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Morphological characterization of Chironomidae (Diptera) larvae in Anzali Wetland, Southwest Caspian Sea: First record of *Chironomus plumosus*

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ABSTRACT

Chironomids (Diptera) include the most abundant group of macroinvertebrates. They are usually collected from aquatic environments for quality evaluation. Tolerant aquatic organisms such as Chironomids are more abundant in polluted sites. This note makes the Chironomids as an excellent bioindicators. This group is not characterized in Anzali Wetland as well. So that, the aim of this study was to recover and characterize chironomid larvae in this wetland. Hence, Chironomid larvae were collected on the seasonal basis from 13 stations in the wetland. The main characteristics for their identification were eye spot, mentum, ventromental plate, antenna, and ventral tubules. The mitochondrial cytochrome oxidase I (COI) gene was applied to simplify the identification of *Chironomus* species by polymerase chain reaction (PCR) and sequencing method. The nucleotide sequence alignments were used for construction of the phylogenetic trees based on maximum likelihood method. Six genera were identified in three subfamilies, including: Chironominae (1 genus), Orthocladinae (1 genus), and Tanypodinae (4 genera). Based on ventral tubules, the dominant population of *Chironomus* larvae found in this study, lie in thummi and plumosus types. Five genera were reported for the first time from Anzali Wetland. The dominant genus was *Chironomus*. These groups of larvae were ultimately identified as *Chironomus plumosus* reporting for the first time from the wetland. It was also found that the Chironomids diversity is higher than those described in few studies before, however, further studies are still needed.

Keywords: Bio-indicator, Chironominae, Cytochrome oxidase I, DNA barcoding, macroinvertebrates.

INTRODUCTION

Wetlands are known worldwide as biodiversity refuges and are among the most biologically diverse in the world. There is huge base of knowledge regarding wetland biodiversity patterns (Orlova *et al*. 2004). Anzali Wetland is made up of large, shallow, eutrophic freshwater lagoons, shallow impoundments, marshes, and seasonally flooded grasslands at the south-western coast of the Caspian Sea. It consists of different aquatic and dry land ecosystems and is a good example of a natural habitat supporting an extremely diverse wetland flora and fauna (Esmaeilzadeh 2016). However, the water quality of the wetland is deteriorating due to the inflow of wastewater and solid waste from neighboring cities, including the provincial capital, Rasht City. The wetland is also getting drier and shrinking due to the inflow of sediment from the catchment area (approx. 3610 km^2). Biological, physical and chemical components of the wetland are linked and interact with each other in a complex manner. Ecological condition of the wetland is maintained based on the delicate balance of those components. Anzali Wetland represents unique and significant ecological as well as economical values.

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However, there are some factors threatening the further sustainability of this commercially- and ecologically-important wetland. It is located in the middle of two flyways including the Africa-Eurasian and Asia-Pacific ones, crossing each other. The ecological values of the wetland can provide unique opportunities for scientific research and education. Similar to the esthetic values of the wetland, these values are also difficult to quantify in monetary term. Most of human activities with negative impacts change water quality and quantity of the wetland, disturb natural habitats and harvest/kill excessive number of wildlife (JICA 2005).

The anthropogenic disturbances strongly affect the species richness of aquatic macro-invertebrates. The macro-benthic fauna are tending to remain in their original habitats with great acclimation capability. They can tolerate any changes in water quality and high loads of pollution. Under polluted conditions, the community structure may simplify in favor of tolerant species, but the abundance of a certain species may increase. Nevertheless, the diversity and species richness decrease. By assessing the diversity and functional groups of the indicator species of the benthic macro-faunal community, it is possible to evaluate water quality. Consequently, they can be considered as good bio-indicators for the environmental changes of aquatic ecosystems (Rosenberg & Resh 1993).

Chironomidae is a major family of the order Diptera including non-biting midges. Members of this group live in natural environment of chironomids and their reproduction region cut across different habitats from the Arctic to Antarctic and also from fresh water sources to very contaminated waters (Failla *et al*. 2015). Chironomids have been used as indicators of water quality (Chutter 1972). Several biological indices concerning evaluation and monitoring of water quality depend greatly on them (Barbosa *et al*. 1997). Variation in species composition, over-lordship of pollution-tolerant species, and repetition of occurring deformities on larval head capsules, are some of the commonly used trait in these types of evaluations (Johnson *et al*. 1993).

Distribution of chironomids and their taxonomy have been studied in some northern and eastern areas of Iran. Most of these studies tended to identify the aquatics as a live food. At present, very few studies have used multiple techniques to differentiate among chironomids in Iran aquatic ecosystems. In this study, we applied morphological methods for characterizing some chironomids and molecular techniques for accurate identification of one *Chironomus* species in Anzali Wetland.

MATERIALS AND METHODS

Study area

Anzali Wetland is located in 37°28´ N and 49°25´ W with an open surface area of 58 km^2. Its southern part is mainly under rice cultivation and patches of woodland, while the northern part is bordered by sand dunes with grassland and scrubby vegetation (Esmaeilzadeh 2016).

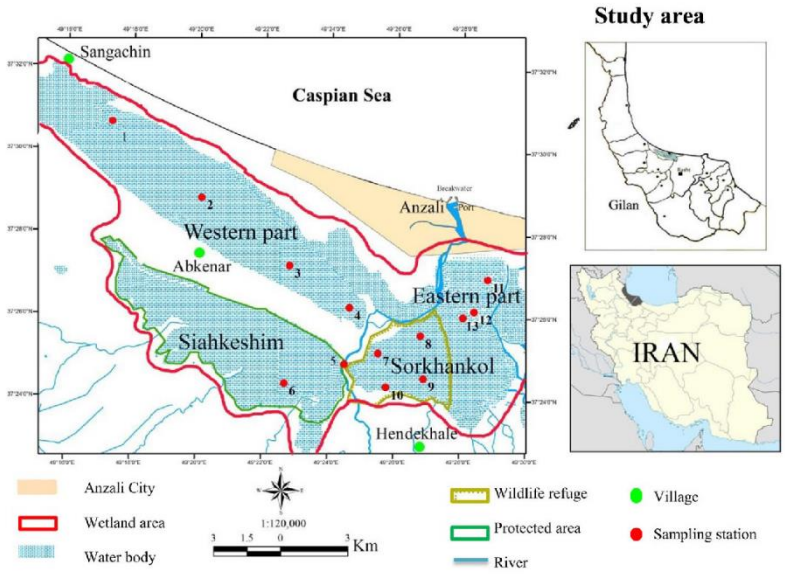

Fig. 1. Study area and sampling stations (Nazarhaghighi *et al*. 2014).

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Sampling and sample processing

Chironomid larvae were collected on the seasonal basis from 13 stations in Anzali Wetland (Fig. 1) from August 2012 to June 2013. The larvae were collected with grab (0.04 m^2) among bryophytes, other submerged plants and bottom surface layer. In the field, the materials were passed through 500µm mesh size sieves, then were placed in 70% ethanol-bottles. Head capsule and terminal abdominal segments with attached tubules were separated and kept for morphological studies, while the remnant was retained for molecular analyses. All measurements and photographs from fixed specimens were taken by 5 MP and processed using TSVIEW software (version 6.2.4.5). Identifications were carried out according to Oliver (1983). Chris Madden (2010) key for genera of larvae in Australian chironomids was employed, allowing identification of genus without the need for routine mounting of larvae. The key provides shortcuts which enable the identification of genus using characters visible on the whole animal, hence reduces the need for mounted larvae in the routine genus identification. In ecological studies, the large larvae (>20 mm) of the *plumosus* group, i.e. those with lateral tubuli, are generally considered to be *C. plumosus* (Kubeka *et al*. 1998).

DNA extraction, PCR and sequencing

Genomic DNA was extracted from the body of larvae by using Tissue & Cell Genomic DNA Purification Kit (GeneMark, Taiwan). A 710-bp fragment of the COI gene was amplified using the flanking primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO 2198 (5'-TAAACTTCAGGGTGACCA-AAAAATCA- 3') (Folmer, 1994), by applying the following PCR profile: 5 min at 94 °C, 30 cycles of 94 °C for 30 s, 49 °C for 30 s and 72 °C for 1 min and a final extension step of 5 min at 72 °C. A typical PCR-protocol is as follows: Template DNA, 5 µL; AmpliTaq Polymerase, 1.25 units; primer concentration, 20 pM; dNTP concentration, 200 mM; $MgCl_2$ concentration, 2 mM; and 5 µL 10X PCR buffer. Sterile water was added to give a total volume of 50 µL. PCR products were confirmed by electrophoresis on a 1% agarose gel with power load dye, and were purified using PureLink Quick PCR Purification Kit (Invitrogen, Germany) for sequencing. Samples were sent to Macrogen Corporation (South Korea) for sequencing. Same forward and reverse primers were used for sequencing of both DNA strands.

Data interpretation

All the DNA-sequences were aligned using the ClustalW alignment program. Analyses were limited to reliably aligned regions from the data set and regions that could not be unambiguously aligned were excluded from the analysis. Sequences from all genes were combined in a single data set. The phylogenetic analyses were carried out using MEGA 5 program by applying maximum parsimony (MP) and neighbor joining (NJ). For the MP trees, 10000 bootstrap replicates were analyzed by heuristic search using close-neighbor-interchange (CNI) branch swapping implemented in the MEGA 5 program.

RESULTS

Species identification

Identifications of species were performed on larvae samples. Six genera were identified in three subfamilies, includeing Chironominae (1 genus: *Chironomus*), Orthocladinae (1 genus: *Cricotopus*), and Tanypodinae (4 genera: *Ablabesmyia*, *Alotanypus*, *Apsectrotanypus*, *Procladius*). Five genera and one species were reported for the first time from the wetland and the dominant genus was *Chironomus*.

Chironomus plumosus: Live specimens were dark pink to red in color, but those preserved in formaldehyde turned into pale. Larval body was usually less than 30 mm in length. The cylindrical body exhibited the bilateral symmetry. Three thoracic and eighth abdominal segments were counted. There were no lateral setae (Fig. 2. A). Eye spots were double and similar in size. One eye-spot was shaped like a kidney (Figs. 2. B and C). Mentum had trifid median tooth and 12 lateral teeth. Widespread anteromedial margin of ventromental plate was smooth (Figs. 2. D, E and F).

Pecten epipharyngis was a broad multitoothed comb (Fig. 2. G, H). Pre-mandible apically was bifid with one additional small tooth near the center (Fig. 2. I). Mandible possesses three dark inner teeth (Figs. 2. J and K). A pair of five segmented antenna is located on the dorsal surface of the anterior end. Antenna length was equal to one-third the width of the head. The first or basal segment was very large and almost three times as long as the

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second segment. A circular hollow called ring organ visible on the basal segment. Antennal blade was on the top of the first segment near the second one which was about the same size as subsegment (Figs. 2 C, L and M). Two pairs of ventral tubules were present on abdominal segment 8. Procercus was prominent, long and colorless. It was attached to rounded extension of the last abdominal segment.

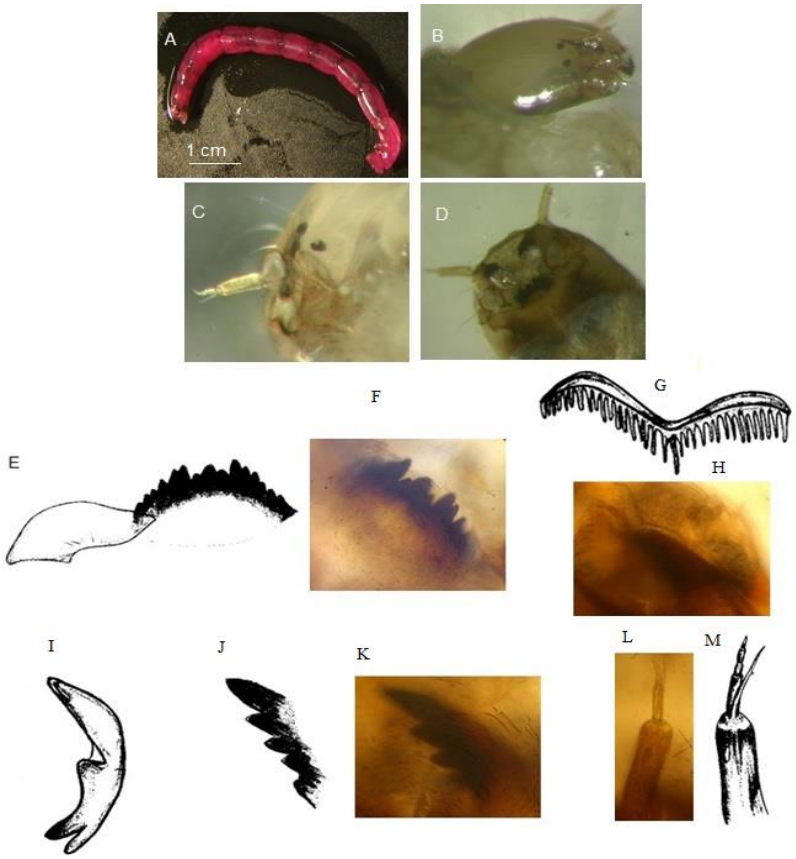

Fig. 2. Larval head parts of *Chironomus plumosus*. A) entire `body; B) lateral view of head; C) eye spot and antenna; D) ventral view of head; E, F) mentum and ventromentum; G, H) pecten epipharyngis; I) premandible; J, K) mandible; L, M) antenna.

Circotopus: Live specimens were less than 1 cm. Antennae were shorter than half the head length. Two separate eye spots were unequal in size. Abdominal body segments were with postero-lateral setae tufts, but they were difficult to see. The actual body appeared not to be covered with setae. Anal prolegs were long and procercus had tuft of setae (Fig. 3).

Ablabesmyia: Live specimens were less than 1 cm. Head was long and with kidney-shaped eye. Two dark simple claws were seen on the posterior prolegs. There were scattered setae on the body (Fig. 3).

Alotanypus: In this specimen, the head was not conical in shape and the ratio of width to length was higher. Mentum had 8 distinct teeth on each side. The teeth were staid in the ascending line on both sides of the front. Posterior prolegs were long. They had multiple and thin claws on it. Body was covered with thin setae (Fig. 4).

Apsectrotanypus: The larvae were small and about 5 mm in length. Mentum had 5 distinct teeth on each side. Procercus had tuft of long setae. There was no claw on the posterior prolegs (Fig. 5).

Procladius: The head and body of these larvae were cream in color. Mentum and ligola were darkened and marked with specific teeth. The mentum was located along two ascending lines on either side of the front mentum. The first segment of the body was swollen, larger and more distinct than other parts of the body. Two brown spots on the back surface were visible (Fig. 6).

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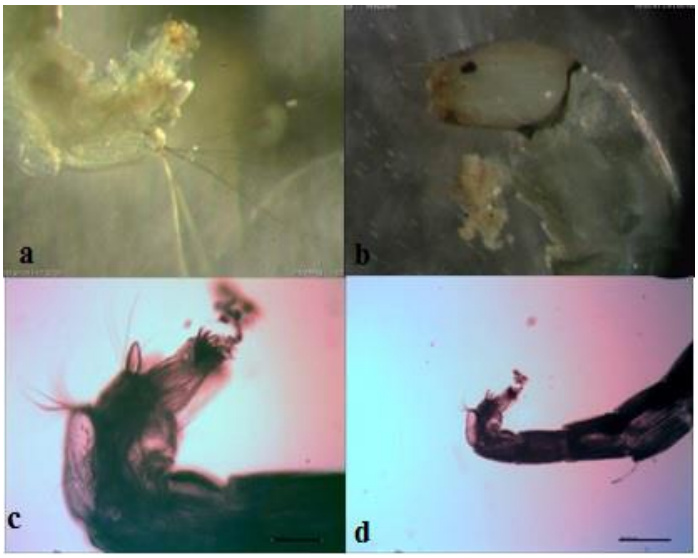

Fig. 3 (a).Tuft of setae in procercus of *Cricotopus*; (b). Larval head of *Ablabesmyia*; (c). Dark claws on posterior prolegs of *Alotanypus*; (d). Abdominal part of body without lateral tubules in *Ablabesmyia*.

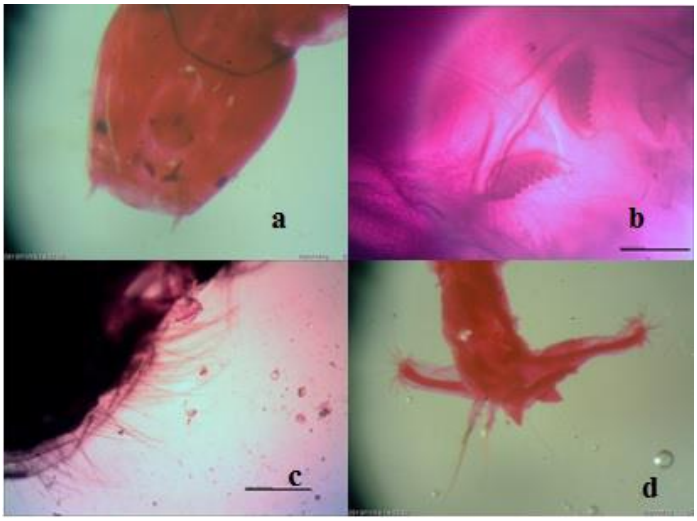

Fig. 4 (a). Larval head of *Ablabesmyia*, (kidney shaped eye, short antenna); (b). Mentum with 8 distinct teeth on each side; (c). Thin setae covered body; (d). Tuft of long setae on procercus and long prolegs.

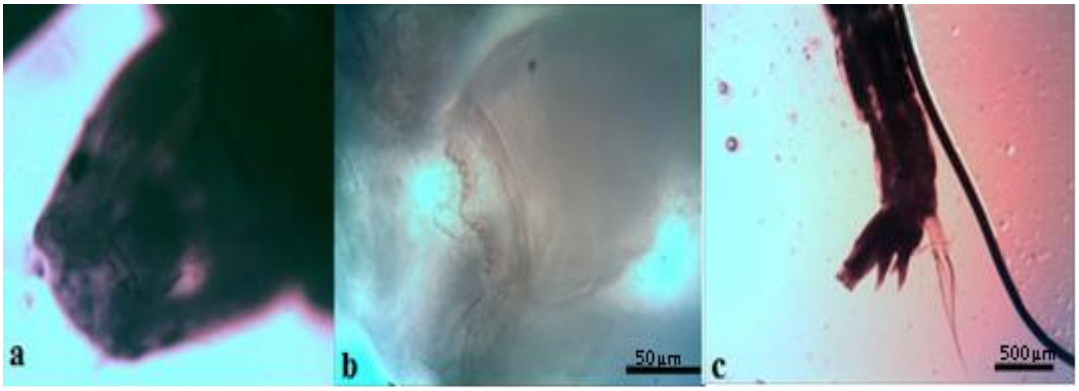

Fig. 5 (a). Larval head of *Apsectrotanypus*; (b). Mentum with 5 distinct teeth on each side; (c). tuft of long setae on long procercus and posterior prolegs without claw.

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Fig. 6 (a). Larval head of *Procladius*; (b). Entire of body (with first swollen and larger segment); (c). Two brown spots on the back surface of first segment.

Amplification and sequencing of mtDNA

PCR amplification of partial fragment of mtDNA cytochrome oxidase subunit I (COI) was successfully achieved (Fig. 7). The sequence was submitted to Genbank database (the accession numbers is KX087217.1). The sequencing showed that the identified *Chironomus* had about 98% homology with *C. plumosus* according to mtDNA cytochrome oxidase subunit 1 (COI) gene.

Analysis of the mitochondrial cytochrome oxidase subunit 1 (COI) sequence from this larva indicated that it differ only 2% from the European *C. plumosus* sequence (GenBank accession number JN016834) (Fig. 8). The gene sequence of the species is reported for the first time in Iran.

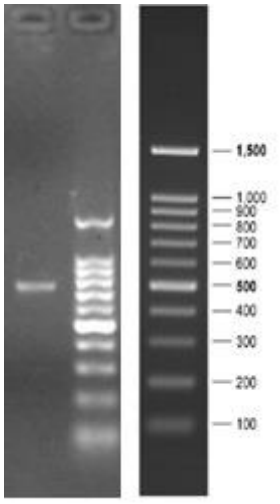

Fig. 7. Gel photograph exhibiting mitochondrial gene amplicon in 1% agarose gel. Left lane is amplified COI gene and right lane is 100 bp ladder.

DISSCUSSION

Three subfamilies: Chironominae, Orthocladinae and Tanypodinae, were identified from the family of Chironomidae in Anzali Wetland. Tanypodinae with 4 genera was a diverse subfamily. However the *Chironomus* was the dominant group among them. Orthocladinae and Tanypodinae were very rare in the study area during the sampling period. Consequently, their identification to species level was not possible. Therefore, this study was focused on *Chironomus* genus. Our efforts for amplification the COI gene from Orthocladinae and Tanypodinae were also not successful. Of course, noteworthy, the *Chironomus* is the most abundant genus of the Chironominae subfamily.

In the past decades, the Russian scientists have studied Chironomidae in the Pontocaspian area. Due to the lack of information on the species of Chironomidae, their classification has become commonplace at the gender level. The following species have been observed in the northeast of Caspian Sea: *Clunio marinus*, *Tanytarsus gr. gregurius kieff*; *Cryptochironomus gr. defectus kieff; Tanytarsus gr. mancus v. d. wulp; Ablabesmyia gr.*

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Tertrasticta kieff. The reports also reveal an uncertain number of *Cricotopus* and *Stictochironomus* genera larvae (Orlova *et al*. 1999). Few studies have been carried out on the Chironomidae in Iran. Marbor is a permanent river in Isfahan Province, Iran. Karami *et al*. (2014) reported the existence of Chironominae (15 genera), Diamesinae (2 genera), Orthocladiinae (17 genera) and Tanypodinae (5 genera) as well as recording *Chironomus*, *Cricotopus*, *Alotanypus*, *Ablabesmia* in this river. Ebrahimnezhad & Allahbakhshi (2013) identified thirty five genera in four subfamilies, including Chironominae (15 genera), Orthocladinae (13 genera), Tanypodinae (5 genera) and Diamesinae (2 genera) along with recording *Cricotopus* and *Ablabesmyia* in Golpayegan River, another river in Isfahan Province, Iran.

Although numerous authors have studied Anzali Wetland from the pollution, faunistic, and ecological aspects (Jafari 2009; Zanjani & Saeedi 2013), however, there are limited data on the benthic species diversity in this wetland.

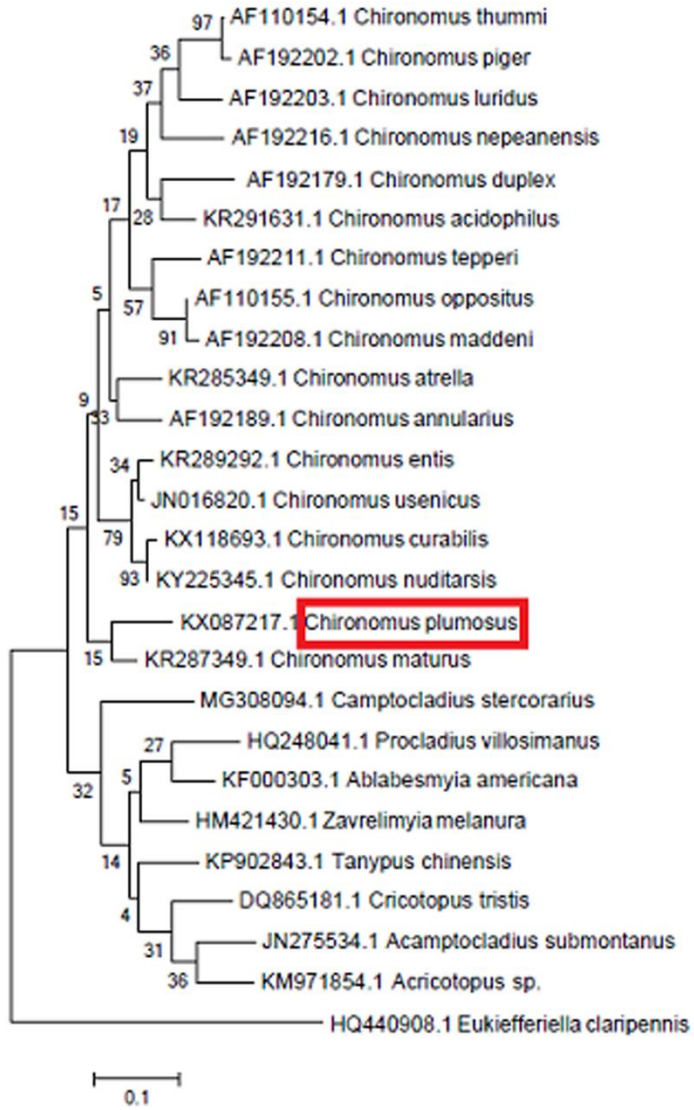

Fig. 8. Phylogenetic relationship of *C. plumosus* isolated from Anzali Wetland with other *Chironomus* species in GenBank database according neighbor-joining bootstrap tree.

Information on Chironomidae species in Anzali Wetland is infrequent. Valypoor (1997) studied the abundance and distribution of chironomid larvae at the family level in this wetland. Another study on integrated management for ecosystem conservation of this wetland by Japan International Cooperation Agency (JICA, 2005) introduced Chironomidae and only *Chironomus*, followed by the final report which also identified

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Chironomus as the dominant genus of the Chironomidae family in this wetland. Due to the large size and abundance of the *Chironomus* genus, the species identification was successful. However, the identification of *Chironomus* larvae to species level can be problematic because there are few conspicuous morphological differences among many *Chironomus* species (Proulx *et al*. 2013). If we cannot correctly identify the *Chironomus* larvae to species level, then it will be difficult to use them to assess environmental impacts. During one year of sampling in Anzali Wetland, *C. plumosus* was reported for the first time in this wetland. Eye spot, mentum, ventromental plate, antenna, and ventral tubules were obvious for identifying the family and the genus. However some features such as the head capsule shape, lateral setae, abdominal segments, and pecten epipharyngis were also used. There have been several efforts to classify *Chironomus* larvae into types, based on the morphology of their ventral tubules (Shobanov 2002; Proulx *et al*. 2013). In the latter study, Proulx *et al.* (2013), have introduced nine types for classification of *Chironomus* larvae based on ventral tubules (salinarius, halophilus, bathophilus, fluviatilis, thummi, reductus, semireductus, melanotus and plumosus). Accordingly, the dominant population of *Chironomus* larvae established in this study, lie in thummi and plumosus types. As stated by Webb & Scholl (1985), the central trifid tooth of the larval mentum pursuant to the degree of fusion of its three component teeth, can be classified to the width of the middle tooth, and the height of the outer teeth relative to the middle tooth. From this point of view, the fourth lateral tooth of the specimen in this study has the same size as the third and fifth lateral teeth. Also, we employed a classification scheme developed by Proulx *et al.* (2013) for *Chironomus* species larvae, based on differences in the central trifid tooth of the mentum and teeth of the mandibles. They observed the range with variation in the above structures. In our *Chironomus* specimen, mentum outer teeth are completely separated from the middle one. Martin (1979) considered the sharpness of the menum teeth for separation of some *Chironomus* species, while Proulx *et al.* (2013), suggested that this feature varied widely within the species. Hence, they did not consider it for separating their examined species. We observed that the coloration of the third inner mandibular tooth was completely dark. It was also separated from the lower mandibular margin.

C. plumosus was previously reported from lakes in Canada (British Columbia, Manitoba, Ontario and Saskatchewan) and the United States (Alabama, California, Colorado, Indiana, Kentucky, Massachusetts, Minnesota, New Mexico, North Dakota, Oklahoma, South Dakota and Wisconsin) at depths up to 23 m (Butler *et al*. 1999; Proulx *et al*. 2013). Proulx *et al.* (2013) found *C. plumosus* at depths ranging from 1 to 8 m, and in oligotrophic to eutrophic lakes ranging in pH from 6.8 to 8.5. In Iran, *C. plumosus* was reported from Tajan River estuary in southern Caspian Sea Basin (Javanshir *et al*. 2008) and from wetlands of the Zarrineh estuary at the south of Urmia Lake (Ahmadi *et al*. 2011) in hypereutrophic condition and polluted water with low quality. Tolerant species such as *Chironomus* are more abundant at eutrophic sites (Proulx *et al*. 2013). It is consistent with the results of several relevant studies conducted on environmental conditions of Anzali Wetland (Mirzajani *et al*. 2010; Esmaeilzadeh 2016). Marques *et al*. (1999), suggested that the *Cricotopus* distribution cannot be associated with water quality. According to Strixino *et al*. (1998), and Henriques-Oliveira *et al*. (2003), habitats with rocky substrate and littoral with sand and pebbles in zones of erosion are favorable for the colonization by *Cricotopus* larvae. The results of study on substrates in Anzali Wetland exhibited that the muddy samples were more frequent than the sandy mud and silt ones in the area (Ghazban & Khosheghbal 2011), which could be the reason for small number of the genus *Cricotopus* in the wetland. In the present study, the *Ablabesmyia* and *Alotanypus* exhibited a lower frequency in Anzali Wetland. *Ablabesmyia* had preference for plots with sandy sediment (Santos *et al*. 2013), while *Alotanypus* was reported from a wide range of standing water environments, such as wetlands containing a higher rate (%) of organic matter in soil. *Alotanypus* is also always associated with surface water or ground water discharge that is characterized by harsh environmental conditions (Siri *et al*. 2011). It is consistent with the results of several relevant studies on such environmental conditions (Mirzajani *et al*. 2010; Esmaeilzadeh 2016). In the recent years, some authors have suggested that the water eutrophication has turned to be a serious environmental challenge in the Anzali Wetland ecosystem. These challenges include the increasing waste loads from industrial, agricultural and human activities (Mirzajani *et al*. 2010). We could not find any morphological variation in these larval specific signs. However, the chironomids were less influenced by morphological alteration and also the habitat structure compared to the other macroinvertebrates (Shobanov 2002). Because there are few obvious structural differences among many *Chironomus* species, identification of *Chironomus* larvae to species level is difficult. Application of molecular

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analysis together with morphological studies basically extended the possibilities for studying sibling and closely related species (Polukonova *et al*. 2009).

The advantage of employing polymorphism in mtDNA genes as a molecular marker is that the mutations are lower than the nuclear genes (Zhimulev *et al*. 2009). The mitochondrial cytochrome oxidase 1 (COI) gene was indicated to be a proper choice for reconstructing phylogenetic relations between closely related families, genera, between species and subspecies of different insects (Boore and Brown 2000). Several species of *Chironomus* genus have been studied according to the COI gene, including C. *balatonicus*, *C. entis, C. plumosus*, *C. muratensis* , and *C. agilis* (Guryev *et al*. 2001). Noteworthy, *C. plumosus* and *C. entis* almost have indistinguishable morphological similarities (Proulx *et al*. 2013). Kiknadze *et al*. (1991) characterized the outer hooks on the anterior margin of the ventromental plates, suggesting that it was shorter and blunter in *C. plumosus* than in *C. entis.* Guryev & Blinov (2002) and Proulx *et al.* (2013) observed the similarities between the COI gene of these two species. Hence, we amplified and sequenced the COI gene of isolated larvae for differentiating between these two species. However, the sequencing and alignment exhibited that the isolated species has 98% homology with the *C. plumosus* in Genbank.

CONCLUSIONS

The chironomids are key organisms in aquatic ecosystem health. The chironomids can be very useful in ranking the health of specific sites. According to the entrance of several pollutants to Anzali Wetland during the last two decades, finding C. *plumosus* susceptible to these pollutants may be a promising issue.

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Conflict of Interest

The authors declare no conflict of interest.

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شناسایی ریخت شناختی نوزادان خانواده شیرونومیده (دیپترا) جدا شده از تالاب انزلی در جنوب غربی دریای خزر: نخستین گزارش گونه *Chironomus plumosus*

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چکیده

شیرونومیده (دیپترا) شامل بزرگترین گروه از بی مهرگان بزرگ هستند که معمولاً از محیط‌های آبی برای پایش کیفیت آب جمع‌آوری می‌شوند. شیرونومیده از جمله فراوانترین گروه آبزیان مقاوم در آبهای آلوده هستند. این امر شیرونومیده را به عنوان یکی از عالی‌ترین شاخص‌های زیستی معرفی می‌کند. این گروه به خوبی در تالاب انزلی شناسایی نشده است. هدف از این مطالعه جداسازی، شناسایی و آنالیز فیلوژنتیکی نوزادان شیرونومیده از تالاب انزلی است. در این تحقیق نوزادان شیرونومیده از سیزده مکان مختلف تالاب انزلی جمع‌آوری شدند. خصوصیات اصلی برای جمع‌آوری این نوزادان شامل لکه چشمی، پوزه، صفحه شکمی-مغزی، آنتن‌ها و توبول‌های شکمی بود. ژن سیتوکروم اکسیداز یک میتوکندریایی برای تسهیل در شناسایی گونه‌های شیرونوموس به وسیله روش واکنش زنجیره‌ای پلیمراز و تعیین توالی به کار رفت. از همپوشانی توالی‌ها برای ساخت درخت فیلوژنی بر اساس روش maximum likelihood استفاده شد. شش جنس در سه زیرخانواده شناسایی شدند. آنها شامل: شیرونومینه (یک جنس)، ارتوکلادینه (یک جنس) و تانی پودینه (چهار جنس) بودند. بر اساس توبول‌های شکمی، جمعیت غالب نوزادان شیرونوموس که در این تحقیق تایید شدند، در انواع plumosus وthummi قرار گرفتند. یکی از این گونه‌ها و پنج جنس برای اولین بار از تالاب انزلی گزارش شدند. جنس غالب، شیرونوموس بود. این گروه از نوزادان در نهایت به عنوان *Chironomus plumosus* معرفی شدند. این گونه برای اولین بار از تالاب انزلی گزارش می شود. این تحقیق نشان داد که تنوع شیرونوموس بیش از آن چیزی است که در چند تحقیق محدود گزارش شده است و به مطالعات بیشتری نیاز دارد.

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