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ORIGINAL ARTICLE

Phylogenetic Relationship of some Indian Chironomids based on mitochondrial DNA Cytochrome Oxidase I

Ramprasad Kuncham[1]*, Tha.Thayumanavan[2] and Gopireddy V. Subba Reddy [3]
[1]Department of Biotechnology, Jawaharlal Nehru Technological University Anantapur, Ananthapuramu 515 002, Andhra Pradesh, India
[2]School of Biotechnology, Dr. G.R. Damodaran College of Science, Coimbatore 641014, Tamilnadu, India
[3]Department of Chemistry, JNTUACE, Pulivendula 516390, Andhra Pradesh, India
*Corresponding Author E-mail: ramprasadbt@gmail.com

ABSTRACT
In this study, two individuals of Tanypus chinesis, Chironomus kiiensis, Polypedilium nubifer and one individual of procladius denticulatus, Glyptotendipes meridionalis, Clinotanypus sp, Arielulus circumdatus were collected from the aquatic ecosystem of varthur lake, Bangalore, India. The mitochondrial DNA has been successfully isolated using modified CTAB method and 650 bp fragment of mitochondrial gene cytochrome oxidase subunit I (COI) has been amplified and sequenced. A phylogenetic relationship among these species has been investigated using MEGA 6 software. Sanger sequence divergence was observed between two individuals of the Tanypus chinensis and it was suggested that might be more than one species was involved. However the intraspecific sequence divergence was lower between Inter species divergence. Interestingly, Arielulus circumdatus showed amazing phylogentic relation to Tanypus chinesis and that presumably reflect co-evolutionary traits of different subfamilies. The sequence of the mtDNA cytochrome oxidase subunit I gene has proven useful to investigate the phylogenetic relationship among the ambiguous species of chironomids.

Key words: *Mitochondrial DNA, COI, Chironomid larvae, MEGA 6,Sequencing*

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INTRODUCTION

Of all major aquatic invertebrate groups, Chironomidae is considered a species-rich family of flies, most abundant and show a wide range of habitat preferences. Chironomidae larvae are inhabitants of organically enriched places such as in flowing water of streams and rivers or standing water of lakes and pools as well as temporary rain-pools. As an important component of the aquatic insect community, Chironomidae has proven useful as biological indicators because of their sensitivity to chemical changes in aquatic ecosystems [1]. Therefore, they are often included in most ecological and toxicological studies [2] and to assess the environment in many lentic environments [3.

Various Chironomidae taxa have been used to identify the general wellbeing of water sources, especially lakes. Studies suggest that relative abundance of various Chironomidae species varies with the change in many factors. These factors include concentration of dissolved oxygen [4], phosphorous and chlorophyll a concentration [5, 6], presence of various metals [7] and amount of organic content in these water bodies [8].

Species of Chironomidae respond differently to a specific pollutant. For example, the frequency of mouthpart deformities for genera like Dicrotendipes and Polypedilum was found to be much higher than that of Orthocladius at the same habitat [9]. The occurrence of mouthpart deformities was found to be near 15% in case of Dicrotendipes and Polypedilum and 2.4% for that of Orthocladius. Characterization of Chironomids on the basis of external morphology has lead to misidentifications in many cases. For instance, *Chironomus sinicus* has been regarded as *Chironomus pulmosus* until now on the basis of

morphology but a study based on karyotype structure and chromosomal polymorphism lead to differentiation of these two species [10]. Such studies indicate that manual identifications and even cytotaxonomic investigations of Chironomids can lead to wrong assignment of species names to Chironomids.

Molecular DNA-based techniques may have the potential to overcome the problems [11] associated with identification of Chironomids and thereby expand their utility in environmental studies. Improvements in the ability to identify Chironomids to species level, where they are most informative, may affirm present taxonomic status or in some cases clarify present taxonomic ambiguities. In order to use DNA–based techniques the choice of appropriate molecular marker is critical. The best molecular markers for species identification correspond to those unconstrained sequences that accumulate numerous substitutions after species divergence. In most molecular studies the mitochondrial DNA (mtDNA) is used and often only one or several genes can act as an effective marker such as cytochrome Oxidase Subunit one (COI). This gene has been widely used in evolutionary studies, population genetics, as well as in species identification due to its relatively high degree of variation [11, 12].

MATERIALS AND METHODS

Sample collection

Two individuals of *Tanypus chinesis*, *Chironomus kiiensis*, *Polypedilium nubifer* and one individual of *Procladius denticulatus*, *Glyptotendipes meridionalis*, *Clinotanypus sp*, *Arielulus circumdatus* Chironomid larvae were collected using aquatic handle net along with the sediment. The sample was washed through 1-mm sieve and the larvae were transferred to a sampling tray with water. Larvae were left unfed in water for 5 days to let the gut contents out [3] and preserved in 70 % alcohol for DNA isolation. Further the head capsules were mounted for morphological identifications. Salivary gland squashes were prepared to confirm the species by cytological methods.

DNA Isolation

The total mitochondrial DNA was extracted using modified CTAB protocol. Preserved 70% alcohol Chironomid Larvae were washed with Ambion nuclease free water and frozen at -15°C for 3 hrs. In 1ml of homogenization buffer containing 0.3M Sucrose, 30mM TrisHcl (PH 7.5) and 10mM EDTA the larvae were homogenized and centrifuged at 1000g for 5 min to remove nuclei n and cellular debris for 3-4 times. The supernatant was collected in 2ml tubes and centrifuged at 13000g for 15 min at 4°C. Discarded the supernatant and pellet was treated with 1 ml of DNase buffer (0.3M Sucrose, 10mM TrisHcl PH 7.5 and 0.05M Mgcl2). 2U of Dnase I (NEB) was added and incubated at 37°C for 1hr and then 25ul of 0.5M EDTA added to stop the reaction. The tubes were centrifuged at 13000g for 10min. Discarded the supernatant and pellet contains mitochondria. 500ul of Lysis buffer containing 0.4M Sucrose, 10mM EDTA, 300mM TrisHcl PH 8, 20ul of 20% SDS, 10ul of 20mg mL-1 Proteinase K (Sigma) was added and the mixture incubated 30 min at 65°C. 100ul of 5M Potassium acetate PH 5 was added and incubated for 30min at -20°C and then Centrifuged at 12000g for 5min. Supernatant was collected, added equal volume of chloroform: Isoamylalcohol (24:1), and centrifuged at 12000g for 15 min. Aqueous phase was collected and added 1/10th volume of 3M sodium acetate and 0.6 Volume of chilled isopropanol. Mixture was incubated at -20°C for 5 hrs, centrifuged at 12000g for 20 minutes. The pellet was washed twice with 70% ethanol. Dehydrated the pellet at room temperature for 5 min and resuspended in 30ul of nuclease free water (Ambion).

Quality and quantity assessment

5ul mitochondrial DNA was loaded onto 1% agarose gel (HIMEDIA) prestained with 0.5ul/100ml of Ethidium bromide (10mg/ml). Gel was run using 1X TAE buffer and image was captured using Gel Doc system (UVITEC Cambridge) (Figure 1).

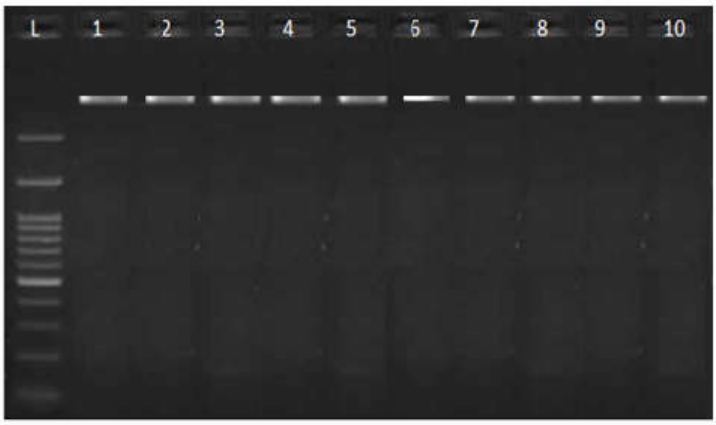

Figure 1: Isolated mt DNA. L-100bp ladder, 1- *Tanypus chinesis 1*, 2- *Tanypus chinesis 2*, 3- *Chironomus kiiensis 1*, 4- *Chironomus kiiensis 2*, 5- *Polypedilium nubifer 1*, 6- *Polypedilium nubifer 2*, 7- *Procladius denticulatus*, 8- *Glyptotendipes meridionalis*, 9- *Clinotanypus sp*, 10- *Arielulus circumdatus*.

The mtDNA concentration was measured using Thermo Scientific NanoDrop 8000 (table 1)

Table 1: NanoDrop profile of mtDNA from Chironomid samples

S.No	Sample Name	A260/280	Conc (ng/ul)	Total Isolation Volume (ul)
1	*Tanypus chinesis 1*	1.88	20.1	30
2	*Tanypus chinesis 2*	1.98	19.7	30
3	*Chironomus kiiensis 1*	1.92	22.3	30
4	*Chironomus kiiensis 2*	1.89	20.8	30
5	*Polypedilium nubifer 1*	1.91	19.4	30
6	*Polypedilium nubifer 2*	1.87	21.1	30
7	*Procladius denticulatus*	1.92	20.5	30
8	*Glyptotendipes meridionalis*	1.84	19.9	30
9	*Clinotanypus sp*	1.89	21.5	30
10	*Arielulus circumdatus*	1.90	19.8	30

PCR

A Conventional PCR was performed following modified PCR analysis [14]. To the MicroAmp® 96-Well reaction Plate (0.2mL), added 3µL buffer, 2µL dntps, 0.3µL Taq DNA polymerase (NEB, USA), 2µL 5M Betain, template 2µL, 20 picomoles concentration of Primer forward 2 µL, primer reverse 2 µL and HPLC water 6.7µL and sealed accordingly with the applicator.

The ±650-bp product of Cytochrome oxidase I mitochondrial gene was amplified by using primer set 911 (TTTCTACAAATCATAAAGATATTGG) and 912 (TAAACTTCAGGGTGACCAAAAAATCA) (Folmer et al. 1994). COI amplification was started with an initial denaturation step (94 °C, 5 minutes). Each cycle consisted of three steps (denaturation, annealing, and extension). Each PCR reaction consisted of 35 cycles of amplification (initial 10 cycles was denaturation at 94°C for 30 sec, annealing at 45 °C for 30 sec, and DNA chain extension at 72°C for 1 minute, last 25 cycles was denaturation at 94°C for 10 seconds, annealing at 50°C for 10seconds, and DNA chain extension at 72°C for 30seconds). A final extension cycle was performed at 72°C for 5 minutes. PCR products were detected by using Agarose gel electrophoresis.

Electrophoresis was performed with 2% Agarose gel (Himedia) prestained with 0.5 µL /100mL of Ethidium bromide (10mg/mL). Gels were run at 80V using 1X TAE buffer and then photographed under UV illumination by using a Gel documentation system (UVITEC Cambridge) (Figure 2).

Sanger Sequencing

Amplified amplicons were purified using QI Aquick PCR Purification kit (QIAGEN, Malaysia). The amplicons were sequenced automatically in both directions using BDT v3.1 chemistry, POP7 Polymer on 3730XL Genetic Analyzer. The thermal program was made up of an initial pre-denaturation step at 95C for 2 min; followed by 30 cycles consisting of a denaturation step at 95C for 10 seconds, annealing step at 52C for 20 seconds and an extension step at 60C for 4 min. The DNA sequences were analyzed using the sequencher 4.8 software. The COI gene sequence obtained was aligned using Codon Code aligner software to obtain the consensus sequence.

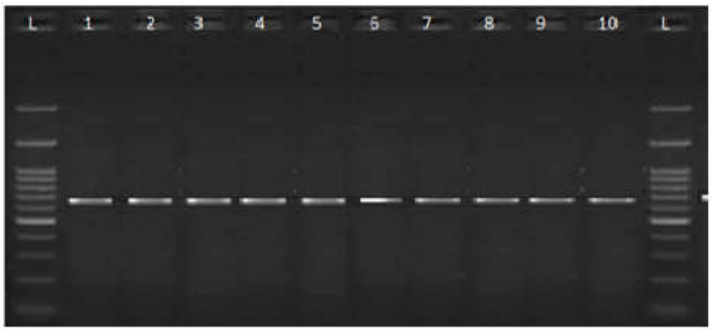

Figure 2: Amplified 650-bp product of Cytochrome oxidase I. L- 100bp ladder, 1- *Tanypus chinesis* 1, 2- *Tanypus chinesis* 2, 3- *Chironomus kiiensis* 1, 4- *Chironomus kiiensis* 2, 5- *Polypedilium nubifer* 1, 6- *Polypedilium nubifer* 2, 7- *Procladius denticulatus*, 8- *Glyptotendipes meridionalis*, 9- *Clinotanypus sp*, 10- *Arielulus circumdatus*.

RESULTS AND DISCUSSION

The isolated mitochondrial DNA using modified CTAB protocol subjected to 1% agarose gel agarose gel electrophoresis (figure 1) and found to be thick bands with good intensity indicating the high copy number. The concentration of mitochondrial DNA of 10 samples were measured using Nanodrop (Thermo) and achieved average concentration of is about 20ng/ul. The Nanodrop profile has revealed the higher purity level of mitochondrial DNA at 260/280 ratio (Table 1).

The universal mtDNA primers, 911 and 912, amplified a 650-bp region of the partial fragment of mtDNA cytochrome oxidase subunit I gene from Chironomid larvae DNA samples (Figure 2). Amplified amplicons were purified using QIA quick PCR Purification kit (QIAGEN, Malaysia) and subjected to Sanger sequencing. Bidirectional reads (Forward and Reverse) were aligned using Codon Code aligner software to obtain the consensus sequence of mitochondrial DNA of 10 samples.

Consensus sequences were used to construct Maximum Parasomy (MP) tree rooted without groups *Culicoides chiopterus* and *Anopheles stephensi*. (Figure 3).

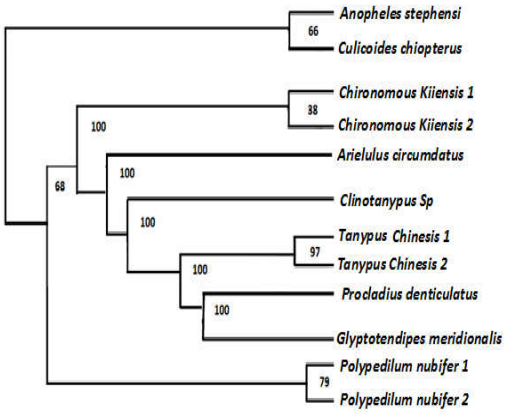

Figure 3: Maximum Parasomy (MP) tree rooted with outgroups *Culicoides chiopterus* and *Anopheles stephensi*

First major cluster divided into two subclusters. The first subcluster grouped *Chironomous Kiiensis* into monophylic clade. The second subcluster grouped *Arielulus circumdatus* as a sister group of the polyphylic group that contains Clinotanypus Sp is a sister group of the monophylic group of Tanypus chinesis, which again subcluster *Procladius denticulatus* and *Glyptotendipus meridionalis*. The second major cluster is a monophylic clade of *Polypedilium nubifer*.

The potential of mitochondrial DNA COI gene, in chironomid identification was investigated. The use of mitochondrial DNA sequence for chironomid identification has been recognized as a

useful tool to resolve the problems of morphological identification [11, 12, 3]. The application of mitochondrial DNA, particularly the Cytochrome Oxidase I andCytochrome Oxidase Il genes, in inferring the phylogeny and genetic divergence of chironomid species have been investigated by several scientists such as Allegrucci *et al*. [13] who concluded that the mitochondrial DNA Cytochrome Oxidase I gene can be used as a tool to investigate the phylogenetic relationship among the chironomid species.

The preliminary results of genetic variation presented that the mitochondrial DNA Cytochrome Oxidase I gene showed high variation among different chironomid species belonging to the two subfamilies, Chironominae and Tanypodinae with divergence level above 0.42 (Table 2). In this study, the inferred phylogenetic tree based on a partial fragment of mitochondrial DNA Cytochrome Oxidase I gene within chironomid species was investigated. The first major cluster was divided into two clades. The first clade grouped into C.kiiensis individuals, a result indicating that closely related taxa.The second clade was made up of apolyphyletic group. In this clade Tp. Chinesis individuals formed an inner monophyletic group with *Arielulus circumdatus*, Clinotanypus sp as asister taxon and outer monophyletic group with *Procladius denticulatus* and *Glyptotendipes meridionalis*. The second major cluster is formed bythe monophyletic clade of *P. nubifer*. This clade was also the basal taxon to all other species. The results showed the monophyletic nature of individuals identified to their species or sibling species based on conventional keys, i.e. all individuals of the presumed *C. kiiensis, Tanypus Chinesis* and *P. nubifes*. Tanypus Chinesis were grouped into their own clusters showing theeffectiveness of this DNA technique in species identification. The sequence alignment strongly suggests that *P. nubifer* and Chironomous Kiiensis had been accurately identified to the species level with low genetic distance values. However, the much higher sequence divergence observed between the two presumed *Tanypus Chinesis* specimens would indicate that more than one species might be involved. Further taxonomic investigation is certainly warranted to elucidate the taxonomic status of this taxon.

In conclusion, mitochondrial DNA region of Cytochrome Oxidase I gene sanger sequence has proven a useful tool in identifying chironomid during certain life stages and offers a reliable and rapid approach for routine identifications of ambiguous species or individuals.

	Culicoides chiopterus	*Anopheles stephensi*	*Chironomus kiiensis1*	*Chironomus kiiensis2*	*Arielulus circumdatus*	*Clinotanypus Sp*	*Tanypus Chinesis 1*	*Tanypus Chinesis 2*	*Procladius denticulatus*	*Glyptotendipes meridionalis*	*Polypedilum nubifer 1*	*Polypedilum nubifer 2*
Culicoides chiopterus	0											
Anopheles stephensi	0.302	0										
Chironomus kiiensis1	0.452	0.428	0									
Chironomus kiiensis2	0.597	0.612	0.014	0								
Arielulus circumdatus	0.598	0.499	0.521	0.542	0							
Clinotanypus Sp	0.681	0.492	0.563	0.495	0.587	0						
Tanypus Chinesis 1	0.653	0.584	0.561	0.524	0.603	0.642	0					
Tanypus Chinesis 2	0.642	0.561	0.494	0.517	0.621	0.642	0.285	0				
Procladius denticulatus	0.637	0.595	0.551	0.559	0.627	0.618	0.173	0.002	0			
Glyptotendipes meridionalis	0.636	0.686	0.561	0.563	0.629	0.642	0.189	0.453	0.431	0		
Polypedilum nubifer 1	0.241	0.277	0.669	0.695	0.63	0.618	0.173	0.321	0.342	0.231	0	
Polypedilum nubifer 2	0.215	0.232	0.542	0.612	0.321	0.421	0.194	0.341	0.241	0.251	0.052	0

Figure 2: Intra and Interspecies genetic distance (MP) of chironomidae species from the partial sequence of COI mtDNA gene for two indivduals *of Tanypus chinesis, Chironomus kiiensis, Polypedilium nubifer* and one individual of *Procladius denticulatus, Glyptotendipes meridionalis, Clinotanypus sp, Arielulus circumdatus* with outgroups *Culicoides chiopterus* and *Anopheles stephensi*

COMPETING INTERESTS

The authors have no competing interests.

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