Molecular identification of Dwarf Bees *Apis florea* species Discovered on the Golden Triangle Area, Red Sea, Egypt

Ali, M.A.[1]*, Mervat A.B. Mahmoud [2] and Sherehan A.R. Salem [2]

[1]*Plant protection Department, Faculty of Agriculture, South Valley University, 83523 Qena, Egypt.*
[2]*Zoology Department, Faculty of Science, South Valley University, 83523 Qena, Egypt.*

Abstract

Honeybees play a crucial role in plant pollination, agriculture's biodiversity, and environmental balance. This study was conducted for the first time in "the Golden Triangle Area". The objective was to identify the bee species that inhibited the Red Sea, focusing on the coast. The work was conducted through intensive and extensive visual observation on a 20 km long and 2.5 km wide area. Morphological measurements using a stereomicroscope and molecular identification were conducted using the mitochondrial *COI* gene. Body, antennal, forewings, hind wings, and hind leg lengths were recorded. The mitochondrial *COI* gene confirmed the species identification of dwarf bees in the studied area. *COI* sequencing produced a nucleotide length of 636 bp. and was deposited in GenBank under accession number (OP797664.1). The *COI* sequence was subjected to BLAST/N at the NCBI and revealed eight species of the genus *Apis*. On average, colonies were found to be about five per square kilometer. Also, the most preferred plantation for the red dwarf honeybees was sider trees. In conclusion, dwarf honeybees moved to the Triangle Golden Zone, which, if taken into consideration, will enrich the agriculture ecosystem. Also, increasing sider trees will increase the natural colonization of dwarf honeybees.

1. Introduction

Apis florea (Hymenoptera: Apidae: Tetragonisca), is the smallest known bee species. They are stingless bees, also called red dwarf honeybees because of their reddish-brown pigmentation on the abdomen and their relatively small size. Despite their diminutive size, these bees are crucial to the region's pollination of both wild and domesticated plants (Oldroyd, 2021). It is native to Asia. It is spread in several regions of the world, such as China and Vietnam, as well as in the Arab Gulf countries such as Oman, Iraq, and Yemen (Wongsiri *et al.,* 1997), and Saudi Arabia (Hepburn *et al.,* 2005). Currently, it is

recorded in countries bordering the Red Sea, such as Jordan and Sudan (Haddad *et al.,* 2008; El-Niweiri *et al.,* 2019). Nowadays, it is recorded in Egypt (Shebl, 2017; Masoud, 2019; Salem, 2020).

Bees play a crucial role in maintaining ecosystem health and global food security (Potts, 2016). Among the various species of bees, *Apis florea*, or the solitary dwarf bee, is a crucial pollinator that can be found in a wide range of environments, from arid deserts to urban areas (Oldroyd and Wongsiri, 2006; Deodikar, 2019). These bees build their nests in cracks and hollow twigs, and unlike regular bees, they do not live in human-made hives. Dwarf bees construct small, spherical nests out of wax and other materials and are known for their ability to adapt to various

*Corresponding author: Mahmoud Abbas Ali
Email: m.abbas@agr.svu.edu.eg

rural, urban, and desert environments, especially in hot climates (Sedighi *et al.,* 2018; Kevan, 2021).

Despite their importance as pollinators, dwarf bees face various threats in the Middle East, including habitat loss, pesticide use, and climate change (El Shafie *et al.,* 2002; Al-Ghamdi *et al.,* 2016). The decline of bees and other pollinators is a significant concern, given their critical role in agriculture and the stability of ecosystems (IPBES, 2016; Rader *et al.,* 2016). Therefore, there has been a growing interest in studying and conserving these bees to safeguard their function in local ecosystems and promote sustainable agriculture and food security in the region (Kremen, 2002; Garibaldi, 2016).

This study aims to identify the bee species present in the Golden Triangle Area, a promising economic zone in Egypt's Red Sea region. The study includes visual observation and molecular identification of the bee species found in the area and their distribution. The collected data will provide valuable insights into the natural insect biodiversity and bee species of the region and promote the use of sustainable agricultural practices that minimize the use of pesticides and maximize the diversity of flowering plants in agricultural landscapes (Garibaldi, 2019). Through this work, we hope to contribute to the conservation of bees and other pollinators, which are critical to the health and well-being of our planet (Potts, 2019; Ollerton, 2021) to encourage environmentally friendly farming practices that reduce pesticide use while increasing the diversity of flowering plants in agricultural settings.

2. Material and methods

2.1. Materials
Visual observation equipment (binoculars, magnifying glass, field notebook, camera), Collecting equipment (insect net, forceps, vials, alcohol), DNA extraction kit, PCR machine, *COI*

gene primers, Gel electrophoresis equipment, DNA sequencing machine.

2.2. Study area
The study area was selected in the promising Egyptian economic zone "the Golden Triangle Area" focusing on the Red Sea region, southeastern Egypt, from February 2021 to December 2021. The study included an extension of 20 km^2 from the city of Safaga to 17 km south towards the city of Al- Quseer. The coastal area and the adjacent mountainous areas were visually observed with a width of about 2.5 km$^{2.}$

2.3. Visual observation
The work was conducted through intensive and extensive visual observation on a 20 km long and 2.5 km wide area to identify the bee species that were inhibited along the coast. Field visits were conducted to buildings within the study area, as reported by residents. These visits aimed to identify and document additional bee specimens. Volunteers also contributed to the collection effort by supplying bee specimens they had discovered in the region. This collaborative approach ensured a comprehensive sampling strategy.

2.4. Vegetation analysis
As an integral part of the field observation, the vegetation spread within the study area was documented. This analysis included recording the types of nectar plants present, which provide essential resources for the local bee population. The prevalence of these plants in the vicinity of the identified insect locations was noted to establish their potential influence on bee distribution**.**

2.5. Laboratory Analysis
All collected samples, including worker specimens and any specimens provided by volunteers were carefully transferred to the laboratory of the Plant Protection Department, Faculty of Agriculture, South Valley University. In the laboratory, rigorous identification protocols were followed to classify the collected

bee species accurately. Molecular techniques were employed to supplement visual and morphological identification, ensuring a comprehensive analysis**.**

2.6. Morphological measurements
Measurements were taken to describe the red dwarf bee's body collected from this specific region: body length, antennal length, antennal segment numbers, proboscis length, thorax length, hind legs length, abdomen length, forewing length, forewing width, hind wing length, hind wing width**.**

2.7. DNA extraction and PCR Conditions
Molecular identification: To have an accurate identification, molecular identification was conducted using the mitochondrial *COI* gene, and genomic DNA was extracted from a fresh sample using the QIAamp DNA Mini kit (Qiagen, Germany) by following the manufacturer's guidelines.

A partial mitochondrial *COI* gene was amplified using forward primer LCO1490 and HCO2198 reverse (Folmer *et al.,* 1994). PCR assays were conducted in 50 µl reactions containing 25 µL PCR master mix, 1 µL of each forward and reverse primer, and genomic DNA. The PCR cycling conditions were performed with an initial denaturation at 94 ℃ for 240 sec., followed by thirty-five cycles including denaturation, annealing, and an extension for 60 sec. at 94 ℃, for 60 sec. at 49℃, and for 60 sec. at 72 °C, respectively. Followed by a final extension at 72 ℃ for 10 min. 1.5 % agarose gel contained ethidium bromide was used to separate the amplified products. For sizing the amplified PCR fragments, 100-bp DNA Ladder RTU (Ready-to-Use), GeneDireX was used.

2.7.1. The Sequencing of PCR
DNA sequencing was achieved by Macrogen (Seoul, South Korea). The sequences were submitted to the National Center for Biotechnology Information (GenBank/NCBI) for obtaining accession numbers.

Sequence alignment was performed using MUSCLE (Edgar, 2004) with default settings. MEGA version 7.0 18 (Kumar *et al.,* 2016) was used to perform the phylogenetic trees analyses using Maximum Likelihood (ML), Neighbour Joining (NJ) Minimum Evolution (ME) methods of tree construction, with one thousand bootstrap iterations (Felsenstein, 1985). The calculation of sequence divergences occurred by utilizing Kimura two-parameter distances (Kimura, 1980). To determine the similarity of our sequence to those already found in the database, we used BLAST searches of the GenBank NCBI database.

2.8. Data analysis
The number of colonies per square kilometer was calculated based on the visual observation data. The preferred plantation for the dwarf honeybees was determined based on the visual observation data. Body measurements were described as the means of six replicates and the standard deviation.

3. Results

3.1. Observation of dwarf bee colonies
Through the survey process, the aggregation of dwarf bee colonies was limited to the center of Safaga City, especially urban locations that contain buildings, abandoned houses, and roofs, where all the insects were visually observed. The average number of colonies was five per km2. *A. florea* nests are noticeable and are made out of a single horizontal comb that is built around and connected to branches or other supports (Figure 1). Nests are often shaded and built into shelters. The most prevalent nectarine plants in the presence of the cults were Sidr trees, *Ziziphus spina-christi* (L.). Also, the continuous movement of the bees has been observed, especially if they are disturbed or people interfere in their places of residence.

Figure 1. Wax comb of dwarf bees.   Curtsy Mahhmoud A. Ali

Figure 2. nest of dwarf bees. Curtsy Mahhmoud A. Ali

Figure 3. Nest of *Apis florea*
Curtsy Mahhmoud A. Ali

Figure 4. Adult *Apis florea* worker
Curtsy Mahhmoud A. Ali

Figure 5. Body parts of *Apis florea* worker, a) Right hind legs, b) Mouth Parts

3.2. Morphological measurements

Data in Table 1 show the body length of an adult red honeybee worker was 7–9.5 mm, with an average of 8.17± 0.75 mm. Body length with wings was 9-11.5 with an average of 10.08±0.73. Antennal length, including all antennal structures, pedicle, scape, and flagellum, was 3 mm, with an average of 3. Antennal segment number was ten. Proboscis length was 1.5-1.8 with an average of 1.55±0.11 with n = 5 each). Thorax length was 2.5-3 with an average of 2.83±0.24. Hind legs length was 6-8 with an average of 7.25±0.80. The forewing length was 7-8 with an average of 7.2±0.37, while the forewing width was 2-2.5 mm. The hind wing length was 5, with an overage of 5, while the width was 1-1.5mm with an average of 1.42±0.19 mm. All measurements were replicated six times.

Table 1. Red dwarf bee body measurements

| No.	Criteria	Average $n \pm$ SD
1	Body Length with wings	8.17± 0.75
2	Antennal length	10.08±0.73
3	Antennal segment number	3±0.00
4	Proboscis Length	10±0.00
5	Thorax length	1.55±0.11
6	Hind legs length	2.83±0.24
7	Forewing length	7.25±0.80
8	forewings width	7.2±0.37
9	Hind wing length	2.17±0.24
10	hind wing length	5±0.00
11	Abdomen length	1.42±0.19
12	Body Length with wings	3.75±0.25

n = 6

3.3. Molecular Identifications

The sequencing of *COI* produced a nucleotide length of 636 bp and was deposited in GenBank under accession number (OP797664.1). The nucleotide frequencies of adenine (A), cytosine (C), guanine (G), and thymine (T) were 33.8, 14, 10.8 and 41.4%, respectively. The A+T content was 75.2, which was higher than the C+G content. The sequence of *COI* was subjected to BLAST/N at (NCBI) and revealed eight species of the genus *Apis*, with three species of the family Chrysididae as outgroups (Table 2). To conduct the phylogenetic tree analysis using (*COI*) sequencing, the sequences of understudied sample *A. florea* was submitted to the analysis together with the eight related species and three out-group species.

Table 2: The understudied *Apis florea* with their related   species and out-group from the GenBank/ NCBI based on (*COI*) sequences.

No.	Species	Accession number
1	*Apis florea* isolate Red Sea Safaga	OP797664.1
2	*Apis andreniformis*	KU212334.1
3	*Apis cerana*	MH588652.1
4	*Apis dorsata*	MF804563.1
5	*Apis florea*	KU737494.1
6	*Apis koschevnikovi*	EU163110.1
7	*Apis laboriosa*	MK904710.1
8	*Apis mellifera*	MW428276.1
9	*Apis nigrocincta*	KY834222.1
10	*Chrysis brasiliana*	KY430790.1
11	*Chrysis ignita*	KY430761.1
12	*Chrysis impressa*	MZ628324.1
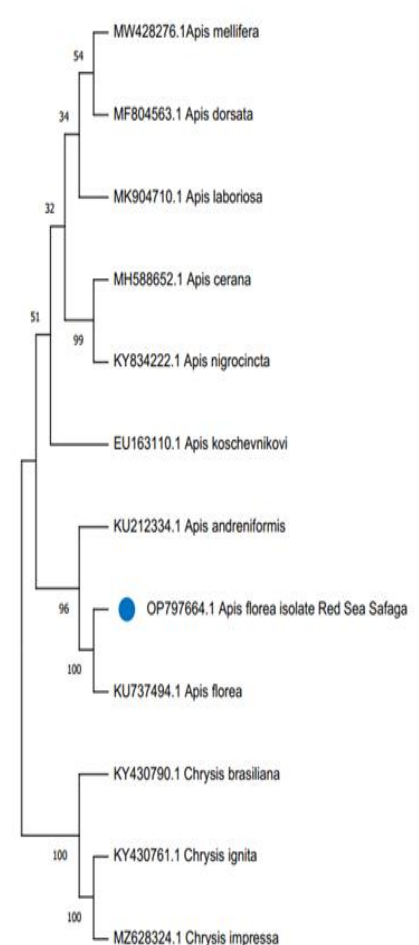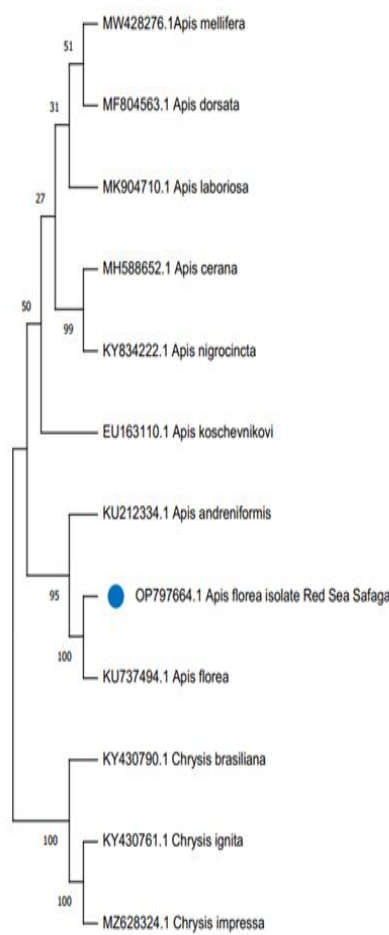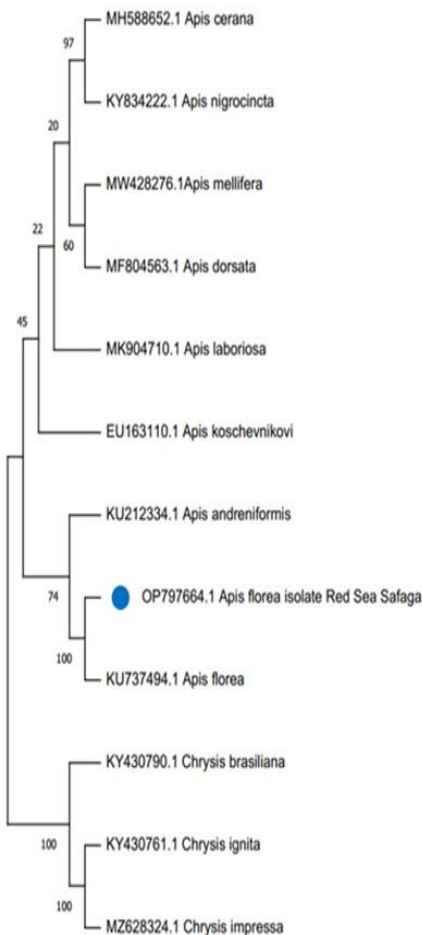
Figure 6. Phylogenetic tree using the Maximum Likelihood method among *Apis florea* with their related species and out-group from the GenBank/ NCBI based on (*COI*) sequences.

Figure 7. Phylogenetic tree using the Neighbour Joining method among *Apis florea* with their related species and out-group from the GenBank/ NCBI based on (*COI*) sequences.

Figure 8. Phylogenetic tree using the Minimum Evolution method among *Apis florea* with their related species and out-group from the GenBank/ NCBI based on (*COI*) sequences.

For more expository phylogenetic relations, we used more than one phylogenetic method; Maximum Likelihood, Neighbour Joining and Minimum Evolution based on (*COI*) sequence. The methods showed nearly the same relations with some differences in support values and revealed that the understudied sample with *Apis florea* formed asister clade with *A. florea* (KU737494.1) from the GenBank/ NCBI (Figures 6-8).

Pairwise genetic distances among the understudied *Apis florea* and the eight species of the genus *Apis* ranged from 0.000 to 0.0195. The most related species to our sample was *Apis florea* (KU737494.1) from the GenBank, where the genetic distance was 0.000, while the genetic distance was 0.0195 for *Apis koschevnikovi*. Overall, the mean distance value was 0.20% (Table 3).

4. Discussions

Through the survey process, it became clear that the aggregation of dwarf bee colonies was limited to the center of Safaga City, especially the areas that contain buildings, where all the insects were observed. The average number of colonies per km^2 was five. These findings agree with (Srinivasan *et al.,* 2000) who reported *A. florae* behavior and habitats. The most prevalent nectarine plants in the area of the presence of the cults were Sidr trees, *Ziziphus spina-christi* (L.). The continuous movement of bees has been observed, especially if they are disturbed or people interfere in their places of residence. Also, (Engel, 2012) reported that they are common near human settlements, man-made structures, forests, or undisturbed natural areas.

The study showed the presence of dwarf bees in the Red Sea region, which may have come from the north, where the Suez Governorate was previously recorded (Shebl, 2017) as this indicates the continuous movement and distribution of dwarf bee colonies, Thus, we can conclude the *widespread* in the future due to what is characterized by this type of abilities to adapt to new environments, and it was found that the most preferred places for dwarf bees were the places containing buildings, especially isolated ones such as the roofs of buildings and neglected tools such as used tires and wooden furniture.

The study showed the presence of dwarf bees in the Red Sea region, which may have come from the north, where the Suez Governorate was previously recorded (Shebl, 2017) as this indicates the continuous movement and distribution of dwarf bee colonies. Thus, we can conclude that the wide spread in the future is due to what is characterized by this type of ability to adapt to new environments, and it was found that the most preferred places for dwarf bees were the places containing buildings, especially isolated ones such as the roofs of buildings and neglected tools such as used tires and wooden furniture.

The current study used DNA sequences of the *COI* gene to describe and identify the prevalent species in species the Golden Triangle area, Red Sea Coast for the first time recorded as *Apis florae*. We believe our finding based on *COI* was correct given that they aligned with multiple GenBank sequences deposited by multiple authors through multiple studies and was designated as *Apis florae*. The results of *COI* confirm that our tested species can be identified as *Apis florae*. Pairwise genetic distances showed that the most related species to our sample was *Apis florea* (KU737494.1) from the GenBank, which is an Indian species (Rakshit) unpupl.) These results in agreement with (Salem *et al.,* 2020), who approved that the *Apis florae* in Suez governorate originated from India. So, the same species moved or migrated from Suez governorate through the coastal border. Through molecular methods, we sought to achieve the understudied species that identified as *Apis florae*. This work contributes valuable knowledge to the scientific community.

Table 3. Pairwise distances among *Apis florea* with their related species and out-group from the GenBank/ NCBI based on (COI) sequences.

	1	2	3	4	5	6	7	8	9	10	11	12
OP797664.1_*Apis_florea*_isolate_Red_Sea_Safaga		0.0000	0.0134	0.0155	0.0164	0.0166	0.0169	0.0175	0.0195	0.0338	0.0347	0.0347
KU737494.1_*Apis_florea*	0.0000		0.0136	0.0155	0.0164	0.0171	0.0170	0.0176	0.0195	0.0340	0.0345	0.0344
KU212334.1_*Apis_andreniformis*	0.0894	0.0909		0.0154	0.0152	0.0157	0.0161	0.0165	0.0168	0.0331	0.0355	0.0358
MK904710.1_*Apis_laboriosa*	0.1062	0.1062	0.1064		0.0137	0.0129	0.0142	0.0151	0.0153	0.0335	0.0353	0.0352
MH588652.1_*Apis_cerana*	0.1178	0.1178	0.1137	0.0858		0.0141	0.0146	0.0098	0.0135	0.0312	0.0342	0.0344
MW428276.1*Apis_mellifera*	0.1234	0.1302	0.1187	0.0877	0.0953		0.0139	0.0158	0.0151	0.0345	0.0365	0.0364
MF804563.1_*Apis_dorsata*	0.1277	0.1302	0.1210	0.0979	0.1054	0.0960		0.0138	0.0159	0.0343	0.0362	0.0362
KY834222.1_*Apis_nigrocincta*	0.1319	0.1344	0.1291	0.1020	0.0520	0.1121	0.0999		0.0154	0.0343	0.0367	0.0368
EU163110.1_*Apis_koschevnikovi*	0.1320	0.1320	0.1078	0.0898	0.0823	0.0948	0.1052	0.0975		0.0356	0.0367	0.0368
KY430790.1_*Chrysis_brasiliana*	0.3229	0.3274	0.3062	0.3106	0.2821	0.3284	0.3313	0.3263	0.2842		0.0197	0.0197
KY430761.1_*Chrysis_ignita*	0.3314	0.3300	0.3377	0.3322	0.3187	0.3511	0.3452	0.3555	0.3118	0.1631		0.0035
MZ628324.1_*Chrysis_impressa*	0.3314	0.3293	0.3407	0.3322	0.3217	0.3534	0.3473	0.3547	0.3118	0.1631	0.0077	

5. Conclusion

This study contributes valuable knowledge to the scientific community regarding the distribution, behavior, and molecular identification of *Apis florea* in the Red Sea region. Further research is needed to monitor the expansion and potential impact of these dwarf bee colonies on local ecosystems and human populations. The study focused on a specific area, so the findings may not be generalizable to other regions. Also, further research is needed to investigate the reasons behind the concentration of natural colonies of dwarf honeybees in households, which could have provided valuable insights into their behavior and habitat preferences. Also, the potential factors that could impact the natural colonization of dwarf honeybees, such as habitat destruction, climate change, and pesticide use.

6. References

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