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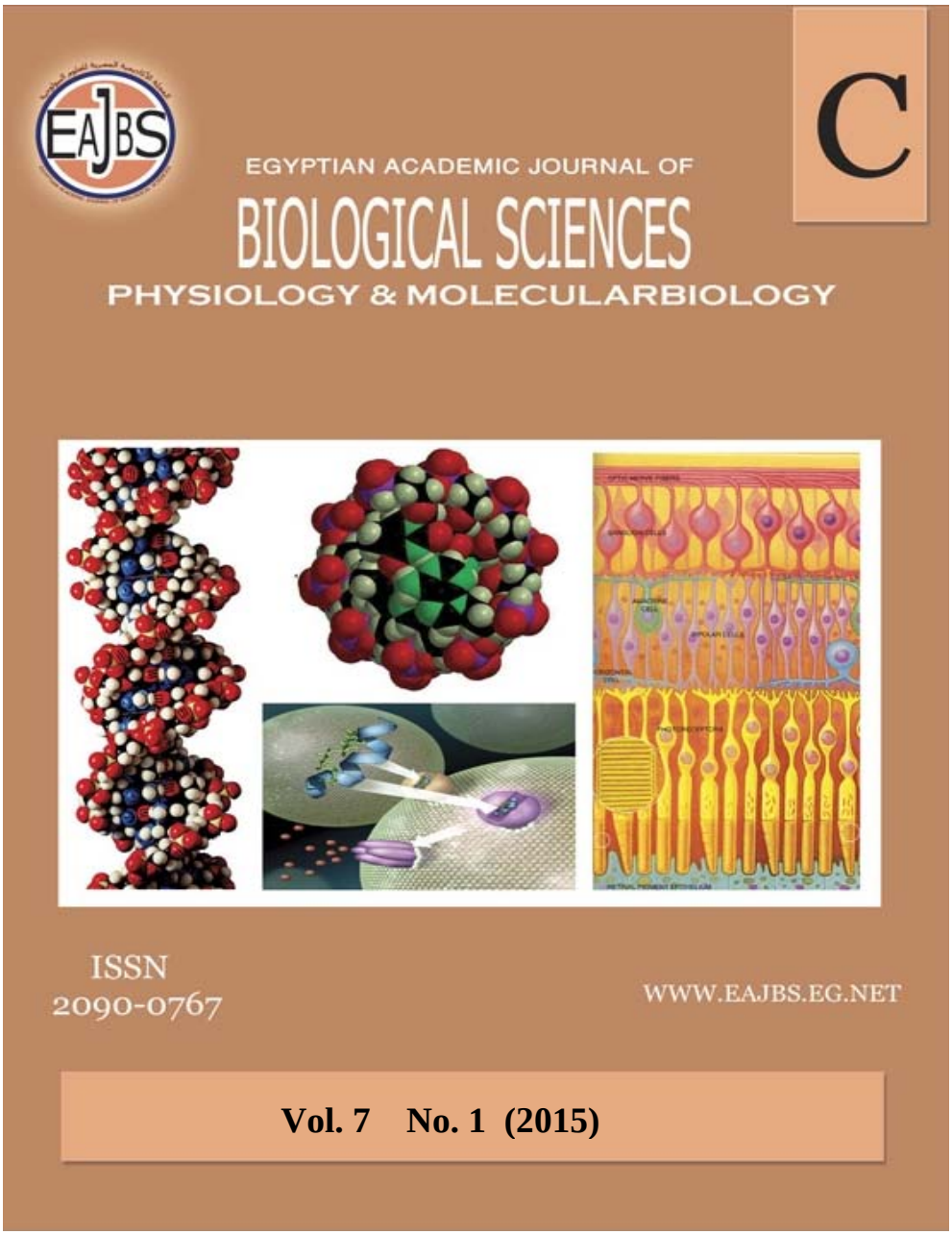

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Molecular phylogenetic relationship among three *Acanthodactylus* species (Reptilia: Lacertidae) in Egypt based on 18S rRNA Sequences

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ABSTRACT

Acanthodactylus is a genus of lacertid lizard, commonly referred to as fringe-fingered lizards or fringe-toed lizards. The systematics of the genus *Acanthodactylus* was classically based on external morphological traits, osteological characters and morphology of the hemipenes. In this study, partial sequences of 18S rRNA gene were used to estimate the relationships among three lacertide species from Egypt: *Acanthodactylus boskianus, Acanthodactylus scutellatus and Acanthodactylus longipes*. These data were used together with morphological information, to evaluate the relationships among these three species. DNA was extracted from the collected specimens, 988 bp fragment of 18S rRNA was amplified using universal primers and sequenced. The obtained sequences were analyzed and a phylogenetic tree was constructed. Results of analysis revealed that *A. scutellatus* and *A. longipes* form a very well supported clade with 86% bootstrap. *A. boskianus* was found to constitute a separate clade. The resultant tree based on DNA sequence data was similar to that produced from a differentially weighted data set of morphological characters. In conclusion, the 18S rRNA gene sequence seems to be a good marker for phylogenetic analysis of *Acanthodactylus* species.

INTRODUCTION

Lizards of the family Lacertidae are widely distributed throughout most of Eurasia and all of Africa. Presently, approximately 259 species of lacertid lizards have been assigned to 24 genera (Fu, 1998). The genus *Acanthodactylus* Fitzinger, 1834, is commonly known as the fringe-fingered lizards and is the largest genus in the family Lacertidae with over 40 described species (Uetz, 2013) and 36 species listed in the EMBL Reptile Database (http://www.embl-heidelberg.de/~uetz/ LivingReptiles.html) Lizards of this genus constitute an important part of the vertebrate fauna in many arid ecosystems in the Middle East and North Africa (Nouira *et al*., 1994, Shenbrot *et al*., 1997), where they are often the most conspicuous diurnal reptiles. As such, they have been the subjects of many ecological (Aljohany *et al*., 1989, Mellado *et al*., 1991, Mellado *et al*., 1992, Perry *et al*., 1994, Belliure *et al*., 1996) and behavioural (Perry *et al*., 1990, Day *et al*., 1999, Sword *et al*., 2000) studies.

Most of the *Acanthodactylus* species inahbit the southern side of the Mediterranean basin and the Arabian Peninsula. One species reaches Europe (Iberian Peninsula), and the genus range extends east to western India and south to the Sahel zone in Africa (Salvador, 1982). Four fundamental studies constructed available systematic knowledge of *Acanthodactylus*, based on external morphology, osteological characters, and the morphology of the hemipenes (Boulenger, 1918, Salvador, 1982, Arnold,1983, and Harris *et al*, 2000). The latter three studies divided the genus into species groups, a division that is commonly used today, although the assignment of some species to groups is debated (*Acanthodactylus blanfordii* Boulenger, 1918, and *Acanthodactylus masirae* Arnold, 1980, Harris *et al*., 2000). The systematics of some species groups is unclear and unstable because of high intraspecific variability of some species and morphological convergence among species (the description of *Acanthodactylus mechriguensis* Nouira *et al*., 1999, Fonseca *et al*., 2008). The distinction among the species within *Acanthodactylus* using superficial features is taxonomically difficult. This is due to the considerable variation in the range of external characters such as body size, size of dorsal scales and absence of enlarged dorsal tubercles when present, their number, size and shape and other morphological characters (Tamar *et al*, 2014).Thus, the most problematic and interesting issues in *Acanthodactylus* systematics are the relations amongst and within species groups, the taxonomy of the genus, and its biogeography.

Acanthodactylus boskianus is the most widespread species of its genus (~8 000 000 km^2; S. Meiri, unpublished data), ranging throughout North Africa and the Sahel, the whole Arabian Peninsula, eastwards to Iran, and northwards to Turkey (Salvador, 1982, Schleich *et al*., 1996, Rastegar *et al*., 1999, Sindaco *et al*., 2000, Sindaco *et al*., 2008). The only molecular phylogenetic study on the entire *Acanthodactylus* genus, however, was published by Harris & Arnold (2000), who suggested that the *A. scutellatus* group includes *A. scutellatus* and *A. longipes*, while *A. boskianus* constitute a separate group. The *A. scutellatus* group is a monophyletic clade consisting of several sand adapted species found in North Africa and northern Arabia. *Acanthodactylus longipes* was described as a variety of *A. scutellatus*. It was first recognized as a full species by Bons & Girot (1962), based on its sympatric occurrence with other members of the *A. scutellatus* group in various parts of its range. The specific status of *A. longipes* is now widely acknowledged (Salvador 1982, Arnold 1983, Crochet *et al*., 2003). Baha el din (1994, 1996) was first reported the species from Egypt, which had been previously overlooked because of its close similarity to *A. scutellatus*. Subsequently other authors adopted this designation for relevant populations in Egypt and Israel (Saleh 1997, Werner 1998, Bouskila & Amitai 2001). The previous molecular studies on *Acanthodactylus* systematic were based on mitochondrial genes (e.g. 12S, 16S and cytochrome b) and nuclear genes as in Tamar *et al*.2014. In this study, we used 18S rRNA for the phylogeny of *Acanthodactylus* species. In fact, Ribosomal RNA is considered as the best target for studying phylogenetic relationship because, it is universal and is composed of highly conserved as well as variable domains (Woese *et al*., 1987, Goerge *et al*., 1977). The ribosomes consist of rRNA and proteins. In all organisms the ribosome consists of two subunits, the small ribosomal subunit (SSU) contains a single RNA species (the 18S rRNA in eukaryotes and the 16S rRNA in others). Moreover, rRNA genes

are evolving more slowly than protein encoding genes and are particularly important for the phylogenetic analysis of distantly related species (Moritz *et al*. 1987).

In order to clarify the systematics and to reveal the phylogenetic relationships between *A. boskianus, A. scutellatus and A. longipes* in Egypt, fragments of ribosomal gene 18S rRNA (18S) is herein sequenced and analyzed for genetic variation. This study introduced the 18S rRNA gene sequence as a good marker for phylogenetic analysis of *Acanthodactylus* species in addition to the morphological characters.

MATERIALS AND METHODS

Specimens:

A total of 40 specimens belonging to the three *Acanthodactylus* species; *A. longipes, A. boskianus,* and *A.* scutellatus were collected from Marsa matruh, Baltim and North Sinai respectively were used in this study (Fig.1). Species as well as collection date and locality for each specimen were recorded. In the laboratory, sex of each of the collected specimens of each species was determined on the basis of morphologic criteria given by Schleich *et al*. (1996). In some cases sex was ascertained by opening the abdomen and checking the gonads.

Morphological analysis:

Morphological traits examined consist of mensural and meristic characters and are summarized in Fig.2. Mensural measurements were taken conventionally using a mm ruler and calipers. Spss 17.0v software was used to assess and compare the taxonomic distances between the three species.

DNA Extraction:

Genomic DNA from specimens of the three Egyptian *Acanthodactylus* species was extracted using a DNeasy blood and tissue kit (QIAGEN) for purification of total DNA from animal tissues (Spin-Column Protocol) following manufacturer's instruction. DNA was then eluted in 200 µl Tris-EDTA (TE) buffer. The quality of the genomic DNA was examined on 0.8% agarose gels stained with ethidium bromide (10µg/ml) (Sambrook *et al*. 1989) and OD260 was determined by Nanodrop.

Amplification of target fragment:

PCR primers pair designated 18S rRNA F **5'-TTAAGCCATGCATGTCTAAG-3'** and 18S rRNA R **5'-GACTACGACGGTATCTAATC-3'**, synthesized by Metabion International AG (Martinsried/Deutschland) were used. Polymerase chain reaction (PCR) with universal primers situated in the flanking regions of the target fragments was performed to amplify the fragments of interest. PCR was performed in a 25 µl volume of solution containing 12.5 µl Go Taq Green Master mix (promega), 2 µl of primer F, 2 µl of primer R (10pmoles), 3.0 µl of template DNA(100 ng) and completed with nuclease free water to reach the required volume. The PCR reaction was performed in a mini thermocycler (Techgene) using the following cycling protocol: initial denaturation at 94^{o}C for 2 min. and then 35 cycles of 94^{o}C for 30 sec, 52^{o}C for 30 sec, and 72^{o}C for 3 min. Final extension was carried out at 72^{o}C for 10 min. Amplified products from the PCR were electrophoresed on 1.5% agarose gels stained with ethidium bromide. A 100 bp ladder (Jena Bioscince, GmbH, Germany) was loaded in each gel then photographed under UV light with a gel documentation system.

Sequencing of 18S rRNA Gene Products:

Each of PCR-product was purified with QIAquick-spin PCR purification kit (Qiagen,Germany). A second PCR was then performed using BigDye Terminator v3.1 cycle sequencing Kit, for a total volume of 20 µl, with each reaction contained 8 µl Terminator ready reaction

mix, 3.2 pmol primer, DNA template (template quantity was calculated according to the PCR product size) and deionized water. Thermal profile for Cycle Sequencing PCR was 1 min at 96°C, 25 cycles (10 sec. at 96°C, 5sec. at 50°C, 4 min at 60°C). After additional step of purification with CENTRI-SEP Columns (Prenciton Separation), DNA sequencing was applied by 3500 genetic analyzer, Applied Biosystems. At least two independent PCR products were used for sequencing per species.

Sequences Analysis:

Chromatograms and sequences were analyzed and edited using BioEdit program®, version 7.2.5 (Hall 1999). The obtained DNA sequences were aligned using Basic Local Alignment Search Tool software [BLAST, version 2.2.22 (http://blast.ncbi.nlm.nih.gov). All new DNA sequences reported in this paper were deposited in GenBank sequence database provided by the National Center for Biotechnology Information (accession numbers; KR054729, KR054730, KR054731, KR054732, KR054733). Multiple alignment analysis was performed with CLUSTALW computer program (Thompson *et al* 1994) in MEGA6 software (http://www.megasoftware.net).

Phylogenetic construction:

UPGMA and Maximum Parsimony trees were constructed using MEGA6 software (http://www.megasoftware.net). In order to improve the homology statements, outgroup (*Mesalina guttulata*) (Accession number AY217917) was included by Basic Local Alignment Search Tool (nBLAST) (www.ncbi.nih.gov/BLAST/) in the NCBI database (National Center for Biotechnology Information, NIH, Bethesda, Maryland, USA) (Tatusova and Madden 1999). Bootstrap analysis employed 500 replicates. For the 18S rRNA gene, the JC (Jukes-Cantor) + G (gamma) model of nucleotide substitution was determined as the most appropriate model according to Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC) (Nei and Kumar 2000). The analysis involved 4 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 954 positions in the final dataset. Genetic distances were created in MEGA6 software (http://www.megasoftware.net).

PCR Products of 18S rRNA gene and sequence data of the *Acanthodactylus* species:

PCR products were amplified from ribosomal gene using synthesized primers set. Across the alignment of 18S rRNA, long region was suitably conserved to allow primer design. The PCR products were approximately 1000 bp for 18S rRNA gene of *A. boskianus*, *A. scutellatus*, and *A. longipes* (Fig. 4).

RESULTS

Morphological analysis:

Differences and similarities among these species are assessed using phenogram analysis based on the morphological taxonomic characters. Phenogram (Fig. 3) shows differences and similarities between three species of *Acanthodactylus*. The phenogramp clearly shows the morphological similarity, as indicated by the short taxonomic distance between the two closely related species *A. scutellatus* and *A. longipes*, and a longer distance between these and *A. boskianus*.

The data set of this study is comprised of 4 sequences of *A. boskianus, A. scutellatus*, *A. longipes* and the outgroup (*Mesalina sp.*). The data set included ribosomal gene fragments of 18S (~961 bp). The number of variable (V) sites were 30, parsimony-informative (Pi) sites were 8, conserved sites were 928 (Fig. 5). Nucleotide composition were T (22.7%), C (25.3%), A (24.0%) and G (28.1%). The Overall average of genetic distance

was 0.019. The level of genetic distance (p-distance) in the 18S rRNA between species is shown in Table 1. The table shows a genetic distance of 1.4% between *A. scutellatus* and *A. longipes*, 2.0% between *A. boskianus* and *A. longipes* and and 2.3% between *Acanthodactylus sp*. and the outgroup.

Phylogenetic construction:

The above analysis resulted in a topology with a mean log-likelihood values lnL= -1610.894, BIC = 3271.294, AICc = 3233.809, nucleotide frequencies: f (A)= 0.250, f (C)= 0.250, f (G)= 0.250, f (T)= 0.250. Our phylogenetic analysis using Maximum Parsimony method distinguishes two major clades (Fig. 6): clade (A) comprises *Acanthodactylus sp.*, clade (B) constitutes outgroup (*Mesalina sp*.). In clade (A), *A. scutellatus* and *A. longipes* form a very well supported clade (high value of bootstrap (86%). While *A. boskianus* constitutes a separate clade. The three Egyptian *Acanthodactylus* gathered in one cluster forming one clade separated from the lacertidae outgroup. Egyptian *A. boskianus* is clearly genetically distinct from other two species. Furthermore, UPGMA method resulted in the same phylogeny as in Maximum Parsimony method, with *A. scutellatus* and *A. longipes* being more genetically related to each other than to *A. boskianus*.

Table 1: Average uncorrected genetic distances (p-distance) between species clades of *Acanthodactylus* from Egypt based on 988 bp fragment of 18S rRNA.

A .boskianus			
A .longipes	0.020		
A. scatellatus	0.015	0.014	
Mesalina guttulata (outgroup)	0.018	0.023	0.023

DISCUSSION

Classical taxonomy relies primarily on morphological characteristics to elucidate the phylogenetic relations among organisms. Recently, the molecular approach based on comparing nucleotide sequences of RNA and DNA and sequences of amino acids of protein open a new era in phylogenetic analysis. Both the classical morphology-based methods and molecular taxonomy methods are of importance as the basic bio-molecular framework of all organisms are similar and morphology of an organism is actually the manifestations of its genome, proteome and transcriptome profiles. A combination of the morphological based methods and molecular analysis based methods, thus strengthens the exercise of the determination of phylogenetic relationships of organisms to a great extent (Patwardhan *et al*. 2014)

In conventional, morphology-based *Acanthodactylus* systematics is fairly easy to assign species to species groups. However, the boundaries between species and relationships within species groups are often unclear and unresolved (Salvador, 1982, Arnold, 1983, Harris *et al.,* 2000, Crochet *et al.*, 2003, Harris *et al.*, 2004, Fonseca *et al*., 2008, 2009). These variations make it hard to construct clear species identification keys. In *Acanthodactylus*, as in many animal groups, molecular phylogenetic analysis offers a decisive method to distinguish species and their taxonomic relations. Our results, based on 18S rRNA data reveal that *A. scutellatus and A. longipes* are monophyletic and *A. boskianus* was genetically distinct from other species used in this study, with *A. boskianus* forming a separate clade.

The 18S rRNA molecule of the ribosomal small subunit is frequently used to infer phylogenetic affiliations of

ancient (>100 Mya) eukaryotic relationships. Its suitability as a phylogenetic tool is two-fold. First, it is a good source of phylogenetic information based on conservation of function, variable mutation rates depending on substructure position, and its ubiquity in all taxa. Second, the molecule provides readily obtainable nucleotide sequences because of high rRNA transcript copy number in eukaryotes and ease of PCR primer design (Woese, 1987Kjer, 1995). The 18S rRNA molecule contains certain stable sections having low substitution rates. As such, 18S rRNAs can provide informative characters for assessing affiliations of evolutionarily distant taxa. However, 18S rRNAs also possess variable regions having high substitution rates. In some organisms these rapidly evolving expansion regions (or helices) provide phylogenetic signals for discerning relationships between evolutionarily closer clades. Calculated genetic distance among the three studied species provide a molecular support to conventional phylogenetic relationship among *Acanthodactylus sp.*

CONCLUSION

Molecular data provide independent evidence that the differential weighting of morphological characters in past analyses was appropriate, where *A. longipes* and *A. scutellatus* are monophyletic group. The 18S rRNA gene sequence is shown to be a good marker for supra-specific differentiation and for taxa grouping purposes. However, additional species and/or additional genes need to be studied for assessment of the true placement of all the *Acanthodactylus* species within Egypt.

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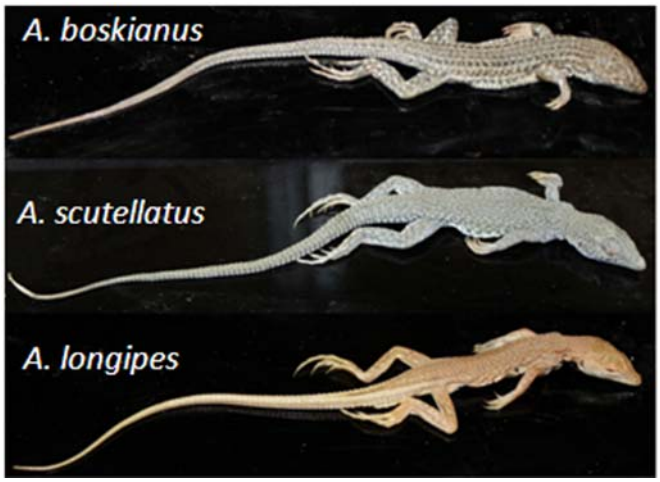

Fig. 1: General view of three *Acanthodactylus sp*. from Egypt.

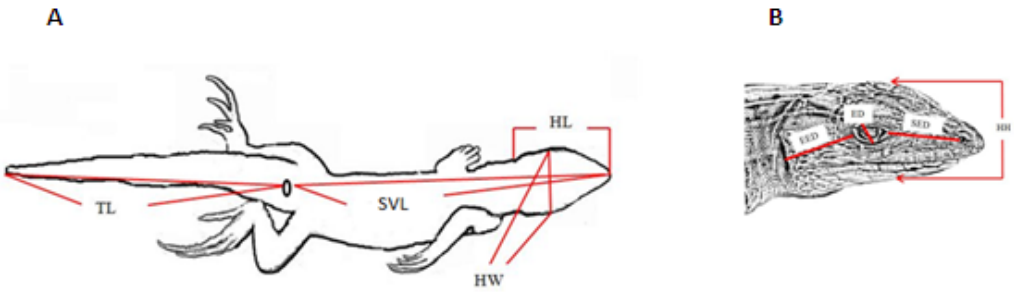

Fig. 2: Morphological characteristics used for the identification of *Acanthodactylus* species. A. Body measurements; Example of the snout to vent length (SVL) measurement. B. Head measurements.

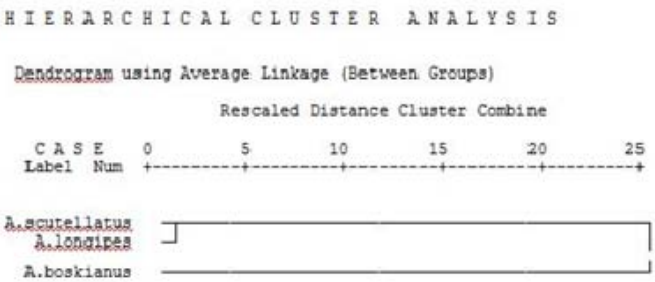

Fig. 3: A phenogram showing the average taxonomic distance (dissimilarity) between three species of *Acanthodactylus* from Egypt.

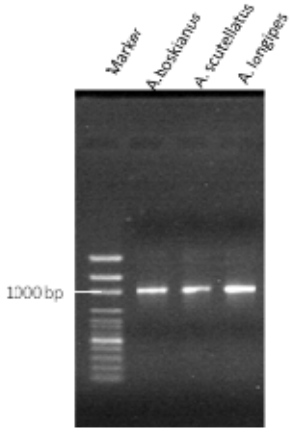

Fig. 4: PCR products (~1000bp fragment) of 18s rRNA gene amplified by universal primers pair from Egyptian lacertides of *Acanthodactylus boskianus*, *Acanthodactylus scutellatus*, *Acanthodactylus longipes* and 100 bp DNA Ladder Marker.

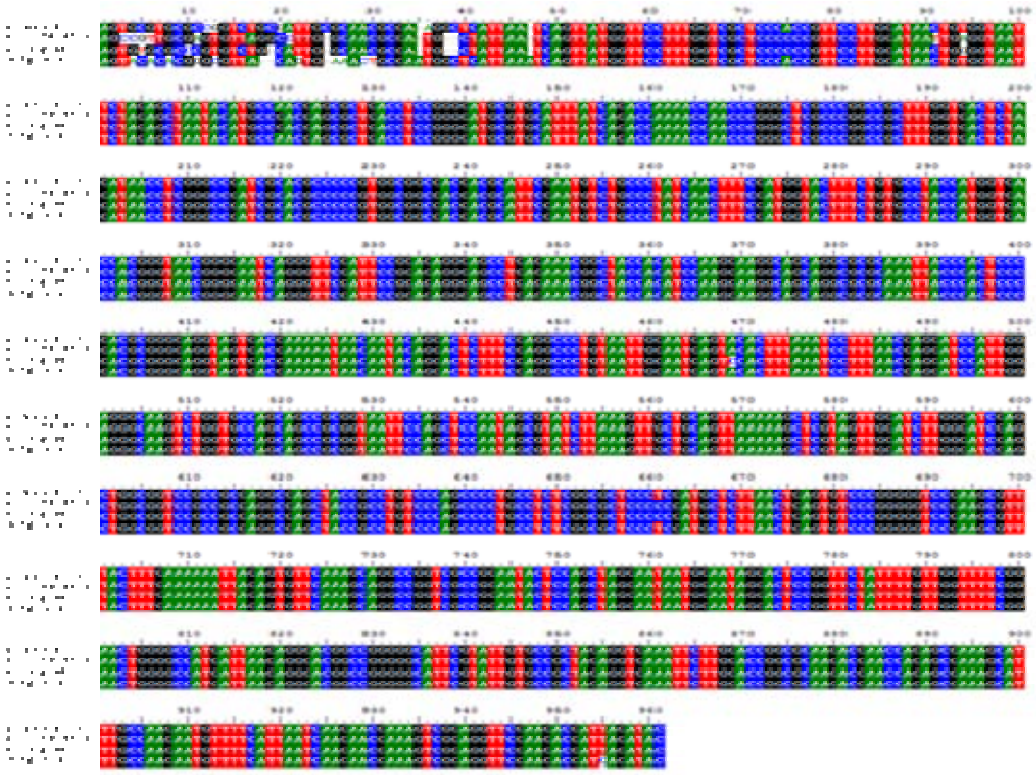

Fig. 5: Nucleotides multiple alignment of partial 18S rRNA gene sequences of three Egyptian *Acanthodactylus*. Colored columns represent identical nucleotide sequences between aligned isolates.

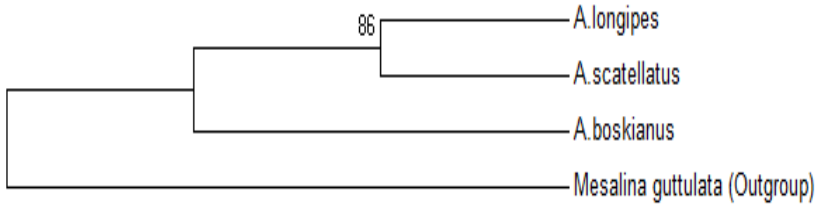

Fig. 6: Rooted phylogenetic tree among the three Egyptian *Acanthodactylus sp*. based on ribosomal gene (18S rRNA) using Maximum Parsimony method.