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RESEARCH PAPER

First appearance of the cave leech *Erpobdella mestrovi* (Annelida: Erpobdillidae) in Iraq (Morphological and Molecular investigation) from Zalm stream, Sulaimani Province, Kurdistan Region, Iraq

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A B S T R A C T:

During survey of different water bodies in Kurdistan Region, Iraq for the presence of leeches, only two distinct specimens with special morphological characters were found in the cave mouth of Ahmadawa Water source in February 2019. The morphological (Body size, oral and posterior suckers characters, coloration and body projections were studied) and molecular study (18S rDNA amplifying with a forward primer C1 (ACCCGCTGAATTTAAGCAT at position 25), and reverse primer C3 (CTCTTCAGAGTACTTTTCAAC at position 390) and sequencing of the amplicons) cleared that the present specimens were belonging to the erpobdillid leeches, *Erpobdella mestrovi*, and the present appearance regard as a first in Iraq and all over the Middle-East for this leech.

KEY WORDS: Cave leech, *Erpobdella mestrovi*, Morphology, Molecular, Iraq.
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1. INTRODUCTION

Leeches origin and their controversial phylogeny within the Clitellata is a considerable debate subject for annelid systematics. Leeches and branchiobdellidans phylogenetic affinities were proposed before 1823 by Odier who described branchiobdellidans (crayfish worms) under leeches (Borda and Siddall, 2004). In early 1990's, evolutionary hypotheses of leeches were depended on plesiomorphic morphological characters interpretation and identification with corresponding of homologies. The morphological analysis supported leeches as, branchiobdellidans and Acanthobdella peledina have a common and molecular data also supported this hypothesis. However, other explanations for these relationships mentioned ectocommensalistic lifestyle adaptation as a cause of these shared ancestry (Holt, 1989; Brinkhurst, 1999).

DNA identification of leeches so useful since the identifications using standard techniques of taxonomy can sometimes be ambiguous since morphological characteristics are useful when dealing with mature specimens, the larval specimens or cocoon identification is enface lots of problems is identification especially for lower taxa (genus and species), also some taxa share many confused morphological and morphometric characters, whereas DNA characterization is

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useful for all taxa and developmental stages (Bely and Weisblat, 2006).

Three genera of erpobdellid leeches, namely *Erpobdella*, *Dina* and *Trocheta* are most species-rich genera. Traditionally can be distinguish by their annulation pattern among them. *Erpobdella* has five un-subdivided annuli per each body somite. Furthermore, the annulation pattern and body coloration patterns has proved to be inappropriate absolutely for the diagnosis of species (Neubert and Nesemann, 1995; Sket and Trontelj, 2008).

Cave leech, *Erpobdella mestrovi* (Kerovec, Kučinić and Jalžić, 1999), first description as a new species were by Kerovec *et al.* (1999) under a synonym *Croatobranchus mestrovi*, from caves in Croatia, Oceguera-Figueroa *et al.* (2005) re-described this species according to their 28S rDNA and changed the genus taxon to *Erpobdella*. This leech species have distinct morphological characters by presence of four oral projections and 12 pairs of body projections but have same basic erpobdillid sexual and digestive properties, some other reports of this leech were done from caves in Slovakia (Sket and Trontelj, 2008). *Erpobdella mestrovi* is a troglobitic leech (A troglobite is a species or population of animals bound to underground habitats (caves and dark wet spaces), living in cold water (4-6°C) (Culver *et al.*, 2009).

All the previous studies were done in Iraq, except that of Bilal *et al.* (2017), were depended only on morphological characters, there for, molecular investigation is a basic need for reviewing of Iraqi Leeches fauna, hence, the present study is the second scientific work deals with this scientific problem.

The present article recorded the first occurrence of *E. mestrovi* in Iraq; the outcome results from morphological examination were confirmed by molecular characterizations of 18S rDNA.

2. MATERIALS AND METHODS

2.1. STUDY AREA AND SAMPLE COLLECTION:

Only two unknown specimens of distinctive leeches were collected from Zalm stream, Sirwan River drainage near Ahmad Awa village (96.1 Km South-East to Sulaimani Province) under the emerged rocks of the cave mouth. Photomicrographs were taken directly for the leeches (as a live with the substratum) for documentation. One captured, leeches were transferred as a live to the laboratory.

2.2 MORPHOLOGICAL INVESTIGATION:

One complete sample and only the anterior and posterior parts of the other were used for morphological study, relaxed in 10% ethanol and put between two glass slides, fixed in 10% formalin, preserved in 70% ethanol. Measurements were taken with ocular micrometers (Kerovec *et al.* (1999; Oceguera-Figueroa *et al.*, 2005).

2.3 MOLECULAR INVESTIGATION were The used sample was the mid-body of one specimen, washed and preserved by deep freeze in ddH2O (-22 C˚). The specimen melted for extraction of the genomic DNA with a DNA extraction kit (GeNet Bio, KOREA). Briefly, macerated in mortar and pestle, transferred into sterile tubes with 200-250 µL tissue lysis buffer, incubated for eight hours. Qualification and quantification of DNA concentration done with Nano Drop (ND- 1000, USA). Samples genomic DNA with (A260–A320) / (A280–A320) ratio was more than 1.7 and outputs more than 30 ng/µL.

Mollaret et al. (2000) methodology was followed for amplification of 18S rDNA region with a polymerase chain reaction (PCR). Universal primers were used, forward primer C1 (ACCCGCTGAATTTAAGCAT at position 25), and reverse primer C3 (CTCTTCAGAGTACTTTTCAAC, position 390) was targeted. PCR reaction and conditions were performed using MJ Research, Applied Biosystem (AB) thermal cycler. The master mix was 50 µL reaction mixture, prepared in PCR tubes containing 2.5 µL DNA templates, 25 µL OnePCRTM master mix (GENEDIREX, KOREA), 1 µL of each primer and 20.5 µL double deionized water (ddH2O). The cycling conditions, 94˚C for 5 min (initial denaturation), 35 cycles of denaturation at 94˚C for 45 sec, annealing temperatures at 51˚C for 45 sec and extension at 72˚C for 45 sec, and 72˚C for 5 min (final extension). Gel electrophoresis was employed to check the validity of PCR products. Five wails with PCR products were prepared and run in 2%

agarose gel, stained with SYBR green that facilitate the DNA visibility with UV light.
The ABI 3130X nucleotide sequence analyzer (SINGAPORE) was used to find the order of nucleotides of 18S rDNA from the specimen. The PCR fragments of the specimens were purified from the agarose and used as a source of DNA template for sequencing.

3. RESULTS AND DISCUSSION

The results of the present study showed the prescience of *Erpobdella mestrovi* (Kerovec, Kučinić & Jalžić, 1999) for first time in Iraq, depending on the following findings:

Only two specimens of *Erpobdella mestrovi* (syname: Croatobarnchus mestrovi) were collected from Zalm stream near Ahmad Awa village under the stones in dark, shadow and cold water sutures.

According to the body length of the two sexually mature collected specimens, is moderately elongated, 28-44 mm long, narrowest body point 3.3-3.6 mm and widest body point 5.4-5.6 mm wide, slight depressed dorso-ventrally, milky to yellow-whitish in color, no visible eyes, the red blood is clearly visible in the ventral side along the lateral coelomic ducts. The total number of annuli 103, five equal annuli comprising the mid-body somite (Fig. 1: A; B; C).

The head region shape is changeable when attach and release, when it free (not attached to the substratum) is slightly expanded, rounded, four pairs of short conical tentacles (0.18-0.21 mm long, 0.11-0.13 mm wide) were extended from anterior and lateral sides, each of them bearing five finger-like papillae (Fig. 1 A; B). These tentacles disappear during sucker adhesion to the substratum. Oral cavity is circular; the mouth have no jaws, is usually widely open (in live and preserved specimens), muscular ridges only visible deep in the pharynx (Fig. 1: A; D).

The body width increases gradually in the caudal direction (very fast in starting of the third body half), clitellar region is slightly swollen (Widest body point without projections) (Fig. 1: A; B).

Gonopores are separated by at least 2.5 annuli, the male gonopore on the annulus anterior to the slightly backward shifted ganglion (probably a2). Studied specimens were having had a typical Erpobdelid male genital atrium with a massive body and conical horns bent distally. The female gonopore starting with somite XVI, (probably b5/b1 = XII/XIII) (Fig. 1: B).

Twelve lateral projections projected from the body, the longest one about 0.5 mm and the shortest 0.04 mm. Two short triangular projections are in the front and behind the longest lateral process. Anal opening is dorsal surrounded by small lips, approximately above the center of the posterior sucker which become a very thin rim when attached (Fig. 1: A; B; C; D).

The sequence from 18S rDNA of *Erpobdella mestrovi* specimens was made of 480 bp (amplified fragment was 521 bp, while after sequencing some miss-nucleotides were excluded, related to quality of sequencing analysis) and put to BLAST then compared with other stored genus of leeches sequences from GenBank database (Fig. 2: A; B; 3).

The blasting of 18S rDNA sequences of the present specimens with that available in Gene Bank showed 98% similarity with the sequences of *Erpobdella mestrovi* (Fig. 2: A; B; 3).

This leech was first described by Kerovec *et al.* (1999) as a new species under the name Croatobranchus mestrovi (Genus name according to the type country Croatia and species name according to the type author Milan Meštrov) during the expedition of Lukina jama. These leeches have a caterpillar like moving by stretching-contracting body. Their body bearing projections look like small legs (most probably auxiliary respiratory organs - branchia). Sket *et al.* (2001) re-described the same species and added some internal characters, and the proposed that this species is belonging to the erpobdillid leeched. Moreover, after DNA analysis, the genus name Erpobdella were fixed by Oceguera-Figueroa *et al.* (2005) and the genus taxon of this species were corrected.

The Morphometric study of the present specimens were fit with that of Kerovec *et al.* (1999) as well as Sket *et al.* (2001), and the investigated body parts were have same morphologic and morphometric characters.

Latter, *E. mestrovi* was found in a few deepest caves of Mt. Velebit in Slovacka jama, Olimp and Velebita. Some undescribed species, including erpobdellid leeches, occur in a number cave of China and the US (Sket and Trontelj, 2008).

According to Mhaisen (2019), 10 leech species were recorded in Iraq including Kurdistan Region before the present work, the first occurrence of leeches was represented as *Piscicola* sp. by (Herzog, 1969); then records continued and arranged alphabetically as, *Acipenserobdella volgensis* on *Carasobarbus luteus* and *Cyprinion macrostomum* (Al-Salmany, 2015); *Cystibranchus mammillatus* on *Mastacembelus mastacembelus* (Bashê, 2008); *Cystibranchus mastacemeli* on *Luciobarbus kersin* (as Barbus kersin) (Al-Jawda et al., 2000) and *Mastacembelus mastacembelus* (as *Mastacembelus simach*) (Rahemo , 1989; 1996); *Cystibranchus* sp. on *Mastacembelus mastacembelus* (as *Mastacembelus simach*) (Rahemo, 1989); *Dina lineata* (Ali and Jaweir, 2013) ; *Dina punctata* (Bilal *et al*., 2017); *Erpobdella octulata* (Ahmed, 2009); *Fadejewobdella quinqueannulata* (Ali, 2010); *Haemopis sanguisuga* (Ahmed, 2009); *Hemiclepsis marginata* on *Cyprinus carpio*, *Luciobarbus xanthopterus* (as *Barbus xanthopterus*), *Mesopotamichthys sharpeyi* (as *Barbus sharpeyi*) (Khalifa, 1985; 1989) and *Planiliza abu* (as *Liza abu*) (Mhaisen *et al.*, 1993) and *Piscicola geometra* on *Arabibarbus grypus* (as *Barbus grypus*) (Al-Jawda *et al.*, 2000), *Leuciscus vorax* (as *Aspius vorax*) (Mhaisen *et al*., 1997) and *Luciobarbus esocinus* (as *Barbus esocinus*) (Adday *et al*., 1999) and *Piscicola* sp. on *Arabibarbus grypus* (as *Barbus grypus*) (Shamsuddin *et al*., 1971), *Luciobarbus esocinus* (as *Barbus esocinus*) (Ali, 1989), *Luciobarbus pectoralis* (as *Barbus schejch*) (Herzog, 1969), *Luciobarbus xanthopterus* (as *Barbus xanthopterus*) (Shamsuddin *et al*., 1971).

Since no previous reports of the *E. mestrovi*, the present occurrence of this leech can be regards as a first in Iraq and all middle-east since no previous reports clearing it is presence in this part of the world.

CONCLUSIONS

First presences of the cave leech (Erpobdella mestrovi) is a main conclusion of the present morphological and molecular investigations for leeches in Iraq and can regard as a 12th species of Iraqi

leech fauna. There is no any report for this leech (Erpobdella mestrovi) in the Middle East at all. Hence, Zalm stream in Sulaimani Province, Kurdistan Region, Iraq regard as a new habitat for this leech in the world.

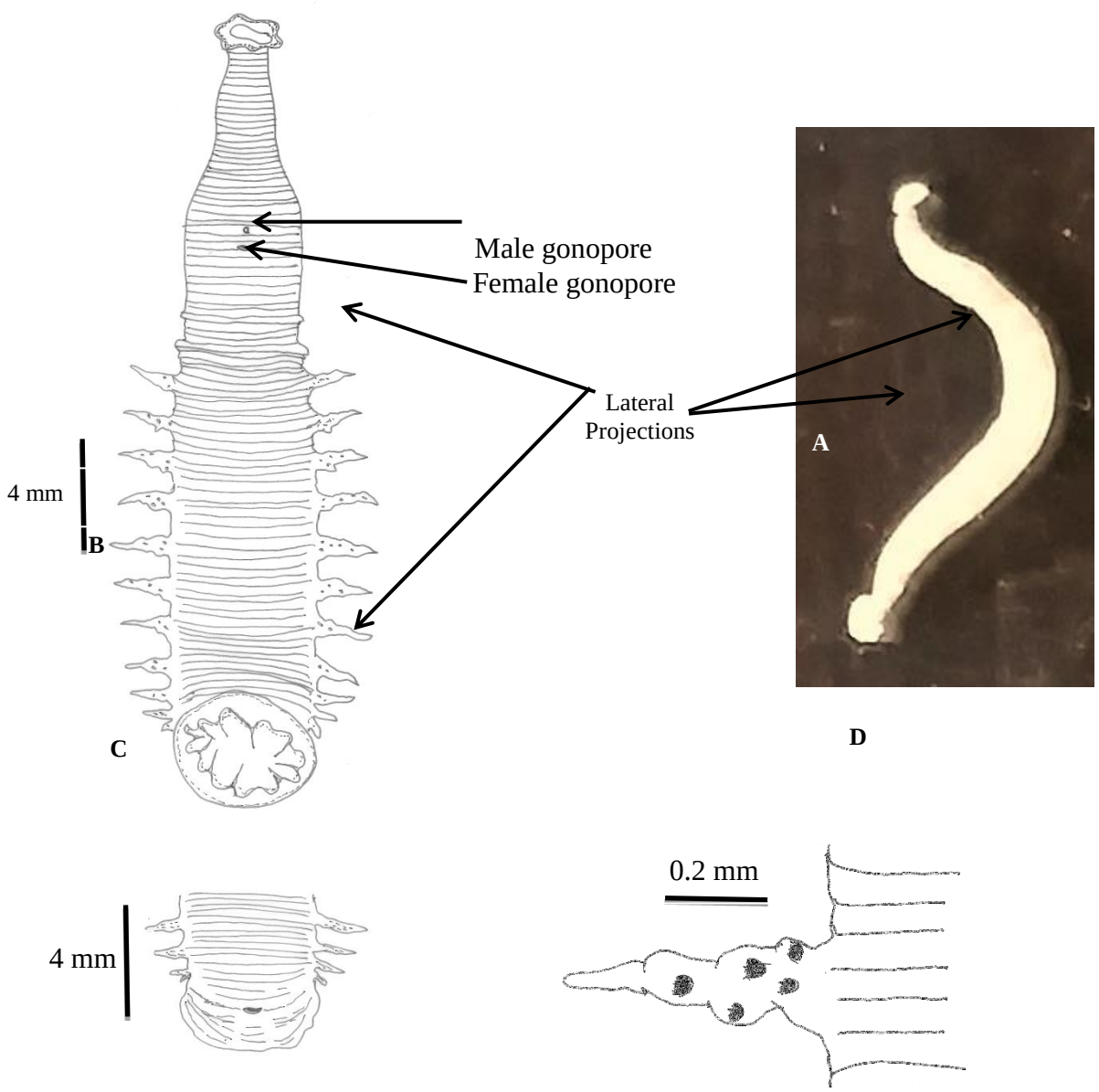


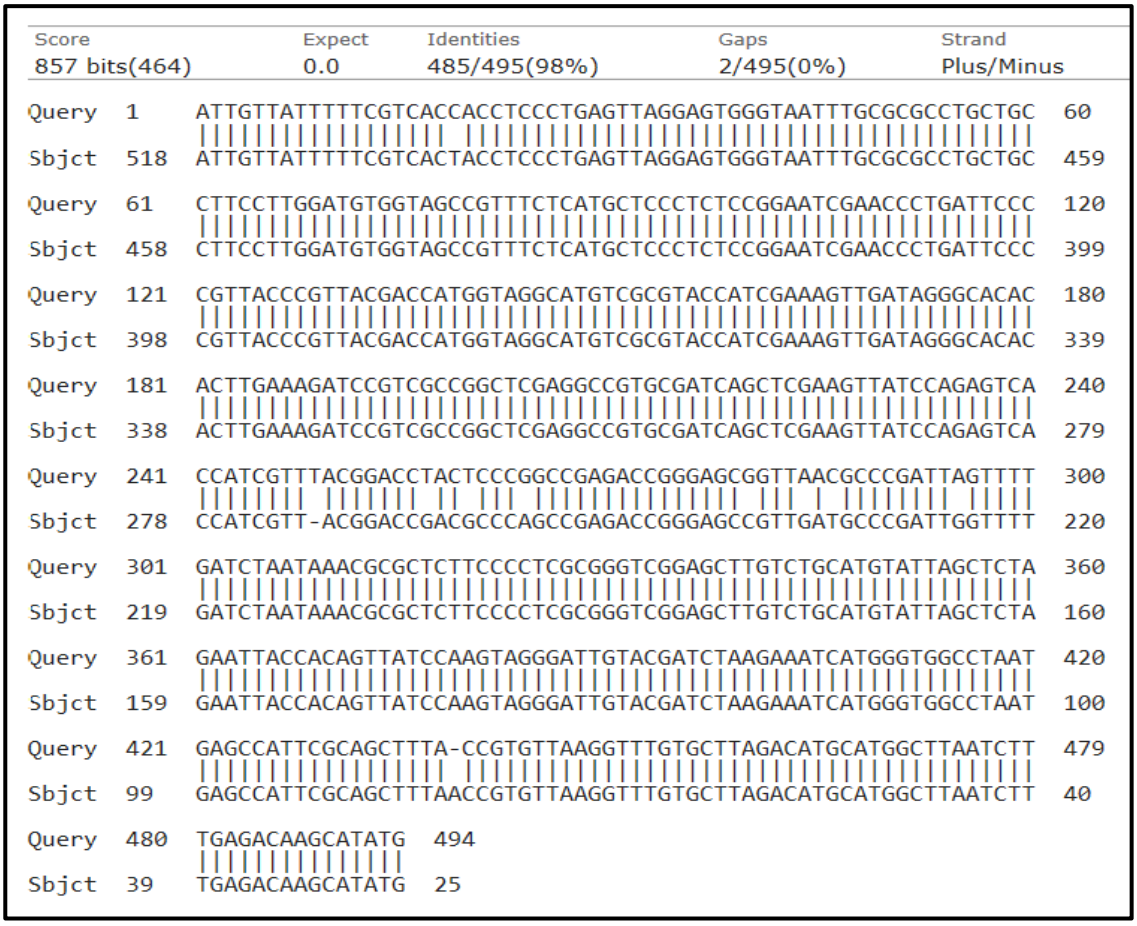
Figure 1: *Erpobdella mestrovi.*

A- Photograph of the fixed specimen whole body (2 X).

B- Lucida drawings, dorsal view.

C- Lucida drawings, dorsal view.

D- Lucida drawings of the lateral projection.



Score	Expect	Identities	Gaps	Strand
857 bits(464)	0.0	485/495(98%)	2/495(0%)	Plus/Minus

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Query  1    ATTGTTATTTTTCGTCACCACCTCCCTGAGTTAGGAGTGGGTAATTTGCGCGCCTGCTGC  60
Sbjct  518  ATTGTTATTTTTCGTCACTACCTCCCTGAGTTAGGAGTGGGTAATTTGCGCGCCTGCTGC  459

Query  61   CTTCCTTGGATGTGGTAGCCGTTTCTCATGCTCCCTCTCCGGAATCGAACCCTGATTCCC  120
Sbjct  458  CTTCCTTGGATGTGGTAGCCGTTTCTCATGCTCCCTCTCCGGAATCGAACCCTGATTCCC  399

Query  121  CGTTACCCGTTACGACCATGGTAGGCATGTCGCGTACCATCGAAAGTTGATAGGGCACAC  180
Sbjct  398  CGTTACCCGTTACGACCATGGTAGGCATGTCGCGTACCATCGAAAGTTGATAGGGCACAC  339

Query  181  ACTTGAAAGATCCGTCGCCGGCTCGAGGCCGTGCGATCAGCTCGAAGTTATCCAGAGTCA  240
Sbjct  338  ACTTGAAAGATCCGTCGCCGGCTCGAGGCCGTGCGATCAGCTCGAAGTTATCCAGAGTCA  279

Query  241  CCATCGTTTACGGACCTACTCCCGGCCGAGACCGGGAGCGGTTAACGCCCGATTAGTTTT  300
Sbjct  278  CCATCGTT-ACGGACCGACGCCCAGCCGAGACCGGGAGCCGTTGATGCCCGATTGGTTTT  220

Query  301  GATCTAATAAACGCGCTCTTCCCCTCGCGGGTCGGAGCTTGTCTGCATGTATTAGCTCTA  360
Sbjct  219  GATCTAATAAACGCGCTCTTCCCCTCGCGGGTCGGAGCTTGTCTGCATGTATTAGCTCTA  160

Query  361  GAATTACCACAGTTATCCAAGTAGGGATTGTACGATCTAAGAAATCATGGGTGGCCTAAT  420
Sbjct  159  GAATTACCACAGTTATCCAAGTAGGGATTGTACGATCTAAGAAATCATGGGTGGCCTAAT  100

Query  421  GAGCCATTCGCAGCTTTA-CCGTGTTAAGGTTTGTGCTTAGACATGCATGGCTTAATCTT  479
Sbjct  99   GAGCCATTCGCAGCTTTAACCGTGTTAAGGTTTGTGCTTAGACATGCATGGCTTAATCTT  40

Query  480  TGAGACAAGCATATG  494
Sbjct  39   TGAGACAAGCATATG  25
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Figure 3: Pair wise alignment of 18S rDNA sequence of *Croatobranchus mestrovi* Query is the study or sample sequence and Sbjct is the GenBank sequence.

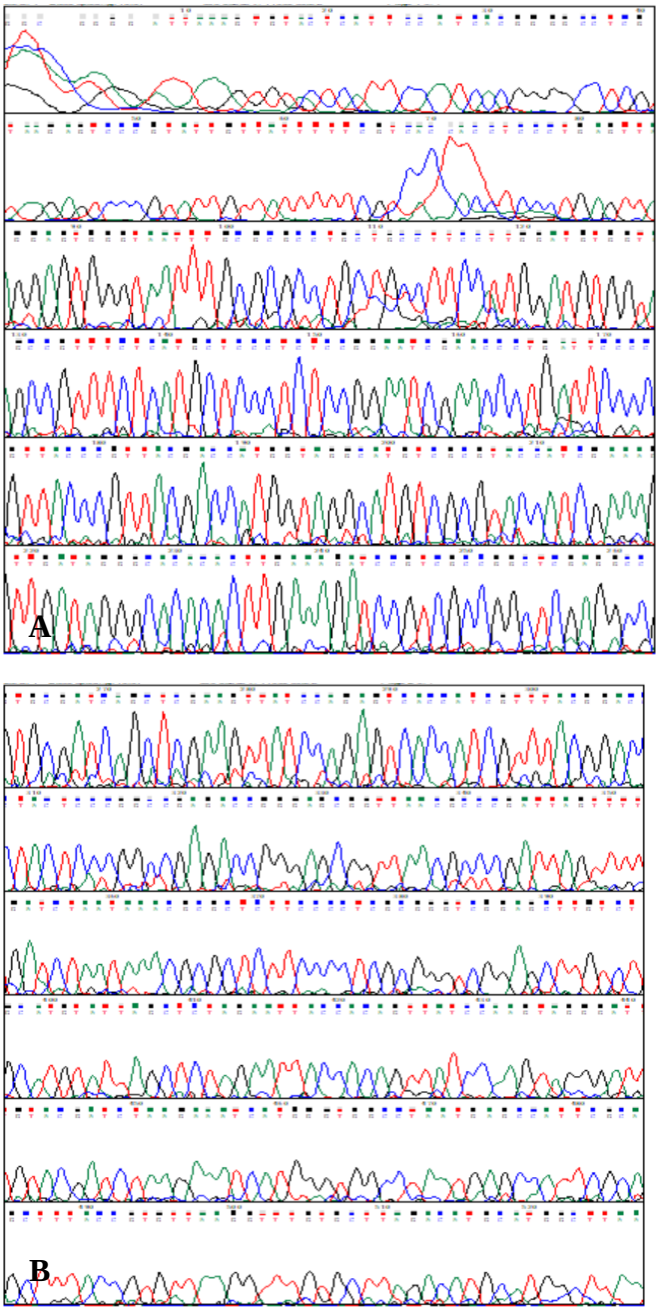

Figure 2: The sequencing result of 18S rDNA of *Erpobdella mestrovi.*

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