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Identification of Genes Associated With Foraging and Stinging Behaviours in Feral Honeybee (*Apis mellifera*) Races in Kenya

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Abstract: **Honeybees from Kenya were evaluated for foraging and stinging behaviours at the colony level. Stability for plenty of honey and/or pollen reserves was established for each colony to prevent bees from absconding. Microsatellite markers were selected by mapping them to honeybees QTLs for stinging and foraging behaviour. Six QTLs namely *sting*-1, *sting*-2, *sting*-3, for stinging response and *pln*-1, *pln*-2 and *pln*-3, for foraging behaviour were considered. Association studies showed that on average colonies stored more nectar (69%) than pollen (31%). The χ^2 correlation coefficient between *sting* and *pln* (0.184458) showed they are positively correlated suggesting that defensive colonies forage for nectar more than the less defensive ones. Candidate gene search identified three genes each associated with foraging (GB46589, GB44258, GB44259) and stinging behaviour (GB48999, GB49000, GB55730). QTLs gene identification amongst African honeybee races is crucial for harnessing their economic important traits for breeding, conservation and productivity efforts.**

Keywords: **Microsatellite, *Apis mellifera*, Quantitative Trait Loci (QTLs), Aggressiveness, Foraging.**

1. INTRODUCTION

Honeybees (*Apis mellifera*) are the most economically valuable pollinators of agricultural crops worldwide (Sinclair *et al*., 2011). The Food and Agriculture Organization (FAO) of the United Nations (UN) estimates that out of some 100 crop species which provide 90% of food worldwide, 71 of these are bee-pollinated (FAO *et al*., 2014). However global honeybee populations are declining (vanEngelsdorp *et al*., 2011) resulting in approximately 1% loss of the earth's biodiversity annually due to habitat loss, pest invasion, pollution (Krupke *et al*., 2012), over-harvesting and diseases (Gallai *et al*., 2009; Civantos *et al*., 2012). African honeybees are better equipped to deal with the challenges of pests and diseases that badly affect *Apis mellifera* in other parts of the world (Frazier, 2010; Karanja *et al*., 2010; Otieno *et al*., 2010). Some African honeybee populations carrying pathogens have been shown to be healthy and do not indicate widespread mortality in Kenya, South Africa, Benin and Uganda (Himberg *et al*., 2009). Honeybees' behavioural traits are as a result of genetic and environmental control. Africa's honeybees tend to have behavioural and genetic characteristics that are different from the European or American bees. For instance, African bees are more aggressive than the docile European honeybees which make them more productive and resilient. Due to these and other attributes, there is a need to protect African honeybees because of their ecological importance (Raina *et al*., 2011; Sinclair *et al*., 2011). African scientists are interested in discovering valuable traits in the African honeybee that other world honeybee populations lack and how those traits could be selected for to sustain their role as pollinators and hive producers (Frazier *et al*, 2010). Once these mechanisms have been discovered, selective breeding of honeybees to bring out those

physiological and behavioural traits could help groups that are currently susceptible to infestation and disease, poor pollinators and low hive producers stay healthy and continue to breed.

Sub-Saharan Africa is yet to breed commercial queens for honey productivity or pollinator colonies (Raina *et al*., 2009). For example, in Kenya, large horticultural and coffee firms who practice beekeeping within their farms, do so without considering whether honeybees are effective pollinators of such crops or not (Kasina *et al*., 2009). There is need to consider selective breeding of African honeybees to augment conservation and increase hive productivity. African honeybees tend to have a high foraging efficiency compared to the European worker bees. African colonies have a greater emphasis on pollen collection, have a more rapid conversion of pollen into brood (Page *et al*., 2000), and devote two to four times as much comb area to brood rearing as compared to European colonies (Fewell and Bertram, 2002). Previously, it has been demonstrated that strains of bees selected for pollen-hoarding, a trait that is enhanced at the expense of nectar foraging (Page and Fondrk, 1995), are better pollinators of cranberries (Cane and Schiffhauer, 2003). This is an important attribute as the bees can be bred specially for pollination of cultivated crops. The amount of pollen a colony collects and stores in its brood nest is a colony-level behavioural phenotype that can be readily quantified. The economically important traits responsible for honey and pollen production in bees are only measurable at the colony level and are greatly influenced by the environment of the hive, both internal and external (Souza *et al*., 2002). Therefore beekeepers can recognize high and low pollen-hoarding colonies and these strains can be selected by intermittent selection on the colony-level phenotype (Page and Fondrk, 1995).

Many economically valuable products such as honey, royal jelly, propolis and pollen are influenced by polygenic traits which can be measured at the colony level (Breed *et al*., 2004; Schneider *et al*., 2004; Oldroyd and Thompson 2007). Bees carry pollen, nectar, or both, as well as propolis (plant resin) or water, back to the hive (Winston 1987). Two major QTLs (*pln*1 and *pln*2) explain 59% of the variation in quantities of pollen stored by honeybee colonies (Lobo *et al*., 2003). These pollen hoarding QTLs influence response thresholds to sucrose of individual bees, confirming that allelic variation influences the behaviour of individual bees in their society (Page Jr. *et al*., 2002). Two QTLs associated with stinging behaviour (*sting*1, *sting*2) (Hunt *et al*., 1998; Lobo *et al*., 2003) have been mapped and are known to influence bee stinging response. Honeybees exhibit defensive behaviour near the nest but highly defensive bees may pursue a prey for considerable distances away from the nest (Hunt *et al* 1998; Guzman-Novoa *et al*., 2003; 2005; Hunt, 2007). This behaviour is not thoroughly characterized in terms of correlated physiological and sensory traits (Hunt *et al*., 1998; Hunt *et al*., 2003; Hunt, 2007). The study of QTLs has a high potential of revealing the genetic architecture of complex traits and propose candidate genes for further study (Phillips, 1999; Page *et al*., 2000; Hunt *et al*., 1998; 1999; 2003).

This study focused on the microsatellite loci linked to the QTLs for the African honeybee populations in Kenya showing dissection in foraging (*pln*1 and *pln*2) and stinging behaviours (*sting*1, *sting*2) in an effort to identify candidate genes responsible for these traits. The candidate genes may assist in marker selection of a honeybee line with specialized hive productivity or better pollinators of crops.

2. MATERIALS AND METHODS

Evaluation of foraging and stinging behaviour:

A total of 47 colonies representing 17 populations and from Central, Eastern, Western and Coastal Kenya were evaluated for stinging and foraging activities. The colony status was assessed for the total number of honeybees, number of occupied frames, sealed brood (s), open brood (o), honey (n) and pollen (p) loads for pollen and nectar foraging behaviour and the observations were recorded. Honeybees colonies were named as either N-nectar foraging, or P-pollen foraging depending on the abundance of pollen or nectar loads. Stinging behaviour was evaluated following the procedure of Collins *et al*. (1984). The characters measured were: the time to respond to the alarm pheromone (isopentyl acetate) (T1S) and the number of stings (SN). An interval of at least 3 days between successive stinging experiments was allowed on the colony to avoid an increase in the number of guard bees. The experiment was replicated 3 times. The number of stings observed and time at first sting were then averaged for each colony. Honeybee colonies were named as either A-aggressive, or M-mild depending on the time of response and the number of stings in each colony. Global positioning system (GPS) coordinates were taken for each sampling location as shown in Table 1.

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Table 1: Geographical Positioning System (GPS) points showing the regions in Kenya from where honeybee samples were collected

Site	Site Name	Region	Altitude	Coordinates	Elevation
1	ICIPE	Central Kenya	High	–1°13.452, 36°53.840	1609m
2	Loresho	Central Kenya	High	–1°01.727, 36°55.553	1638m
3	Kikuyu	Central Kenya	High	–1°14.203, 36°41.275	1918m
4	Thika	Central Kenya	High	–0°59.867, 37°04.700	1715m
5	Dabaso	Coast Kenya	Low	–3°20.357, 39°59.248	5m
6	Chambuko	Coast Kenya	Low	–3°22.327, 39°47.162	142m
7	Hewani	Coast Kenya	Low	–2°14.190, 40°10.687	13m
8	Mituki, Taita Hills	Coast Kenya	Mid	–3°25.467, 38°20.550	1522m
9	Ronge, Taita Hills	Coast Kenya	Mid	–3°21.008, 38°25.023	1284m
10	Chawiya, Taita Hills	Coast Kenya	Mid	–3°28.740, 38°20.443	1494m
11	Bidii	Eastern Kenya	Mid	–0°45.649, 38°09.372	1021m
12	Kasanga	Eastern Kenya	Mid	–0°46.488, 38°08.955	929m
13	Kathiani	Eastern Kenya	Mid	–0°36.453, 38°01.430	1009m
14	Mumoni	Eastern Kenya	Mid	–0°32.948, 38°00.240	1051m
15	Mathiakani	Eastern Kenya	Low	–2°15.000, 38°22.000	573m
16	Isiekuti	Western Kenya	Mid	0° 14.936, 34° 53.488	1596m
17	Makuchi	Western Kenya	High	0°04.000, 34°46.000	1696m

Collection of honeybee specimens:

Initially the hives were smoked slightly using a smoker to mesmerize the bees. Adult worker honeybees were then collected by allowing individual bees from the hive entrance to enter into labelled 28 ml universal Bijou bottles placed upside down. The bottles containing bee samples were then instantly added ~15 ml of 95% ethanol to kill and preserve them. They were then transported to the laboratory for storage at 4°C before DNA isolation.

Total DNA extraction:

Total DNA were isolated from the entire bee without a head by a deproteinization method of proteinase K digestion using DNeasy Blood and Tissue kit (Qiagen). Initially, individual worker bees were sterilized in 5-10% bleach for 10 minutes to remove impurities and contaminants. They were then rinsed in sterilized double distilled water and dried at room temperature for one hour. These were then ground individually by hand using a polypropylene pestle in a 1.5 ml eppendorf (microcentrifuge) tube containing ATL lyses buffer (Qiagen). Ground samples were digested in proteinase K by incubating at 65^{o}C for between 30 minutes to 1 hour. The rest of the protocol followed Qiagen DNeasy® Blood and Tissue handbook protocol for tissue extraction. The extracted DNA was suspended in 100 µl of AE elution buffer from the kit.

Microsatellite markers for QTL analysis:

Twelve of sixteen microsatellite markers were selected by mapping them to honeybee QTLs for stinging and foraging behaviour. Four markers were not attached to any of the QTLs. Six QTLs namely *sting*-1, *sting*-2, *sting*-3, *pln*-1, *pln*-2 and *pln*-3 with high LOD scores were considered based on Hunt *et al*., (2007). Sting-1 had the highest LOD score for colony stinging response and was also the only QTL associated with initiation of stinging at the individual-bee level. The *sting*-2 region contains two candidates for modulation of response to moving visual targets and alarm pheromone which are the primary stimuli that elicit stinging behaviour. *Sting*-3 like *sting*-2, has genes with the potential to modulate sensitivity to visual and olfactory stimuli. *Pln*-1, *pln*-2 and *pln*-3 are QTLs based on colony pollen storage. For each QTL, and based on available resources, two polymorphic microsatellite loci from the microsatellite database were selected by scanning the genome sequences linked to QTLs for repeat sequences using web-based tandem repeat finder and comparing with isolated markers by Solignac *et al*. (2003). Overall twelve microsatellite markers associated with QTL for stinging and foraging were selected. The primers were fluorescently labelled as Ned (yellow), Vic (green), 6-fam (blue) and Pet (red) according to the size range of the expected amplified fragments. The marker information and PCR conditions are shown in Table 2.

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Microsatellite amplification:

The PCR amplification for each sample was performed in a total reaction volume of 10 µl consisting of 1 × PCR buffer (10 × Buffer with $(NH_4)_2SO_4$, MBI Fermentas®), 1.0 µl template DNA solution, 200 µmol each dNTP, 0.8 µmol of each forward and reverse primer, 0.4 Units of Taq DNA polymerase (MBI Fermentas®). Magnesium chloride salt concentrations differed for each primer reaction. Touchdown PCR reaction at an initial denaturation at 95°C for 5 minutes, then 5 cycles at 94°C for 30 seconds, 30 seconds at 5°C above the primer specific annealing temperature and decreasing by 1°C per cycle and 72°C for 1 minute, then 30 cycles of denaturation at 95°C for 30 seconds, primer specific annealing temperature for 30 seconds and extension temperature of 72°C for 1 minute. The last cycle was followed by a 10 minutes extension step at 72°C. Two microliters were electrophoresed on a 2% agarose gel containing Ethidium bromide and prepared in 1 × TAE buffer to confirm the presence, size, intensity and the quality of the amplicons. The bands were visualized under an ultraviolet transilluminator (KODAK® Gel Logic 200). The remaining PCR products were kept at -20°C before genotyping. An individual was declared null (not amplified at a locus) only after two or more amplification failures.

Table 2: Primer sequence information for microsatellite markers (Solignac *et al*, 2003)

Marker	Region	Repeat motif	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')	QTL	Size range (bp)	Ta (°C)	$MgCl_2$ (mM)
AJ509390	LG1	$(CT)_{11}$	Ned-CCATTCTTCCTCGATAACACG	AGGGCGTCAGGAAGGAAG	Pln-3	240-280	55	1.2
AJ509721	LG2	$(CT)_{11}$	6-Fam-GAATATGCCGCTGCCACC	TTTCGTTGCATCCGAGCG	Sting-3	150-190	55	1.2
AJ509362	LG3	$(AG)_{24}$	6-Fam-GCGAAAATTGCCGGGTTATA	TGCAACTTTATCGTTTCGACGT	Sting-2	140-170	50	1.5
AJ509384	LG7	$(CT)_{15}$	6-Fam-AAGGGTATCGCGGCGTAG	TTCGGTTTGACGAATGCG	Sting-1	270-300	50	1.5
AJ509439	LG7	$(CT)_{10}$	Vic-ACGGAGGGAAAATGGAGAG	GTTCGGTCACGTTAAACGG	Sting-1	110-140	50	1.5
AJ509486	LG3	$(CT)_7$	Pet-ATATCCCGGTGGCCACGT	TGTCGCCACGCATAACTCG	Sting-2	110-140	55	1.2
AJ509655	LG2	$(GGA)_8A(GGA)_2$	Vic-CGCCGATCTGGATGGAAC	CTTGCCAAGTTCACTGCACTG	Sting-3	160-180	50	1.5
AJ509311	LG2	$(AG)_6AT(AG)_3$	6-Fam-GTACACCAGAAGCGTCCCA	ACAGACTTGGGAGCATCGA	Pln-1	100-130	60	1.2
AJ509387	LG2	$(CA)_{14}$	Vic-CGCAACGCTTACTTACGG	AGCTCGAATCCAATTCGC	Pln-1	200-220	55	1.5
AJ509441	LG1	$(GA)_{14}$	Ned-GCTGCGGCCAACGCTAAC	GACACGGCTCGCGACCA	Pln-2	130-150	55	1.5
AJ509729	LG1	TC-rich	Ned-GTCGGACGGTGTTCGGTC	AGAACAGGCGGAACGTGC	Pln-2	170-200	55	1.5
AJ509307	LG1	$(GT)_5(GA)_5AA(GA)_6$	Pet-CGGAAGCGTAAATAGAGAAG	AAATGGAAAGTAGATGTGCG	Pln-3	130-150	56	1.2
AJ509690	LG13	$(AG)_{10}$	Vic-TGAACGTAAACAGAAATACGCG	GACATTGTGTGGGAGCGTG	None	135-160	50	1.5
AJ509635	LG16	$(GAA)_{12}...(GAA)_5$	Ned-GATCGTGGAAACCGCGAC	CACGGCCTCGTAACGGTC	None	140-170	55	1.5
AJ509381	LG12	$(GA)_9$	Pet-GATTAGAGGCAGGAATTCGCA	CGCGAAACGGCTTACATTC	None	150-180	50	1.2
AJ509656	LG9	$(CATA)_7$	Pet-CCGGTCTCTCGATATTTTTATC	AGCAATTGGCATCGATACAC	None	190-220	50	1.5

QTL-Quantitative Trait Locus; Ta-Annealing Temperature

Microsatellite genotyping:

The standard/formamide/PCR products mixture was separated on a DNA automated sequencer (ABI PRISM® 3730*xl* DNA analyzer, Applied Biosystems). Reliable genotyping or sizing was considered under the 3rd order Least Squares Method which uses regression analysis to build a best-fit cubic function curve from the internal-lane size standards used for size calling. The data points of the unknown fragments were compared to the size calling curve. The end result was an electropherogram with a series of peaks that represented different alleles according to the size, peak height and peak area detected by the software. The results of genotyping data were collected by GeneMapper® software (version 3.7, Applied Biosystems) in excel format for statistical analysis.

Association between variables:

Statistics of association within and between quantitative and qualitative variables was characterized using Torocor software version 1.0 (Hardy, 2009) which also performs tests of spatial autocorrelation using randomization. The four quantitative variables that were analysed are as follows: average sting number (AvSN), average time at first sting (AvTS), sealed-to-open brood (sos) and nectar-pollen ratio (npn). The qualitative variables that were analysed are as follows: A-aggressive, M-mild, N-nectar foraging, and P-pollen foraging. Bilateral tests were used for Pearson's correlation coefficients between quantitative variables, whereas unilateral tests were used for the χ^2 statistics and the intra-class correlation coefficients. Using randomization tests statistically significant values were marked by * ($P<0.05$ and at least 99 randomizations), ** ($P<0.01$ and at least 499 randomizations), or *** ($P<0.001$ and at least 4999 randomizations) and preceded by + or – to indicate whether the observed value was higher or smaller than the mean value after randomization. Tests of spatial autocorrelation were done using complete randomization (5000 replicates) with null hypothesis (Ho) of no spatial autocorrelation between variables.

Association between phenotypic variables:

Basic statistics for quantitative variables nectar occupied combs (npn), sealed combs (sos), Average sting number (AvSN) and average time in seconds for sting (AvTS) were determined by the program "AutocorQ" ver. 2.00. The spatial autocorrelation of each variable were tested using complete randomizations (5000 replicates), whereby the values of a variable are randomly shuffled among all samples. *I*(*d*), as well as the regression slopes *b*lin and *b*log, were recomputed for many randomized data sets to assess their distributions under the null hypothesis (*H*o) that there is no spatial structure. Bilateral tests were used for Moran's I while unilateral tests were employed for regression slopes. All sample pairs were considered to give Moran's *I* in three classes of 2, 4 and 8 km distances. A matrix of Pearson's correlation coefficients between quantitative variables was given showing variables that are positively correlated. Chi-square (χ^2) coefficient between variables showed statistically significant correlated variables.

Marker-Trait Association:

Microsatellite Analyzer (MSA) software (Dieringer and Schlötterer, 2003) determined the allele number, allele frequency, gene diversity, polymorphism information content (PIC) and gene frequency (Breseghello and Sorrells, 2006). Trait Analysis by aSSociation, Evolution and Linkage (TASSEL) program, version 2.1 (Bradbury *et al*., 2007) was used to analyze the marker properties, Linkage Disequilibrium (LD), principal component (PC) matrix, hierarchical clustering, and Q+K mixed model. Association between markers and traits was analyzed using a general linear model (GLM) and a mixed linear model (MLM) method in TASSEL. TASSEL is an open-source software package that uses genetic markers to evaluate associations with traits, identify evolutionary patterns, and analyze linkage disequilibrium. TASSEL requires three types of data primarily for the analysis (i) marker segregation data (ii) phenotype data and (iii) Ancestry coefficient data (Q matrix). In order to identify if there was any structure/grouping in the population for association mapping analysis, a Bayesian phylogenetic method using STRUCTURE software (Pritchard *et al*., 2000) was used. This software was used to obtain an optimum population structure by determining a value for K and an estimate inferred ancestry (Q matrix) of individuals suitable for TASSEL analysis. For each K, ten runs were performed separately, 100,000 iterations and a burn-in period of 100,000 were carried out for each run. A value of *K* (number of clusters) was selected when the estimate of InPr(X|K) peaked in the range of 1 to 10 sub-populations. To reduce an elevated false-positive rate (spurious associations), the relative kinship matrix (K matrix) was estimated by SPAGEDi software (Hardy and Vekemans, 2002) which uses markers to develop an estimate of the identity by descent (IBD) relationship matrix. Kinship or relatedness is estimated by the "coefficient of relatedness", which is defined as the probability that the alleles of a locus chosen at

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random from two individuals are identical by descent. Kinship coefficients were used in association analysis (MLM) to refine the results. The significant marker-phenotype associations were declared by P≤0.001 and the magnitude of the QTL effects were evaluated by R^2-marker. P-values larger than 1e-3 or 0.001 were not considered.

Candidate genes search:

Based on association mapping results, the candidate genes were determined. To identify putative genes that flank microsatellites, identified microsatellites were mapped on the *Apis mellifera* genome at www.beebase.org using the blastn tool. For each microsatellite, a genome browser on the website was used to determine upstream and downstream genes. Further, the putative names of the resulting genes were identified by a search on the non redundant protein database of National Centre for Biotechnology Information (NCBI) using blastp. Gene ontology was done using Blast2GO® (Cornesa *et al*., 2005) programme.

3. RESULTS

Basic statistics on phenotypic variables:

The mean, standard deviation and the range (minimum and maximum values) for the four quantitative variables are shown in Table 3 below. Approximately 69% of the honeybee combs were occupied by the nectar while 31% were occupied by pollen stocks for all the colonies studied. The sealed combs were only 43% compared to the 57% opened combs for all the colonies suggesting that honeybees leave combs open in readiness for the storage of honey. The average time in seconds for sting (AvTS) was 11.87 seconds for the populations studied showing that the honeybee colonies responded relatively fast. The average sting numbers (AvSN) recorded were 21.46 stings within the first one minute of the experiment. Overall the standard deviation indicates that for the quantitative variables the values were widespread within their ranges. Honeybees were either aggressive (A) or mild (M) depending on the number of stings and response time of the first sting. The frequency of the aggressive state (A) was 0.5532 while for the mild bees (M) was 0.4468. Hence relatively more colonies were aggressive compared to mild colonies. Honeybees were either nectar foraging (N) or pollen foraging (P) depending on the nectar or pollen comb numbers recorded. The frequency of the nectar foraging was 0.7447 while that of pollen foraging was 0.2553. This means that on average colonies foraged for and stored more honey than pollen in the hives.

Table 3: Basic statistics for quantitative and qualitative variables

		Mean	Standard deviation	Min value	Max value
Quantitative	npn	0.69	0.24	0.05	1.00
	sos	0.43	0.19	0.00	0.82
	AvTS	11.87	10.89	2.00	37.00
	AvSN	21.46	10.82	6.00	40.30
Qualitative	Aggressive	0.5532			
	Mild	0.4468			
	Nectar foragers	0.7447			
	Pollen foragers	0.2553			

Correlation between variables:

There was a correlation between sealed brood and the nectar foraging variables (sos vs npn at -0.03051) as shown in a matrix of Pearson's correlation coefficients between quantitative variables in Table 4 below. A correlation was also observed between sealed brood and the average time of the first sting (sos vs AvTS at 0.14329) quantitative variables. Average time of the first sting (AvTS) and average sting numbers (AvSN) also showed correlations with nectar foraging variable (npn). The Pearson correlation coefficient between average time of the first sting (AvTS) and average sting numbers (AvSN) (0.817063***) was statistically significant at P<=0.001. This shows that the stinging response corresponds to a high number of stings. The Chi-square (χ^2) coefficient between *sting* and *pln*, qualitative variables (from contingency tables) was 0.184458 showing that they are positively correlated although this was not statistically significant. This means that stinging response correlates with amount of nectar or pollen in the colony.

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Table 4: Matrix of Pearson's correlation coefficients between quantitative variables

	npn	sos	AvTS	AvSN	sting
npn	0.00000				----
sos	0.03051	0.00000			----
AvTS	0.115781	0.14329	0.00000		----
AvSN	0.035495	0.113362	0.817063***	0.00000	----
pln	----	----	----	----	0.184458

Codes: * for P<=0.001 and >=4999 replicates; ** for P<=0.01 and >=499 replicates; * for P<=0.05 and >=99 replicates (bilateral tests)**

Marker polymorphism:

A total of 16 SSR markers across 235 honeybee samples from 47 colonies were used for population structure assessment and association analysis. The number of alleles per locus ranged from 6 to 32 with an average of 14.63. The average polymorphic information content (PIC) value was 0.72 and ranged from 0.21-0.87 as shown in Table 5 below.

Table 5: Polymorphic statistical information of markers

Item	Information for all markers
Number of marker loci	16
Sample size	235
Number of alleles	235
Variation of allele number	6-31
Average number of alleles	14.63
Unbiased heterozygosity	0.74
Variation in PIC	0.21-0.87
Average PIC	0.72

Population structure analysis:

Subpopulations were confirmed by Evanno plot of deltaK against K which gave a value of K at 8 (Figure 1). Similarly a plot of mean of estimate Likelihood (Ln) probability of data against L(K) showed a maximum value of K at 8 while a simulation summary obtained from structure results alson gave K=8

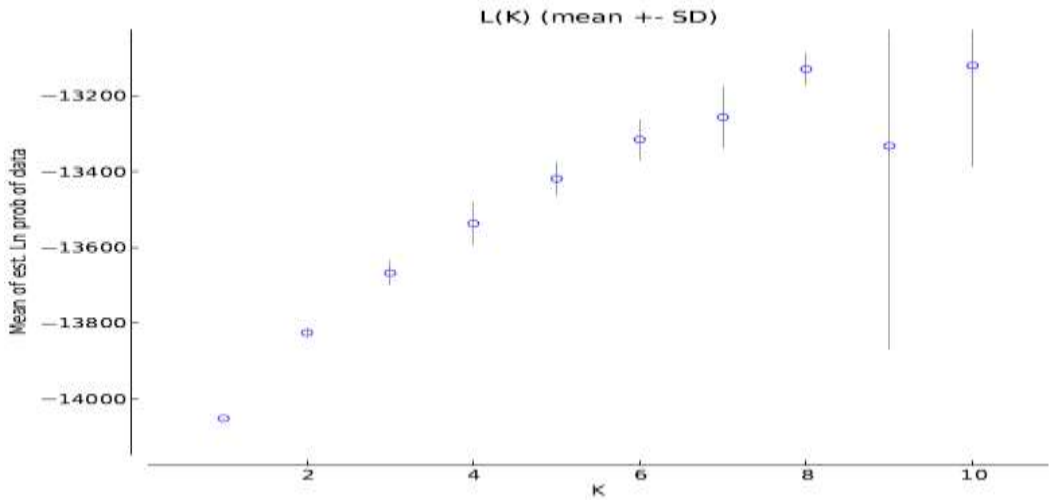

Figure 1: A plot of mean of estimate Ln probability of data against L(K) to determine K clusters

Linkage disequilibrium:

The critical values, r^2, D' and P-values were obtained by analysis of 16 SSR markers. At the highly significant threshold of $r^2 \geq 0.1$, none of SSR marker pairs remained in perfect LD. The pairs of loci with significant P-values less than 0.001 (P<0.001) were 6.7% representing 8 out of 120 pairwise comparisons (green and red boxes in Figure 2). These were as

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follows; AJ509635 vs AJ509439, AJ509655 vs AJ509656, AJ509390 vs AJ509655, AJ509690 vs AJ509387, AJ509390 vs AJ509384, AJ509390 vs AJ509635, AJ509690 vs AJ509655 and AJ509486 vs AJ509721. Figure 2 shows a graphical representation of the linkage disequilibrium statistics and the P-values showing the levels of significance. None of the pairs of loci shows complete linkage disequilibrium with D'=1. D' values ranged from 0 to 0.90 while r^2 values for all pairs of loci were below 0.01. Therefore there was no significant linkage disequilibrium between the markers studied.

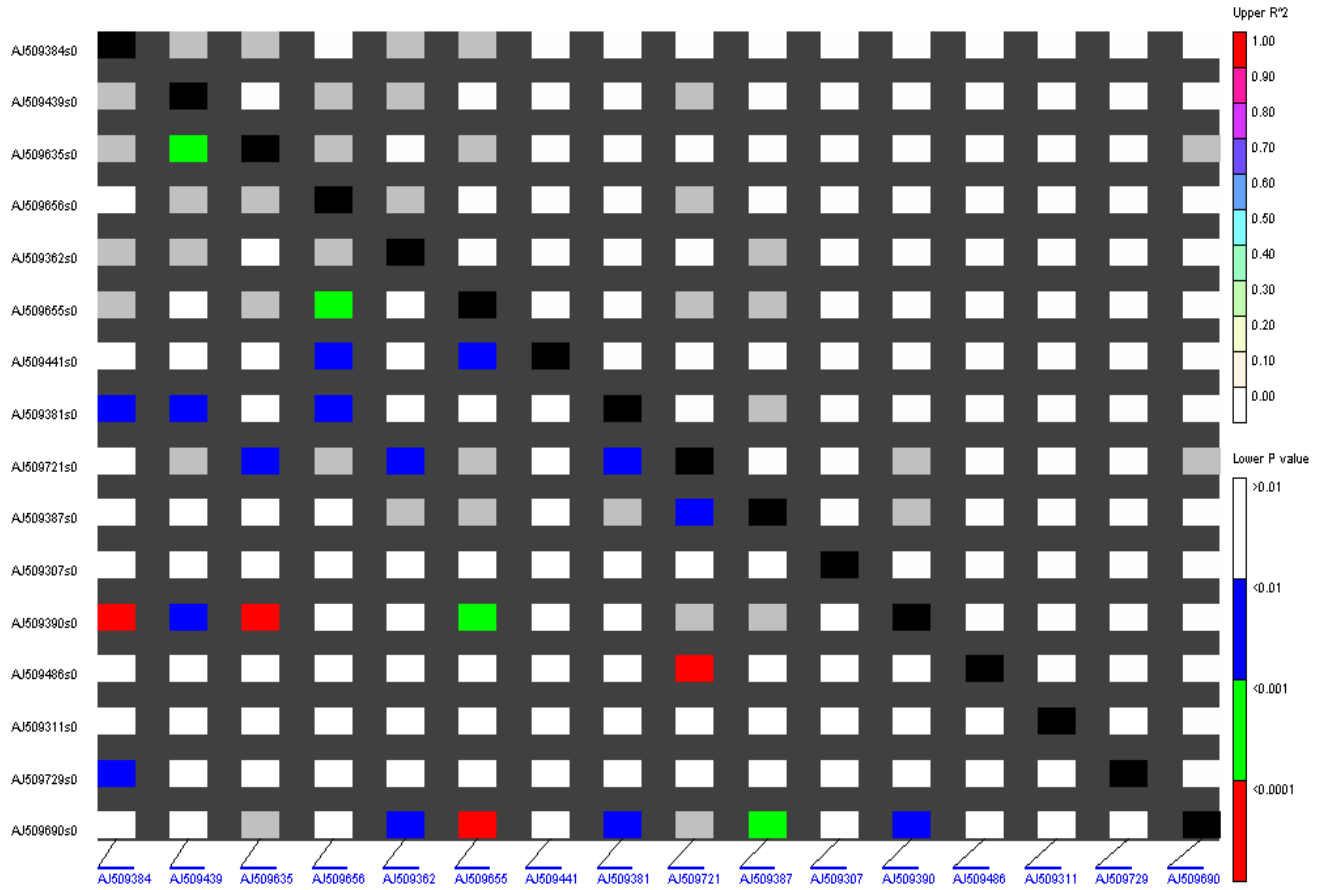

Figure 2: Graphic representation of linkage disequilibrium statistics

Identification of marker-phenotype association:

The results of General Linear Model using TASSEL ver 2.1 programme are presented in Table 6. The table displays the F-statistics and p-values for the F-tests. In addition it contains information about degrees of freedom, the error mean square for the model, R-square of the model, and R-square for the marker. The model R-square is the portion of total variation explained by the full model. The marker R-square is the portion of total variation explained by the marker but not by the other terms in the model. The #perm_Marker is the number of permutations run, pperm_Marker is a test of individual markers, and p-adj_Marker is the marker p-value adjusted for multiple tests. The p-adj_Marker value is a permutation test derived using a step-down MinP procedure and controls the family-wise error rate (FWER). For example, if only markers with p-adj values of 0.05 or less are accepted as significant, then the probability of rejecting a single true null hypothesis across the entire set of hypothesis is held to 0.05 or less. This test takes dependence between hypothesis into account and does not assume that hypotheses are independent as do other multiple test correction procedures. Out of 16 markers tested four markers showed a significant correlation with the phenotypes and are associated with their respective phenotypes. Marker AJ509381 (LG12) was found to be strongly associated with three of the four phenotypes namely: the Average Sting Number (AvSN), Average Time of the first Sting (AvTS) and sealed brood (sos). Marker AJ509307 was significantly associated with Average Sting Number (AvSN). Finally markers AJ509384 and AJ509721 were significantly associated with nectar foraging (npn) phenotype. Initially, selection of AJ509384 (LG7) was associated with sting-1 QTL, AJ509721 (LG2, position 195.8cM) was associated with sting-3 QTL, AJ509307 (LG1, Grp 1.24) was associated with pln-3 QTL and AJ509381 (LG12) was not associated with any of the QTLs but was found to have a correlation with all of the phenotypes.

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Table 6: GLM result file from TASSEL v 2.1

Trait	Locus	df	F	p_value	#perm	p-perm	p-adj	df_Model	df_Error	MS_Error	Rsq_Model	Rsq
AvSN	**AJ509381**	2 9	2.44 41	1.62e-04	5000	6.00e-04	2.00e-04	36	198	0.0426	0.3648	0.2274
AvSN	**AJ509307**	1 6	2.76 19	4.81e-04	5000	8.00e-04	0.0022	23	211	0.0449	0.2868	0.1494
npn	**AJ509384**	6 1	1.93 69	5.05e-04	5000	4.00e-04	0.0014	68	166	0.0254	0.4743	0.3742
npn	**AJ509721**	6 6	1.85 24	9.19e-04	5000	1.00e-03	0.005	73	161	0.0255	0.4885	0.3884
sos	**AJ509381**	2 9	2.51 43	1.00e-04	5000	2.00e-04	2.00e-04	36	198	87.2428	0.3672	0.233
AvTS	**AJ509381**	2 9	3.94 78	3.94e-09	5000	2.00e-04	2.00e-04	36	198	75.5281	0.4451	0.3208

The results of Mixed Linear Model (MLM) analysis are shown on Table 7 below. Markers AJ509307, AJ509384 and AJ509721 identified in GLM analysis were found not to be associated with any of the four phenotypes when kinship data from SPAGeDi was included in the analysis showing that only marker AJ509381 had a significant correlation with all the four phenotypes observed in this experiment at a p-value less than 0.05. However this marker was strongly associated with the Average time of the first Sting (AvTS).

Table 7: Mixed Linear Model (MLM) result file

Trait	Locus	df_Marker	F_Marker	p_Marker	lnLikelihood
AvSN	**AJ509381**	27	1.8208	0.0111	39.2977
npn	**AJ509381**	27	1.8921	0.0074	85.3007
sos	**AJ509381**	27	1.5547	0.0473	-7.01e+02
AvTS	**AJ509381**	27	2.7257	3.81e-05	-6.82e+02

Candidate gene identification:

The results of marker effects on MLM analysis show four microsatellites namely AJ509381, AJ509384, AJ509721 and AJ509307 that were mapped on the *Apis mellifera* genome at www.beebase.org using the blastn tool.

Table 8: Upstream and downstream candidate genes identified

Marker	Upstream	Downstream
AJ509381	GB55730: CUGBP Elav-like family member 4-like isoform X1	GB55732: Protein coding
AJ509384	GB44258: aryl hydrocarbon receptor nuclear translocator homolog isoform X5	GB44259: aryl hydrocarbon receptor nuclear translocator homolog isoform X4
AJ509721	GB46589: SAGA-associated factor 29 homolog	GB46588: amino acid ABC transporter substrate-binding protein
AJ509307	GB48999: helix-loop-helix protein 11	GB49000: TBC1 domain family member 13-like

GB46589: The beebase identifies saga-associated factor 29 homolog as GB46589 with a gene identification symbol LOC408702 found in *Apis mellifera*. This is a protein coding type gene also known as GB18746 and located in the linkage group 2 (LG2). A sequence length of 293 amino acids and approximately 20 hits were observed. The predicted associated function is similar to that of humans.

GB55730: The beebase identifies CUGBP elav-like family member 4-like as GB55730 with a gene identification symbol LOC10353 found in *Apis mellifera* as a protein coding gene also known as GB17926 located in chromosomal linkage group 12 (LG12; NC_007081.3). A sequence length of 404 amino acids and approximately 20 hits were observed. In gene id in NCBI is 410353.

GB46588: The sequence name GB46588 found on bee base did not match any associated gene sequence in the database and therefore no function was associated with it.

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GB55732: The sequence name GB55732 found on bee base did not match any associated gene sequence in the database and therefore no function was associated with it.

GB48999: The beebase identifies GB48999 as the helix-loop-helix protein 11 with transcription factor ap-4. A basic helix-loop-helix (bHLH) is a protein structural motif that characterizes a family of transcription factors. The motif is characterized by two α-helices connected by a loop. In general, transcription factors including this domain are dimeric each with one helix containing basic amino acid residues that facilitate DNA binding. bHLH transcription factors are often important in development or cell activity that include; neurogenesis, myogenesis, hematopoiesis, sex determination and gut development. Approximately 51 bHLH transcription factors have been identified in honeybees. Transcription factor ap-4 of the bHLH in NCBI ID is 726729, Uniprot is H9KBF8 and is found in the chromosomal linkage group 1 (LG1). The sequence is 537 amino acids long and 20 hits were recorded. The alternative gene id is GB14420 with gene identification symbol LOC100679356.

GB49000: The bee base identifies the gene GB49000 as tbc1 domain family member 13-like which is a member of Rab family of proteins. Rab family of proteins is a member of the Ras superfamily of monomeric G proteins. There are approximately 70 different Rabs that have been identified in humans thus far. They are mostly involved in vesicle trafficking, defining the identity and routing of vesicles. Rab GTPases regulate many steps of membrane traffic, including vesicle formation, vesicle movement along actin and tubulin networks and membrane fusion. These processes make up the route through which cell surface proteins are trafficked from the Golgi to the plasma membrane and are recycled. In honeybees the gene identification symbol is LOC408604 which is a protein coding gene type with an alternative gene id as GB13802. The gene is located in the chromosomal linkage group 1 (LG1, NC_00707.3). Rab family of proteins are peripheral membrane proteins, anchored to a membrane via a lipid group covalently linked to an amino acid. Specifically, Rabs are anchored via prenyl groups on two cysteines in the C-terminus.

GB44258 and GB44259: GB44258 and GB44259 in beebase represent an aryl hydrocarbon receptor nuclear translocator homolog (ARNT) which is associated with ahr-1 (aryl hydrocarbon receptor-1) and has a role in cellular differentiation. It is located in the nucleus and functions in DNA binding in the biological processes of transcription and transcription regulation. The isoform has been chosen as the canonical sequence. The gene is required for pharyngeal development and is therefore expressed in many cell types throughout development, including hypodermal cells, intestinal cells, pharyngeal cells, and neurons (Huang *et al*., 2004). Basically, it is expressed in every cell during embryo development. If impaired or unavailable, it leads to abnormal cell morphology in developing neurons and arrested development at larval life stage due to its requirement in the pharynx and aggregation behaviour is diminished in *C. elegans*.

Blast2GO programme identified the functions of the upstream and downstream genes as shown in Table 9 below.

Table 9: Description of gene id and the associated gene ontology

Name	Description	Length (aa)	#Hits	min. eValue	mean Similarity	#GOs	Gene Ontology
GB46589	saga-associated factor 29 homolog	293	20	0	86.45%	0	transcriptional regulation
GB55730	cugbp elav-like family member 4-like isoform x1	404	20	0	97.05%	0	Protein coding
GB48999	transcription factor ap-4	537	20	0	80.95%	14	Defense response
GB49000	tbc1 domain family member 13-like	396	20	0	87.75%	3	Regulation of Rab GTPase activity
GB44258	aryl hydrocarbon receptor nuclear translocator homolog isoform x1	78	14	2.10e-31	86.50%	10	Sequence-specific DNA binding transcription factor activity
GB44259	aryl hydrocarbon receptor nuclear translocator homolog	614	20	0	93.95%	10	

4. DISCUSSION AND CONCLUSION

We have studied the stinging and foraging behaviour in regard to feral African honeybees. The study identified important associations between stinging and foraging phenotypes with the markers genotype and has proposed candidate genes that could play a vital role in these behaviours. Using linkage disequilibrium (LD) we identified a few markers that are coinherited. This small percentage of the overall marker combinations can be explained by the rate of LD decay which is dependent on multiple factors, including the population size, the number of founding chromosomes in the population, and the number of generations for which the population has existed. We expect a fairly constant rate of LD decay associated with honeybees due to their swarming and hybridization behaviour. Different honeybees' subpopulations have different degrees and patterns of LD. The large population sizes found in bees partly explains why honeybees have a small percentage of LD. However, despite a high rate of recombination events, we expect honeybees to have genes that are in linkage disequilibrium though this information was not captured by our analysis. Further development in this area is required.

The average nectar stored in combs (npn) was high at 0.69±0.24 suggesting that on average honeybee colonies foraged for and stored more honey than pollen in the hives when stability is established. Honeybees respond quickly to availability of floral resources and therefore seasonal patterns may be used to explain the nectar storage (Hepburn and Radloff, 1998). In tropical regions, floral resources are available throughout the year, but are not always sufficient to warrant colony growth. Therefore once established, honeybees' colonies respond quickly to the changing environmental conditions rapidly by adjusting the brood size and numbers of sexuals produced and honey produced. The average time in seconds for the first sting (AvTS) was 11.87 seconds for the populations studied showing that the African honeybee colonies respond relatively fast to the aggression. This is also consistent with the average sting numbers (AvSN) recorded at 21.46 stings within the first one minute of the experiment. This means that most African honeybees are aggressive which is consistent with the need to protect the stored honey (food) in the combs and the developing brood. This implies that African honeybees with a high guarding and stinging behaviour have more nectar stored or developing brood in their colonies.

Out of 16 genetic markers tested four markers showed a significant association with the phenotypes. Marker AJ509381 (LG12) was found to be strongly associated with three of the four phenotypes namely; the Average Sting Number (AvSN), Average Time of the first Sting (AvTS) and sealed brood (sos). The gene associated with this marker is CUGBP Elav-like family member 4-like isoform which is characterized as protein coding gene in *Apis mellifera* (Kaplan and Linial, 2006). It is not clear which role this gene plays in honeybee stinging or foraging behaviour. Marker AJ509307 was significantly associated with Average Sting Number (AvSN) which had two genes; tbc1 domain family member 13-like and transcription factor ap-4. TBC1 domain family member 13-like is a member of Rab family of proteins mostly involved in vesicle trafficking, defining the identity and routing of vesicles but no apparent role has been identified in honeybees. Transcription factors are often important in development or cell activity that include; neurogenesis, myogenesis, hematopoiesis, sex determination and gut development. These factors could have an overall effect of age of first foraging in honeybees. Finally markers AJ509384 and AJ509721 were positively associated with nectar foraging (npn) phenotype and have aryl hydrocarbon receptor nuclear translocator homolog isoform and SAGA-associated factor 29 homolog associated with them respectively. Aryl hydrocarbon receptor nuclear translocator has been shown to interact with many other factors and is involved in many important biological processes such as response to hypoxia, embryonic placenta development, positive regulation of endothelial proliferation. We therefore predict that this gene is important not only to foraging but also to the stinging behaviour observed in honeybees. Precise role needs further investigation for a conclusive function. SAGA-associated factor 29 homolog in *Apis mellifera* is predicted to be a component of Ada2/Gcn5/Ada3 transcription activator complex (or SAGA complex) involved in transcriptional regulation through association with methylated histone residue binding in chromatin remodelling. It promotes acetylation, eviction, and methylation of nucleosomes in transcribed coding (Mosesson *et al*., 2014). The role of SAGA complex in honeybees foraging and stinging behaviours is not precisely known. The role of the genes discovered cannot be conclusively get discussed in this paper. Identification of genes and analysis demonstrated above shows that association mapping analysis can help identify the genes that are significantly linked to the traits of interest which may further be investigated for their role in hive productivity or in pollination activities. Although this needs additional work, the genes may further be selected for breeding, conservation and productivity efforts

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