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Research Article

Identification of *Baetis* (*Rhodobaetis*) *braaschi* (Zimmermann, 1980) from Hablehrood River (Iran) using COI barcoding

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Abstract

Baetis (*Rhodobaetis*) *braaschi* is an Ephemeropteran species with a vast territory in the world with its eastern limit ends in Iran. This study was conducted to investigate the COI gene sequences of *B. braaschi* from Hablehrood River, Tehran, Iran. In this study, a molecular database of 780 bp was analyzed. The intraspecific genetic difference was found to be insignificant. The phylogenetic analysis identified that four haplotypes (58227, 23419, 28407, and 34719) from Hablehrood River highly supported clusters belonging to *B. braaschi.* Topology of the evolution tree on the basis of maximum likelihood method showed that the samples collected in this study were monophyletic with the samples recorded for the Czech Republic.

Keywords: DNA barcoding, Ephemeropteran, Phylogeny, Taxonomy

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Introduction

The genus *Baetis* is a worldwide group of Baetidae, which is divided into three sub-genera including *Baetis* (Leach, 1815), *Rhodobaetis* (Jacob, 2003), and *Tenuibaetis* (Kang and Yang, 1994). *Rhodobaetis* is distinguished by some specific morphological features such as the presence of a villopore on femur, larval antennae never brought together, no keel on frons, stick-shape prostheca of mandibles, no seta on mandibles, 3-segmented labial palp, and specific projection on the 2nd segment of labial palp (Sroka *et al.,* 2012b). The genus has been reported from different parts of Iran. Tahmasebi *et al.* (2020) reported *Rhodobaetis (Baetis) rhodani* (Pictet, 1834) from Jajrood River, Tehran, Iran. Bojkova *et al.* (2018) in a complete review of Iranian mayflies, mentioned that Iran would be the easternmost limit of this genus distribution area. Some species of the genus have been reported from different parts of the country such as *B. rhodani* from West Azerbaijan, Guilan and Ardebil provinces, *Baetis ilex* (Jacob and Zimmermann, 1978) from Tehran, Guilan and Ardebil provinces, *Baetis vadimi* (Godunko, Palatov and Martynov, 2015) from Guilan and Ardebil provinces and *Baetis braaschi* from Guilan and Ardebil provinces.

Baetis braaschi (Zimmermann, 1980) is previously defined as a subspecies of *B. rhodani* and recently has been categorized as *Rhodobaetis* subgenus (Sroka *et al.,* 2012a). The species is a microhabitat specialist, which is mainly abundant in cobbles, boulders, and rapid currents (Vilenica *et al.,* 2018), especially in mountain areas (Martynov, 2013). It is known that the species is distributed mainly in central Asia to Crimea. The species has been reported from Uzbekistan, Turkmenistan and Tajikistan (Kluge, 1982), Crimea (Godunko *et al.,* 2004), Ukraine (Martynov and Godunko, 2010), Turkey (Kazanci and Turkmen, 2012; Salur *et al.,* 2016), Georgia (Gabelashvili *et al.,* 2018), Iraq (Al-Saffar, 2016; Khudhur and Sroka, 2021) and Iran (Keikhosravi *et al.,* 2022; Koroojdehi *et al.,* 2022). Staniczek *et al.* (2020) reported *B. braaschi* from the southern (Khuzestan) and northern (Mazandaran) provinces of Iran as permanent habitats. Khodhur and Sroka (2021) suggested that the species would have been delivered to Iraq through water flows from Turkey or Iran.

The species has been considered as a part of the materhithral community of Crimmean rivers which has been mostly described (Godunko *et al.,* 2004). In a COI barcoding study, populations of three different geographical regions (Crimean Peninsula, Eastern Ukraine, and Caucasus) with nine sampling stations were investigated and the intraspecific distance showed seven haplotypes for *B. braaschi* which did not reflect any differences in morphological features; so it was concluded that variability in the morphological characters of *Baetis* species, especially in subgenus *Rhodobaetis* species do not follow differences in COI sequences (Sroka *et al.,* 2012b). In addition, morphologically, the species shows

minute differences against other *Rhodobaetis* species according to the shape of the bristles on the larva femora, spatula on larva antennae, and the shape of labial palp. Therefore, it is suggested to consider COI sequences to increase the accuracy of the phylogenetic analysis and species identification (Tiunova *et al.,* 2021).

In a similar study, Al-Saffar (2016) investigated the COI sequence of *B. braaschi* in Iraq and found consistent matching between Iraq population of the species with the previous sequence from Europe and Turkey in Genbank. Tiunova *et al.* (2021) reconstructed the phylogenetic relationships of COI sequences belonging to all *Baetis* species collected from Russian Far East; *B. braaschi* made a polytomy node on the Bayesian tree with *B. ilex* due to COI barcoding and morphological data.

In a recent study, Keikhosravi *et al.* (2022) investigated the morphological and molecular features of *B. braaschi* populations from different parts of Iran and showed that Iranian specimens had 99% molecular similarity with their European conspecifics. Due to the lack of enough information about the genomic knowledge of *B. braaschi,* this study was conducted to investigate the COI barcoding of the species collected along Hablehrood River which is located in the Southern Central Alborz mountain, Tehran, Iran.

Materials and methods

The sampling area was selected as a length of 27 km along Hablehrood River bank limited between two geographical points the longitude of 52˚15′30″E to 52˚40′56″E and the latitude of 35˚31′34″N to 35˚39′11″N. Four sampling points were considered along the river named Simindasht, Mazdaran, Khomadeh, and Namroud; the geographical characteristics of the sampling points are presented in Table 1 and Figure 1. *Baetis* nymphs were collected manually by a minute pence from pebbles and the bottom surface of rocks during the spring and summer of 2022 and preserved in ethanol 70%.

DNA was extracted using the DNA tissue kit (MasterMix-MBST, Iran) according to the manufacturer's instructions. The kit has been designed on the basis of the selective binding of nucleic acids to a silica based membrane. Ethanol-based samples were used for DNA isolation. Formalin is often used to preserve insect samples for DNA extraction. 10 specimens of *B. braaschi* nymphs were placed in AT1 lysis buffer for 5 min and 5µL proteinase K for 20 min at 60˚C. After adding 450µL AT2 binding buffer and incubating for 5 min at 60˚C, 450µL ethanol was added. The mixture was diluted in 48µL water and 2µL RNAase. Then, the content was transferred to the column and subsequently, 700µL of the isolating buffer, AT4, was added to extract the DNA and centrifuged for one min. Finally, 50-100µL of the elution buffer, AT5 was added to elute the DNA.

Table 1: Geographical situation of the sampling points along Hablehrood River, Tehran, Iran.

Sampling points	Longitude (E)	Latitude (N)	Altitude (m)
Simindasht	52˚29'50"	35˚31'38"	1489
Mazdaran	52˚36'05"	35˚34'51"	1611
Khomadeh	52˚41'07"	35˚40'03"	1732
Namroud	52˚40'52"	35˚43'54"	1846

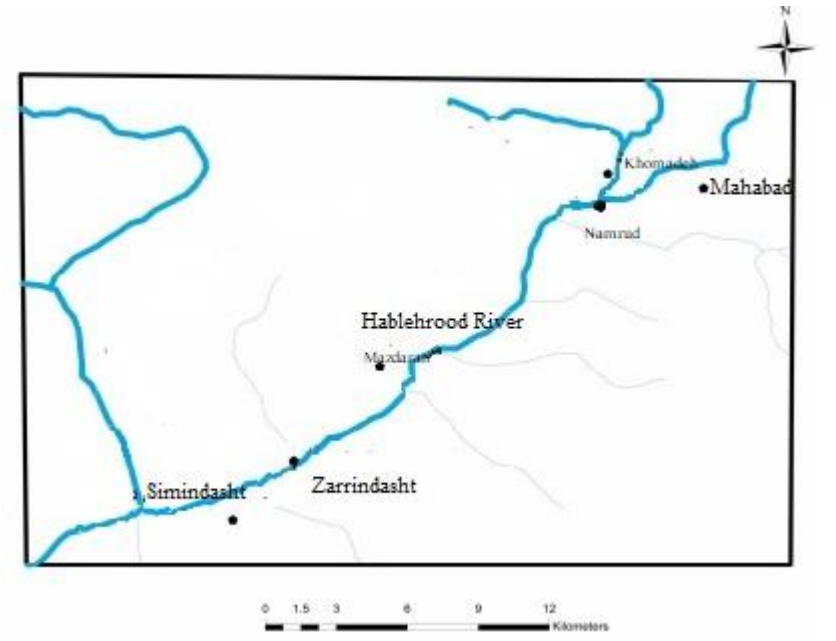

Figure 1: The sampling points along Hablehrood River.

Polymerase Chain Reaction was conducted in a volume of 25µL, consisting of 3µL DNA template, 1µL of 10 pmol/µL of each primer, 12.5 µL Romax 2x MasterMix, and 7.5µL DEPC water. The primers used for this study was designed according to the cytochrome oxidase I sequence belonging to insects taxa within the DNA sequence between forward and reverse sequences on GenBank as follows:

Forward sequence: GGTCAACAAATCATAAAGATATTGG with accession No. LCO1490
Reverse sequence: TAAACTTCAGGGTGACCAAAAAATCA with accession No. HCO2198

Optimized PCR conditions included initial denaturation at 94°C for 3 min, 5 cycles of denaturation at 94°C for 20 sec, annealing at 40°C for 20 sec, and extension at 72°C for 45 sec, with a final extension at 94°C for 20 sec. As suggested for DNA extraction in some other arthropods (Becker *et al.*, 2021), the cycle was repeated again with 32 cycles of annealing at 51°c for 30 sec, extension at 72°C for 40 sec, and the final extension at 72°C for 5 min. Through spectrophotometry, the quantity of the DNA in the solution was calculated from the absorbance of 260

nm (A260) and the purity was calculated by the ratio of A260/A280.

Finally, 10µL of PCR product was run on a 1.5% agarose in TBE buffer. 10µL of DNA Green Viewer™ (Parstous, Iran) was added to the solution and after swirling the flask, poured into the gel tray. After visualization of the positive band under UV, the PCR product was extracted from the gel using DNA extraction kit from agarose gel. In this study, DNA Green viewer protocol was used to visualize DNA band under UV which is an alternative to the traditional Ethidium bromide stain for detecting DNA (Cunha *et al*., 2020).

Molecular reconstruction was conducted on nine obtained sequences in accordance with the 18 reference sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank) to represent the species from the study area.

All GenBank accession numbers and collecting details of the specimens are given in Figure 2. Sequence chromatograms were inspected and edited using Chromas v. 2.5. Alignment, reconstruction, and genetic distance calculations were conducted in MEGA v.7.0 (Kumar *et al.*, 2016). Maximum Likelihood analysis for each node was conducted with 1000 bootstrap replicates. All PCR products were sequenced by Macrogen Sequencing Service (Seoul, South Korea), the sequence data were deposited in the GenBank database with the assigned accession numbers: OP263724, OP263725, OP263726, OP263727, OP263728, OP263730, and OP251141. Multiple alignments of the sequences were performed with ClustalW in Mega 4.1 to assess nucleotide composition and variable sites. Phylogenetic analysis of the samples was conducted using Maximum Likelihood (ML) algorithm 5.

Results

In this study, a molecular database of 780 bp for *B. braaschi* was analyzed. The intraspecific genetic difference was found to be insignificant. The phylogenetic analysis identified that four haplotypes (58227, 23419, 28407, and 34719) from Hablehrood River highly supported clusters belonging to *B. braaschi* (similarity of 97.23, 96.94, 97.78, and 98.42. The topology of the evolution tree on the basis of maximum likelihood method (ML) showed that the samples collected in this study were monophyletic with the samples recorded for the Czech Republic (Fig. 1).

The partial COI sequences of *B. braaschi* collected in this study and 11 sequences from GenBank (JN164283, JN164279, JN164278, JN383393, JN164281, JN164282, JN164280, JN184284, JN383339, JN383392, and JN383390) were used for further phylogenetic analyses. A sequence from GenBank (AY326863) was also used as an out-group.

Phylogenetic analysis and ML showed that 28 sequences of COI had partially different topologic patterns for the evolutionary tree. As previously mentioned, the phylogenetic tree of COI haplotypes at 1000 bootstrap rate showed clustering into four major clades with the bootstrap index of 75, 97, 83,

and 64 for the samples recorded on Genbank.

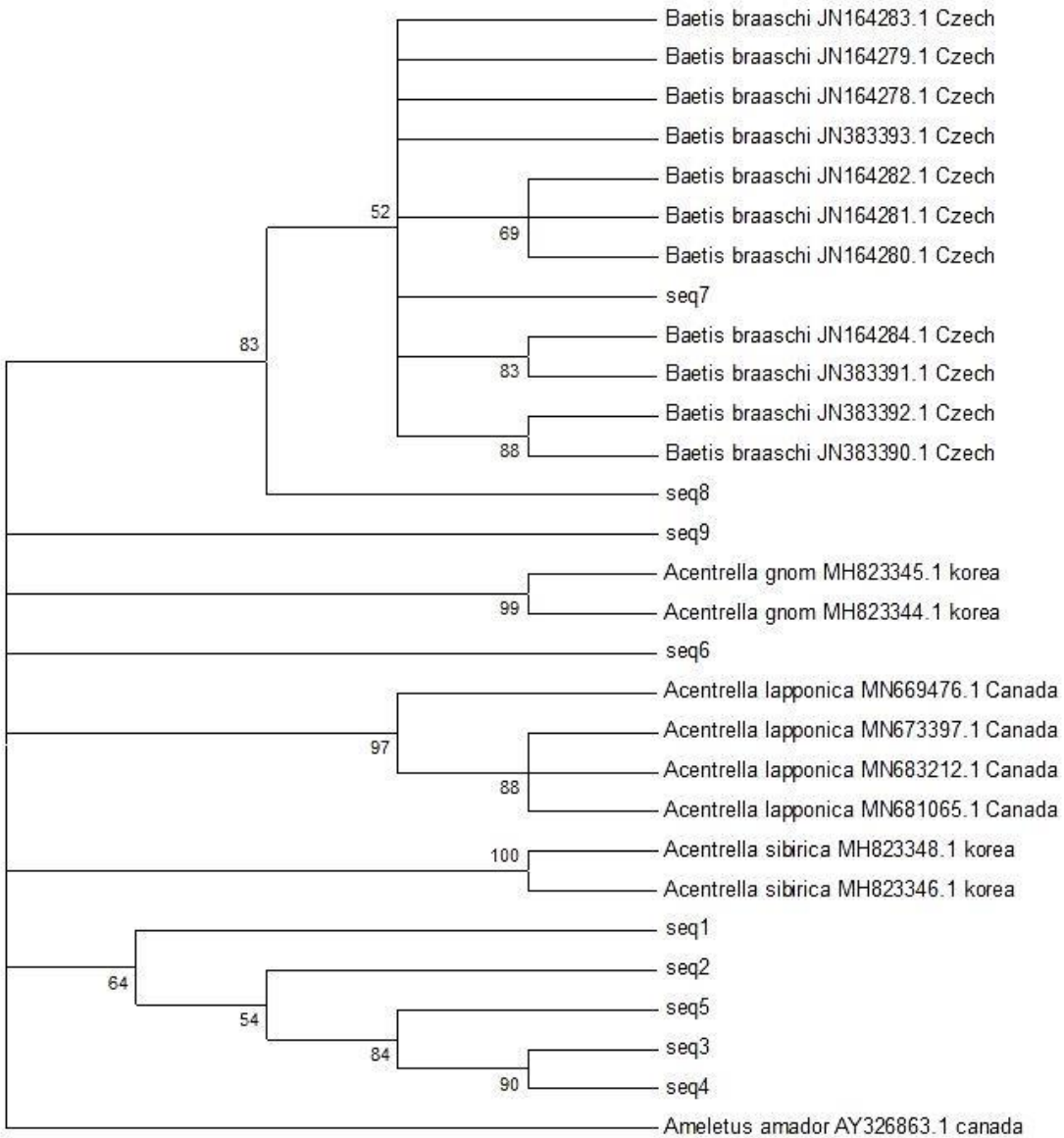

Figure 2: Phylogenetic tree of mitochondrial haplotypes of *B. braaschi* from Hablehrood River.

The partial COI sequences derived in this study are recorded on GenBank as seven accession numbers as follows: OP263730 (647 bp), OP263725 (574 bp), OP263728 (637 bp), OP263726 (628bp), OP263724 (636 bp), OP251141 (467 bp), and OP263727 (652 bp).

Discussion

Four haplotypes were found in nine samples of *B. braaschi* obtained along Hablehrood River, which showed a difference in their abundance. In a similar study, Keikhosravi *et al.* (2022) studied different populations of *B. braaschi* from varied parts of Iran and found that the Iranian haplotypes showed high similarity to East Europe

samples; similar results were obtained in this study. It can be concluded that geographical distribution had insignificant effects on the genotype of the species but as Avolio *et al.* (2012) indicated previously, spatiotemporal isolation of the same species in different locations could preserve genetic variation which would lead to raise in the haplotypes differentiation. The same fact showed up in the results of this study. Our results revealed that the Iranian population of *B. braaschi* along Hablehrood River, grouped with samples from the Czech Republic. They may have originated from a very prevalent ancestor. As the species has been reported from the northwest part of Iran, Guilan, and Ardebil provinces, it can be assumed that the population in Hablehrood River is a result of water flow from Alborz mountain southward. On the other hand, due to the short lifespan of Ephemeropteran adults, populations of the species in this order are not capable of flying long distances; so there is no way for species divergence or genetic drift. In another similar study, Koohpayma *et al.* (2021) indicated that flying would be a strong way to distribute insects` genotypes toward new locations.

Most of the studies about *B. braaschi* and totally *Baetis* species have been done in Europe and unfortunately, there is a great lack of information about the species, especially from the Middle East. Khudhur and Sroka (2021) in their recent checklist of mayflies in Iraq suspected that *B. braaschi* would be misidentified as *B. vernus* Curtis, and insisted that for the correct identification of the genus relying on the molecular methods. Due to the results of Sroka *et al.* (2012a), several studies revealed that several specimens of the same species would represent different COI sequences, but *B. braaschi* can be a much reversed situation where some specimens significantly differ in morphological aspects but the COI sequences are uniform. This study admitted the high need for molecular methods for the correct identification of Ephemropteran species. Due to many challenges in identifying the species based on the morphological features, DNA barcoding would be an effective alternative.

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