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Full Length Article

Morphological and Molecular Analysis of some Bee Species of the Subgenus *Taeniandrena* (Hymneoptera: Andrenidae) from Northern Egypt

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Abstract

Andrenids is one of those groups which have some taxonomic impediments. *Andrena ovatula* represents such taxonomic impediment with great individual variations in size, body length and even in coloration. Several species were collected and identified on the basis of morphological characters. This study involved DNA sequencing of COI region of the mitochondrial DNA genome of *A. ovatula* to determine the population genetics and bio-geographical structure from three different locations in Egypt (Alexandria (A), Monofia (M) and Suez Canal (S)). Each population had three females as a preliminary study of this group for barcoding in Egypt. All investigated samples belonged to nine mtDNA haplotype. The phylogentic analysis showed that there are two groups of *A. ovatula* as some samples matched 100% with *Andrena intermedia* not known or recorded in Egypt so far. A large sampling scale of these species which is widely distributed in Asia, Europe and Africa could show more population differentiation. Further studies can help for more clarifications of the species identity of the genus *Andrena* worldwide. © 2019 Friends Science Publishers

Keywords: Solitary bees; Apoidea*; Andrena*; Barcoding; DNA sequences; mtDNA; Phylogenetic; Population genetics

Introduction

Andrenidae is one of the bee families, divided into four subfamilies, two of which (Andreninae and Panurginae) are widely distributed in the Palaearctic Region. Genus *Andrena* is one of the largest bee genera and is known as sand bees or solitary mining bees with over 3000 known species worldwide (Michener, 2007). Species of the genus *Andrena* fly from early spring to early summer (Dubitzky *et al*., 2005), and very few in summer or autumn seasons. Several species of *Andrena* are generalists on their floral resources and gather pollens from several plant species (polylectic species) but some others considered as oligolectic species (Shebl *et al*., 2015). Family Andrenidae is a monophyletic group and few efforts have been made to trace its phylogeny (Alexander, 1995; Larkin, 2002). The subgenus *Taeniandrena* is primarily Palaearctic including 23 medium size bee species, are hard to distinguish morphologically due to overlapping characters (Shebl, 2010). *Andrena* (*Taeniandrena*) *ovatula* (Kirby, 1802), one of the most common bee species of this subgenus; is a good pollinator of some cultivated crops, is widespread all over the world (Asia, Africa and Europe) with some known subspecies. In Egypt, this species is known across the country around the Nile Delta and other regions of the desert (Shebl *et al.,* 2013; Shebl and Farag, 2015). Yet the phylogenetic position of this genus, as well as their relationships with other bee families has not been satisfactorily analyzed**.**

Andrenidae is poorly studied group in terms of their molecular phylogeny. There were very few studies attempted to tackle this genus (Danforth, 1999). Mitochondrial cytochrome oxidase I (COI) is a mitochondrial protein-coding gene and has been tested successfully with a variety of insect phylogenetic analysis (generic and subgeneric levels) (Simon *et al.,* 1994) and has been found suitable for obtaining a good resolution on the subgeneric level within bees. There are very few molecular and phylogenetic studies of the subfamilies, tribes, and genera of Andrenidae (Danforth *et al.,* 2013). Recently, Kek *et al.* (2017) used mitochondrial cytochrome oxidase subunit I (COI) gene region to identify and classify bees.

The aim of the current study was to examine nucleotide diversity of CO1 mtDNA of *A. ovatula* collected from different locations in northern Egypt, ecotypes as the initial step of molecular characterization, taxonomic position and population differences among the individuals from different geographical areas. Moreover, it addresses other questions to find out if there are any genetic differences between different geographical populations or not? This is the first time to study *Taeniandrena* using DNA sequences approach in Egypt, although there are some published data on some other subgenera (Larkin *et al*., 2005; Reemer *et al.*, 2008), which could lead to further studies on the phylogeography, population genetics and DNA barcoding of Egyptian bees.

Materials and Methods

Specimen Collection, Identification and Description

Bee individuals were collected by sweeping from different locations across Egypt during several field collections of bees from several plants such as *Triforium alexandrinum, Vicia faba* and *Brassica* spp. The specimens were killed, pinned and deposited at the sec author's private collection. The specimens of Andrenids were collected from three major cities Ismailia (Eastern part of Egypt), Minoufia (Nile Delta) and Alexandria (Western part of Egypt) as shown in Fig. (1) during spring seasons in the years 2011–2017.

The specimens were identified based on the taxonomic key of the Egyptian species (Moustafa *et al.*, 1979) then sorted out into three groups of *A. ovatula* based on locality (3 to 5 specimens per locality or per group, first group Minoufia 1–3; sec group Ismailia 4–6 and third group Alexandria 7–9. Specimens were examined and described using a stereoscopic microscope (OPTOMETRON D-81829). Photos of specimens were taken and illustrated using ADOBE PHOTOSHOP and PHOTOSCAPE software (Fig. 2). Male terminalia was pulled out from fresh captured specimens for examination and compared with the illustration published by Shebl (2010). The morphological terminology was adopted from Shebl and Tadauchi (2011). Terms and abbreviations used are as follows: T: tergum, S: sternum, HL: head length, HW: head width, FL1: first flagellum length, FL2: sec flagellum length, FL3: third flagellum length.

DNA Extraction, Amplification, and Sequencing

DNA was extracted from wholly legs from each population. Standard protocol for animal tissues of DNeasy Tissue Kit (QIAGEN) was followed for DNA extraction. Concentration and purity of DNA of each sample was measured by a Nanodrop-photometer (IMPLN Germany). Primers used for PCR amplification in this study were 5'–CCACATTTATTTTGATTTTTTGG–3' as a forward 5'–TCCAATGCACTAATCTGCCATATTA–3' as a reverse

Fig. 1: Geographical distribution of the experimental location

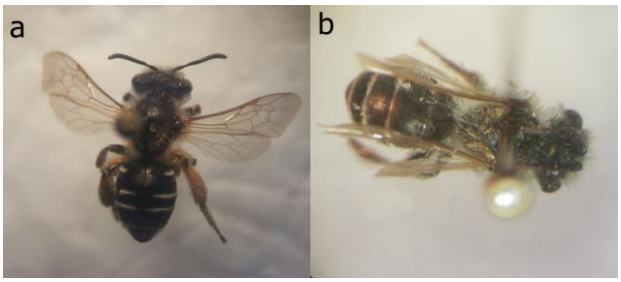

Fig. 2: a) Andrena ovatula ovatula female; **b**) Andrena ovatula ovatula male
Male. BL 6-6.5 mm; WL 4.5.5.5 mm

(Petersen *et al.*, 2007).

PCR amplification of the mitochondrial cytochrome oxidase I (COI) gene was performed in a total of 50 μl reaction volume using water, 10x PCR buffer, $MgCl_2$, dNTP (2 m*M* each), primers (10 pm/μL each), 0.6 U of *Taq* polymerase and 2 μL template DNA. The conditions for PCR were the following: initial denaturation (95°C, 3 min) followed by 35 cycles of denaturation (94°C, 45 s), primer annealing (56°C, 45 s), and DNA extension (72°C, 1 min). The size of the amplified COI- region was determined using a 7 μL aliquot of the PCR product electrophoresed on 1.2% agarose gel. The ladder used was 100bp. All sequencing reactions were prepared using a Big Dye Terminator Ready Reaction according to the manufacturer's recommendations. Termination PCR reactions were performed in a programmable thermocycler under the following conditions: 10 s denaturation at 96°C, 5 s annealing at 50°C and 4 min extension at 60°C, repeated for 30 cycles. The amplified, fluorescently labeled and terminated DNA was salt-precipitated and analyzed on the automated sequencer ABI PRISM 310.

Data Analysis

Sequences of CO1 mtDNA (590 bp) were aligned with published sequences of nine different haplotypes of *A. ovatula* populations. Sequence alignments were done using the Bio Edit 7.0.5 software (Hall, 1999). Neighbour Joining (NJ) analyses were performed using ClustalX software (Thompson *et al.*, 1997). The number of haplotypes and

their frequencies were determined both visually and with the program DnaSP version 4 (Rozas *et al.*, 2003), DNAsp also estimated the following variables: haplotypic diversity (Hd), nucleotide diversity Pi (π), based on Nei (1987). Nucleotide diversity was interpreted as the average proportion of nucleotide differences between all possible pairs of sequences in the sample (Hartl and Clark, 1997), the mean number of pairwise nucleotide differences (K) according to Tajima (1983). The parameter theta (θ) is the proportion of nucleotide sites that are expected to be polymorphic in any sample from this region of the genome was estimated based on Hartl and Clark (1997). Our data was compared with the similar genome of the Gene Bank (International Nucleotide Sequence Database Collaboration).

Results

Systematic of the Species *Andrena ovatula* Kirby

A. ovatula is a widespread bee species that visits a wide range of flowers so the identity of the species is very important for any pollination study. So, it is important to provide full taxonomic characters to help non specialists to identify this species easily. This study provides a full description of both sexes of this species as it wasn't presented in previous literature dealing with Egyptian Andrenids.

Redescription of the Female

Color: Flagellum brownish beneath; mandibles black reddened apically; facial fovea brownish above, whitish below; wing membranes subhyaline, moderately brownish, veins and pterostigma brownish; femur, tibia, a tarsus of hind leg brownish; tibial spur yellowish.

Pubescence: Hairs on head sparse except antennal area with dense hairs, long, whitish; those on clypeus sparse, short, whitish, those on vertex dense, slightly long, whitish, those on genal area dense, whitish, long, Hairs on mesoscutum sparse, dense laterally, short, whitish; those on scutellum scanty, dense laterally, short, whitish, those on mesepisternum dense, long; whitish, propodealcorbicula developed, whitish, with internal simple, sparse hairs, trochanteral floccus perfect, whitish, femoral floccus dense, long, whitish, tibial scopa long, simple, silver whitish hairs. Hairs on metasomalterga T_{1-2} with interrupted whitish hair bands, T_{3-4} with completed whitish hair bands; caudal fimbria brownish; S_{2-5} with long, sparse, whitish subapical fimbriae.

Structure: Head length 2.7–3 mm, Head width 3.5–4 mm, HL/HW = 0.75–0.77. Vertex flat in frontal view, tessellate, shiny, with small PP. FL1> FL2+FL3, FL2 < FL3 which are broader than long. Eyes with inner margins subparalleled. Supraclypeal area slightly flat rather convex, shiny, with small PP. Facial fovea long, broad, occupying 3/4 the space between the eye and lateral ocellus, much extending to a line at lower margins of antennal fossae. Face above antennal fossae with fine longitudinal rugulae and weak interrugal PP, shagreening. Facial quadrangle broader than long. Clypeus flattened, densely tessellate, punctate with slightly dense, obscure PP, with longitudinal median impunctate space. The process of labrum broad, trapezoidal, not emarginated apically.

Lower paraocular area punctate with dense, shiny with min PP. Malar space linear, broad. Genal area slightly broader than the eye, tessellate and shiny with sparse, min PP. Mesosoma: Pronotum with lateral suture and without humeral angle, densely tessellate, shiny with fine PP. Mesoscutum densely tessellate, punctate, shiny with fine, obscure, small PP. Scutellum tessellate, punctate, shiny with dense, obscure, fine, small PP. Propodeal enclosure triangular, weakly indicated, rugulose basally, tessellate apically; dorsal face of propodeum roughened with fine, small PP. Mesepisternum tessellate, roughened, with fine, small PP. Vein 1st*m-cu* meeting sec submarginal cell at the middle of the cell. Metasoma: Metasoma condensed oval, metasomalterga densely tessellate, punctate, nearly shiny, T_{1-4} with finely dense, small PP; posterior depressions of terga weakly indicated; pygidial plate V shaped without raised internal triangular area. S_{2-5} tessellate, shiny with dense, min PP.

Redescription of the Male

Color: Flagellum brownish beneath; mandibles black reddened apically; wing membranes subhyaline, brownish, veins and pterostigma red brownish; femur, tibia and tarsus brownish, tibial spur pale brownish.

Pubescence: Hairs on head dense, whitish; those on clypeus dense, slightly long, whitish, with lateral fringes, those on vertex slightly long, whitish, those on the genal area slightly dense, whitish, long. Hairs on mesoscutum dense, long, whitish; those on scutellum dense, long, , whitish, those on mesepisternum dense, long, whitish. Hairs on metasomalterga T_{1-2} with dense, long, whitish hairs, T_{3-4} with scanty, suberect, short hairs, T_{1-4} with completed whitish hair bands; caudal fimbria whitish; S_{2-5} with long, slightly dense, whitish subapical fimbriae.

Structure: Head length 2–2.5 mm, Head width 2.5–2.7 mm, HL/HW = 0.8–0.9. Vertex flat in frontal view, tessellate, shiny, with fine PP. FL1<FL2+FL3, FL2 = FL3 which are longer than broad. Eyes with inner margins subparalleled. Supraclypeal area slightly flat rather convex, shiny, with small PP. Face above antennal fossae with fine longitudinal rugulae and weak interrugal PP, shagreening. Facial quadrangle broader than long. Clypeus flattened, weakly tessellate rather smooth, punctate with dense, obscure PP, with longitudinal median impunctate space. The process of labrum broad, trapezoidal, not emarginated apically. Lowerparaocular area punctate with dense, shiny with min PP. Malar space linear, broad. The genal area as broad as eye, weakly tessellate and shiny with min PP. Mesosoma:

Pronotum with lateral suture and without humeral angle, densely tessellate, shiny with min, fine, sparse PP. Mesoscutum densely tessellate, punctate, shiny with fine, obscure, small PP. Scutellum weakly tessellate, punctate, shiny with dense, fine, small PP. Propodeal enclosure triangular, weakly indicated, rugulose basally, tessellate apically; dorsal face of propodeum roughened with fine, small PP. Mesepisternum densely tessellate, with fine, small PP. Vein 1^{st}*m-cu* meeting sec submarginal cell at the middle of the cell. Metasoma: Metasoma condensed oval, metasomalterga densely tessellate, punctate, nearly shiny, T_{1-4} with finely dense, small PP; posterior depressions of terga weakly indicated. S_{2-5} strongly tessellate and shiny with dense, min PP.

Male genitalia and male sternitesstructreis are shown in Fig. 3–4. Specimens examined. [Egypt] 1 female, Suez, Amer Village., 18. iii. 2014 (M. Shebl); 1 female, Ismaila, Suez Canal University Campus., 17. i. 2018 (M. Shebl); 1 male, Ismaila, Suez Canal University Campus., 17. i. 2018 (M. Shebl); 2 males, Ismaila, Suez Canal University Campus., 14. i. 2014 (M. Shebl); 3 males, Ismaila, Masaid, El KantraGharb, 3.ii.2018.

Floral records. *Triforium alexandrinum*, *Vicia faba*, *Brassica* spp.

Distribution. N Africa, Europe and Asia

Remarks: Add here morphological comparison of this species as you have re-described this species. Please compare your re-description with previous descriptions mentioning your additions and previous shortcomings of description.

Molecular Analysis

The molecular analysis of *Andrena* comprises nine individuals representing three different locations from Egypt (three individual per each region) as shown in (Fig. 1), based on differences between COI mtDNA sequences in *A. ovatula* from Egypt. Population from Minoufia (seq1, seq2, and seq3), Ismailia (seq4, seq5 and seq6) and Alexandria (seq7, seq8 and seq9) generated 20 nucleotide substitutions. DNA sequences initially produced 850 bp of mitochondrial COI. Overall aligned sequences which were used for the analysis totaled 590 bp based on the shortest and clear one. The number of sequences were nine with 590 bp in length; total number of sites (excluding sites with gaps/missing data) were 590 out of which 570 were invariable (monomorphic) sites.

The variable (polymorphic) sites were 20; out of the five were singleton variable sites at (site positions): 135, 185, 222, 326 and 438 and 14 parsimony informative sites. Parsimony informative sites (two variants): 15 at site positions: 33, 99, 138, 315, 339, 378, 384, 393, 426, 459, 519, 523, 531 and 537 beside parsimony informative sites (three variants) at site positions: 108. However, there are no variable sites with four variants Fig. (5) and Table (1).

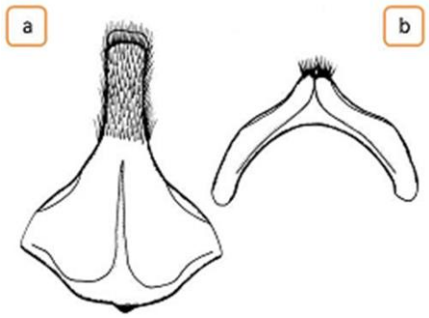

Fig. 3: a) Structure of 8^{th} sternum; **b**) Structure of 7^{th} sternum of male *A. ovtaula ovatula*

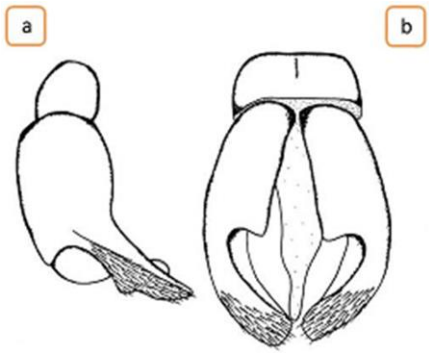

Fig. 4: a) Lateral view; **b**) Doral view of male terminalia of *Andrena ovatula ovatula*

Based on nucleotide diversity seq1 differ from seq2 and 3 with 3 nucleotides (Minoufia population). Seq4 differ from seq5 and 6 (*A. ovatula*) with 6 nucleotides (Ismailia population). Seq7 differ from seq8 and 9 with 4 nucleotides (Alexandria population) as shown in Table (1). This shows some differences among the species or ecotype of *A. ovatula* from each population and population from Ismailia (seq4-, seq5 and 6) showed high nucleotide variation than other populations as appears from phylogenetic tree Figs. (6) and (7). From aforementioned information beside phylogenetic tree it clear that the population (seq4., seq5 and seq6) from Ismailia seems to be highly diverged and isolated than other populations from Alexandria and Minoufia because of barrier and long distance between those areas and Ismailia (Fig. 6 and 7).

Number of Haplotypes, h: 9 and haplotype (gene) diversity, Hd: 1.000. The variance of Haplotype diversity was 0.003. The standard deviation of haplotype diversity was 0. 052. Moreover, nucleotide diversity, Pi (π) was 0.015 and theta (θ) (per site) was 0.013. An average number of nucleotide differences, k: 8.78.

The amount of phylogenetically informative characters of the sequenced part of COI was noticeably very low (5.7 %) for *Andrena*.

Comparing the genomic sequences of *A. ovatula* using BLAST revealed that some species are matching with *A.*

Table 1: Nucleotides at 19 variable positions of COI mtDNA region, differentiating haplotypes of *Andrena* populations from Minoufia (seq1-, seq2 and 3), Ismailia (seq4-, seq5 and 6), and Alexandria (seq7, seq8 and 9)

Nucleotide position Haplotype	33	99	108	135	138	185	222	315	326	339	378	384	393	426	438	459	519	523	531	537
seq 1	T	T	T	A	A	T	T	C	C	T	C	C	T	T	T	C	C	C	T	C
seq 2	.	.	.	.	.	.	.	T	.	.	T	.	.	.	.	.	.	.	.	.
seq 3	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T
seq 4	C	C	A	G	G	A	.	.	T	C	T	.	C	C	C	T	.	T	C	T
seq 5	C	C	A	.	G	.	.	.	.	C	T	T	C	C	.	T	.	T	C	T
seq 6	.	C	A	.	G	.	.	.	.	C	T	T	C	C		T		T	C	T
seq 7	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.	A	.	.	.
seq 8	.	.	C	.	.	.	.	T	.	.	.	.	.	.	.	.	A		.	T
seq 9	.	.	C	.	.	.	.	T	.	.	.	.	.	.	.	T	A	.	.	.

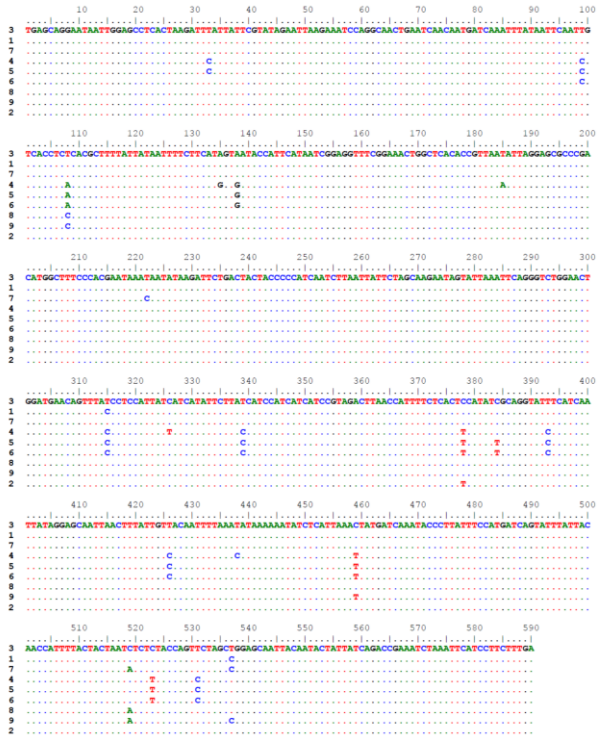

Fig. 5: Multiple sequence alignment of nine individual represent tree populations of COI mtDNA sequences in *Andrena*) from Egypt**.** Minoufia (seq1, seq2 and seq3), Ismailia (seq4, seq5 and seq6) and Alexandria (seq7, seq8 and seq9)

Table 2: Identity of the *Andrena* species form Egypt, checked with the bold engine using BALST

Sequence matching percentage	*A.* intermedia	*A.* ovatula	*A. wilkella*
1 Minoufia	99.83%	96.92%	97.08%
2 Minoufia	99.83%	97.09%	96.9%
3 Minoufia	100%	96.92%	97.08%
4 Ismailia	97.44%	98.8%	95.21%
5 Ismailia	97.78%	99.15%	95.9%
6 Ismailia	97.95%	98.97%	95.8%
7Alexandria	100%	---	96.92%
8 Alexandria	99.66%	---	97.08%
9 Alexandria	99.49%	---	97.08%

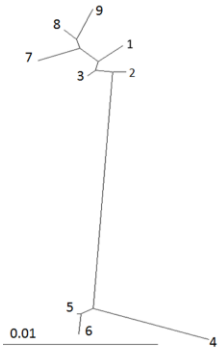

Fig. 6: Neighbor-joining (NJ) unrootedtree based on differences between COI mtDNA sequences in *Andrena* populations from Egypt. Minoufia (1, 2 and 3), Ismailia (4-, 5 and 6), and Alexandria (7, 8 and 9)

intermedia, the matching percentage is ranging from 99.49 to 100% in the population collected from Alexandria and Minoufia while the Ismailia population is ranging from 99.2 to 99.8% (Table 2).

Discussion

Taeniandrena Hedicke, 1933 is a primarily Palaearctic subgenus including 11 species from Europe, two species from North Africa, five species from Central Asia and 8 species from East Asia (Tadauchi and Xu, 2003). Almost all species resemble each other and very hard to differentiate morphologically. In addition, the subgenus has taxonomic impediment with some close species such as *A. ovatula*, *A. wilkella*, *A. intermedia*. *etc*. *A. ovtaula* is one of the most common species which have a wide range of distribution in Asia, Europe and Africa. Several subspecies were recognized in *A. o. ovatula* and *A. o. heliopolis* (Egypt), *A. o. poupilleri* Dours1872) (Libya and Crete) and *A. o. transcaspiensis* Osytshnjuk (Grace, 2010; Shebl, 2010; Shebl *et al*., 2013).

Andrenidae has been a challenging group in view of phylogeny, the known subfamilies has wide-ranging from two to five subfamilies (Almeida, 2008). The results related to molecular analysis showed that (1) *Andrena* from Ismailia (Eastern part of Egypt), contain six highly divergent mitochondrial lineages. The difference in the nucleotide sequence occurred naturally due to various causes of intrinsic and extrinsic variations. These variations

called mutations or reshuffling of existing genes during sexual reproduction (Gregory, 2009) resulting in changes in the genome and consequently the formation of new species or sub-species. The different sequences between selected *A. ovatula* are only 3, 6 and 4 for populations from Minoufia (Nile Delta), Ismailia (Eastern part of Egypt), and Alexandria (Western part of Egypt), respectively. Moreover, some genetic variation of any species displays undergoing genetic variation, a process which can be driven by ecological, evolutionary or historical factors (Oliveira *et al.*, 2004). Also, our results suggest that *A. intermedia* is newly recorded from Egypt around river Nile and western part as shown in Table (2). Although some bee fauna were studied extensively morphologically in some regions but the discovery of new species and new records of bees is still running in the Palaearctic region unclear (Shebl *et al.*, 2016; Engel *et al.*, 2017; Shebl, 2017; Shebl *et al.*, 2018).

We recommend further genomic and proteomic studies to support the results of this study and to confirm the status of *A. intermedia* and *A. ovatula*. This information indicates that COI will provide more help to unify the above concept. A further study incorporating more genetically markers is needed to substantiate the findings of this research work. This study will contribute to increase the awareness, importance and effectiveness of using DNA barcodes in species level identifications of Egyptian bees. This protocol has been used successfully in several cases of bees and revealed some cryptic species of some bee groups (Carman and Packer, 1996; Pederson, 1996; Danforth *et al.*, 1998; Zayed and Packer, 2007; Rehan and Richards, 2008; Schaefer and Renner, 2008; Sheffield *et al.*, 2009). Here, with our recent finding, probably the species of *A. ovatula* identified morphologically has some species complex and more species of the same subgenus could be discovered. So, with more sampling efforts from several countries the speciation and subspeciation of this group might be clarified.

Conclusion

The Middle East, including North Africa and Saudi Arabia, is a very rich area with bee fauna. Although many species of Andrenids are known in Egypt as well as in North Africa and Saudi Arabia, but still there is limited data and lack of information about Andrenidae family. Therefore, more sampling efforts might help to clarify the status of *A. ovatula* complexity. Based on the results presented in this study, *A. intermedia* is a newly recorded bee species in Egypt based on CO1 which was identified previously as *A. ovatula* based on morphological characters. So this research will encourage some similar studies on bees in Egypt and other countries such as Saudi Arabia.

Acknowledgments

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