(共1页)

DNA collection from cuticle of virgin queen to detect chalkbrood resistance SNP (C2587245T)

Virgin queen cuticle collection

DNA extrction from Virgin queen cuticle





200 μL 5% Chelex100

10 μL 10mg/mL 蛋白酶K

7 μL DTT

55℃ 20h

↓

100℃水浴变性 8min

↓

4℃ 13000g离心 3min

上清液可直接用来进行PCR测序，或调整浓度后通过快速检测的手段来鉴定处女王基因型

Assistant breeding on chalkbrood resistance with SNP (C2587245T)

★ Predit colonies' chalkbrood-resistance level and facilitate chalkbrood resistance breeding by analyzing P_C of each colony.

★ C/C queen at C2587245T will potentially generate the offspring with high level of resistance to chalkbrood.

★ Useful to minimize the influence of chalkbrood on managed honey bees.





Fengqian No.1 Honeybee was test and popularized in Henan, Zhejiang, Anhui, Jiangsu, Hubei, Yunnan, Fujian, Hainan provinces in China

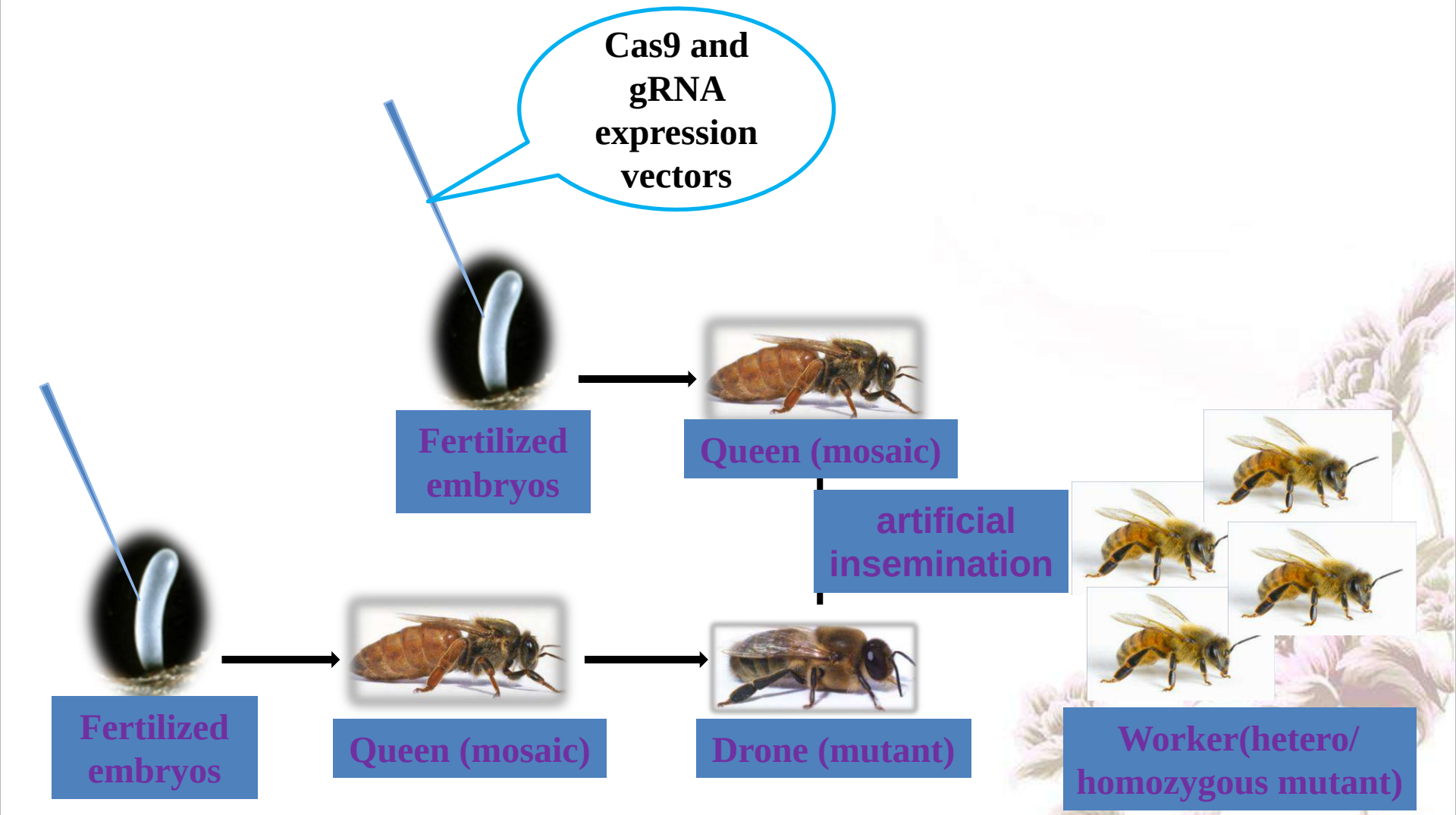
4. Prospective

- We focused on SNP candidates located on two studied chromosomes rather than all of them.

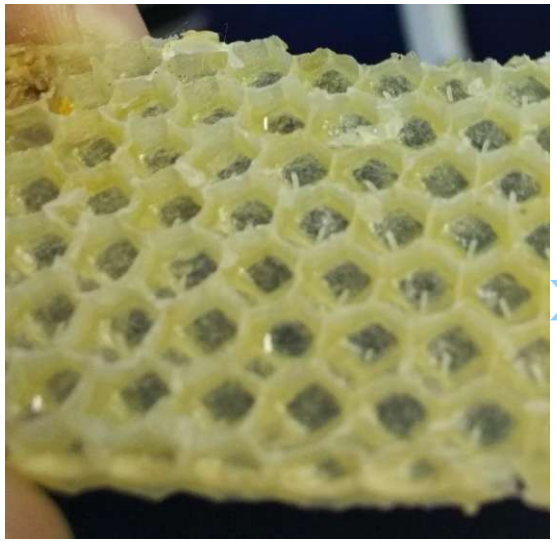
- *MRJP* family may have important functions in honey bee physiology besides the nutritional roles for larvae (Buttstedt *et al*., 2014; Bíliková *et al*., 2009).

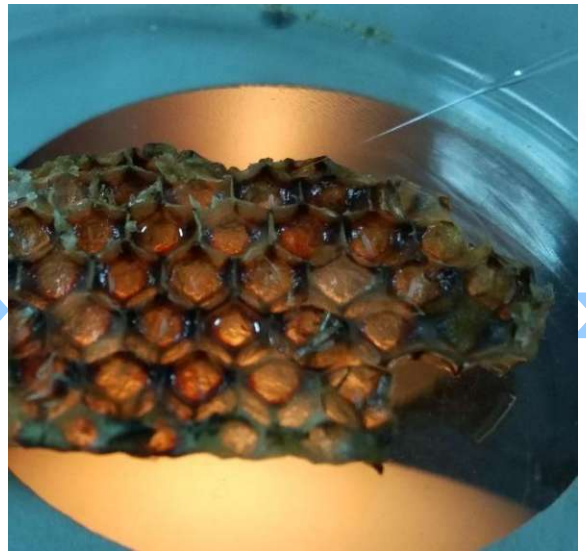
- Though the role of SNP C2587245T/ the intron in susceptibility to chalkbrood disease remains unknown, detail mechanisms still need further research, CRISPR/Cas Systems (Mali et al., 2012).

Honeybee CRISPR/Cas9 System ……



Honeybee egg micro-injection and hatching ……





Collecting eggs injection embryo hatching

Honeybee CRISPR/Cas9 System ……*white* gene knockout

A

组别	注射物质	蜂卵注射数目	幼虫孵化数目(%)	嵌合体(%)
对照组	无菌水	20	16/20(80)	_
注射组	Cas9 蛋白 mRNA	40	11/40(27.5)	10/11(90.9%)

B

C

(W.T) TTTTATTACAGTATGCGGGGTTGCGTATCCAGGTGAATTGTTGGTAATTA

(A_{2-4}) TTTTATTACAG -GTTGGTAATTA (-28)

(A_{2-5}) TTTTATTACAGTATGCGGGGTTGCGTGAAGTGTTCCAGGTGAATTGTTGGTAATTA (+5)

(A_{3-2}) TTTTATTACAGTATGCGGGGTTG-G-ATTGTTGGTATGAATTGTTGGT

(A_{3-6}) TTTTATTACAGTATGCGGGGTTGC- - -TCCAGGTGAATTGTTGGTAA

(A_{3-7}) TTTTATTACAGTATGCGGGGTTGC- - - ---- GGTGAATTGTTGGTAA

(A_{3-9}) TTTTATTACTTTATGCGGGGTTGC - - - ---- GGTGAATTGTTGGTAA

(A_{5-2}) TTTTATTACAGTATGCGGG-TT----------GTAA

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