



## PHYLOGENETIC ANALYSIS OF *Sphaerostris picae* (ACANTHOCEPHALA: CENTRORHYNCHIDAE) BASED ON LARGE AND SMALL SUBUNIT RIBOSOMAL DNA GENE

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**Abstract-** The purpose of the present study was to add new 18S and 28S DNA gene sequences data to *Sphaerostris picae* (Rudolphi, 1819) Golvan, 1960 and analyze the generated sequences to define the taxonomic placement of genus *Sphaerostris* and providing a better resolution inside the Palaeacanthocephala. Two regions: 18S and 28S of nuclear ribosomal DNA of *S. picae* were amplified using polymerase chain reaction and sequenced following the instructions of GATC German company facility. Malign module in the DNASTar Lasergene V7 was used to design a forward and reverse primer of 28S DNA gene. 18S and 28S DNA gene sequences of *S. picae* were aligned with sequences for both genes of Palaeacanthocephalans retrieved from GenBank. Results were analyzed using distance matrix methods UPGMA. The resulting phylogenetic trees suggest a paraphyletic arrangement of the two Palaeacanthocephala orders; Echinorhynchida and Polymorphida depending on the placement of the three echinorhynchids, *Transvena*, *Rhadinorhynchus* and *Gorgorhynchoides* in the polymorphid clade. The present study is the first to generate gene sequences of genus *Sphaerostris* and discuss its relationships within Palaeacanthocephala. Further comprehensive studies should be done for other species of genus *Sphaerostris* and family Centrorhynchidae as all based on molecular phylogenetic analysis to solve their taxonomic overlapping.

**Keywords-** *Sphaerostris picae*, Acanthocephala, Ribosomal DNA, Degenerate primer, Phylogenetic analysis

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### Introduction

Acanthocephalans have been related to Rotifera, Nematoda, Nematomorpha and Gastrotricha [1]. Most phylogenetic analyses of phylum Acanthocephala, based on structural and molecular data, have focused on its relationships to other invertebrate phyla, however studies deal with the inter-relations between different Acanthocephala families and species with uncertain taxonomy are still out of focus.

Verweyen, et al. [2] reported that the first molecular phylogenetic analyses of Acanthocephala made by Garey, et al. [3] confirmed the major taxonomic grouping of the traditional classifications. In this grouping, Palaeacanthocephala is placed close to the Eoacanthocephala, with the Archiacanthocephala being the most basal taxon. According to Herlyn, et al. [4], the monophyly of the Palaeacanthocephala is still a matter of debate. Consequently, the assumed phylogeny of taxa within the Acanthocephala varies, placing either archiacanthocephalans [1,5] or palaeacanthocephalans [6] at the base of the taxon.

Molecular data using 18S and/or 28S DNA gene sequences of family Centrorhynchidae are very limited and has indicated that the

classification based on the structure of the proboscis and shape and number of its hooks is not consistent with phylogenetic relationships [3].

Amin, et al. [7] reported that, since the erection of *Sphaerostris* (family Centrorhynchidae) as a subgenus of *Centrorhynchus* Lühe, 1911 [8], its taxonomy has been in a state of confusion and largely dependent on the use of proboscis armature, especially the number of proboscis hooks rows. This character has proven to be extremely variable through the genus, so a good number of synonymies were made. The authors accelerated that this genus is in need to a serious taxonomic revision that will be enhanced by different types of analyses based on the molecular criteria.

The purpose of the present study was to add new 18S and 28S DNA gene sequences data to *S. picae* as a member of the family Centrorhynchidae (Palaeacanthocephala). Such data may help in understanding and defining the taxonomic placement of genus *Sphaerostris* and providing a better resolution inside the Palaeacanthocephala. The phylogenetic relationships of *S. picae* were examined with 21 acanthocephalan species belonging to 9 related families that all are belongings to the class Palaeacanthocephala,

as inferred from partial 18S and 28S DNA sequences. The study used an algorithm ClustalW2 for designing degenerate primers which are of particular value in amplifying homologous genes from different organisms.

## Materials and Methods

### Specimen and DNA Isolation

*S. picae* was collected from the small intestine of the Hooded crow *Corvus corone cornix*, Linnaeus 1758, which is a common resident inhabiting cultivated land and wooded terrain in Nile Delta and valley in Egypt. For identification, worms were preserved in 70% ethanol, stained with Mayer's carmine, mounted in Canada balsam [9] and identified according to Dimitrova, et al. [10,11]. Specimens were stored at -20°C until nucleic acid was extracted. DNA extraction followed Chloroform-Isoamyl alcohol method [12]. Three replica each of one worm were homogenized on ice in TE buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM EDTA) and digested by adding 400µl of TE buffer, 40µl 10% sodium dodecyl sulphate (SDS) and 10µl of 20mg/ml proteinase K, then incubated in 55°C overnight. The supernatant was extracted twice with buffered phenol (pH 8.0) and once with chloroform/isoamyl alcohol (24:1). The aqueous layer (~ 400µl) was collected and 16µl of 5 M NaCl and 420µl ice cold isopropanol were added. The sample was cooled in the refrigerator at -20°C for 10 min and centrifuged for 5 min at 15,000 rpm. The supernatant was poured off and after complete drying of the pellet, 50µl of the TE buffer was added and left for 30 min to complete dissolving. The sample was stored at -20°C till use.

### Designing the Degenerate Primers of 28S DNA Gene

The DNA sequences of 28S DNA gene of 23 species accession numbers from AB500157.1 to AB500179.1 were retrieved from the sequence database of NCBI. FASTA file format of the previous sequences was used in the algorithm type of the Blast (Basic local alignment search tool). Expected values (E-values) was calculated to infer homology and distance of similarity between the sequences [13].

The mealign module implemented in the DNASTar Lasergene V7 used the Clustal W<sub>2</sub> algorithm for alignment collected sequences and produced the conserved blocks, which were used to design the degeneracy primers. All primer characteristics were adjusted. Primer Blast tool [14] was used to detect the homology between the designed primers and targeted templates in the GenBank database (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>), the position of degeneracy nucleotides, and the uniqueness for the forward and reverse primers.

The PCR insilico module (Ruslan- Kalendar version 4.0.8 university of Helsinki, Finland) was used to produce all the details of the PCR program such as the optimum temperature for the forward and reverse primer, the predict PCR product, and the positions of primers on the template sequences.

### DNA Amplification and Sequencing

Two regions of nuclear ribosomal DNA were amplified using polymerase chain reaction (PCR). The first is the 18S DNA using the primers:

forward.5' GGGCCGTAGACGACCAAGTGT -3' and reverse. 5'GGAAAGCGCTCGCCAAGTTATT 3' [2].

The second is 28S DNA gene using degenerate designed primers:

forward 5'GTAAIG CGGCGAGTGAAGTGGGAAGITA -3' and reserve 5'TAICACTCGGACTAGAA AIC C AIC CA 3'.

PCR mixture (50µl) contained 25µl Maxima Hot Start PCR Master Mix (Fermentas), 1µl final concentration of each primer, 17µl nuclease-free and 6µl of template DNA solution. PCR cycling parameter for 18S DNA gene included initial denaturation period at 95°C for 10 min, annealing at 58°C for 1 min and extension at 72°C for 45 seconds followed by another 35 cycles at the same denaturation, annealing and extension temperatures and finished with a 10 min final extension at 72°C (after the modification of Garey, et al. [2]). A negative control (no template) was also performed for every PCR run to determine any potential contamination of the PCR products.

PCR products were evaluated by electrophoresis of 4µl PCR mixture through 1% agarose gel against 1 Kb ladder (Fermentas), and cleaned up using GeneJET™ PCR purification Kit (fermentas) and the purified DNA was stored at -20°C. 2µl of PCR product was sequenced using an ABI big dye terminator kit following the instructions of GATC German company facility and an ABI 3730xl DNA sequencer .

### Phylogenetic Analysis

In addition to the 18S and 28S DNA gene sequences generated in this study, additional 21 sequences for 18S DNA and 16 for 28S DNA genes of other Paleacanthocephalans and one out group were obtained from the GenBank [Table-1]. The ClustalW<sub>2</sub> algorithm was used to visualize the consensus sequences and the dendrogram. The dendrograms were built depending on the molecular based evolution set by Pearson and Lipman [15]. The matrix methods UPGMA (Unweighted-Pair-Group Methods with Arithmetic Mean) was used to present the dendrograms and the relation between homologous sequences based on the produced multiple sequence alignment [16].

## Results

### DNA Sequencing and Data Set

The sequence obtained for 18S DNA is a 385 nucleotides long including primers with 59% GC content [Fig-1], while 28SDNA sequence is a 282 nucleotide long, including primers with 58% GC content [Fig-2]. The 18S and 28S DNA sequences for *S. picae* are in the process of publication in the nucleotide data set in GenBank (NCBI). The homologous sequences for the two generated genes were retrieved from the GenBank and processed all together by mealign tool of DNASTar Lasergene V7 to produce the multiple sequence alignment. Highly conserved blocks were detected, the best position of consensus sequence was assigned.

### Phylogenetic Analysis

Molecular analyses of both 18S and 28S DNA using matrix unweighted pair-group method with arithmetic mean (UPGMA) revealed the degree of genetic similarity between *S. picae* and all retrieved species [Fig-2], [Fig-3] using full optimization and 1000 bootstrap replicates.

Table 1- Species related to class Palaeacanthocephala and represented in the phylogenetic analyses with Gene bank accession numbers.

| Order            | Family                         | Species                              | 18rDNA        | 28rDNA        |
|------------------|--------------------------------|--------------------------------------|---------------|---------------|
| Polymorphida     | Centrorhynchidae               | <i>Centrorhynchus</i> sp.            | AY830155      | AY829104      |
|                  |                                | <i>Sphaerirostris picae</i>          | Present study | Present study |
| Polymorphida     | Polymorphidae                  | <i>Polymorphus</i> sp                | AF001838      | -             |
|                  |                                | <i>Profilicollis botulus</i>         | EU267805      | EU267818      |
|                  |                                | <i>Pseudocorynosoma constrictum</i>  | EU267800      | EU267812      |
|                  |                                | <i>Corynosoma strumosum</i>          | EU267804      | EU267816      |
|                  |                                | <i>Andracantha gravida</i>           | EU267802      | EU267814      |
|                  |                                | <i>Southwellina hispida</i>          | EU267807      | -             |
|                  |                                | <i>Arhythmorhynchus brevis</i>       | AF064812      | -             |
|                  |                                | <i>Ibirhynchus dimorpha</i>          | GQ981436      | GQ981437      |
|                  |                                | <i>Hexaglandula corynosoma</i>       | EU267808      | EU267817      |
| Polymorphida     | Plagiorrhynchidae              | <i>Plagiorrhynchus cylindraceus</i>  | AF001839      | AY829102      |
| Echinorrhynchida | Rhadinorrhynchidae             | <i>Gorgorhynchoides bullocki</i>     | AY830154      | AY829103      |
|                  |                                | <i>Rhadinorrhynchus</i> sp           | AY062433      | AY829099      |
| Echinorrhynchida | Transvenidae                   | <i>Pararhadinorrhynchus</i> sp       | HM545903      | -             |
|                  |                                | <i>Transvena annulospinosa</i>       | AY830153      | AY829098      |
| Echinorrhynchida | Cavisomidae                    | <i>Filisoma bucerium</i>             | AF064814      | AY829110      |
| Echinorrhynchida | Arhythmacanthidae              | <i>Acanthocephaloides propinquus</i> | AY830149      | AY829100      |
| Echinorrhynchida | Pomphorhynchidae               | <i>Pomphorhynchus laevis</i>         | AY218124      | -             |
| Echinorrhynchida | Echinorrhynchidae              | <i>Echinorrhynchus gadi</i>          | AY218123      | AY218146      |
|                  |                                | <i>Acanthocephalus dirus</i>         | AY830151      | AY829106      |
| Echinorrhynchida | Unclassified Echinorrhynchida  | <i>Echinorrhynchida</i> sp.          | EU732662      | -             |
| Out group        | Trhypochthoniidae (Arthropoda) | <i>Trhypochthonius tectorum</i>      | HQ711371      | -             |

|     |            |            |            |            |
|-----|------------|------------|------------|------------|
| 1   | TTATGNTTGT | CTCTTGTGAT | GCGCTTCATT | GTGTGTCTCG |
| 41  | GTGAAGAGCC | ACTGTTCACT | TTGAAAAACT | CAGTGTGCTC |
| 81  | AATGCAGGCT | AACTGCTCGA | ATTACGCTGC | ATGGGATGAT |
| 121 | GAAATGGGGT | CTCCGCTCCG | TTTCGTTGGT | TTACNAAAGC |
| 161 | AAAGAACATG | ATTAATCGGG | ACAGACGGGG | CATTCTGTAT |
| 201 | TGCGGTGCTA | AAATTGAAAT | TCTGTGACCA | TGCGAAAACA |
| 241 | AACAACGCA  | AAAGCATTTG | CCAAAAATGT | TTTCTTTAAT |
| 281 | CACAAACAAA | AGTTAAAGGA | TCAAAAAACA | TAAATACCG  |
| 321 | TCCTATTCT  | AACGGTAAAC | TGTGCCGGCC | AGGAATTCCC |
| 361 | CAACGCAATA | ACTTGCAAN  | CCCTT      |            |

Fig. 1- Nucleotides sequence of 361 bp fragments of 18S DNA gene of *Sphaerirostris picae*.

|     |            |            |            |            |
|-----|------------|------------|------------|------------|
| 1   | CCACGTTTAT | TCATGCGGTG | GTTAGTATGA | GTGATAATGC |
| 41  | GTGCTTGTCC | AGGTGTGCTG | CTTCTCCAGC | TATCCGCATT |
| 81  | TGTAAGTTCG | CTTGACTGTA | GTAACCTTGA | AAGATGGTGC |
| 121 | GAGACCTGCA | TGAGTGGGTG | GTGGTTGCCG | ATATTGTTTT |
| 161 | CTGCTTCAGA | GTCGAGTTGC | CTGGGAACGC | ATCTCAAAGT |
| 201 | CGTTGGTAAA | CCTCATCTAA | AGCTAAATAT | GACTTCGAGA |
| 241 | CCGATACCGA | ACAACCTACG | TGAGGAAAAT | TTGCCAATAA |
| 281 | CT         |            |            |            |

Fig. 2- Nucleotides sequence of 281 bp fragments of 28S DNA gene of *Sphaerirostris picae*.

both *Pomphorhynchus* (family Pomphorhynchidae) and *Echinorrhynchus* (family Echinorrhynchidae) with a genetic similarity 95.7%. *Acanthocephalus* (family Echinorrhynchidae) and *Filisoma* (family Cavisomidae) have high genetic similarity 96.8%, the clade that carries both generasisters to that of *Echinorrhynchida* (family Echinorrhynchidae) and *Acanthocephaloides* (family Arhythmacanthidae). The clade carries *Transvena* and *Pararhadinorrhynchus* (family Transvenidae) and *Rhadinorrhynchus* (family Rhadinorrhynchidae) appears separate from the other 2 major clades.

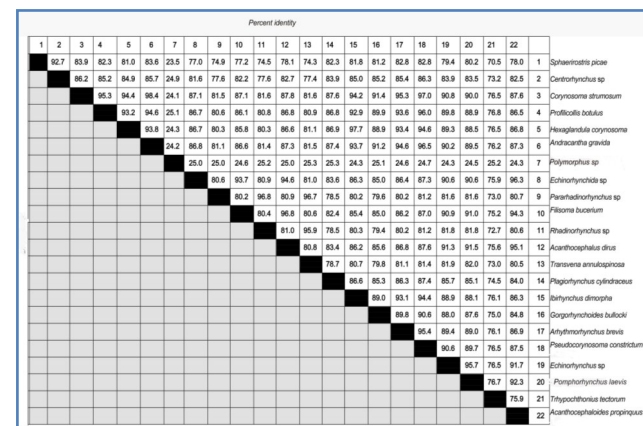
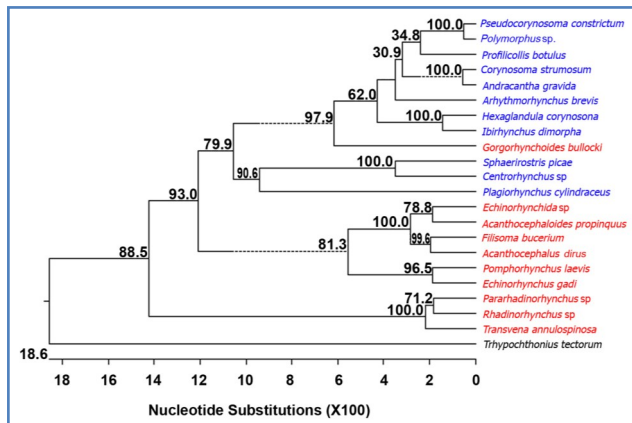


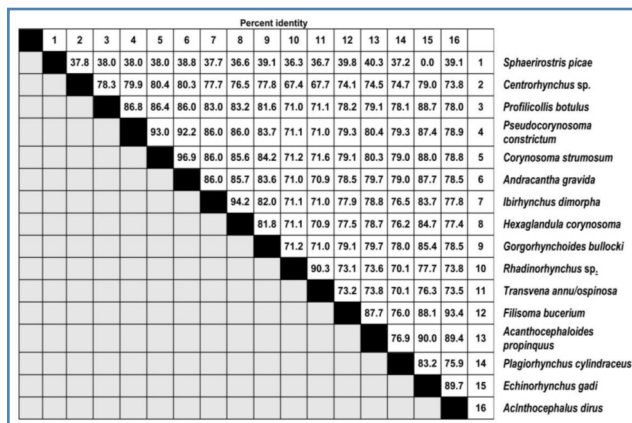
Fig. 3- Percent identity based on 18S DNA gene between *Sphaerirostris picae* and twenty two species belong to orders Echinorrhynchida and Polymorphida (Palaeacanthocephala) retrieved from GenBank.

The cluster of Polymorphida includes one echinorrhynchid namely *Gorgorhynchoides*. The most basal genus in the polymorphid clade is *Plagiorrhynchus*, followed by the clade of both *Sphaerirostris* and *Centrorhynchus* (family Centrorhynchidae) with a genetic similarity 92.7%. *Plagiorrhynchus* and *Sphaerirostris* have 82.3% similarity distance. Two clades in the cluster of family Polymorphidae carry taxa with high genetic similarity; the first carries *Ibirhynchus* with

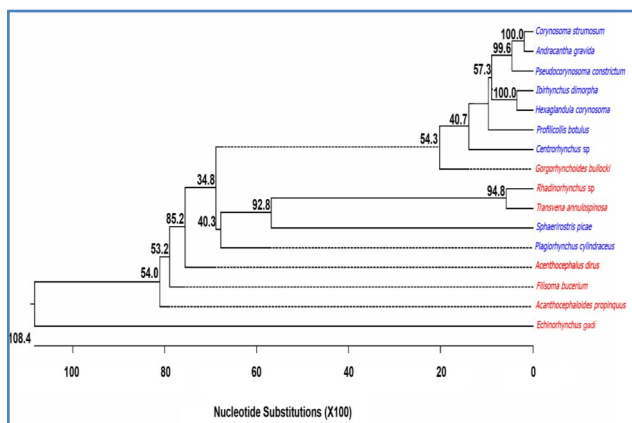
*Hexaglandula* (97.7%) and the second includes *Andracantha* with *Corynosoma* (98.4%).



**Fig. 4-** Percent identity based on 28S DNA gene between *Sphaerirostris picae* and sixteen species belong to orders Echinorhynchida and Polymorphida (Palaeacanthocephala) retrieved from GenBank.



**Fig. 5-** Phylogntic dendrogram for Palaeacanthocephala relationships based on the 18S DNA data set. Arthropoda is used as out-group. The tree illustrates that the order Echinorhynchida (red) and Polymorphida (blue) have a paraphyletic arrangement. The branch length scale is the number of substitutions per site.



**Fig. 6-** Phylogntic dendrogram for Palaeacanthocephala relationships based on the 28S DNA data set. The tree confirms the paraphyletic arrangement of order Echinorhynchida (red) and Polymorphida (blue). The branch length scale is the number of substitutions per site.

Analysis based on 28S DNA gene [Fig-5], [Fig-6] represents two major clades of Palaeacanthocephala. The first is of Echinorhynchida, which lacks the two closely related genera; *Transvena* and *Rhadinorhynchus* (90.3 %genetic similarity), in addition to *Gorgorhynchoides* which are all allocated in the polymorphid clade. The clade of *Transvena* and *Rhadinorhynchus* formed a sister group to *Sphaerirostris* (family Centrorhynchidae). *Echinorhynchus* is the most basal genus in the echinorhynchids. It has 89.7% genetic similarity with *Acanthocephalus* which is related to the same family (Echinorhynchidae), being relatively more close to *Acanthocephaloids* (Arhythmacanthidae) (90% genetic similarity).

Depending on 18S DNA gene analysis, *S. picae* has high genetic similarity ratio (92.7%) with *Centrorhynchus* sp (Centrorhynchidae) [Fig-3], [Fig-4], while in that of 28S DNA gene [Fig-5], [Fig-6], both species are separated by a Echinorhynchids clade encloses *Transvena*, *Rhadinorhynchus* and *Gorgorhynchoides*.

### Discussion

The relationship of the Acanthocephala to other invertebrate phyla has been estimated recently by analysis of structural and molecular data. The first molecular phylogenetic analyses within Acanthocephala [2] confirmed the major taxonomic grouping of the traditional classifications. In that analysis, Palaeacanthocephala was placed close to the Eoacanthocephala, while Polyacanthocephalans appeared sister to the Eoacanthocephalans with the Archiacanthocephala being the most basal taxon [3]. Previous studies of acanthocephalans combined data sets of both, 18S and 28S DNA [2,17]. The present study data set adds to the most recent analyses of Palaeacanthocephala relationships by Garcia-Varela and Nadler [17], Garey, et al. [2] and Verweyen, et al. [3].

Palaeacanthocephala include two orders; Echinorhynchida and Polymorphida. According to Verweyen, et al. [3], these species rich taxa include 83 genera and 594 species of Acanthocephalans. Both orders demonstrate high morphological diversity, which may explain why traditional identification keys have distinguished taxa according to their final hosts. Based on traditional classification, Echinorhynchida is divided into 10 families and 339 valid species, while Polymorphida includes only three families and a total of 255 valid species [3].

The present study analyzed the relationships between the three polymorphids families and six of the echinorhynchids families using both 18S and 28S DNA gene analytical methods which revealed the paraphyletic assembly of Palaeacanthocephala. This finding is incorporated by the previous analysis of Herlyn, et al. [4] and Verweyen, et al. [3], however both studies depended only on the analysis of 18S DNA gene. Otherwise, phylogenetic analysis based on morphological and ecological characters of Palaeacanthocephala [5,18] revealed the monophyletic form of this class.

The present 18S DNA gene analysis indicates that the family Rhadinorhynchidae (Echinorhynchida) is paraphyletic, where the analysis allocates *Gorgorhynchoides bullocki* in the Polymorphida cluster. This replacement is confirmed by the present analysis based on 28S DNA gene, where *Rhadinorhynchus* sp which belongs to the same family (Rhadinorhynchidae), presents together with *Gorgorhynchoides bullocki* in the same position. This finding was in agreement with the previous reports of Verweyen, et al. [3], Herlyn,



et al. [6] and. García-Varela and Nadler [17]. In addition, Shih, et al. [19] traditionally classified *Rhadinorhynchus pristis* as a member of order Polymorphida. It is worth mentioning here that *Gorgorhynchoides* has trunk hooks on its praesoma (only some echinorhynchid acanthocephalans have irregularly arranged hooks on the trunk) while the regular distribution of hooks on the trunk is one of the most common features within the polymorphids.

Based on 18S and 28S DNA gene analysis, the polymorphid; *Plagiorhynchus cyndraecus* is placed between Polymorphid and Echinorhynchid clades. Morphologically, this species has some Echinorhynchid characters, where the cylindrical trunk has anterior hooks and the males have six cement glands [3].

An interesting finding in the present study, revealed by 18S DNA gene analysis, is the relatively close relation of *Sphaerostris picae* (family Centrorhynchidae) with *Plagiorhynchus cylindraceus* (Plagiorhynchidae) when compared with the relation of *S. picae* with the other species. Morphologically, the later species is characterized by cylindrical fusiform aspinose trunk, slender lemnisci, and 6 elongate tubular cement glands in the males. *Sphaerostris* has the same characters except only 4 cement glands. Otherwise, Amin, et al. [7] reported the presence receptacle process at the anterior end of the proboscis receptacle of *S. picae*, this trait distinguishes this species from all other species of its genus. A similar structure was reported previously in the proboscis of *Plagiorhynchus digiticephalus* (Plagiorhynchidae) [20], These facts might introduce a justification of the close relation of both genera.

The present analysis of 18S DNA gene shows high similarity ratio (92.7%) between *Sphaerostris* and *Centrorhynchus*. Dollfus and Golvan [21] listed both *Centrorhynchus teres* Westrumb, 1981 and *Centrorhynchus (Sphaerostris) picae* Rudolphi, 1819 as valid species. The present analysis of 28S DNA gene separates *Sphaerostris* from *Centrorhynchus* by two clades represents three Echinorhynchid species. Morphologically, Golvan [8] erected *Sphaerostris* Rudolphi, 1819 as subgenus of *Centrorhynchus* Lühe, 1911, later he [22] listed 26 species of *Sphaerostris* by reversing the synonymies, where the differentiation was based on the proboscis armature [7]. Relations between the two main genera of Family Centrorhynchidae need further analysis based on both morphological and molecular data.

To the best of our knowledge, no data set are available for either 18S or 28S DNA genes of *Sphaerostris*, the present study is the first to add gene sequences to this genus. As the taxonomy of *Sphaerostris* was largely based on the proboscis armature, and this character has proven to be extremely variable [7], further comprehensive studies should be done for other species of this genus based on molecular analysis to solve their taxonomic overlapping.

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