The first record of *Apis mellifera* jemenitica IN EGYPT AS AN EXOTIC RACE

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The western honeybee (*Apis mellifera* L.) is the predominant species within a global population of honeybees, which typically consists of seven to twelve distinct species. Up to 27-31 subspecies of *A. mellifera* are distributed all over the world and differ significantly in their morphology, behavior, biology, and physiology according to the environmental conditions to which they have adapted (Chen *et al*., 2016; Eimanifar *et al*., 2018 and El-Bermawy *et al*., 2012). In the past, the Egyptian bee race (*A. mellifera* lamarckii) was the most widespread in Egypt, but at this time, the Carniolan (*A. mellifera* carnica) and the Italian (*A. mellifera* ligustica) races are the most prevalent (Eid *et al*., 2011). *A. mellifera* jemenitica is a distinct subspecies of the western honeybee, sometimes referred to as the Arabian or Nubian honeybee. It originated in Sudan, south of the Sahara, Somalia, and the southern Arabian Peninsula (Horth *et al*., 2017; Al-Ghamdi and Nuru, 2013 and Alabdali *et al*., 2021). Before this study, no one was able to prove the appearance of this race in Egypt. This study is considered the first discovery of this race, which was easy to discover once it appeared because of its unique and distinctive features, like being smaller, slender, shorter, and pale yellow compared to both native subspecies in Egypt, *A. m. carnica* and *A. m.* ligustica (Khan *et al*., 2017 and Ruttner, 1988).

Several studies have reviewed the identification key of this subspecies in terms of morphological and physiological properties. According to the findings of Al-Ghamdi and Nuru (2013) and Alattal *et al.* (2014) and Ahmed *et al.* (2012), the Saudi Arabian race (*A. m.* jemenitica) is the smallest honeybee species in the Ara-

bian region and is distinguished by its pale-yellow coloration. Other studies have reported that *A. m.* jemenitica is considered a native race to this region due to the unique climatic conditions they are suited to Alqarni *et al*. (2011), Taha and Al-Kahtani (2019) and Al-Ghamdi *et al*. (2021). Although Al-Ghamdi *et al*. (2013) and Alattal *et al*. (2014) considered this subspecies as a wide geographical range subspecies, Al-Ghamdi *et al*. (2020) noted that this subspecies is beginning to spread beyond its known geographic boundaries at a rate that may seem rapid, possibly due not only to beekeepers but also to climate change. In this regard, the current study aimed to prove the first record of *A. m.* jemenitica in Egypt as an exotic race.

MATERIALS AND METHODS

Area of study

In this study, four honeybee samples were collected (Table 1); three of them were obtained from three different commercial apiaries located in Shibin El-Kom, Menofia Governorate, Egypt (Fig. 1a). Another sample (frozen honeybee workers) was obtained by Al-Asal Al-Barri Company (Riyadh, Saudi Arabia) from the Jazan Government, Jazan region, Saudi Arabia (Fig. 1b) as a reference strain.

Honeybee sampling

Bee samples were obtained in February 2023. A total of 60 worker bees per sample was collected, and one sample was collected from three colonies/apiary and 20 worker bees/colony (Table 1). The required studies have been done at the Bee Research Laboratory, Plant Protection Department, Faculty of Agriculture, Benha University, Egypt.

Honeybees are collected live, labeled, and then divided into two parts. The first part was kept in glass jars containing 70% ethyl alcohol and glycerol and kept at room temperature until morphological measurements were performed. In comparison, the second part was kept directly in a deep freezer at -20 ℃ for molecular analysis.

Morphometric measurements

The collected honeybees belonging to the subspecies *A. mellifera* jemenitica were morphologically recognized according to the methodology outlined by Ruttner *et al*. (1978) and Ruttner (1988).

Each honeybee worker was dissected, divided into various parts, and then put on glass slides. To improve the measuring process, a glass cover was used to enclose all components, with the exception of the tergites and sternites, which were positioned on glass rods to ensure their consistent morphology. Subsequently, the slides were subjected to a drying process inside an oven maintained at a temperature range of 40℃ to 45℃.

The morphometric measurements were performed with a stereo binocular microscope equipped with an ocular scale that had been calibrated against a stage micrometer. In the current study, the esti-

mated measures included body size (BODY), proboscis length (PROBL), flagellum length (FLL), tibia length (TIBL), forewing length (FWL), femur length (FEML), metatarsus width (METW), wax mirror width (WMW), cubital index (CI), forewing width (FWW), hind leg length (HLL), metatarsus length (METL), number of hamuli (HN), and length of metasomal terga III and IV (T3L+T4L).

Molecular analysis

In this study, two molecular marker techniques, namely inter-simple sequence repeats (ISSRs) and *mt* 16S-specific PCR, were used to investigate the genetic similarity between bees from Saudi Arabia (the reference sample) and the other three samples from Menofia, Egypt.

Genomic DNA Extraction

The honeybee workers' sample tissue should be ground into a fine powder using a mortar and pestle in the presence of liquid nitrogen. Subsequently, 0.2 grams of the tissue powder should be transferred to a 2 ml Eppendorf tube. The mitochondrial and total genomic DNA of bees was extracted using two specific DNeasy® mini-Kit (Qiagen Santa Clarita, CA), which are often used for DNA isolation from insect tissue.

Inter-simple sequence repeats (ISSRs)

DNA samples with a concentration of about 10 ng/µL were used in conjunction with a PCR marker. The oligonucleotide sequences of the ten ISSR primers utilized in this investigation were chosen from a collection of Operon kits (Operon Technologies Inc., Alameda, California, USA). The nucleotide sequence of the forward and reverse primers employed for detecting the COI-COII specific intergenic region of the mitochondrial DNA is shown in Table (2) (Cornuet *et al.*, 1991; Garnery *et al.*, 1998).

The 25 µl reaction volume used for the amplification reaction contained 12.5 µl of the Sigma Master Mix: 3 µl ISSRs primer; 1.5 µl of reverse primer, and 1.5 µl of forward primer for COI-COII intergenic region-PCR), 3 µl (µl =10 ng) of template DNA, and 6.5 µl of dH_2O. The PerkinElmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) was used to carry out the PCR amplification. The system is designed to execute a denaturation cycle lasting 5 min at a temperature of 94°C. This is followed by 35 cycles, each consisting of a denaturation step at 94°C for 50 sec, an annealing step at a temperature range of 45°C to 50°C (depending on the primer used) for 1.30 min, and an elongation step at 72°C for 1 min. The primer extension step in the last cycle was extended to 10 min at 72°C. The amplification products were separated by ethidium bromide (0.5 µg/ml) on a 3% agarose gel in 1X TBE buffer at 120 volts. Paplauskiene *et al.* (2006).

The data was analyzed using the Gel-Doc 2000 instrument, equipped with the Diversity Database Fingerprinting Software (version 2.1, Bio-Rad), to construct the dendrograms and determine the

genetic relationships of the Saudi Arabia sample and other relative forms of bees in Egypt.

RESULTS AND DISCUSSION

Morphometric measurements

Results of Egypt honeybee samples revealed that the average total body size (BODY) ranged from 3.935 (HS3) to 3.940 (HS4) with an average of 3.938 mm, which is essentially identical to the measurements of *A. mellifera* jemenitica (the reference strain, HS1), which recorded 3.927 mm (Table 3). Moreover, the mean FLL of three samples collected from Egypt was 2.383 mm, while the reference strain from Jazan was 2.32 mm. Results also indicated that the PROBL was 5.31.

In the current study, the FEML ranged from 2.39 to 2.44 mm, with a total average of 2.41 mm, while the reference strain recorded 2.38 mm. Regarding the TIBL, the lowest value was recorded in the reference strain from Jazan by 2.80 mm, while the strains collected from Egypt have tibia with lengths ranging from 2.84 to 2.90 with an average of 2.87 mm (Table 3).

For metatarsus measurements of length and width, results showed that the gross mean of METL and METW of the three samples from Egypt were 2.27 and 1.06 mm, respectively. In contrast, the reference strain from Jazan was 2.22 and 1.0 mm, respectively. The HLL ranged from 7.1 to 7.2, with an average of 7.17 mm for three samples from Egypt and 7.0 mm for the reference strain from Jazan. A high hooks (hamuli) number (HN) was recorded in the current study at 22.4. Additionally, among the metasoma measurements, the length of 3 and 4 metasomal terga (T3L+T4L) was 3.82 mm, which was higher than the reference strain from Jazan by 3.80 mm. On the other hand, the WMW ranged from 2.0 to 2.03 mm with an average of 2.02 mm, which was higher than those obtained for the reference strain from Jazan by 2.0 mm. For the forewing measurements (length and width), the gross mean for the three samples from Egypt was 7.80 and 3.25 mm for FWL and FWW, respectively. Also, the CI value ranged from 2.11 to 2.14 with a gross mean of 2.13, which was higher than those for the reference strain, which was found to be 2.10 (Table 3).

Western bees (*Apis mellifera* L.) are common in Egypt in the form of several races and hybrids, of which the Carniolan, Italian, Caucasian, and Egyptian bees are the most important. Many races of bees have entered Egypt legally or illegally, and we have recently noticed that the Arabian or Nubian honeybee race (*A. m.* jemenitica) is entering some Egyptian areas. To confirm this, we performed morphometric measurements and genetic analyses of samples from these bees. The morphometric study of all samples may have proven their identification as *A. m.* jemenitica, but, likely, they varied genetically. Therefore, genetic analyses should be carried out to corroborate the morphological findings (Alghamdi and Alattal, 2021). In the current study, it was proven

that honeybee workers randomly collected from three apiaries located in Egypt were identical to the reference honeybee sample obtained from the Kingdom of Saudi Arabia in morphological terms and according to the standard morphometric measurements by Ruttner (Ruttner *et al*., 1988) using fourteen morphological measurements. The presence of *A. m.* jemenitica in three distinct regions of the Menoufia Governorate (Egypt) was clearly and significantly established based on genetic findings derived from ISSR and PCR methods .

Results of Egypt honeybee's samples revealed that the average total body size (BODY) was 3.938 mm, which is essentially identical to the measurements of the reference strain, HS1 (*A. m.* jemenitica), which recorded 3.927 mm. This result indicates that it conforms to the specifications of the Arabian or Nubian honeybee (*A. mellifera* jemenitica**)** compared to standard numbers by Ruttner (1988) of 3.82 mm. Similar findings were reported by Ahmed *et al*. (2012) and Alabdali *et al*. (2021), who found that the collected and recognized *A. m.* jemenitica bees had bodies measuring of 3.92 and 3.80 mm, respectively (Table 3). Moreover, the mean FLL and PROBL of three samples collected from Egypt, as well as the reference strain from Jazan, were typically similar to those obtained by Alabdali *et al*. (2021) and Alqarni (2011).

Regarding the leg measurements, FEML, TIBL, METL, and METW, as well as the HLL of samples collected from Egypt (HS2, HS3, HS4), were compared to the measurements of the reference strain from Jazan (HS1), so the results confirmed that the collected samples were *A. m.* jemenitica according to Ruttner's numbers (Ruttner, 1988) and the native strain by Alabdali *et al*. (2021). These results were confirmed by the ISSR technique, which showed a very high genetic similarity between the standard bee sample obtained from Jazan, Saudi Arabia, and the samples taken from the Menofia Governorate. A specific PCR technique was then used to confirm the high genetic similarity due to the presence of the genetic sequence of the *mt*DNA COI-COII intergenic region gene in all samples under study as compared with the reference sample.

Genetic similarity and relationships between bees s amples

Genetic diversity assessment plays a pivotal role in understanding the similarities and variations within honeybee races, and this study utilized the power of 10 ISSR primers to analyze genetic similarity among bee samples. These primers were selected to estimate the genetic similarity between different locations in the Menofia governorate and compare them with the obtained reference sample (Saud Arabian bees). The amplification of reproducible fragments using the 10 primers resulted in a total count of 88 bands, 16 of which were identified as polymorphic fragments. The observed polymorphism level of 18.18% suggested a relatively low degree of polymorphism and a high level of simi-

larity across the bee samples under investigation. The amplified fragments exhibited variation in size across multiple primers, with a range of 200 to 1800 bp, as seen in Fig. (1a).

The selected ISSR primers produced several bands consisting of a variety of DNA fragments, ranging from 6 to 9 bands per primer, with an average of 7.5 bands per primer. The ISSR 29 primer yielded the highest number of bands (9), while the ISSR 05 primer resulted in the lowest number of bands (6). The genetic similarity of the four bee samples was evaluated by computing the similarity matrices using Dice's similarity coefficients (DSC's) based on the scored ISSR data matrix. The genetic similarity across the four bee samples varied from 81% to 94%, with an average value of 87.5%, according to ISSR data analysis. In addition to ISSR analysis, Table (4) and Fig. (1b) show that the Saudi Arabia reference sample, HS1 and HS2, had the greatest degree of genetic similarity (94%), whereas the HS1 and HS4 samples had the lowest genetic similarity (81%).

The dendrogram partitioned the genotypes of workers into three main clusters, with four samples being considered. The first cluster consisted of the HS2 sample obtained from Egypt, while the second cluster included the HS1 bee workers gathered from Jazan, Saudi Arabia. In contrast, the second cluster contained the remaining two samples (HS3 and HS4), as shown in Fig. (1c). On the other side, total genomic DNA from bee samples was analyzed by PCR using primers specific to the coding region of the COI–COII intergenic region. The analysis of DNA amplification in all samples collected from Egypt demonstrated the detection of a DNA amplification fragment measuring 600 bp. Those bands were also detected in the Saudi Arabia sample (HS1), aligning with findings from a prior investigation conducted on the Syrian honeybee belonging to the *Apis O lineage* in Jordan (Alburaki *et al*., 2011; Alattal *et al*., 2014; Haddad *et al*., 2009).

Similar results were found by Paplauskiene *et al*. (2006) when they used eleven ISSR primers on *Apis mellifera* caucasica and *Apis mellifera* carnica, along with three lines of the latter. On average, 6.8% of the cases generated five or more different types of DNA profiles, 70.4% of cases produced two or more types of profiles, and 22.8% of cases showed no polymorphism between the bee races and within the lines tested.

Although Ahmed *et al.* (2012) and Alqarni (2011) recorded lower values for all morphometric measurements compared to the current study and Alabdali *et al*. (2021), they found that the tested strains were *A.m.* jemenitica. This can be attributed to the existence of a permissible range of differences in measurements. In such cases, genetic analyses are the decisive factor that confirms or denies the morphological findings, as reported by Shohani *et al*. (2014), where they found that the application of ISSR in honeybee identification estimated the genetic variation be-

tween 5 different isolates of *Apis mellifera* from Kurdistan, Markazi, Khuzestan, Isfahan, and Fars despite identical morphological measurements and obtained that the ranges were between 250 and 1000 bp. Two main groups were obtained according to the use of cluster analysis, where the first contained samples from Kurdistan and Khuzestan while the central, Fars, and Isfahan samples were summarized in the second group, indicating that the ISSR molecular markers can separate different strains of honeybees.

High values for the number of hooks (Hamuli) (HN), the width of the wax mirror (WMW), and the length of 3 and 4 metasomal trogs (T3L+T4L) were recorded in the current study compared to the reference strain from Jazan and the local strains tested by Alabdali *et al*. (2021) and Alqarni (2011). Whereas the overall average of the front measurements (length and width) for the three samples from Egypt (HS2, HS3, and HS4) was less than the values recorded by Alabdali *et al*. (2021) and Alqarni (2011). In contrast, Alabdali *et al*. (2021) recorded a higher cubic index (CI) value compared to the current study. These findings might be confirmed by the ISSR technique, as reported by Rouhani *et al.* (2018). They used six out of nine (ISSR) primers to detect the genetic variation within and among different groups of pistachio hard scale samples of honeybee (Kerman, Iran) and showed the best polymorphism using Ntsys (ver. 2.02) and PopGene (ver. 1.31) software to analyze the obtained data. Three groups were clustered: group A, which consists of Bardsir, Sirjan, Baft, Shahrbabak, and Zarand, collected samples; Group B, including south Kerman, Rafsanjan, north Kerman, and Kabootarkhan collected samples; and finally Group C contained Anar collected bees.

Finally, according to the morphometric and molecular data of the present study, the results demonstrated the typical homology between the samples collected from Egypt and the reference sample from Saudi Arabia, which supports our visual observations of Yemeni bees in Egypt.

1. Conclusion

This research is regarded as the first where no data or information was available to record the emergence of Yemeni bees in Egypt. However, we noticed its appearance visually. Consequently, a group of morphological and molecular studies were conducted on honeybee samples collected from Menoufia Governorate in Egypt compared to those obtained from Jazan, Saudi Arabia, as a reference strain. According to the results of the developed morphometric measurements, all collected samples were typically identical to the reference race in body size and length of the flagellum, proboscis, femur, tibia, metatarsus, hind leg, metasomal terga III and IV (T3L+T4L), and forewing. As well as the width of the metatarsus, wax mirror, and forewing were identical. Furthermore, the number of hamuli and cubital index were equal. In addition, the results of molecular analysis using two types of markers (Inter Simple

Sequence Repeat and COI–COII intergenic region) confirmed the morphological measurements. They showed a high level of similarity between the collected samples from Egypt and the reference race from Saudi Arabia.

In conclusion, we documented the beginning of the appearance of Yemeni bees in Egypt, and this may be due to the climate changes that the world is witnessing, which qualify Egypt to become a suitable breeding region for this race during the coming years.

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Availability of data and materials

Data are available upon request from the authors.

Authors' contributions

Conceptualization: E. E. Nowar; Saud, A. M. Aljuweer and S. I. Kasem. Investigation: E. E. Nowar and T. A. Elakkad. Methodology: E. E. Nowar and T. A. Elakkad. Resources: S. I. Kasem and A. M. Aljuweer. Software: E. E. Nowar and T. A. Elakkad. Writing-original draft: E.E. Nowar and T. A. Elakkad. Writing, review, and editing: E.E. Nowar. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

SUMMARY

The Arabian or Nubian honeybee, scientifically known as Apis mellifera jemenitica (A. m. jemenitica), is a distinct subspecies of the western honeybee. A. m. jemenitica is indigenous to several tropical locations, including Saudi Arabia, Sudan, and Somalia. A. m. jemenitica has not been recorded before in Egypt. Therefore, this work aimed to validate the visual observation of the beginning of the appearance of Yemeni bees in Egypt. Fifteen morphometric measures and two molecular markers (Simple Sequence Repeats; SSRs and 16SrRNA) were used to establish morphological and genetic identity among A. m. jemenitica samples collected in Egypt and those acquired in Saudi Arabia as a reference. Morphometric and molecular analyses revealed close similarities between the studied bees, which confirm our hypothesis about the presence of Yemeni bees in Egypt.

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Table (1): Geographical details of four locations.

Samples	Region	Coordinates	No# samples (3 colonies/apiary)
HS1	Jazan (Jazan)	16°53′21″N 42°33′40″E	60
HS2	Menofia (Shbin El-Kom)	30°33′31″N 31°0′36″E	60
HS3	Menofia (Shbin El-Kom)	30°33′31″N 31°0′36″E	60
HS4	Menofia (Shbin El-Kom)	30°33′31″N 31°0′36″E	60

Table (2): Primer names and sequences used in ISSRs and 16S-PCR detetion.

Primers	Sequence 5' to 3'	T (°C)
ISSR- 04	5'-AGAGAGAGAGAGAGAGYC-3'	54
ISSR- 05	5'-AGAGAGAGAGAGAGAGYG-3'	45
ISSR- 09	5'-ACACACACACACACACYT-3'	47
ISSR- 11	5'-ACACACACACACACACYG-3'	48
ISSR- 12	5'-GTGTGTGTGTGTGTGTYG-3'	52
ISSR- 16	5'-CGCGATAGATAGATAGATA-3'	50
ISSR- 17	5'-GACGATAGATAGATAGATA-3'	52
ISSR- 23	5'-AGACAGACAGACAGACGC-3'	54
ISSR- 29	5'-GATAGATAGATAGATAGC-3'	47
ISSR- 31	5'-GACAGACAGACAGACAAT-3'	47
COI	(E2) 5'- GGCAGAATAAGTGCATTGGGC-3'	50
COII	(H2) 5'- CAATATCATTGATGACCTTA-3'	

Table (3): Morphometric measurements of three collected samples according to Ruttner's numbers compared to the reference strain.

Morphological measurements	Ruttner' No.	Alabdali *et al*. (2021)	Reference strain* (HS1)	Samples under study**			
				HS2	HS3	HS4	Average
BODY	(9+6)	3.92	3.927	3.938	3.935	3.940	3.938
FLL	-	2.30	2.32	2.36	2.39	2.40	2.383
PROBL	4	5.31	5.32	5.32	5.30	5.31	5.31
FEML	5	2.40	2.38	2.44	2.41	2.39	2.41
TIBL	6	2.85	2.80	2.88	2.90	2.84	2.87
METL	7	2.23	2.22	2.25	2.30	2.27	2.27
METW	8	1.01	1.00	1.00	1.08	1.10	1.06
HLL	-	7.02	7.00	7.10	7.20	7.20	7.17
HN (No.)	-	22.35	22.20	22.40	22.50	22.30	22.4
T3L+T4L	(10+11)	3.82	3.80	3.82	3.84	3.81	3.82
WMW	13	2.01	2.00	2.00	2.03	2.02	2.02
FWL	17	7.74	7.61	7.64	7.92	7.85	7.80
FWW	18	3.21	3.20	3.23	3.24	3.27	3.25
CI (degree)	(29+30)	2.15	2.10	2.14	2.11	2.13	2.13

All measurements were estimated as mm except HN and CI

**Apis mellifera* jemenitica from Jazan ** Samples from Egypt

Table (4): Genetic similarity matrices within and among the four samples of *A. mellifera* jemenitica.

	HS1	HS2	HS3	HS4
HS1	1.00			
HS2	0.94	1.00		
HS3	0.82	0.88	1.00	
HS4	0.81	0.86	0.93	1.00

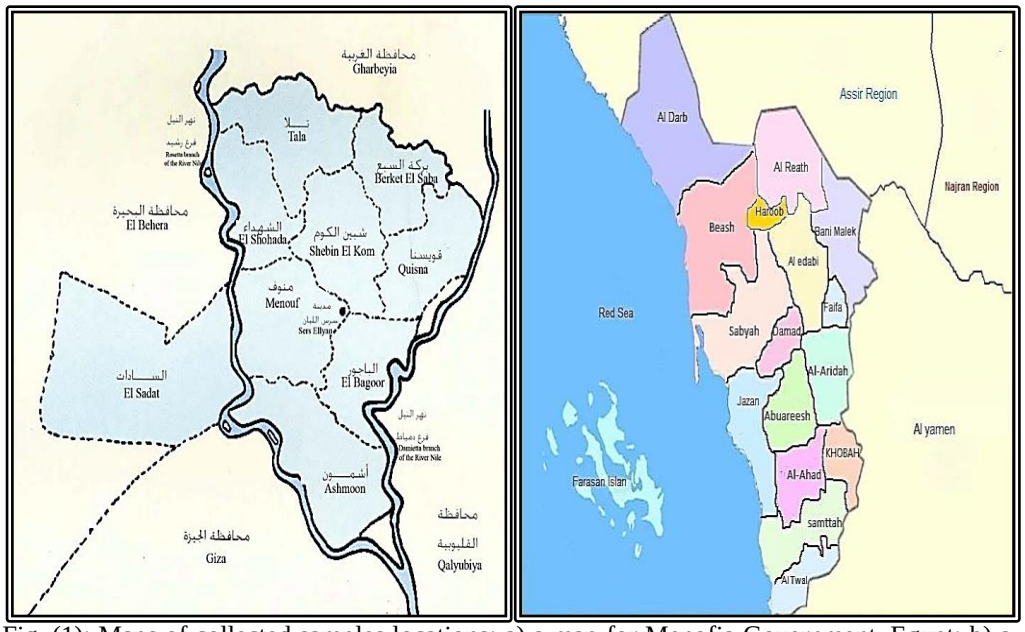

Fig. (1): Maps of collected samples locations: a) a map for Menofia Government, Egypt; b) a map for Jazan Government, Jazan region, Saudi Arabia (the area of study containing a red circle).

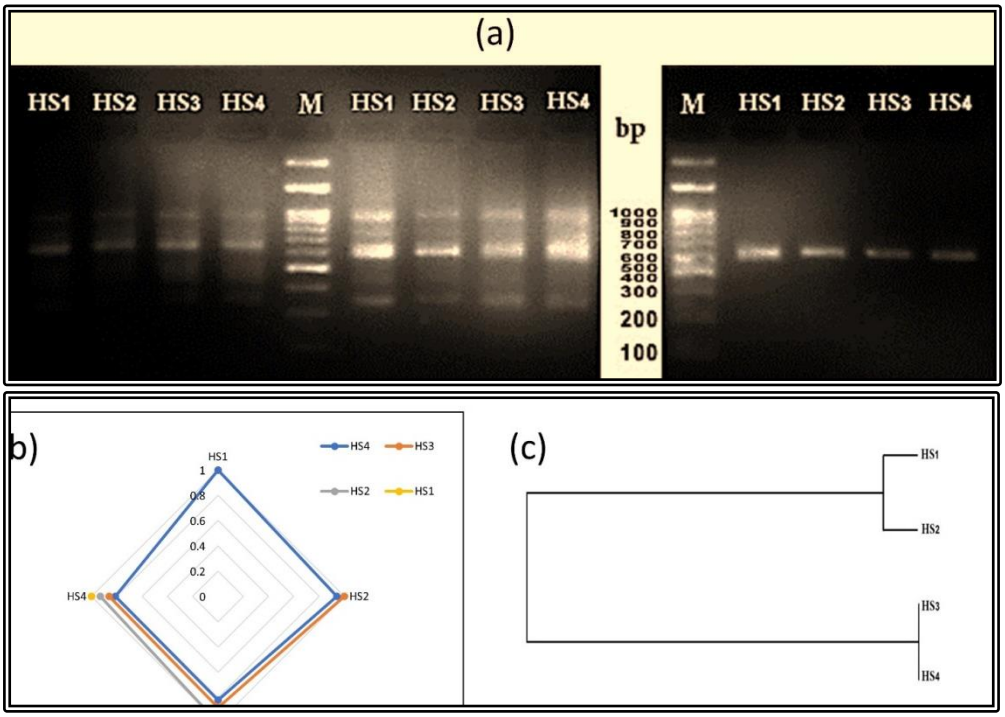

Fig. (2): (a) ISSR profiles of the four honeybee samples as detected with ISSR primers and COI–COII intergenic region–specific PCR. (M) DNA molecular weight standards 100 bp DNA ladder; (b) An illustration of the genetic similarity matrices inside and between the four bee samples; (c) dendrogram for the four bee samples produced using ISSRs generated data using the UPGMA algorithm and similarity matrices derived using DSC's.