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STUDIES OF DNA BARCODING AND MOLECULAR PHYLOGENY OF *ECHINORHYNCHUS VELI*

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ABSTRACT
Phylogenetic relationships of Phylum Acanthocephala have remained unresolved. The phylum has traditionally been divided into three classes, Archiacanthocephala, Palaeacanthocephala and Eoacanthocephala; a fourth class, Polyacanthocephala, has been proposed later. The present study was to DNA barcode the acanthocephalan parasite, *E. veli*, and to ascertain its phylogenetic position within the phylum Acanthocephala. Sequence analysis of 18S rRNA gene proved the identity of *E. veli* as a distinct species in Palaeacanthocephala. *E. veli* was found to be a sister species to *Pomphorhynchus laevis* and *P. tereticollis*. Analyses support the hypothesis that Acanthocephala is an independent and monophyletic group that includes four subclades that too are monophyletic. Based on inter group genetic divergence, the Palaeacanthocephala seemed to be more closely related to Archiacanthocephala than to Eoacanthocephala or Polyacanthocephala. With regard to the relationship with rotifers, the findings conclusively showed that Acanthocephala enjoys the status of a sister group to Rotifera.

KEYWORDS: acanthocephalan parasite, genetic divergence.

INTRODUCTION

Molecular phylogeny deals with the structure and function of biological macromolecules and how they change over a period of time so as to give information on the evolutionary relationships. With the advent of molecular biological techniques like electrophoresis, protein sequencing, PCR and DNA sequencing, largely assisted by the advanced computers over the past 30 years, Life Science researchers have been able to contribute much to understand the evolutionary kinship between organisms at molecular level. Modern theory of evolution says that all organisms on earth have originated from a common ancestor; it means that any set of species is related. This relationship is called phylogeny and is represented by phylogenetic trees which graphically represent the evolutionary history related to the species of interest based on morphological, physiological and molecular features. Parasite species have been identified and described based on the traditional Linnean species concept based on differences in morphological features and to a lesser extent, the host identity (Mayr, 1963). Genetic analysis through the application of molecular technique can elucidate the micro-evolutionary process and provide a correct and confirmative identification of a specimen (McManus and Bowles, 1996; Blaxter, 2001). Today the best tool available for this analysis is DNA sequencing. It is the process of determining the precise sequence of nucleotide in a short stretch of DNA. The basic idea behind the process is to compare specific sequences to find out differences and similarities, thereby assessing the phylogenetic relationship among related species. This is the basis of the now famous DNA taxonomy or DNA barcoding (Tautz *et al.*, 2003). Based on the DNA sequence divergence of nuclear and mitochondrial genes, Perrot and Minnot (2004) and Moret *et al.* (2007) reported genetic polymorphism in *Pomphorhynchus laevis* collected from different geographical sites. In their attempt to study the phylogeny, Steinauer *et al.* (2005) sequenced the complete mitochondrial genome of the acanthocephalan *Leptorhynchoides thecatus*. Molecular phylogenetic studies based on DNA sequencing have been elucidated in different acanthocephalans; García *et al.* (2005) in *Corynosoma luhe*; García and Gonzalez (2008) in *Leptorhynchoides* and *Pseudoleptorhynchoides*; Martinez *et al.* (2009) in *Neoechinorhynchus golvani* and García *et al.* (2011) in *Southwellina dimorpha*.
The present study aims at DNA sequencing of 18S rRNA genes of *E. veli* to ascertain its phylogenetic position within the phylum Acanthocephala.

MATERIALS & METHODS

Specimens of *Echinorhynchus veli* were collected from their natural fish host, *Synaptura orientalis*. Worms were washed 3 times in 0.7% saline, preserved in absolute ethanol and stored at 4ºC.DNA was isolated from *E. veli* using QIAGEN-Dneasy blood and tissue kitas per manufacturer's instruction. 25 mg tissue of the parasite was used to extract the DNA.

Quantification and Purity of DNA

- Diluted DNA sample (20µL of the sample was made up to 2000 µL with sterile distilled water) was prepared.
- The spectrophotometer was turned on (and the UV bulb) for about 15 minutes to warm up the machine and the wavelength was set at 260 nm.
- Distilled water was used as the blank.
- Recorded the OD at 260nm and 280nm.

Primers used in sequence analyses

The details of the primers used in the sequence analyses are given in Table 1.

TABLE 1: Primers used in sequence analyses

Gene	Primer sequence	Product length	Reference
18S rRNA	F - 5' – AAG GTG TGM CCT ATC AAC T – 3' R - 5' – TTA CTT CCT CTA AAC GCT C – 3'	1843 bp	Song *et al.*, 2008

PCR amplification of 18S rRNA gene

PCR was performed in reaction mixture of 25 µl with the thermo cycling conditions for the amplification of 18SrRNA gene fragment at 95°C for five min. followed by 10 cycles at 95°C for 30 seconds, 45°C for 40 seconds, 72°C for 90 seconds, followed by 30 cycles of 95°C for 30 seconds, 51°C for 40 seconds, 72°C for 90 seconds, and a final extension step at 72°C for five min. in a DNA thermocycler (Eppendorf). The details of Primer and PCR mixture and the thermocycling parameters are given in Tables 2 and 3. Sequencing was carried out at Sci Genom Labs Pvt. Ltd. Cochin.

Sequence Submission to GenBank

The sequences obtained from the study were submitted to GenBank as sequin files. The sequences obtained were used for a BLAST search at NCBI and 21 sequences with the highest similarity belonging to class Palaeacantho cephala were selected, followed by one sequence from Polyacanthocephala, two sequences from Eoacanthocephala, three sequences from Archiacantho cephala, three sequences from Rotifera and one outgroup *Phyllodesmium colemani* belonging to Gastropoda (Mollusca) were also selected for a meaningful analysis.

Sequence Alignment

Sequences were multiple aligned with CLUSTAL Programme and adjusted manually to maximize alignment using BioEdit 7.00 (www.mbio.ncsu.edu.).

Phylogenetic Analyses

Phylogenetic analyses were performed using different methods like Maximum Likelihood (ML) and Maximum Parsimony (MP) to check the consistency of the topology of the resulting tree. *Phyllodesmium colemani* (Mollusca) was used as the outgroup for all the analyses.

The most fitting nucleotide substitution model was chosen using the model test performed using MEGA ver. 5 (Tamura *et al.*, 2007; Kumar *et al.*, 2008). The Maximum Likelihood and the Maximum Parsimony analyses were also performed using the same software package and the branch support for Maximum Likelihood and Maximum Parsimony was evaluated using 1000 bootstrap replicates (Felsenstein, 1985). Pairwise genetic distances between the clades were determined using the Kimura 2-parameter method (Kimura, 1980; Smith *et al.*, 2008).

TABLE 2: PCR mixture details for 18SrRNA gene amplification

Components	Final Concentration in Single PCR Tube	Volume (µl)
Milli Q or Triple distilled water		13µl
Taq Assay Buffer (10X)	1X	2.5µl
$MgCl_2$ (25 mM)	2.5 mM	2.5µl
dNTPs Mix (10 mM each)	2.5 mM	0.5µl
Primer (10 µmole/µl)	(10 µmole/µl)	0.25µl F 0.25µl R
Taq polymerase (3U/µl)	1U/ µl	1µl
DNA Template	10-30 ng	5µl
Total		25 µl

TABLE 3: Thermal cycle conditions for 18SrRNA gene amplification

Step	Denaturation		Annealing		Polymerization	
	Temp	Time	Temp	Time	Temp	Time
First step	95ºC	5 min				
10 Cycles	95ºC	30 sec	45ºC	40 sec	72ºC	90 sec
30 cycles	95ºC	30 sec	51ºC	40 sec	72ºC	90 sec
Last step	Final extension 72ºC for 5 min					

RESULTS

In this study, five samples of *E. veli* were used for phylogenetic analysis using partial sequences of 18S rRNA nuclear genes. The sequences generated in the present samples were submitted to GenBank under accession numbers KP260659, KP278482, KP278483, KP278484 and KP278485.

Agarose gel analysis of total genomic DNA isolated from *E. veli* and agarose gel electrophoresis showing amplification products of nuclear 18S rRNA gene fragment are shown in Fig. 1 and 2. Accession numbers of acanthocephalan species retrived from GenBank including those of the present species are given in Table 4. Maximum Likelihood and Maximum Parsimony Trees are shown in Figs. 3 and 4. Table 5 shows the genetic distance (%) calculated and compared with other species and Table 6, the mean genetic distance between groups.

Multiple sequence alignment and genetic diversity analyses

The 18S rRNA gene sequences of 32 species were selected for the analysis (31 from the GenBank and one from current study); of the selected, 22 species represented

Palaeacanthocephala, one species Polyacanthocephala, two species Eoacanthocephala, three species Archiacanthocephala, three species Rotifera and one mollusc as outgroup. The sequence was aligned using CLUSTALW multiple sequence alignment software package. The final alignment dataset obtained after discarding the potentially misaligned sequence consisted of a total of 867 sites of which 307 sites were conserved, 393 sites were variable. Out of the variable sites 271 (69%) were parsimony informative. The K2+G nuclear substitution model was selected for 18S RNA gene. The phylogenetic trees constructed with Maximum Likelihood and with Maximum Parsimony were very similar, revealing distinct clade bootstrap support.

Palaeacanthocephala forms a monophyletic group with two distinct clades. The first clade consists of species : JX442166: *Bolbosoma turbinella*, JX014225: *Bolbosoma vasculosum*, JX442170: *Corynosoma validum*, EU267804: *Corynosoma strumosum*, AF001837: *Corynosoma enhydri*, EU267803: *Corynosoma magdaleni*, EU267802: *Andracantha gravid*, JX442169: *Corynosoma obtuscens*, JX442168: *Corynosoma austral*, EU267801: *Pseudocorynosoma anatarium*, EU267800: *Pseudo corynosoma constrictum*, JX442173: *Polymorphus trochus*, EU267807: *Southwellina hispida*, JX442171: *Polymorphus brevis* and EU267805: *Profilicollis botulus*. The second clade is represented by the following: AY218123: *Echinorhynchus gadi*, AY830156: *Echinorhynchus truttae*, KF559309:*Pomphorhynchus laevis*, AY423347: *Pomphorhynchus tereticollis*, JX014226: *Rhadinorhynchus pristis*, JX014224: *Rhadinorhynchus lintoni*,KP260659, KP278482 to KP278485: *Echinorhynchus veli*.

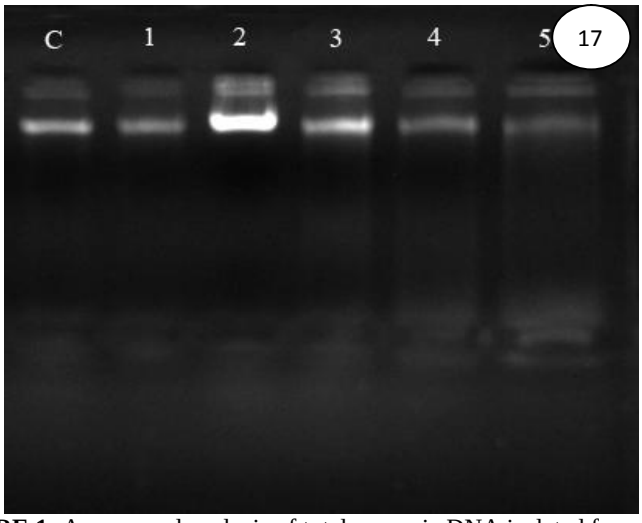

FIGURE 1: Agarose gel analysis of total genomic DNA isolated from *E.veli*
C- control DNA; 1-5, DNA isolated from *E. veli*

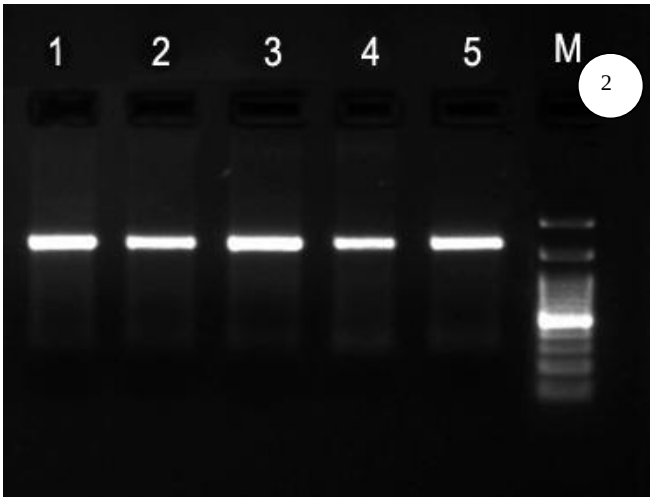

FIGURE 2: Agarose gel electrophoresis showing PCR amplification products of nuclear 18S rRNA gene fragment of *E. veli* (1-5), M-100bp size ladder

TABLE 4: Accession numbers of acanthocephalan species retrieved from GenBank for the current study

Acanthocephalan species	Accession Numbers
Floridosentis mugilis	AF064811
Neoechinorhynchus crass	AF001842
Macraacanthorhynchus ingens	AF001844
Oligacanthorhynchus tortuosa	AF064817
Mediorhynchus grandis	AF001843
Pomphorhynchus laevis	KF559309
Pomphorhynchus tereticollis	AY423347
Rhadinorhynchus pristis	JX014226
Rhadinorhynchus lintoni	JX014224
Echinorhynchus veli 1	KP260659
*Echinorhynchus veli*2	KP 278482
Echinorhynchus veli 3	KP 278483
Echinorhynchus veli 4	KP278484
Echinorhynchus veli 5	KP278485
Echinorhynchus gadi	AY218123
Echinorhynchus truttae	AY830156
Pseudocorynosoma anatarium	EU267801
Pseudocorynosoma consrictum	EU267800
Polymorphus trochus	JX442173
Corynosoma validum	JX442170
Bolbosoma turbinella	JX442166
Corynosoma obtuscens	JX442169
Bolbosoma vasculosum	JX014225
Corynosoma strumosum	EU267804
Corynosoma enhydri	AF001837
Corynosoma magdaleni	EU267803
Andracantha gravida	EU267802
Corynosoma australe	JX442168
Southwellina hispida	EU267807
Polymorphus brevis	JX442171
Profilicollis botulus	EU267805
Polyacanthorhynchus caballeroi	AF388660

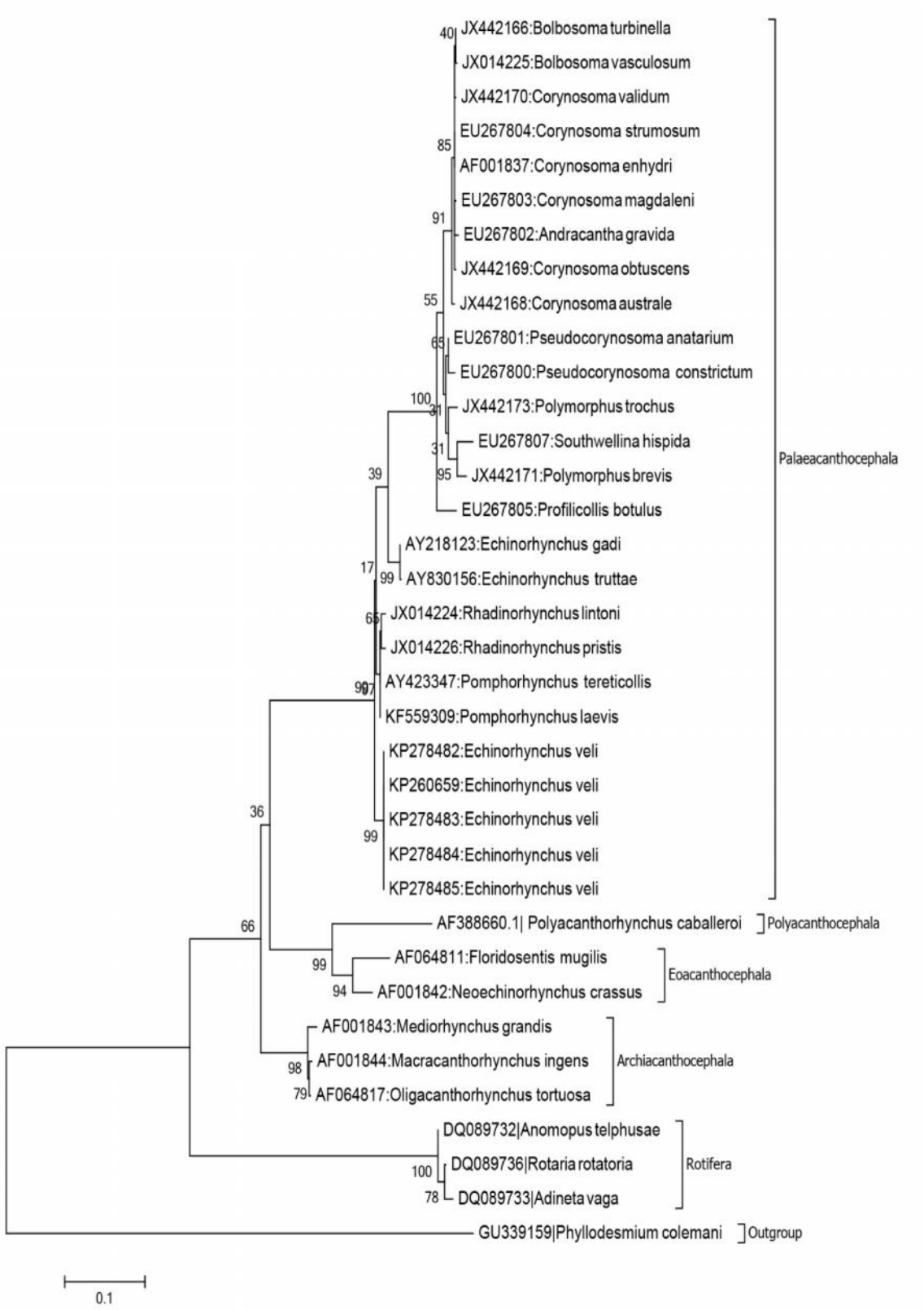

FIGURE 3: Maximum Likelihood Tree based on Kimura 2-parameter genetic distances of 18S rRNA gene sequences of species belonging to Palaeacanthocephala, Archiacanthocephala, Eoacanthocephala, Polyacanthocephala, Rotifer and Mollusc (*Phyllodesmium colemani*) as outgroup. Numbers at branches indicate bootstrap support (%).

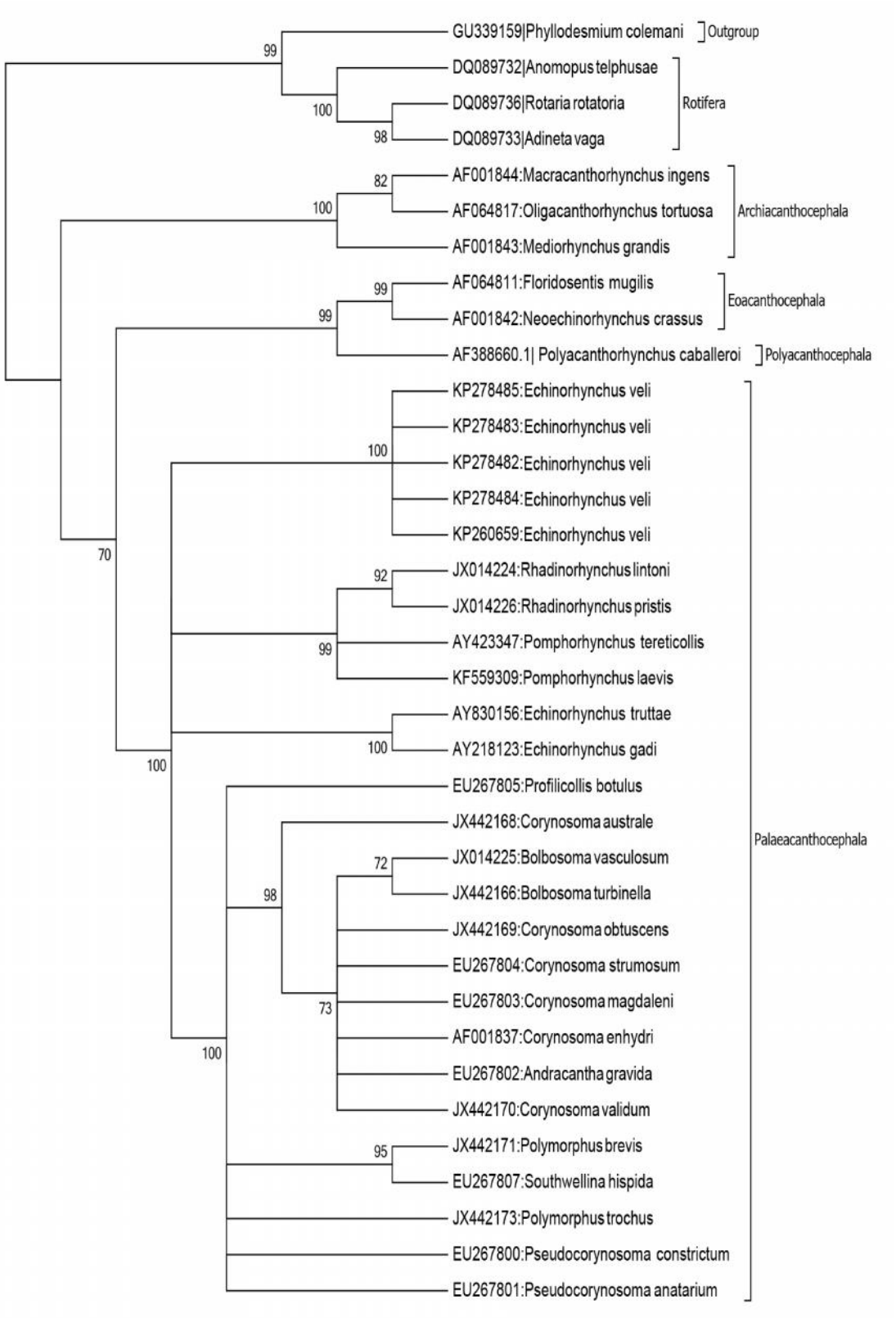

FIGURE 4: Maximum Parsimony Tree based on Kimura 2-parameter genetic distances of 18S rRNA gene sequences of species belonging to Palaeacanthocephala, Archiacanthocephala, Eoacanthocephala and Polyacanthocephala; Rotifera and Mollusc (*Phyllodesmium colemani*) as outgroup. Numbers at branches indicate bootstrap support (%)

TABLE 5: Genetic distance (%) calculated using Kimura 2-parameter model based on 18S rRNA sequences of individuals of Palaeacanthocephala, Archiacanthocephala, Eoacanthocephala and Polyacanthocephala; Rotifera and Mollusc (*Phyllodesmium colemani*) as outgroup compared with 18S rRNA sequence generated from five samples of *E.veli*

Accession No.	Species	Genetic Distance		
KP260659	*Echinorhynchus veli* {Palaeacanthocephala}			
KP278482	*Echinorhynchus veli* {Palaeacanthocephala}	0.000		
KP278483	*Echinorhynchus veli* {Palaeacanthocephala}	0.000	0.000	
KP278484	*Echinorhynchus veli* {Palaeacanthocephala}	0.000	0.000	0.000
KP278485	*Echinorhynchus veli* {Palaeacanthocephala}	0.000	0.000	0.000
AF064811	*Floridosentis mugilis*{Eoacanthocephala}	0.214	0.214	0.214
AF001842	*Neoechinorhynchus crassus* {Eoacanthocephala}	0.201	0.201	0.201
AF001844	*Macracanthorhynchus ingens* {Archiacanthocephala}	0.171	0.171	0.171
AF064817	*Oligacanthorhynchus tortuosa* {Archiacanthocephala}	0.169	0.169	0.169
AF001843	*Mediorhynchus grandis* {Archiacanthocephala}	0.174	0.174	0.174
KF559309	*Pomphorhynchus laevis* {Palaeacanthocephala}	0.016	0.016	0.016
AY423347	*Pomphorhynchus tereticollis* {Palaeacanthocephala}	0.016	0.016	0.016
JX014226	*Rhadinorhynchus pristis* {Palaeacanthocephala}	0.023	0.023	0.023
JX014224	*Rhadinorhynchus lintoni* {Palaeacanthocephala}	0.023	0.023	0.023
AY218123	*Echinorhynchus gadi* {Palaeacanthocephala}	0.033	0.033	0.033
AY830156	*Echinorhynchus truttae* {Palaeacanthocephala}	0.035	0.035	0.035
EU267801	*Pseudocorynosoma anatarium* {Palaeacanthocephala}	0.062	0.062	0.062
EU267800	*Pseudocorynosoma constrictum* {Palaeacanthocephala}	0.065	0.065	0.065
JX442173	*Polymorphus trochus* {Palaeacanthocephala}	0.065	0.065	0.065
JX442170	*Corynosoma validum* {Palaeacanthocephala}	0.071	0.071	0.071
JX442168	*Corynosoma australe* {Palaeacanthocephala}	0.067	0.067	0.067
JX442166	*Bolbosoma turbinella* {Palaeacanthocephala}	0.069	0.069	0.069
JX442169	*Corynosoma obtuscens* {Palaeacanthocephala}	0.071	0.071	0.071
JX014225	*Bolbosoma vasculosum* {Palaeacanthocephala}	0.069	0.069	0.069
EU267807	*Southwellina hispida* {Palaeacanthocephala}	0.079	0.079	0.079
EU267804	*Corynosoma strumosum* {Palaeacanthocephala}	0.069	0.069	0.069
AF001837	*Corynosoma enhydri* {Palaeacanthocephala}	0.069	0.069	0.069
EU267805	*Profilicollis botulus* {Palaeacanthocephala}	0.073	0.073	0.073
EU267803	*Corynosoma magdaleni* {Palaeacanthocephala}	0.071	0.071	0.071
EU267802	*Andracantha gravid* {Palaeacanthocephala}	0.071	0.071	0.071
JX442171	*Polymorphus brevis* {Palaeacanthocephala}	0.077	0.077	0.077
AF388660	*Polyacanthorhynchus caballeroi* {Polyacanthocephala}	0.222	0.222	0.222
GU339159	*Phyllodesmium colemani*;Mollusca{Outgroup}	0.638	0.638	0.638
DQ089732	*Anomopus telphusae* {Rotifera}	0.388	0.388	0.388
DQ089736	*Rotaria rotatoria* {Rotifera}	0.395	0.395	0.395
DQ089733	*Adineta vaga* {Rotifera}	0.395	0.395	0.395

TABLE 6: Between group mean genetic distance (%) of Palaeacanthocephala, Archiacanthocephala, Eoacanthocephala, Polyacanthocephala, Rotifer and Mollusc (*Phyllodesmium colemani*) calculated using Kimura 2-parameter model based on of 18S rRNA sequences

		1	2	3	4	5
1	Palaeacanthocephala					
2	Archiacanthocephala	0.193(19.3)				
3	Eoacanthocephala	0.224(22.4)	0.152(15.2)			
4	Polyacanthocephala	0.239(23.9)	0.193(19.3)	0.143(14.3)		
5	Rotifera	0.419(41.9)	0.327(32.7)	0.389(38.9)	0.421(42.1)	
6	Outgroup	0.663(66.3)	0.635(63.5)	0.665(66.5)	0.700(70.)	0.718(71.8)

Figures in parenthesis indicate percentage.

DISCUSSION

The phylogenetic analyses were made using 18S ribosomal RNA (18S rRNA) gene sequences generated in the present study as well as those retrieved from GenBank. In addition to the sequences generated from *E. veli*, sequences of 27 acanthocephalans belonging to the four classes Archiacanthocephala, Eoacanthocephala, Polyacanthocephala and Palaeacanthocephala and three rotifers and one mollusc (outgroup) were used for the analyses. The main objective of the analysis was to ascertain the phylogenetic position of *E. veli* among the acanthocephalans.

Based on the morphological features, the position of *E. veli*is well rooted among Palaeacanthocephala. The mean genetic divergence calculated among the acanthocephalan species selected for the current analysis and the phylogenetic trees constructed based on18S rRNA gene sequences fully justify the identity of *E.veli* as a distinct species in this class *E. veli* was found to be a sister species to *Pomphorhynchus laevis* and *P. tereticollis* with just 1.6% genetic divergence.

The present analyses based on the near-complete sequences of 18S rRNA genes support the hypothesis that Acanthocephala is an independent and monophyletic group that includes four subclades, each of them representing a class and is in agreement with current classifications of this parasitic group (Amin, 1987). The phylogenetic trees deduced from the analyses of the sequences exhibited bifurcations consistent with the division into Palaeacanthocephala, Eoacanthocephala, Polyacanthocephala, Archiacanthocephala, Rotifera and the outgroup Mollusca. Of these six clades, four correspond to Acanthocephala and one each to Rotifera and outgroup Mollusca. Currently the species *Polyacanthorhynchus caballeroi* is placed in the class Palaeacanthocephala. But, Amin (1985) had placed it along with a few other species in a new class Polyacanthocephala created by him. However, in all the phylogenetic trees produced by the analysis of 18S rRNA gene sequences, the species *P. caballeroi* stands out as a separate and distinct clade with high bootstrap value of 100. These results are consistent with the view that Polyacanthocephala represents an independent class within Acanthocephala. Moreover, within the context of sampled taxa, all phylogenetic analyses are consistent with monophyly of the major taxonomic groups of the Acanthocephala, suggesting that the current higher order classification comprising four classes (Amin, 1987) is natural. The present data set gathers support from the more recent analyses of acanthocephalan relationships by Garey *et al.* (1996), García and Nadler (2005) and Verweyen *et al.* (2011) proposing a monophyletic origin of acanthocephalans and their separation into four distinct classes. However, the current finding is in disagreement with the results of Herlyn *et al.* (2003) who, based on partial SSU sequences, suggested paraphyly to Palaeacanthocephala.

Traditionally, the taxonomic groups in Acanthocephala have been identified based on morphological features and host characteristics (Bullock, 1969). In general, there is a large degree of congruence between morphological and molecular phylogenetic hypotheses of Acanthocephala, as well as congruence between phylogenetic hypotheses and traditional taxonomic classifications (Near *et al.,* 1998; García*et al.,* 2000; Monks, 2001).

In this study the phylogenetic relationships inferred by Maximum Likelihood and Maximum Parsimony analyses support the hypothesis that Archiacanthocephala is the most basal and the earliest divergent group within the phylum, followed by Eoacanthocephala, Polyacantho cephala and Palaeacanthocephala (Fig. 4). This finding is in agreement with the reports of García *et al.* (2000), Near *et al.* (1998) and Monks (2001). In addition, as evident from Table 6, the inter group genetic divergence observed in the study shows that Palaeacanthocephala is more closely related to Archiacanthocephala (19.3%) than to Eoacanthocephala (22.4%).

Monophyly of the Palaeacanthocephala was supported by Maximum Likelihood and Maximum Parsimony tree inference, and the palaeacanthocephalan clade was strongly supported by bootstrap analyses. Palaeacantho cephala is the most derived clade and shows the highest diversity compared to the other three classes.

Acanthocephala- Rotifera relationship has been a topic of discussion that still awaits conclusion. The current analysis of the 18S rRNA gene sequences of acanthocphalan species, rotifers and a molluscan outgroup clearly indicates and supports the view that acanthocephalans might have diverged from Rotifera sufficiently early with respect to the genetic distance (Table 6). The observed genetic divergence of 41.9% from rotifers and 66.3% from the molluscan outgroup, suggests that acanthocephalans form a group independent from rotifers.

In conclusion, the 18S rRNA genes have been used successfully not only to illustrate the phylogenetic relationships of Acanthocephala but to establish the phylogenetic position of *E. veli* within the class Palaeacanthocephala too. However, with regard to the origin of *E. veli*, further studies are needed using additional molecular markers from multiple genes and addition of sequences from more species, as most of the species described from geographic area of *E. veli* are yet to be sequenced.

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