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# Molecular Identification and Evolutionary Relationship of the New Record *Callistethus sp.7VF-2014* (Coleoptera:Scarabaeidae:Reutelinae) in North of Iraq

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**Abstract**

A total of 15 specimens of the *Callistethus sp.7VF-2014* were collected from different parts in Duhok Governorate during period extended from May to July 2015. The present study was done for molecular identification and evolutionary relationships according to DNA sequence and RNA secondary structure of *Callistethus sp.7VF-2014*. The partial nuclear 28S ribosomal (rDNA) sequence (365 bps) of this species was amplified with universal primers and sequenced by monitoring genetic analyzer. Molecular identification and phylogenetic relationships were carried out using 28S rDNA region. The outcome demonstrated that the query sequence was more than 98% identical to this species of the insect. The phylogenetic tree revealed 96- 98% relationships of the sequence of *Callistethus sp.7VF-2014* and 28S rDNA regions for other Gen Bank stored species of *Callistethus*. Likewise, the present evolutionary finding was affirmed by the molecular morphometrics, according to the secondary structure of 28S rDNA region. The topology investigation created same information as the gained tree. In conclusion, DNA sequence analysis recorded the species *Callistethus sp.7VF-2014* for the first time in Duhok City, Kurdistan region, Iraq. Phylogenetic evolutionary as indicated by both; DNA primary sequence and RNA secondary structure can be considered as a valuable tools for separating species of *Callistethus*.

**Keywords:** *Callistethus sp.7VF-2014*, 28S rDNA, molecular identification, phylogeny, molecular morphometric.

## 1. Introduction

The genus *Callistethus* was proposed by Blanchard (1851). Potts (1974) considered *Callistethus* as a synonym of *Anomala* when working on Neotropical Anomalini. Morón (1997) listed six Mexican species of Anomalini as members of *Callistethus*. However, in phylogenetic studies in view of species of 18 genera of Anomalini from around the globe supported the monophyly of *Callistethus* permitting another recognizable proof for it (Ramírez *et al.*, 2009). The monophyly of the genus *Callistethus* and its definition in relation to the genus *Anomala* had been widely questioned, but no consensus has been reached (Jameson *et al*., 2003).

Based on the checklist by Krajcik (2007), the genus *Callistethus* comprises 142 species distributed in Asia (85 species) and America (57 species), although their real diversity is probably much higher, included only one species from Nepal, *C. consularis* Blanchard, 1851. Since then, a few other Asiatic species have been described in this genus, such as *C. stoliczkae* Sharp, 1878, *C. seminitidus* Fairmaire, 1889, *C. pterygophorus* Ohaus, 1903, and *C. umidicauda* Arrow, 1912. Ramírez-Ponce and Morón, (2012) described a new species *Callistethus tlapanecus*, in eastern mountains of the Guerrero, Mexico.

Based on molecular data, only broader studies not focused on Anomalini (García *et al*., 2013 and Ahrens *et al*., 2014). Uses of molecular markers in the phylogenetic studies of various organisms have become increasingly important in recent times. Although widely practiced even now, traditional morphology based systems of classification of organisms have some limitations. On the other hand, it appears that the use of molecular markers, though relatively recent in popularity and are not free entirely of flaws, can complement the traditional morphology based method for phylogenetic studies (Patwardhan *et al*., 2014). Filippini *et al.* (2015) carried out another phylogenetic analysis of *Callistethus* and *Anomala* species, based on sequences of one nuclear (a

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fragment of 28S) and two mitochondrial (16S and COI) genes, described 11 new species from the revision of the genus *C. allistethus, C. carbo*, *C. flavodorsalis*, *C. fuscorubens, C. lativittis, C. levigatus*, *C. macroxantholeus*, *C. microxantholeus*, *C. multiplicatus*, *C. parapulcher*, *C. pseudocollaris* and *C. stannibractea*, and also propose a new synonym, new combinations, from Costa Rica.

28S rRNA gene sequence for many major metazoan groups has become available in the recent years. Also, efforts to align sequences according to the secondary structure model for 28S rRNA of these organisms have become valuable for the purpose of phylogenetic evolutionary analyses (Manzari *et al*., 2002 and Schmidt *et al*., 2006).

*Callistethus sp.7VF-2014*, recently identified as a new species, was described in National Center for Biotechnology Information (NCBI) (*https://www.ncbi.nlm.nih.gov/Taxonomy/Browser /wwwtax.cgi?lvl=0&id=1447034*) and Universal Protein Resource (UniProt) (*http://www. uniprot. org/taxonomy/1447034*). In the present study, the first objective is to confirm molecularly the presence of *Callistethus sp.7VF-2014* in Kurdistan region, Iraq. This goes more specifically to describe the molecular identification of *Callistethus sp.7VF-2014* using 28S rDNA sequence. The study was also set out with the aim of performing evolutionary development with other species of the *Callistethus* and to do the secondary structure modeling as a support of the primary molecular finding.

## 2. Material and Methods

### *2.1. Study Area and Sample Collection*

Fifteen specimens of the *Callistethus sp.7VF-2014* collected from different parts in Duhok Governorate by light traps from Zawita 19/5 /2014, Bamarne 22/5 /2014, Sarsank 15/6 /2014, Swartka 21/6/2014 and Sarke 25/6/2014


**Figure 1.** Map of Iraq showing Duhok city and the red color points showing exact location of samples.

### *2.2. Extraction, Amplification and Sequencing of DNA*

The genomic DNA of *Callistethus sp.7VF-2014* samples was obtained by employing AccuPrep® Genomic DNA Extraction Kit (BIONEER, KOREA) according to the manufacture's instruction with few modifications (incubation time of tissue lyses step was extended into 3 hours and utilized absolute ethanol instead of isopropanol for DNA precipitation). The samples were macerated in mortar and pestle, and the contents were transferred into sterile tube contain 200 µl tissue lysis buffer and kept in incubator at 60 ˚C for 3 hrs. Qualification and quantification of DNA concentration was determined by using NanoDrop (ND- 1000, USA).

The targeted region of partial 28S rDNA was amplified by PCR using universal primers, forward primer C1 (5′-ACC CGC TGA ATT TAA GCA T-3′ at position 25), and the reverse primer C3 (5′-CTC TTC AGA GTA CTT TTC AAC-3′ at position 390), they were designed and selected by (Mollaret *et al*., 2000). A total of 50 µl volume of reaction mixture was prepared to contain 2 µl DNA template, 25 µl OnePCR$^{TM}$ master mix (GENEDIREX, KOREA), 1 µl for each primers and 21 µl double deionized water (dd$H_2O$) in a thermocycler MJ Research, Applied Biosystem (AB). The cycling profile consisted of an initial denaturation step of 5 min at 94˚C followed by 35 cycles at 94˚C, 45 sec. at 51˚C, 45 sec. at 72˚C 45 sec., and final extension 5 min at 72˚C. The PCR products were run in 2% agarose gel electrophoresis. Bands stained with SYBR green were visualized on an UV transilluminator using 100 bp ladder (GENEDIREX, KOREA), expected size of the PCR amplicon was 365 bps.

DNA sequencing, using both forward and reverse primers was performed separately by ABI 3130X genetic analyzer (SINGAPORE). The PCR fragments of the *Callistethus sp.7VF-2014* were excised from the agarose gel and used as a source of DNA template for sequence specific PCR amplification.

### *2.3. Sequence and Structure-Based Phylogeny*

Muscle multiple sequence alignment program (http://www.drive5.com/muscle/) was employed to compare consensus sequences with the neglectful gap and expansion penalties. The results of 28S rDNA regions were entered in the MEGA 6.0 program (http://en.bio-soft.net/tree/MEGA.html) for constructing the evolutionary developmental trees. The phylogenetic tree of the *Callistethus sp.7VF-2014* was constructed using character state method (maximum likelihood). Branch support was given, employing 1000 bootstrap replicates. Secondary structures of 28S rDNA regions of *Callistethus sp.7VF-2014* were expected by the online MFold package (version 3.5) (*http://unafold.rna.albany.edu/?q=mfold*). MFold is the broad employed algorithms for secondary structure of RNA expectation that are dependent on a search for the minimal free energy state.

## 3. Results

### *3.1. Molecular Identification*

The expected amplicon size for 28S rDNA of *Callistethus sp.7VF-2014* is supposed to be 365 bp (Figure 2), but the results obtained from sequencing were only 349 bp (missing 19 bp due to the quality of amplified sequence). BLAST program from Gen bank (*http://blast.ncbi.nlm.nih.gov/*) was used to compare our amplified sequences with other stored species of *Callistethus* sequences. The results got from the BLAST indicated that the query sequence was more than 98% identical to *Callistethus sp.7VF-2014* (Figure 3).

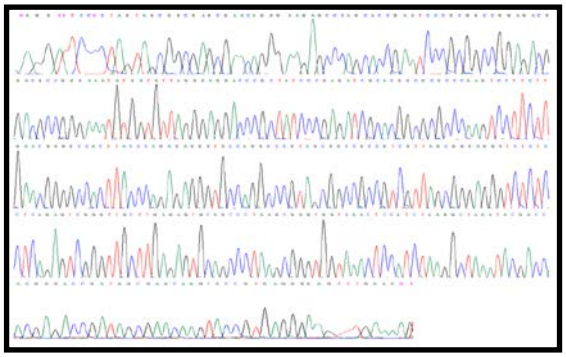

**Figure 2.** The chromatography sequence result of 28S rDNA sequence of *Callistethus sp.7VF-2014*

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Query  1    AACTAACTAGGATTCCCCTAGTAGCGGCGAGCGAACAGGGAAGAGCCCAGCACCGAATCC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20   AACTAACTAGGATTCCCCTAGTAGCGGCGAGCGAACAGGGAATAGCCCAGCACCGAATCC  79

Query  61   CGCGGCCGGTTGACGGACGCCGGGAAATGTGGTGTTAGGGAGGACCCGCTATCCCGAGAT  120
            ||||||||||||||||||||||||||||||||||||||||||||| |||| ||| |||||||
Sbjct  80   CGCGGCCGGTTGACGGACGCCGGGAAATGTGGTGTTAGGGAGGATCCGCCATCTCGAGAT  139

Query  121  CGCACGGCGCGCCCAAGTCCTTCTTGAACGGGGCCACTTACCCAGAGAGGGTGCCAGGCC  180
            || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  140  CGTTCGGCGCGCCCAAGTCCTTCTTGAACGGGGCCACTTACCCATAGAGGGTGCCAGGCC  199

Query  181  CGTAGTTGGCCGCCGTCGTTAGCGGGAGGGTCTCTCCTCAGAGTCGGGTTGCTTGAGAGT  240
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  200  CGTAGTTGGCCGCCGTCGATCGCGGGAGGATCACTCCTCAGAGTCGGGTTGCTTGAGAGT  259

Query  241  GCAGCCCTAAGCGGGTGGTAAACTCCATCTAAGGCTAAATACGACCACGAGACCGATAGC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  260  GCAGCCCTAAGCGGGTGGTAAACTCCATCTAAGGCTAAATACGACCACGAGACCGATAGC  319

Query  301  GAACAAGTACCGTGAGGGAAGTTTGAAAAG  330
            |||||||||||||||||||  ||||||||
Sbjct  320  GAACAAGTACCGTGAGGGAAAGTTGAAAAG  349
```

**Figure 3.** Pairwise alignment of 28S rDNA sequence of *Callistethus sp.7VF-2014*. Query is the study or sample sequence and Sbjct is the GenBank sequence

### *3.2. DNA Sequence Based Phylogeny*

Phylogenetic tree according to primary sequence showed 96-98% relationships in comparing the sequence of *Callistethus sp.7VF-2014* and stored 28S rDNA regions for the other species of *Callistethus*. Phylogenetic analysis employing the diverse method, maximum likelihood method as mentioned in Figure 4. It was revealed and indicated that the topology was the same among acquired trees with significant bootstrap support for the clades. For the bootstrap analysis, the values of 70% and above represented the accuracy of evolutionary development and showed reliable grouping among various species of *Callistethus*. The tree topologies according to phylogenetic analysis showed *Callistethus sp.7VF-2014* as molecularly closely related with *Callistethus sp*. 6VF-2014 and *Anomala sp*- 206 species (Figure 4).

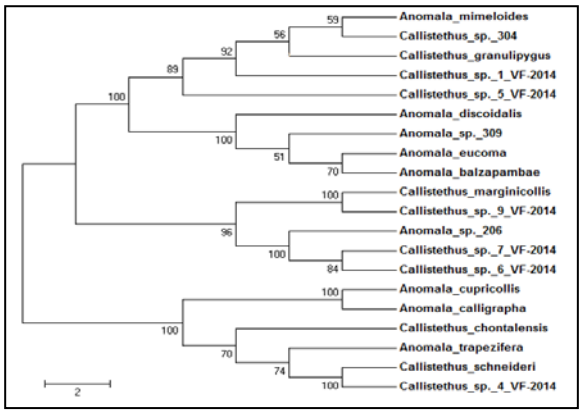


**Figure 4.** Phylogenetic positioning of *Callistethus sp.7VF-2014* according to sequences of 28S rDNA employing maximum likelihood method, (Tamura 3-parameter model with invariant sites)

### *3.3. RNA Structure-Based Phylogeny*

Predict 28S rDNA secondary structural properties with the highest negative free energy ΔG= -131.60 kcal/mol of *Callistethus sp.7VF-2014* to provide the principal information for evolutionary developmental analysis (Figure 5). The secondary sequence properties of 28S rDNA structure, as represented in the figure, were analyzed according to loops and conserved stems. In the structure of *Callistethus sp.7VF-2014* the loops arrangements exhibit according to their numbers and classified into: interior loop, hairpin loop, bulge loop, multi loop and exterior loop (Table 1). The topology was only based on the expected RNA secondary structure of 28S rDNA region which determined most associations among the species researched.

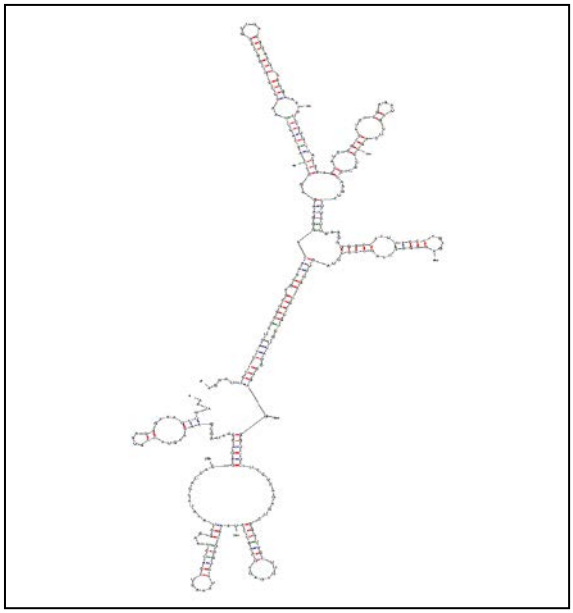

**Figure 5.** Schematic representation of the 28S rRNA expected secondary sequence of *Callistethus sp.7VF-2014*

**Table 1.** Arrangement of various loops of 28S rDNA sequence region of *Callistethus sp.7VF-2014*

| Type of loops | Number of loops |
|---|---|
| Interior | 8 |
| Hairpin | 6 |
| Bulge | 5 |
| Multi | 3 |
| Exterior | 1 |

## 4. Discussion

*Callistethus sp.7VF-2014* species genetically distinguished from the other available species of *Callistethus* previously identified with the same rDNA region marker, available at the GenBank in National Center for Biotechnology Information (NCBI). The primary sequence analysis using universal primers of study sample revealed that the insect belongs to species *Callistethus sp.7VF-2014* (Figure 3).

Phylogeny is the history of descent of a group of taxa such as species from their common ancestors including the order of branching and sometimes the times of divergence.

In many cases, evolution of nucleotide sequences is more or less affected by various kinds of constraints. In order to compensate for such factors and to estimate precise relationships among taxi, a large variety of phylogenetic technique has been devised (Simon *et al*., 1994 and Mindell *et al.*, 1996). For phylogenetic inference, the obtained data sequences were analyzed, using Maximum Likelihood method (ML) analysis.

Dendrogram in (Figure 4) shows how closely these species are related to each other's. The aim of producing a dendrogram is to visualize the best relationships of the phylogenetic (Evolutionary history) between the studied species *Callistethus sp.7VF-2014* and the other 19 species which belong to the same tribe: Anomalini, subfamily Reutelinae and family: Scarabaeidae. *Callistethus sp.7VF-2014*, *Callistethus sp.*6VF-2014 and *Anomala sp*. 206 species form monophyletic group, because they are descended from a single common ancestor. This result agrees with that of Ramírez *et al.* (2009) who supported the monophyly of the *Callistethus* species. Jameson *et al*. (2003) also supported monophyly of the genus *Callistethus* and genus *Anomala*. In another study, Filippini *et al*. (2015) recorded that the *Callisthethus* is subordinate in *Anomala* and thus most likely *Anomala* has either to be split in several genera or *Callisthethus* to be included in Anomala with increasing phylogenetic knowledge.

Furthermore, phylogenetic discoveries were affirmed utilizing secondary structure investigation as a tool of computational biology, RNA loops were used for amendment the alignment. Molecular morphometrics is expected to analyze phylogenetic relationships depend on similarities among some structural proprieties of folded nucleotide molecules (Bernard *et al.*, 2000). Molecular morphometrics has been suggested to be the most strengthened tool in comparison to classical primary structure analysis, due to the only consideration of the size variations for homologous structural segments, whereas molecular morphometrics representing the folding pattern of RNA molecule (Bernard *et al.*, 2000). The topology of the present study, based on the expected RNA secondary sequence of the 28S rDNA structures, revealed and identified most associations among the species studied; for instance the structure of *Callistethus sp.7VF-2014*, total number loops preference were 23 loops, types of the loops according to their numbers were exterior loop (1), multi loop (3), bulge loop (5), hairpin loop (6) and interior loop (8), respectively as presented in Figure 5 and Table 1. Incorporation of secondary structure information allows improved estimates of phylogeny among several *Callistethus* species.

In conclusion, DNA sequencing of the sample study revealed the first recording of *Callistethus sp.7VF-2014* in Duhok City, Kurdistan region, Iraq. Phylogenetic evolutionary as indicated by both, DNA primary sequence and RNA secondary structure can be considered as valuable tools for separating species of *Callistethus*.

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