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Original article

Multiple *Wolbachia* strains in *Apis mellifera capensis* from South Africa*

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Abstract – Eggs of the honeybee *Apis mellifera capensis* from South Africa were screened for *Wolbachia* using degenerate primers designed to amplify a segment of the *wsp A* gene sequences. This strategy resulted in the identification of two new strains (*w*Cap-B2, and -A1) in addition to the one (*w*Cap-B1) characterized earlier from *A. m. capensis* and *A. m. scutellata*. Strain-specific primers were designed and used to assay eggs from both *A. m. capensis* and *A. m. scutellata*. The *w*Cap-B1 sequence was amplified consistently from both *A. m. capensis* and *A. m. scutellata*, but the *w*Cap-B2 and -A1 sequences were amplified sporadically only from *A. m. capensis*. This indicates that the *w*Cap-B1 strain could be present at a higher titer or that *w*Cap-B2 and -A1 are present only in some individuals in the wider *A. m. capensis* population. The detection of these new *Wolbachia* strains suggests that additional investigations are required to determine the role of *Wolbachia* in the biology of *A. m. capensis* workers.

***Apis mellifera capensis* / new *Wolbachia* strains / degenerate primers / universal primers / high-fidelity PCR**

1. INTRODUCTION

The Cape honeybee *Apis mellifera capensis* Escholtz in South Africa is unique in that, under queenless conditions, workers lay unfertilized eggs that typically develop into diploid females (= thelytokous parthenogenesis) (Onions, 1912; Anderson, 1963). In all other subspecies of *Apis mellifera* L. only males are produced from worker-laid eggs (= arrhenotokous parthenogenesis) (Ruttner, 1988). This has allowed Cape workers to be successful parasites of other honeybee subspecies, notably the neighboring Savanna honeybee (*A. m. scutellata* Lepetelier) (Neumann and Hepburn, 2002). Although the natural populations of Cape and Savanna honeybees in South Africa appear stable (Beekman et al., 2008), the movement of Cape honeybees by beekeepers outside their natural range has repeatedly resulted in Cape honeybees successfully parasitizing Savanna honeybee colonies (Allsopp, 1992; Martin et al., 2002).

Thelytoky in the Cape honeybee has been shown to be genetically determined by a single recessive allele (Ruttner, 1988; Lattorff et al., 2005), and the same locus influences the onset of oviposition and pheromone production in Cape workers (Lattorff et al., 2007). These results notwithstanding, there remains the possibility that endosymbiotic bacteria such as *Wolbachia* could be involved in thelytokous parthenogenesis in Cape honeybees. *Wolbachia* bacteria are widespread and common among arthropods (Jeyaprakash and Hoy, 2000), although none had been found in Cape honeybees before 2003 (Wenseleers and Billen, 2000).

Hoy et al. (2003) used a more sensitive method to screen *A. m. capensis* and

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Table I. Tests of *A. m. capensis* and *A. m. scutellata* eggs for *Wolbachia* strains using high-fidelity PCR. + indicates amplification and – indicates no amplification.

Date collected	Sample	Subspecies	No. of eggs	*w*Cap-B1	*w*Cap-B2	*w*Cap-A1
2/10/2003	1	*A. m. capensis* Pretoria 'clone'	20	+	+	+
	Negative control		0	-	-	-
08/14/2003	2	*A. m. scutellata*	50	+	-	-
	3	*A. m. scutellata*	50	+	-	-
	4	*A. m. capensis*	50	+	+	+
	5	*A. m. capensis*	50	+	-	-
	Negative control		0	-	-	-
10/15/2003	6	*A. m. scutellata*	40	+	-	-
	7	*A. m. capensis*	40	+	+	+
	Negative control		0	-	-	-
02/09/2004	8	*A. m. scutellata*	10	+	-	-
	9	*A. m. scutellata*	10	+	-	-
	10	*A. m. scutellata*	10	+	-	-
	11	*A. m. scutellata*	10	+	-	-
	12	*A. m. capensis*	10	+	+	-
	13	*A. m. capensis*	10	+	-	-
	14	*A. m. capensis*	10	+	-	+
	15	*A. m. capensis*	10	+	-	-
	Negative control		0	-	-	-

A. m. scutellata for *Wolbachia*, which resulted in the finding of a single *Wolbachia* strain (*w*Cap-B1) based on identical *wsp* sequences obtained from both subspecies. Because no unique *Wolbachia* were detected from *A. m. capensis*, the search was further expanded to identify additional bacterial species present in *A. m. capensis* using amplified 16S rRNA sequences. This resulted in the identification of *Bifidobacterium*, *Lactobacillus* (Gram-positive bacteria), *Gluconacetobacter* (Gram-negative bacteria), *Bartonella* (α-proteobacteria), *Simonsiella/Neisseria* (β-proteobacteria) and *Serratia* (γ-proteobacteria) (Jeyaprakash et al., 2003). However, all were present in both *A. m. scutellata* and *A. m. mellifera* Linnaeus, and it remained unlikely that they had a role in the unusual reproductive behavior of *A. m. capensis* (Jeyaprakash et al., 2003; Cox-Foster et al., 2007).

A problem with these microbial surveys is that they were all conducted using universal primers (Braig et al., 1998; Weisburg et al., 1991), which may amplify sequences from the majority of bacterial species present in *A. m. capensis*, but not amplify all. Hence, designing degenerate primers using conserved gene regions could allow detection of additional bacteria. The objective of this project was to design degenerate *Wolbachia wsp* primers and screen *A. m. capensis* for the presence of additional strains of *Wolbachia*.

2. MATERIALS AND METHODS

2.1. Colony sources and DNA extraction

The following honeybee eggs were collected in South Africa and shipped in 95% ethanol to the USA (Tab. I): (a) worker-laid eggs from an *Apis mellifera capensis* laying worker colony (= clone) (Baudry et al., 2004) in Pretoria; (b) queen-laid eggs from seven queenright *A. m. capensis* colonies in Stellenbosch; and (c) queen-laid eggs from seven queenright *A.m. scutellata* colonies in Kenhardt. Genomic DNA was extracted by grinding pooled eggs (from 10 to 50 per sample) in 600 μL of PUREGENE cell lysis solution (Gentra System, Minneapolis, MN), digesting with Protease K (1 mg) (Roche Diagnostics, Indianapolis, IN) at 37 °C for 16 h, and precipitating with 200 μL

Table II. List of primers used to amplify *Wolbachia* from the eggs of *A. m. capensis* and *A. m. scutellata*.

Primer name	Primer sequence	Expected size of products
Degenerate primers		
wsp-deg F	5'-GCA/GTTTGGT/CTAT/CAAAATGGA-3'	~ 0.32 kb
wsp-deg R	5'-GCACCAT/AAAGAACCA/GAAA/GTA-3'	
Specific primers		
*w*Cap-B1 F	5'-GGCTAAAGATACAGATGTAGTA-3'	273 bp
*w*Cap-B1 R	5'-CGAGCTCCAGCAAAGAGTTT-3'	
*w*Cap-B2 F	5'-GACGTTAGTGGTACAACATTTA-3'	263 bp
*w*Cap-B2 R	5'-CGAGCACCAGCATAAAGCTT-3'	
*w*Cap-A1 F	5'-GTAACATTTGACCCAGCAAATA-3'	258 bp
*w*Cap-A1 R	5'-CGAGCTCCAGCATAAAGTTTG-3'	

PUREGENE protein precipitation solution over ice for 10 min. The supernatant (~ 750 μL) was collected after centrifugation at 14 000 rpm for 10 min at 4 °C and genomic DNA precipitated by mixing with 600 μL 2-propanol at −20 °C for 16 h. The genomic DNA was recovered by centrifugation at 14 000 rpm for 20 min at 4 °C, washed with 70% ethanol, dried, dissolved in 50 μL sterile water and stored at −20 °C.

2.2. Primers

The *Wolbachia wsp A* sequences of A and B reference strains were obtained from GenBank (Accession Nos. AF020058, AF020061, AF020063, AF020070, AF020071, AF020073, Af020077, AF020079, AF020082-AF020085, AF071910, AF071911, AF071916-AF071918, AF071923, AF071924, AF217724 and AF217725) and their open reading frames aligned using CLUSTAL X. Two conserved amino acid regions (AFGYKMD and YFGSF/YGA) were detected and used to design degenerate primers (Tab. II). *Wolbachia* strain-specific primers were subsequently designed from the *wsp* sequences obtained from the honeybees (Tab. II).

2.3. High-fidelity PCR and cloning

High-fidelity Polymerase Chain Reactions (Hf-PCR) were performed using a 50-μL reaction volume containing genomic DNA (1 μL), 50 mM Tris pH 9.2, 16 mM ammonium sulfate, 1.75 mM $MgCl_2$, 350 μM dATP, dGTP, dCTP, and dTTP, 400 pM of forward and reverse primers, 1 unit of *Tgo* and 5 units of *Taq* DNA polymerases (Roche Diagnostics, Indianapolis, IN) (Barnes, 1994). Three linked profiles were used for amplification in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA); (i) One cycle consisting of denaturation at 94 °C for 2 min, (ii) 10 cycles consisting of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 68 °C for 30 s, and (iii) 20 cycles consisting of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 68 °C for 30 s with 20 s added to every consecutive cycle. The amplified PCR products were cloned into pCR2.1 TOPO using the reagents provided by manufacturer (Invitrogen, Carlsbad, CA) and plasmids isolated from several independent *Escherichia coli* (Migula) Castellani and Chalmers transformants were sequenced. The sequences obtained were aligned using CLUSTAL X.

3. RESULTS AND DISCUSSION

Genomic DNA extracted from 20 *A. m. capensis* (Pretoria 'clone') eggs was amplified using the degenerate primers (wsp-deg F and wsp-deg R) (Tab. II) and a 0.3-kb DNA band was produced. Twenty independent clones generated by cloning the Hf-PCR products were completely sequenced and compared. This resulted in the identification of four *wsp* sequences; (i) Thirteen sequences (313 bp) were identical and displayed similarity to a sequence obtained earlier from *A. m. capensis* and *A. m. scutellata* (*w*Cap-B1) (GenBank accession No. AF510085), which clustered within the Con group of B-*Wolbachia* (Hoy et al., 2003), (ii) Five sequences (316 bp) were identical (*w*Cap-B2), not represented

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                    10          20          30          40
wCap-B1     TGACATTAGA GTTGATGTTG AAGGGCTTTA CTCAAAATTG
wCap-B2     C..T..C..G .......... .G........ C...C.AC.A
wCap-A1     C..C..C..G .......... .A........ T...T.CC.A

          50          60          70          80          90
GCTAAAGATA CAGAT-GTAG TA-AATACTT -CTGAAACAA ---ATGTTGC
AAC...A.CG AC.T.AGTGG .GC..CAT.. ACTCC.A... ---C.G....
AAC...A.TG AT.T.AAAGA .GT..CAT.. GACCC.G... ATAC.A....

        100         110         120         130         140
AGACAGTTTA ACAGCATTTT CAGGATTGGT TAACGTTTAT TACGATATAG
.A.....G.G GC....C... .......G.. T.....T... ..........
.G.....G.A AT....A... .......A.. G.....G... ..........

        150         160         170         180         190
CGATTGAAGA TATGCCTATC ACTCCATACG TTGGTGTTGG TATTGGTGCA
.G........ .......... .........G .......... .G........
.A........ .......... .........A .......... .G........

        200         210         220         230         240
GCATATATCA GCAATCCTTC AAAAGCTGAT GTAGTTAAAG ATCAAAAAGG
..A.....C. ...A.....C AG..G.TAG. .CAGT.A.AG .TC...AAGG
..G.....T. ...C.....T GG..C.CGC. .TGAA.G.TC .AA...GTAA

        250         260         270         280         290
ATTTGGTTTT GCTTATCAAG CAAAAGCTGG TGTTAGCTAT GATGTAACTC
.......... ...TA..... C......... ......T... ........C.
.......... ...GG..... T......... ......T... ........T.

        300         310         320
CAGAAATCAA ACTCTTTGCT GGAGCTCGT
.....A.... G..T.A.... ..T......
.....G.... A..T.A.... ..A......
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Figure 1. CLUSTAL X alignment of the *wsp* sequences amplified from *A. m. capensis*. Dot denotes matching nucleotides and hyphen denotes deletion.

in GenBank, and clustered within the Pip group of B-*Wolbachia* (GenBank accession No. FJ438823), (iii) One sequence (316 bp) displayed a 1-bp difference from *w*Cap-B2 and contained a 'G' instead of 'A' at position 37 (Fig. 1), which could be a *Taq*-generated error, and (iv) One sequence (319 bp) (*w*Cap-A1) was different from all others, not represented in GenBank, and clustered within the *w*Mel group of A-*Wolbachia* (GenBank accession No. FJ438824) (Fig. 1). Thus, it appears that multiple *Wolbachia* strains exist in *A. m. capensis* worker eggs, which was unexpected, and confirms our suspicion that our earlier assays for *Wolbachia* using universal primers failed to amplify all *Wolbachia* sequences (Hoy et al., 2003).

Alignment of the sequences from the three *Wolbachia* strains from *A. m. capensis* revealed sequence differences between them (Fig. 1), and allowed designing *Wolbachia* strain-specific primers for *w*Cap-B1, -B2 and -A1 (Tab. II).

Fresh samples of both *A. m. capensis* and *A. m. scutellata* eggs collected from Stellenbosch and Kenhardt, South Africa, and received in three separate shipments, were assayed for the three different *Wolbachia* strains (Tab. I). A Hf-PCR assay of both *A. m. capensis* and *A. m. scutellata* eggs indicated that the *w*Cap-B1 sequence was consistently amplified from all samples screened (100%). However, the *w*Cap-B2 or -A1 sequences were amplified from *A. m. capensis* less consistently (Tab. I), and never from any *A.m. scutellata* samples. This suggests that the titer of the *w*Cap-B1 *Wolbachia* might be higher than that of the *w*Cap-B2 and -A1 *Wolbachia* in

A. m. capensis, or that primer interference may prevent efficient amplification, or that *w*Cap-B2 and -A1 *Wolbachia* might be present in some *A.m. capensis* colonies or individuals, but not in others. Hf-PCR is efficient in amplifying sequences from as little as 1 fg of plasmid DNA (~ 100 copies) (Jeyaprakash and Hoy, 2000, 2004), which suggests that *w*Cap-B2 or -A1 sequences could consist of less than 100 copies.

These results suggest that *Wolbachia* might yet be involved in the thelytokous reproduction exhibited by *A. m. capensis*, even if the characteristic itself is genetically inherited. It is interesting to note that all three *Wolbachia* types were present in the Pretoria clone eggs (Tab. I), but that *w*Cap-B2 and -A1 *Wolbachia* were relatively rare in the wider Cape honeybee population. There are no known egg parasitoids present in the study area and therefore we concluded that these *Wolbachia* strains are specific to Cape honeybee. The presence of these additional *Wolbachia* strains might determine which *A. m. capensis* workers and colonies are able to be successful parasites, and ultimately determine the 'winning' clone (Baudry et al., 2004).

Currently, there is no evidence that these *Wolbachia* strains are responsible for the production or success of diploid females by *A. m. capensis* workers, and additional work will be required to determine if these *Wolbachia* have a role in this behavior. Furthermore, these data suggest that, although arthropods are routinely screened for *Wolbachia* and other bacteria using universal primers, the universal primers may not be sufficiently sensitive and their use could be underestimating the number of bacteria present.

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Présence de souches multiple de *Wolbachia* chez *Apis mellifera capensis* d'Afrique du Sud.

***Apis mellifera capensi* / *Wolbachia* / nouvelle souche / amorce dégénérée / amorce universelle / PCR haute fidélité / parthénogenèse thélythoque / femelle diploïde**

Zusammenfassung – Vorkommen mehrerer *Wolbachia* Stämme in *Apis mellifera capensis* aus Südafrika. Arbeiterinnen der südafrikanischen Kapbiene sind in der Lage, Weibchen zu produzieren, während Arbeiterinnen aller anderen Subspezies der Honigbiene nur Männchen produzieren können. Dieses Merkmal erlaubt es Arbeiterinnen der Kapbiene, in andere Bienenvölker einzudringen und die Königin zu ersetzen, was letztendlich eine Reduktion der Honigproduktion zur Folge hat. Obwohl es inzwischen Erkenntnisse über die genetischen Grundlagen dieses Verhaltens gibt, kann nicht ausgeschlossen werden, dass hierbei auch *Wolbachia*-Bakterien eine Rolle spielen können. Frühere Untersuchungen mittels Universalprimern detektierten den *Wolbachia*-Stamm *w*Cap-A1. Da aber erstens dieser Stamm sowohl in Kap- als auch in Scutellata-Bienen gefunden wurde, und zweitens Universalprimer zwar Sequenzen der Mehrzahl aber nicht aller *Wolbachia*-Stämm amplifizieren können, entwickelten wir degenerierte Primer für die *wsp A* Gensequenzen aller A und B Referenzstämme (Tab. II), um zu sehen, ob in Kapbienen zusätzliche Stämme gefunden werden können. Die Amplifikation der *wsp* Sequenzen aus Eiern der Kapbiene mittels dieser degenerierten Primer ermöglichte die Identifizierung zweier zusätzlicher Stämme (*w*Cap-B2 and –A1) (Abb. 1). Für diese wurden sodann spezifische Primer entwickelt (Tab. II), mit denen Eier aus verschiedenen Völkern untersucht werden konnten. Wir fanden, dass der *w*Cap-B1 Stamm sowohl in Kap- als auch in Scutellata-Völkern konstant präsent war, während die Stämme *w*Cap-B2 und *w*Cap-A1 vereinzelt in Kapbienen detektiert wurden (Tab. I). Es ist daher möglich, dass entweder der Titer für den *w*Cap-B1Stamm höher ist, oder dass die Stämme *w*Cap-B2 und *w*Cap-A1 nur in einzelnen Arbeiterinnen der Kapbiene vorkommen. Zusätzliche Untersuchungen sind notwendig, um der Frage nachzugehen, ob diese *Wolbachia*-Stämme im Verhalten der Arbeiterinnen der Kapbiene eine Rolle spielen.

***Apis mellifera capensis* / Neue *Wolbachia Stämme* / Degenerierte Primer / Universalprimer / High-fidelity PCR**

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