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Abstract Polymerase Chain Reaction (PCR) and subsequent separation of PCR products on polyacrylamide gels to find Single Stranded Conformation Polymorphisms (SSCP) was used for three loci, known from studies in North America to affect defensive behavior (Quantitative Trait Loci (QTL) sting1–3), on samples of Iranian honey bee (*Apis mellifera L.*). In the present study these loci were amplified with specific primers for sting1–3. The analysis of these loci using SSCP, created different patterns among samples. The polymorphisms observed in this study of the Iranian population of honey bees are a first step toward the eventual implementation of a genetic marker-based selection program to reduce defensive behavior. Now, because of this study, the markers are available to conduct more research so that the association of these polymorphic loci and aggressive traits in the Iranian honey bee population can be determined.

Keywords Defensive behavior · honey bee · SSCP · polymorphism

Introduction

Unlike other livestock, the productivity of honey bee colonies does not depend primarily on individual physiological traits, like growth rate, but on social traits, like honey and pollen hoarding (Page et al. 2000) as well as defensiveness. Even a trait like disease resistance is strongly influenced by social factors for example hygienic behavior (Spivak and Rueter 1998), as well as by the individual's innate immune system. Thus, when

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beekeepers seek to genetically improve honey bee stocks, they are primarily interested in improving colony-level behavioral traits, rather than physiological ones of individual workers (Oldroyd and Thompson 2007).

The usual selection criteria for commercial breeding programs are honey production, disposition (i.e. low nest defense), and perhaps disease resistance. Assays used to evaluate colonies for selection are labor intensive and are problematic because of variable environmental factors. Recent advances in the behavioral genetics of bees should soon see the development of genetic markers for various traits including stinging behavior (Lobo et al. 2003). This provides the possibility for enhancing the effectiveness of selection programs via the use of marker-assisted selection. Breeders could graft from selected queens and then determine the markers carried by the resulting virgin queens. This will allow the breeder to choose from among the virgin daughters those that carry the preferred allele at the target loci. Such a program would increase the strength of selection, while reducing the number of colonies evaluated in expensive field evaluations. Marker assisted selection would be particularly useful when screening for new genetic material from outside a closed population (Oldroyd and Thompson 2007).

One of the important traits in this regard is defensive behavour of honey bees. Defensive behavior of honey bee colonies may have implications for the fitness of the colonies, for human and animal health and for honey bee breeding. If the defensive behavior of honey bees is reduced, it is possible that the colonies might become less fit in some environments in which benefits associated with defensiveness for reducing predation outweigh the disruption of colony activities. However, beekeepers can provide primary protection of the hives in order to reduce predation and thus reduce the advantage of defensive behavior. Reduced defensiveness would make it easier to manange bees, there would be fewer stinging incidents of people and livestock so hives could be kept near to human habitations where they would presumably be safer from predation.

Honey bee nest defense is an extremely complex colony-level character. Most honey bees exhibit defensive behavior near the nest, but highly defensive bees may pursue intruders for considerable distances away from the nest. Defensive behavior involves at least two tasks: guarding behavior at the hive entrance and flying out and stinging. Guard bees in the entrance defend the hive from insect invaders including "robbing" bees from other colonies. Guards specialize in exploratory behavior in the nest entrance and learn to recognize the hydrocarbon blends in the cuticles of their nestmates by olfaction, and reject non-nestmates by biting or stinging. Only 10–15 % of workers have been observed to guard the entrance during their lifetime. Also in any colony some (5–10) or many (>100) workers will stand near the entrance and act as guards (Moore et al. 1987; Breed et al. 1992). In general vigorous defense against other insects attempting to invade the hive is of benefit to the colony.

Guard bees respond to mammalian predators by flying from the hive and attempting to sting the intruder. Honey bees responding in this manner, sometimes referred to as soldiers, attack the nose, eyes, ears and mouth of the intruder and will continue to harass the intruder even after stinging. Each honey bee that stings releases a plume of alarm pheromone that attracts more colony defenders as the attack on the intruder escalates. Finally, once the intruder is driven away, bees continue to harass it until it is some distance from the hive. Each of the behaviours exhibited in the defense

response can be quantified (Breed et al. 2004; Oldroyd and Thompson 2007; Moore et al. 1987). Stinging behavior is generally quantified by measuring the time to first sting after the presentation of a stimulus, the number of stings in a target provided near the colony under test, and some measure of the duration of attack after the stimulus is removed (Stort 1974; Collins and Kubasek 1982). One measure of duration involves determining how far bees follow a retreating intruder. Extreme forms of defensive behavior are undesirable in managed hives.

Honey bee colonies vary greatly in their stinging behavior and defensiveness has a strong genetic component (Collins et al. 1984; Breed and Rogers 1991; Guzman-Novoa et al. 1999). To study the genetic basis of the defensive behavior preliminary studies have been done on of the Africanized honey bees. Honey bee queens were imported into Brazil from Africa, hence, the name Africanized honey bees, in 1956 and have since spread throughout most of the Americas. They were detected in Mexico in 1986 and in the United States in 1990. Africanized honeybees appear to have retained their highly defensive behavior and predominantly African genotype (Guzman-Novoa et al. 2002).

High defensiveness is the most noticeable characteristic of Africanized bees (Guzman-Novoa and Page, 1993, 1994a). In Mexico, these bees have caused thousands of stinging incidents that resulted in the deaths of more than 300 people as well as in several thousand animal fatalities (Guzman-Novoa and Page 1994b). Africanized bees react to a visual stimulus 20-times faster than European bees, and deposit eight-times as many stings in experimental targets (Collins et al. 1982). The defensive behavior of Africanized bees is heritable in the broad sense (Collins et al. 1984; Guzman-Novoa and Page 1994a). It has shown that individual differences in guarding behavior, as least partly is under genetic control. Moreover, an increase in the level of African parentage will increase colony defensive behavior (Guzman-Novoa and Page 1993, 1994a).

While honey bees in Iran rarely exhibit extreme defensive responds, there are many stinging incidences and this character makes it hard to work with colonies. Iranian beekeepers are dissatisfied with their honey bees because of their stinging behavior. So having less aggressive bees is an interesting issue in this country.

Known Loci Affected on Honey Bee Defensive Behavior Crosses involving highly defensive African-derived honeybees and low-defensive European races were used to map putative "sting" Quantitative Trait Loci (QTLs) based on colony-level stinging assays at hive entrances (Hunt et al. 1998). Hunt et al. (1998) identified five potential QTLs (designated sting1–sting5) that are apparently involved in the degree of stinging behavior exhibited by colonies. The existence of sting-1, sting-2 and sting-3 was subsequently confirmed in independent crosses (Guzman-Novoa et al. 2002; Arechavaleta-Velasco et al. 2003; Arechavaleta-Velasco and Hunt 2004).

Several studies showed that quantitative trait loci (QTLs) affecting stinging behavior that were mapped as a colony trait (Hunt et al. 1998) influence the expression of guarding behavior of individual bees (Guzman-Novoa et al. 2002; Arechavaleta-Velasco et al. 2003). In one of these studies, it was found that guards were genetically different from other types of bees in the colony, including nurses, foragers, and stingers, based on the allelic frequencies of four sequence tag sites (STSs) linked to stinging behavior QTLs (Arechavaleta-Velasco et al. 2003). It has been reported that further eight loci may also affect this trait (Arechavaleta-Velasco and Hunt 2004).

The QTL that had the largest effect on the phenotypic variance of colony stinging responses, sting-1, was shown to influence individual stinging behavior in two independent studies (Guzman-Novoa et al. 2002; Arechavaleta-Velasco et al. 2003). Sting-1 influences both the degree of colony-level stinging behaviour (time to first sting) and the probability of being a guard. Although results of some studeies suggest that the defensive behaviour in honey bees may be affected by paternal effects (Guzman-Novoa and Page 1993; Guzman-Novoa et al. 2005), but Guzman-Novoa et al. (2002) have reported that the tendency to sting is apparently the dominant allele at this locus. Sting-2 was mapped to the honey-bee linkage group III (Hunt and Page 1995) on the basis of a colony-level behavioral trait, namely, the number of stings per minute. In this segment, the *sts/a11-.31* marker is at the LOD-score peak corresponding to the most likely position of gene/s influencing this behavior and that of the linked markers tested in QTL confirmation studies. In reciprocal backcross families, alleles of *sts/a11-.31* inherited from a defensive parent were found to associate with defensive guarding behavior (Arechavaleta-Velasco et al. 2003).

SSCP is a simple and reliable technique, based on the assumption that changes in the nucleotide sequence of a PCR product affect its single strand conformation. Molecules differing by as little as a single base substitution should have different conformers under non-denaturing conditions and migrate differently. Therefore, those differences can be detected as a shift in the electrophoretic mobility (Hayashi 1991).

For the present study three known loci (sting1–3) were evaluated for the presence of polymorphisms and the possible use of these polymorphisms in a marker-based selection regime is discussed. Furthermore, the variants, polymorphisms, revealed in this study were sequenced in order to attempt to see their underlying genetic differences.

Material and Methods

Sample Collecting

Adult honey bee samples were collected from several locations of Iran (Karaj (n=40), Chalous (20), Golestan (20), Isfahan (40) and southern part of Sistan and Baluchestan (40)).

Workers were collected in 96 % ethanol and held in −20° of centigrade until used. Total DNA was extracted from each worker using CTAB method (Hunt 1997) with minor modification.

The reaction PCR condition for all loci was as below:

Each PCR reaction (25 μl) contained: 1× PCR buffer, 2 μl MgCl2, 2 μl dNTPs,1 μl of each primer, 0.3 μl (5 U/μl) of *Taq* polymerase, 10 mM dNTPs and 2 μl DNA. Materials required for PCR were obtained from Cinnagen Company. A total of 35 cycles were adapted for each basic program. Bioer PCR equipment used in this research was purchased from Techne Company. After proliferation, 5 μl of each PCR product was electrophoresed in a 2 % agarose gel in order to check the result of the reaction. The PCR conditions and primer sequences of each used marker are listed in Table 1 (Arechavaleta-Velasco and Hunt 2004).

For SSCP analysis, 2 μl of the PCR product was mixed with 18 μl of mixed of denaturation solution and loading buffer (95 % formamide deionized, 25 mM EDTA,

Table 1 Primer sequences and PCR condition used for amplification of studeid seqments

Genomic region	Primer sequences	Thermoprofile PCR
sting1 STS A17.080	Forward primer: TGG TGG AAG GTT TGT ATA TTC G Reverse primer: AAGTTT CTT ACC ACG AGC CTG T	Denaturation:94 °C, 30 s Annealing:55 °C, 60 s Extention:72 °C, 120 s
sting2 STS A11.310	Forward primer: ACT TTT GAG GCG AAG AGG AAT AC Reverse primer: CTTGTC CAC GAC GAT TAC TTT TC	Denaturation:94 °C, 30 s Annealing:58 °C, 60 s Extention:72 °C, 120 s
Sting3 STS A64.084	Forward primer: ATC CAG AGG ATT GAT CTC GAT G Reverse primer TGCAAC ATT TGT CTC TGT GAT G	Denaturation:94 °C, 30 s Annealing:56 °C, 60 s Extention:72 °C, 120 s

0.025 % xylene-cyanole and 0.025 % bromophenol blue), denatured for 15 min at 96 °C, rapidly chilled on ice block and loaded onto 12 % polyacrylamide gel (37.5 1). Electrophoresis was performed in 1× TAE buffer. The gels were run in the following conditions: 300 V for 24 (sting1)-30 (sting2 & sting3) hours. Temperature was held at 8 °C during running. The gels were stained with silver nitrate solution. The patterns of DNA was observed and photographed.

Samples from each different SSCP pattern were sequenced by BioNeer Company. Alignment of the sequenced segments was done by BioEdit program (Hall 1999).

Results and Discussion

Alignment of the variants with the reference sequence revealed variations among the samples.

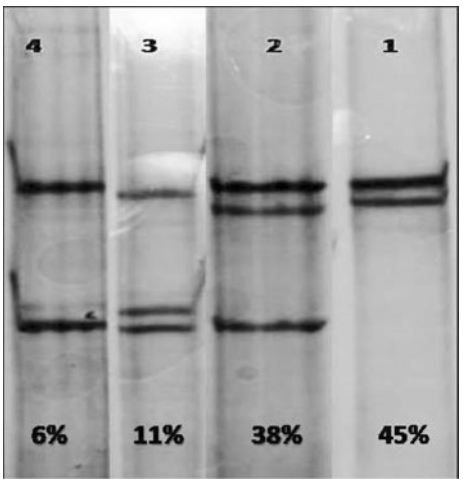

Fig. 1 Result of SSCP analysis of sting1, the patterns are marked with no 1–4

Fig. 2 Multiple Sequence alignment of sting1—Edited. (*H* stands for heterozygote genotype)

Sting-1

Results of sting-1 SSCP analysis are shown in Fig. 1 and the multiple aligning of sequenced segment is shown in Fig. 2. For this segment the reference sequence was NW-003377954.1. For all segments in this study alignment were done with the segments between forward and reverse primers and so numbering is from begin of forward primer. Because the reference sequences were so long that our programs couldn't handle aligning such a long sequences. A total of 4 patterns were observed in these samples and were numbered as **# 1 to # 4.** The results of multiple aligning with

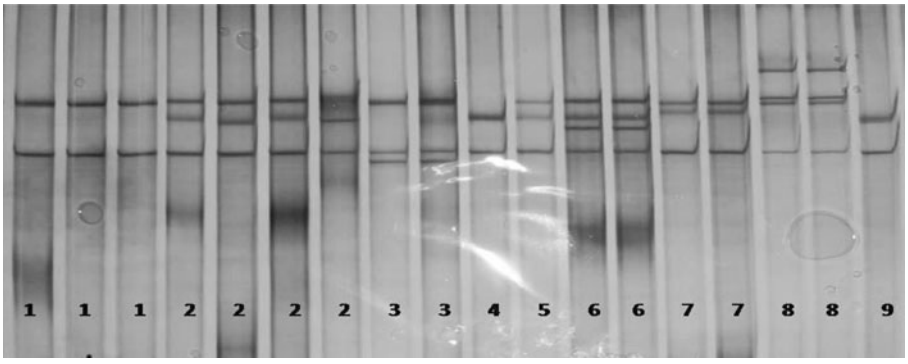

Fig. 3 Result of SSCP analysis of sting2, the patterns are marked with no 1–9

the reference sequence are shown in Fig. 2. The used nucleotide base codes are based on International Union of Pure and Applied Chemistry (IUPAC) codes.

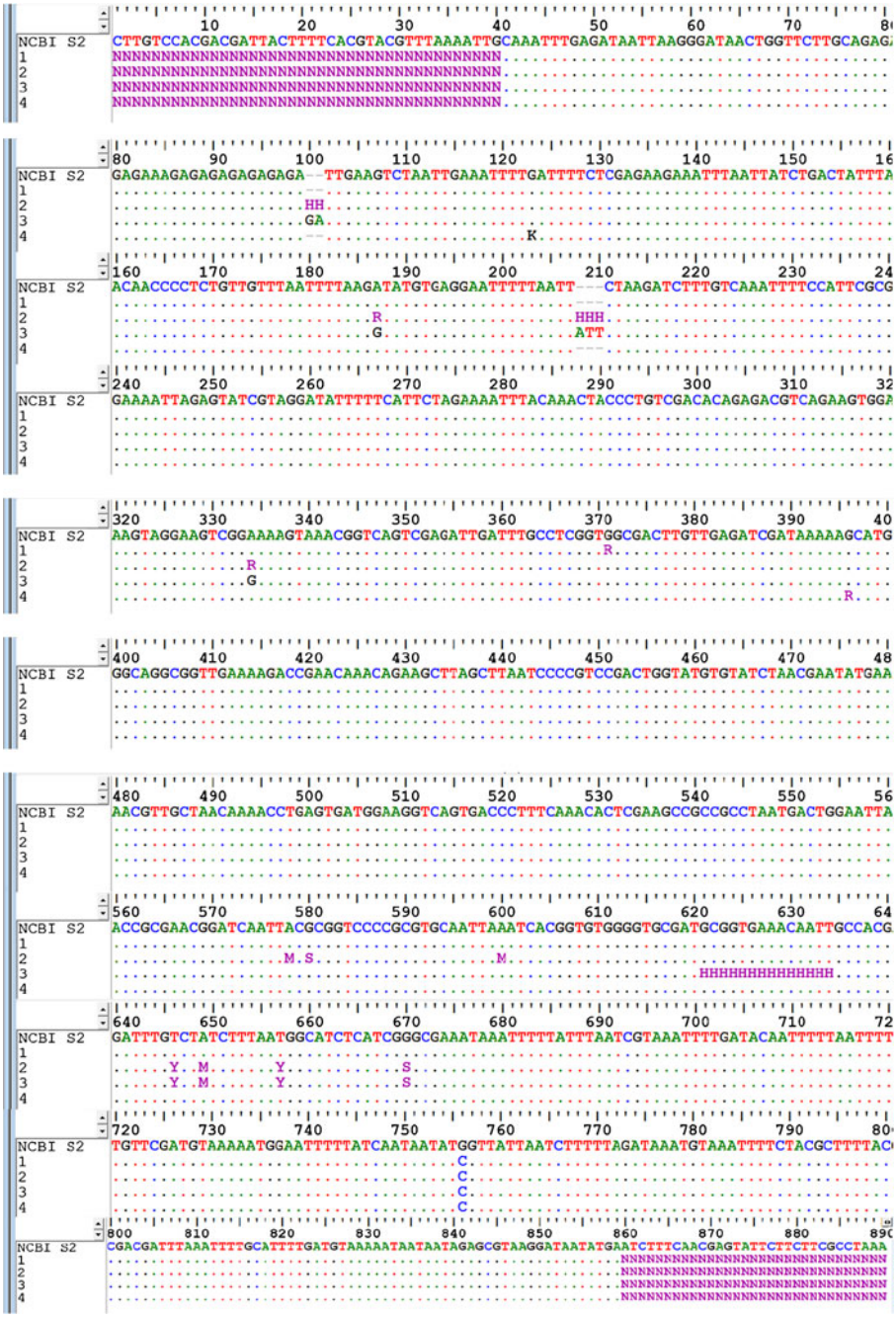

Fig. 4 Multiple Sequence alignment of sting2—Edited. (*H* stands for heterozygote genotype). 621:634 in pattern 3: Deletion of a 14 nucleotide segment in a heterozygous genotype

Sting2

For the sting2 segment a total of 9 different SSCP patterns was observed (Fig. 3), two of them had very low frequency and due to limitation in financial resource we didn't send them for sequencing. Among others, for some of the samples the results of sequencing were such that we couldn't align them to other sequences. Due to the existence of several InDels (Insertion/Deletion) in the segment it was not possible to align them using this procedure. The results of multiple aligning with the reference sequence (NW-003377943.1) are shown in Fig. 4. In this segment also there was some observable insertion/deletion.

Sting3

SSCP analysis of sting3 segments revealed 4 patterns (Fig. 5). Result of multiple aligning with reference sequence (NW-003377928.1) is shown in Fig. 6. Also in Fig. 6 we have presented a segment of detected sequence by the sequencer system. For this segment also there were some nucleotide substitutions relative to reference sequences (Fig. 7).

The Sequences in our samples have relatively large difference with the reference sequences. Also, large differences can be observed among the studied samples. Surely if more samples were examined, more patterns could be observed.

Accumulation of mutations in some areas of sequences was more than other areas. Unfortunately, there was no functional information available about these genomic regions, so we were unable to explain the possible cases of such differences. It is possible that the segments which have many variations belong to introns or from another point of view it could be the result of variations which are functional and may be maintained in the population.

There is also a high degree of heterozygosity. Heterozygote advantage may maintain the diversity in the population which is especially important at the sex determination

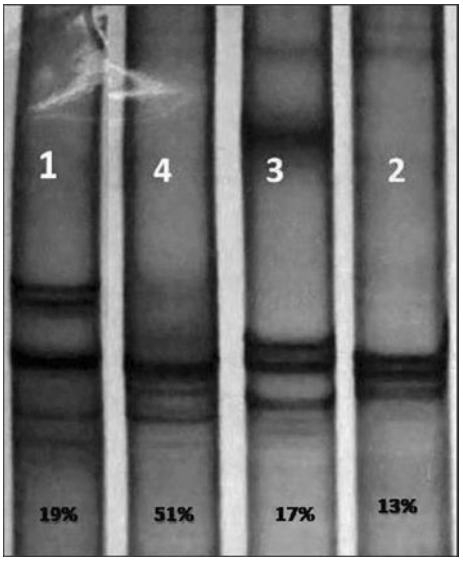

Fig. 5 Result of SSCP analysis of sting3, the patterns are marked with no 1 to 4

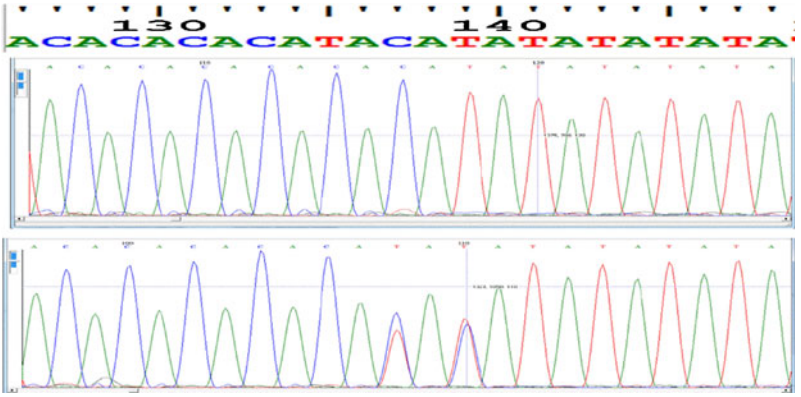

Fig. 6 A segment of direct sequencing of sting3 PCR products for two samples

locus. However, it may be a feature of the honey bee genome. The special mating system of bee colonies also could be important because the unmated queen flies out from the hive and is mated by many males. The genotype of queen has the major influence on the

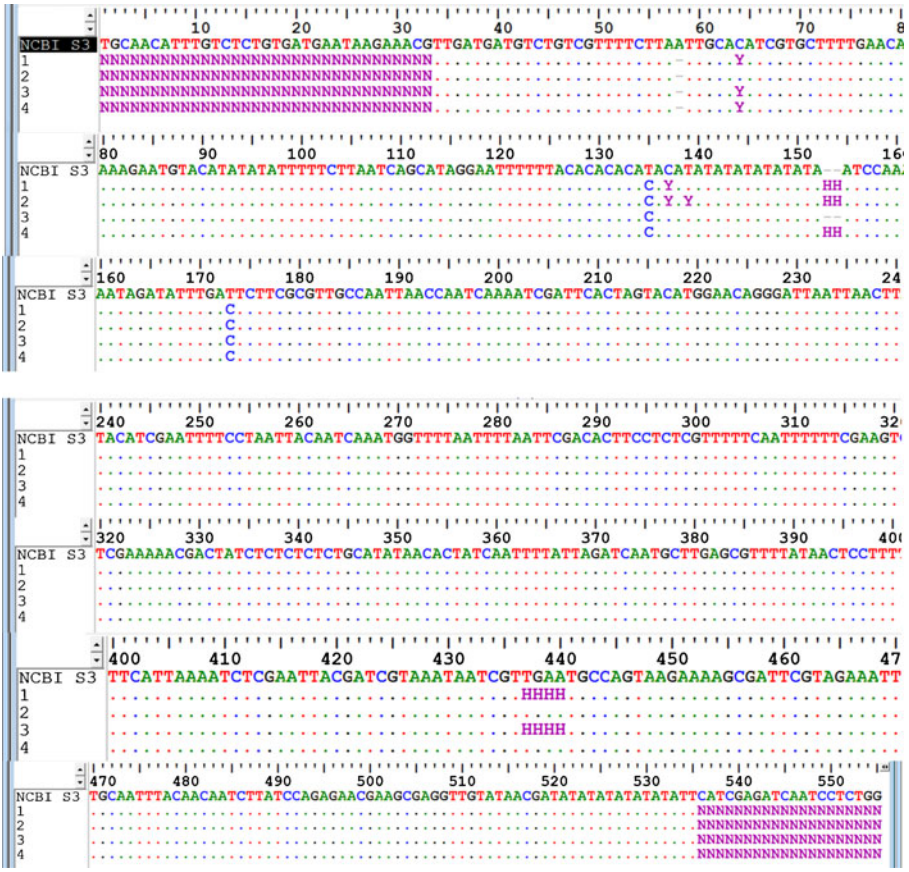

Fig. 7 Multiple Sequence alignment of sting3—Edited. 152:153 in patterns 1,2 and 4: Insertion of TA in heterozygote genotype. 437:440: Deletion of a TGAA segment in patterns 1 and 3 as heterozygous genotype

colony's genotype; the quality of the queen in beekeepers colonies can greatly affect the composition of the population. In the other hand it is believed that drones and virgin queens from the same colony have different behaviors in selecting the mating area they fly. Virgins seem to fly out further than the drones, probably in response to some evolutionary instinct to prevent inbreeding (Connor 2006). This can create also high level of variation in the honey bee genome.

The polymorphisms observed in this study of the Iranian population of honey bees are a first step toward the eventual implementation of a genetic marker-based selection program to reduce defensive behavior. Now, because of this study, the markers are available to conduct more research so that the association of these polymorphic loci and aggressive traits in the Iranian honey bee population can be determined. In general, these results indicate that there is a great variety in the studied genomic regions. This means that it is possible to use of this type of information in breeding programs with the goal of modifying undesirable aggressive behavior.

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