JEB™
Website : www.jeb.co.in
E-mail : editor@jeb.co.in

Journal of Environmental Biology

ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

Inter and intraspecific diversity of Chironomid larvae using COI and RAPD markers

Ramprasad Kuncham[1]*, Tha.Thayumanavan[2] and Gopireddy V. Subba Reddy[3]

[1]Department of Biotechnology, Jawaharlal Nehru Technological University Anantapur, Ananthapuramu-515 002, India

[2]School of Biotechnology, Dr. G.R. Damodaran College of Science, Coimbatore-641 014, India

[3]Department of Chemistry, JNTUACE, Pulivendula-516 390, India

*Corresponding Author E-mail: **ramprasadbt@gmail.com**

Publication Info

Paper received:
13 June 2016

Revised received:
21 July 2016

Re-revised received:
05 August 2016

Accepted:
05 August 2016

Abstract

Cytochrome Oxidase I (COI) and Random Amplified Polymorphic DNA (RAPD) markers have become widely applied tools for quick and accurate identification of species and to resolve intra and inter specific relationship in many insect groups. In the present study, these markers have been used to infer genetic relatedness of intra and inter specific populations collected randomly from different Indian geographic regions. Initially, mitochondrial DNA isolated using modified CTAB method and 480bp fragment of mitochondrial gene cytochrome oxidase subunit I (COI) has been amplified and sequenced. Sanger sequencing data of 19 species of Chironomidae including 20 populations of *Chironomous circumdatus* obtained and inter and intra specific phylogenetic relationship was investigated using MEGA 6 software. Interspecific divergence was found to be 0.578 as compared to intra specific divergence (0.042). Further, RAPD analysis of 20 population of *Chironomous circumdatus* was carried out using RAPD markers for reliable information about genetic diversity. Total DNA extracted and PCR amplified using 2 arbitrary decamer primers, which produced 180 reproducible and scorable bands ranged from 300bp to 1300bp. Dendrograms with genetic distance coefficient were performed with the procedure of Nei and Li (Dice) using UVIsoft analysis software taking low range DNA ruler as molecular weight marker. Relationships inferred from RAPD UPGMA tree were partially similar to the results obtained from sequences of cytochrome oxidase I of mitochondrial DNA (COI).

Key words

Chironomid larvae, Cytochrome oxidase I, Genetic divergance, RAPD, Mitochondrial DNA,

Introduction

Two general approaches using invertebrates are being employed worldwide to conduct biological assessments of aquatic systems. One is taxonomic, and the other is functional. The first involve measures, such as species density, specific diversity or richness, while the sec is focused on food webs and energy flow (Cummins *et al.,* 2005). The use of functional approach may be more adequate if the goal is to characterize ecosystem condition. Within this context, the concept of guild has become useful, since it can be considered as a functional unit in community analysis, making it unnecessary to consider each and every species as a separate entity.

The Chironomidae family is widely distributed and is the most abundant group of insects found in freshwater environment. It plays a vital role in aquatic food webs, representing a major link between producers, such as phytoplankton and benthic algae and secary consumers. These organisms can occupy important position in the trophic dynamics of aquatic ecosystems, due to their numeric abundance and role in nutrient cycling. Chironomids act as brigde in supplying important organic nutrients for predators

(Sankarperumal and Pandian, 1992). Moreover, due to their long life cycle and low mobility, chironomids integrate various biological processes and may be used as indicators of environmental conditions (Kuhlmann *et al.,* 2001). Due to the amplitude of their feeding habit and their adaptive strategies during different stages of their aquatic life, chironomid larvae are one of the most important groups of aquatic insects (Ferrington, 2008).

DNA barcoding is a molecular diagnostic method that employs a short DNA sequence to rapidly and accurately identify a species. This method has revitalized traditional taxonomy and allows for a better understanding of organisms and their relationships (Hebert and Gregory, 2005; Hebert *et al.,* 2003; Kim *et al.,* 2011; Yoo *et al.,* 2006). However, DNA barcoding methods may not provide adequate resolution to identify recently diverged species, species complexes, or groups with a slow evolutionary rate (Kerr *et al.,* 2007; Radulovici *et al.,* 2010). Such cases require suitable markers for accurate species identification (Park *et al.,* 2007).

Mitochondrial DNA data can be powerful in resolving species-level phylogenies. The order of genes in the mitochondrion is variable, and are separated by large regions of noncoding DNA. The mitochondrial genome rearranges itself frequently so that many rearranged forms can occur in the same cell. The use of mtDNA has become increasingly popular in phylogenetics and population genetic studies due to developments in methodology for mtDNA isolation, use of restriction enzymes to detect nucleotide differences, the development of PCR methodologies and applicability of universal primers for amplification of DNA (Brown *et al.,* 1982). The COI gene of mitochondrial DNA is slowly evolving compared to other protein coding mitochondrial genes and is widely used for estimating molecular phylogenies (Russo *et al.,* 1996). This gene has been widely used in evolutionary studies, population genetics, as well as in species identification due to relatively high degree of variation (Carew *et al.,* 2003; Sharley *et al.,* 2004).

Inter-and-intraspecific identification can be identified using the random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) (Semagn *et al.,* 2006). RAPD-PCR utilizes short, synthetic oligonucleotides of random sequences as a single primer that is able to anneal and prime at multiple locations throughout the genome of an organism; a spectrum of amplification products are produced that are characteristic of template DNA (Welsh and McClelland, 1990; Williams *et al.,* 1990). The presence and absence of a specific PCR product is diagnostic for the oligonucleotide- binding sites on genomic DNA (Williams *et al.,* 1995) and, therefore, can serve as useful molecular markers for taxonomic and population genetic studies (Feng *et al.,* 2009).

However, the combined use of different markers can provide more reliable information about genetic diversity when compared to use of only one marker. The expectation is that some errors or problems presented by a certain marker can be minimized using other markers (Demeke *et al.,* 1997; Saker *et al.,* 2005; Souza *et al.,* 2008). Thus the objective of the study was to characterize genetic divergence among 19 species of chironomids collected from different nature adaptations using RAPD and COI markers and comparing their consistency.

Materials and Methods

Sample collection : Chironomid larvae (Table 1 and 2) were made between 2014 and 2015 by 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 (pfenninger *et al.,* 2007) 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.

Isolation of DNA : Total mitochondrial DNA was extracted by modified CTAB protocol (Kuncham *et al.,* 2016). The concentration of mitochondrial DNA samples was measured by Thermo Scientific NanoDrop8000 and achieved average concentration of about 20 ng ul^{-1}. Total DNA for RAPD analysis was extracted using Macherey-Nagel Nucleospin tissue kit and concentration obtained about 100ng μl^{-1}.

Table 1 : Collection location of Inter species analyzed in this study

Species	Gen BankAccession Number
Chironomous circumdatus	KX271850
Kiefferulus calligaster	KX271851
Chironomus flaviplumus	KX271852
Kiefferulus barbatitarsis	KX271853
Nilodorum tainanus	KX271854
Glyptotendipes tokunagai	KX271855
Microchironomustener	KX271856
Benthalia dissidens	KX271857
Einfeldiapagana	KX271858
Zavrelimyia species	KX271859
Thienemannimyia species	KX271860
Procladius culiciformis	KX271861
Procladius species	KX271862
Procladius denticulatus	KX271863
Glyptotendipes meridionalis	KX271864
Clinotanypus species	KX271865
Tanypus chinensis	KX271866
Arielulus circumdatus	KX271867
Polypedilum sp	KX271868

Mitochondrial COI amplification and sequencing : A conventional PCR was performed by modified PCR analysis (Murugkar *et al.,* 2003). To a 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 2µl, primer reverse 2µl and HPLC water 6.7µl and sealed accordingly with the applicator. The 480bp product of Cytochrome oxidase I mitochondrial gene was amplified by using primer set COIS 5'GGATCACCTGATATAGCATTC CC) and COIA (CCCGGTAAAATTAAAATATAAACTT C) (Folmer *et al.,* 1994). COI amplification was started with an initial denaturation step (94 °C, 5 min). 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 50 °C for 30 sec, and DNA chain extension at 72°C for 1 min, last 25 cycles was denaturation at 94°C for 10 sec, annealing at 55°C for 10sec, and DNA chain extension at 72°C for 30 sec). A final extension cycle was performed at 72°C for 5 min. PCR products were detected by using Agarose gel electrophoresis.

The amplicons of COI were purified to remove contaminants. DNA sequencing reaction of COI genes carried out with forward and reverse primers using BDT v3.1 Cycle sequencing kit, POP7 Polymer on 3730XL Genetic Analyzer. Consensus sequence of COI genes were generated from forward and reverse sequence data using aligner software. DNA sequencing data was analyzed using BLAST with NR database of NCBI Genbank. 19 species sequences were submitted to NCBI Genbank trough Bankit tool and obtained accession number (Table 1).

RAPD-PCR amplification : RAPD was carried out with 2 decameroperan random primers (OPA–TCGGCGATAG, OPB-GTTTCGCTCC) and amplification started with an initial denaturation step (94°C, 2 min). Each cycle consisted of three steps (denaturation, annealing and extension). Each PCR reaction consisted of 40 cycles of amplification. Initially, 10 cycles were started using denaturation at 94°C for 1 min, annealing at 35 °C for 1 min, and extension at 72°C for 2 min, last 30 cycles were maintained using denaturation at 94°C for 30 sec, annealing at 35°C for 30 sec, and DNA chain extension at 72°C for 1 min). A final extension cycle was performed at 72°C for 5 min. PCR products were detected with 100bp ladder (Invitrogen) on Agarose gel electrophoresis. Electrophoresis was performed with 2% Agarose gel (Himedia) prestained with 0.5 µl of ethidium bromide (10 mg ml^{-1}). Gels were run at 80V using 1X TAE buffer and then photographed under UV illumination by using a Gel documentation system (UVITEC Cambridge) (Fig. 4a and b).

Results and Discussion

The universal mtDNA primers, COIS and COIA, amplified a 480bp region of the partial fragment of mtDNA cytochrome oxidase subunit I gene from Chironomid larvae DNA samples (Fig. 1a and b). Amplified amplicons were purified using QIAquick 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 39 samples. 39 mtDNA cytochrome oxidase I (COI) sequences were achieved successfully and genbank accession number were obtained for 19 species. Consensus sequences were used to construct phylogenetic tree for inter as well as intra populations. (Fig. 2a and b).

Phylogenetic tree analysis indicates two major groups 1 and 2. In group 1, *Procladius culiciformis* and *Procladius denticulatus* form monophyletic clade with high boot strap value 95 and also contained two sister groups i.e., *Tanypus chinesis* and *Arielulus circumdatus*. Group 2 was further divided into 3 subgroups 2a, 2b and 2c. 2a subgroup consisted *Thienemannimyia* and *Procladius species* that formed monophyletic clade with bootstrap value 84 and contained sister groups *Nilodrum tainanus*, *Glyptotendipes tokunagai*, *Glyptotendipus meridionalis* and *Kiefferulusbar batitarsis*.

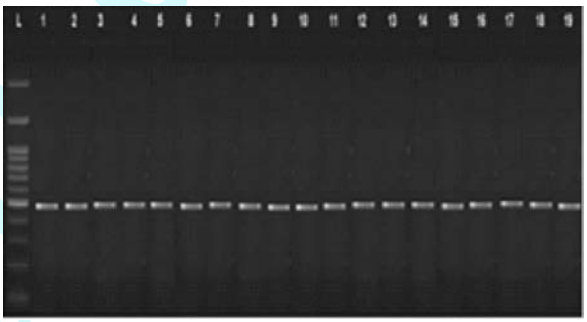

Fig. 1 (a) : Amplified products of COI of 19 species 1-19 samples, L = 100 bp Ladder

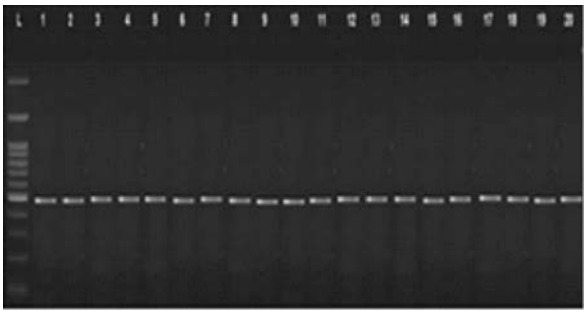

Fig. 1 (b) : Amplified products of 20 populations of *Chironomus circumdatus*. 1-20 samples, L = 100 bp Ladder

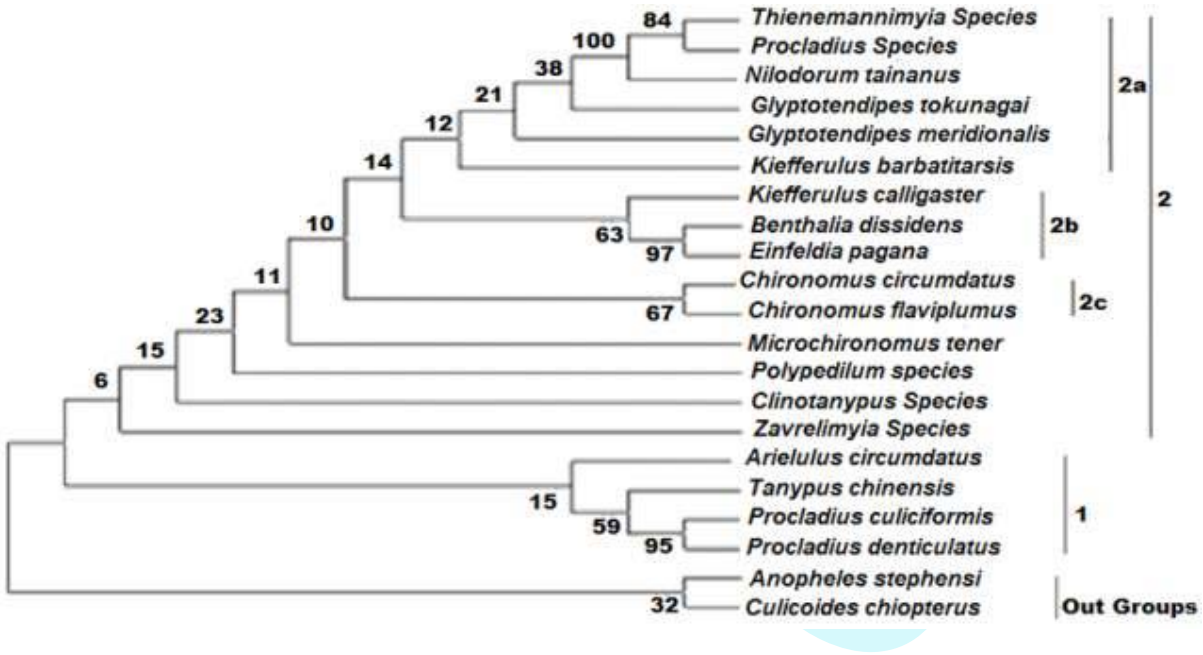

Fig. 2 (a) : Maximum Likelihood (ML) phylogenetic tree for inter species of Chironomidae inferred from COI gene fragment rooted with outgroups of Anopheles stephensi and Culicoideschiopterus

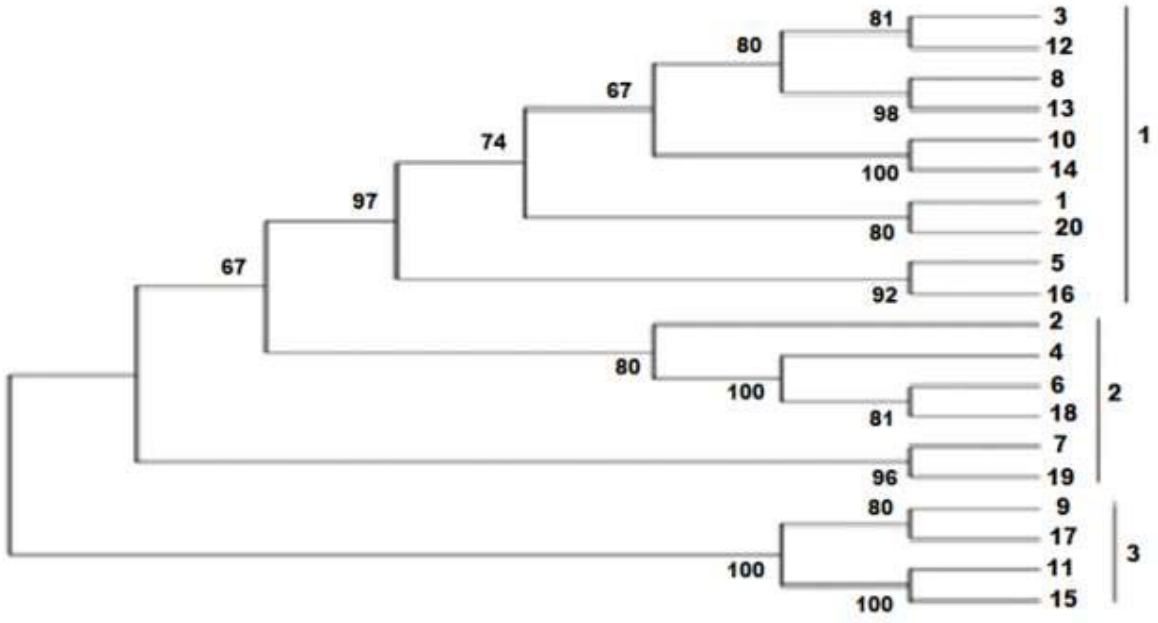

Fig. 2 (b) : Maximum Likelihood (ML) phylogenetic tree for intra species of Chironomidae inferred from COI gene fragment

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	*Anopheles stephensi*																					
2	*Culicoides chiopterus*	0.275																				
3	*Chironomus circumdatus*	0.247	0.341																			
4	*Kiefferulus calligaster*	0.246	0.369	0.190																		
5	*Chironomus flaviplumus*	0.255	0.339	0.115	0.155																	
6	*Kiefferulus barbatitarsis*	0.268	0.414	0.235	0.148	0.226																
7	*Nilodorum tainanus*	1.773	1.413	1.236	1.266	1.362	1.261															
8	*Glyptotendipes tokunagai*	0.286	0.387	0.164	0.147	0.140	0.147	1.035														
9	*Microchironomus tener*	0.275	0.391	0.208	0.252	0.208	0.241	1.434	0.215													
10	*Benthalia dissidens*	0.228	0.325	0.173	0.085	0.155	0.117	1.290	0.163	0.207												
11	*Einfeldia pagana*	0.228	0.325	0.173	0.085	0.155	0.117	1.290	0.163	0.207	0.000											
12	*Zavrelimyia Species*	0.284	0.297	0.234	0.310	0.271	0.351	1.483	0.270	0.233	0.311	0.311										
13	*Thienemannimyia Species*	1.528	1.331	1.777	1.389	1.686	1.361	0.283	1.333	1.643	1.209	1.209	1.991									
14	*Procladius culiciformis*	0.218	0.345	0.246	0.172	0.271	0.225	1.752	0.282	0.253	0.182	0.182	0.232	1.663								
15	*Procladius Species*	1.664	1.499	1.387	1.442	1.522	1.361	0.247	1.330	1.683	1.441	1.441	1.628	0.207	1.801							
16	*Procladius denticulatus*	0.246	0.391	0.255	0.226	0.301	0.216	1.569	0.291	0.311	0.200	0.200	0.259	1.522	0.070	1.851						
17	*Glyptotendipes meridionalis*	0.237	0.368	0.173	0.199	0.190	0.182	1.115	0.156	0.253	0.182	0.182	0.299	1.533	0.295	1.294	0.293					
18	*Clinotanypus Species*	0.284	0.359	0.198	0.224	0.260	0.264	1.387	0.216	0.271	0.216	0.216	0.232	1.683	0.215	1.702	0.235	0.282				
19	*Tanypus chinensis*	0.209	0.337	0.198	0.262	0.279	0.264	1.454	0.264	0.331	0.245	0.245	0.204	1.933	0.147	1.586	0.148	0.244	0.215			
20	*Arielulus circumdatus*	0.458	0.465	0.505	0.415	0.532	0.431	1.516	0.428	0.564	0.416	0.416	0.448	1.387	0.361	1.455	0.381	0.419	0.416	0.403		
21	*Polypedilum species*	0.313	0.366	0.215	0.243	0.262	0.233	1.237	0.206	0.262	0.216	0.216	0.272	1.777	0.291	1.652	0.293	0.224	0.253	0.322	0.476	

Fig. 3 : Distance matrix between Inter species

2b subgroup consisted of *Benthali adissidens* and *Einfeldiapagana* as a monophyletic clade with sister group of *Kiefferlus calligaster*. 2c group consisted *Chironomos circumdatus* and *Chironomous flaviplums* with boot strap value of 67. *Microchironomoustener*, *Polypedilum species*, *Clinotanypus species* and *Zavrelimyia species* isolated separately but fall into major group 2.

Procladius culiciformis and *Procladius denticulatus* formed monophyletic clade and showed high similarity with boot strap value and the distance matrix between these two species was 0.070, but *Procladius species* formed monophyletic clade with *Thienemannimyia species* and showed high divergence with *Procladius culiciformis* and *Procladius denticulatus*, *Glyptotendipes tokunagai* and *Glyptotendipes meridionals* isolated separately and didn't form monophyletic clade but fall in 2a group. The results clearly indicated that there was high divergence in the same genus species. The overall distance average of the species analyzed was 0.578. The highest divergence found between *Thienemannimyia species* and *Zavrelimyia species* was 1.991 (Fig. 3).

Phylogenetic tree for the population of *Chironmous circumdatus* revealed 3 major groups. Group 1 consisted of 5 monophyletic clades with boot strap value higher than 80. Group 2 consisted of 2 monophyletic clades with 2 sister groups. Group 3 consisted of 2 monophyletic clades. All the species were closer with more than 80% similarity and 20% divergence and the average distance was 0.042. So, more than 50% of total diversity was found in inter specific population and was less than 20% in intra species.

RAPD was also performed for intraspecific population to attain more accuracy and for reliable information about genetic diversity. The expectation was that some errors or problems presented by a certain marker could be minimized using other markers (Demeke *et al.,* 1997; Saker *et al.,* 2005; Souza *et al.,* 2008).

RAPD is a dominant expression marker, hence a dominant allele at a particular locus was denoted by the presence of a band. Whereas, absence of a band is a manifestation of a homozygous recessive allele at that locus, average heterozygosity and clustering of the species. Several studies have shown that the number of loci amplified with RAPD primers depends on factors such as reagents and reaction conditions, sample conditions, DNA quality and extraction methods (Khandka *et al.,* 1997). Variations were minimized and amplification artifacts in the RAPD profiles were prevented by removing larval gut contents and frozen larvae, by using similar amount of template DNA for PCR runs.

Band detection and their molecular weight interpretation was determined by UVIsoft (IM000040) analysis software taking low range DNA ruler as molecular weight marker. On the basis of presence or absence of bands in 20 population of *Chironomus circumdatus*, a data matrix was prepared. Only those bands which were present in more than 70% population were considered for calculation. Smears were not taken into consideration. Two decamer random primers generated too many bands were eliminated, and only those that produced clear, distinct, and reproducible bands were considered. At least 6 PCR repetitions per sample were

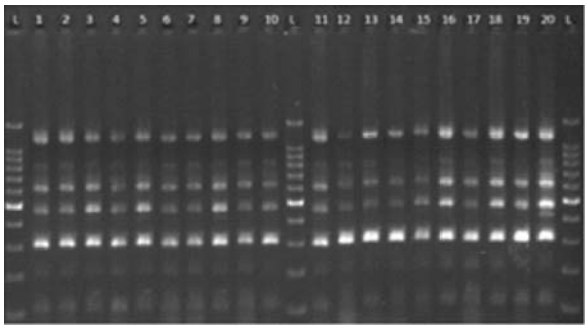

Fig. 4 (a) : Amplification of total DNA for RAPD analysis with primer OPA. 1-20 = samples, L = 100 bp Ladder

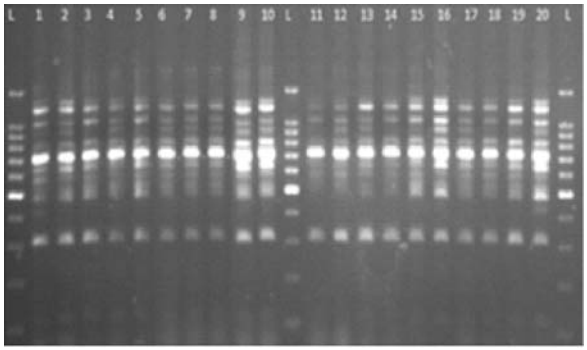

Fig. 4 (b) : Amplified of total DNA for RAPD analysis with primer OPB. 1-20 samples, L = 100 bp Ladder

performed, and 2 batches of primers were used to ensure reproducibility of results.

The RAPD profiles obtained with OPA and OPB primers is shown in Fig. 4a and b respectively. Amplified fragments ranged from 300 to 1300bp, and the number of distinct bands for each primer varied from 4 to 5. Each RAPD band was treated as a separate character and scored 1 (present) or 0 (absent), and a rectangular binary data matrix was obtained. A similarity matrix was obtained by Nei-Li (Dice) similarity index and a dendrogram was constructed by UPGMA procedure (Fig. 5a and b).

Dendrogram (UPGMA) of primer OPA was similar to the primer OPB, but with little variation which might be due to poor resolution of agarose gel and more manual work involved in the experiment. But RAPD marker results didn't correlate completely with COI marker analysis. Only closely related population gave similar results like 5 and 16, 7 and 9, 8 and 13, 9 and 17, 10 and 14, 11 and 15. Whereas, sample 1 formed monophyletic clade with 20 in COI phylogenic tree but dendrogram (UPGMA) results showed that sample 1 was closely related to 2 and formed monophyletic clade, sample 20 formed sister group with monophyletic clade of 1 and 2. Samples 3 and 12 were isolated in dendrogram (UPGMA),

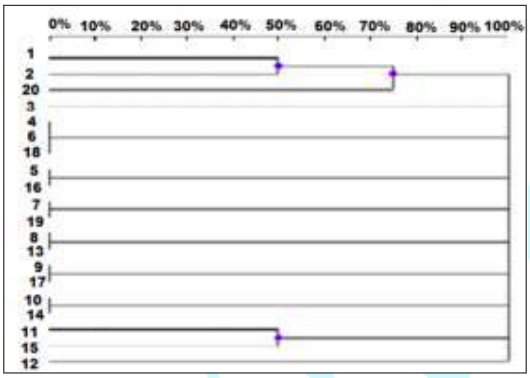

Fig. 5 (a) : Dendrogram (UPGMA) showing the genetic relationships between 20 populations of *Chironomus circumdatus* using primer OPA

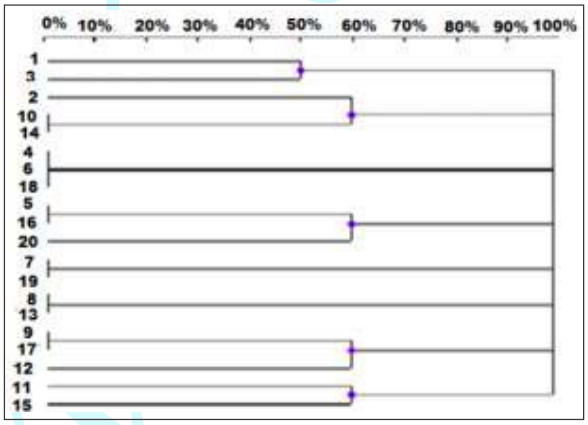

Fig. 5 (b) : Dendrogram (UPGMA) showing the genetic relationships between 20 populations of *Chironomus circumdatus* using primer OPA

but in COI marker analysis these two samples led to the formation of monophyletic clade with boot strap value of 81. The samples 6 and 18 formed monophyletic clade with 2 and 4 sister groups falling in the major group 2 in the COI phylogenetic tree. Whereas in RAPD dendrogram samples 4,6,and 18 were closely related and belonged to a single clade, but sample 2 was more divergent from these.

These markers have proved to be accurate defining the species and its genetic diversity owing to the confusion of identification using DNA barcoding with COI and RAPD. Comparison of RAPD dendrogram with COI based phylogenetic analysis indicates partial similarity in terms of formation of major groups, monophyletic clades and isolated clades. The purpose of this study was to investigate whether the COI barcode provided sufficient resolution to identify Chironomids. The mitochondrial marker cytochrome c oxidase I (CO1) has been successfully used for barcoding of some invertibrates (Taylor *et al.,* 2012).While the COI barcode region alone is not intended to be used to resolve

taxonomic relationships, it appears to contain enough phylogenetic signal to delineate close relationships within and among chironomid species.

There is a distinct barcode gap between the intraspecific and interspecific divergences of Chironomids from South India. Although, RAPDmarkers are cost-effective and useful, COI markers are more effective in identifying the species and divergence. This study provides an advanced understanding of this valuable taxa and points to productive new avenues for further research on this important organism.

Acknowledgment

The authors are grateful to Mr. Maddi Reddy for helping in the collection of samples, preparation and revising the manuscript.

References

Brown, W.M., E.M. Prager, A. Wang and A.C. Wilson: Mitochondrial DNA sequences of primates: tempo and mode of evolution. *J. Mol. Evol.,* **18**, 225-239 (1982).

Carew, M.E., V. Pettigrove and A.A. Hoffmann: Identifying Chironomids (Diptera: Chironomidae) for biological monitoring with PCRFLP. *Bull. Entomol. Res.,* **93**, 483-490 (2003).

Cummins, K.W., R.W. Merrit and C.N. Andrade: The use of invertebrate functional groups to characterize ecosystem attributes in selected streams and rivers in south Brazil. *Stud. Neotr. Fauna Environ.,* **40**, 71-90 (2005).

Demeke, T., B. Sasikumar, P. Hucl and R.N.Chibbar: Random amplified polymorphic DNA (RAPD) in cereal improvement. *Maydica.,* **42**, 133-142 (1997).

Feng, K., S. Wang, D.J. Hu, F.Q. Yang, H.X. Wang and S.P. Li: Random amplified polymorphic DNA (RAPD) analysis and the nucleosides assessment of fungal strains isolated from natural Cordycepssinensis. *J. Pharm. Biomed. Anal.,* **50**, 522–526 (2009).

Ferrington, L.C.: Global diversity of non-biting midges (Chironomidae; Insecta-Diptera) in freshwater. *Hydrobiologia,* **595**, 447–455 (2008).

Hebert, P.D.N., S. Ratnasingham and J.R. deWaard: Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. *Proc. R. Soc. Lond. B.,* **270**, 96–99 (2003).

Hebert, P.D.N and T.R. Gregory: The promise of DNA barcoding for taxonomy. *Syst. Biol.,* **54**, 852–859 (2005).

Herbert, P.D.N., A. Cywinska, S.L. Ball and J.R. deWaard: Biological identifications through DNA barcodes. *Proceed. Royal Soc. London B Biol. Sci.*, **270**, 313-322 (2003).

Kim, S., C.B. Kim, G.S. Min, Y. Suh, J. Bhak, T. Woo, H. Koo, J.K. Choi, M. K. Shin, J. Jung, K.H. Song, H. Ree, U. W. Hwang, Y. Park, H.S. Eo, J. Kim, S. M. Yoon, H. S. Rho, S. H. Kim, H. Lee, M.S. Min, J. K. Kim, C. Sim, Y.J. Won, J. Lee, S. Cho, J.E. Seo, S. Lee, J.I. Song, H.Y. Han, S. Shin, S.J. Song, J.K. Park, K. S. Eom, J.H. Park and W. Kim: Korea Barcode of Life Database System (KBOL). *Animal Cells and Systems*, **16**, 11-19 (2011).

Kerr, K.C.R., M.Y. Stoeckle, C.J. Dove, L.A. Weigt, C.M. Francis and P.D.N. Hebert: Comprehensive DNA barcode coverage of North American birds. *Mol. Ecol. Notes,* **7**, 535–543 (2007).

Khandka, D.K., M. Tuna , M. Tal, A. Nejidat and A. Golangoldhisrh: Variability in thepattern of random amplified polymorphic DNA. *Electrophoresis,* **18**, 2852–2856 (1997).

Kuncham, R., T. Thayumanavan and G.V. Subbareddy: Development of a modified mitochondrial DNA extraction method from Chironomid larvae. *J. Environ. Biol.,* **37**, 473-478 (2016).

Murugkar, H., H. Rahman and P. Dulta: Distribution of virulence genes in *Salmonella serovars* isolated from man and animals. *Ind. J. Med. Res.,* **117**, 66-70 (2003).

Park M.H., C.J. Sim, J. Baek and G.S. Min: Identification of genes suitable for DNA barcoding of morphologically indistinguishable Korean Halichondriidae sponges. *Mol. Cells.,* **23**, 220–227 (2007).

Pfenninger, M., C. Nowak, C. Kley, D. Steinke and B. Streit: Utility of DNA taxonomy and barcoding for the inference of larval community structure in morphologically cryptic *Chironomus* (Diptera) species. *Mol. Ecol.,* **16**, 1957-1968 (2007).

Radulovici, A.E., P. Archambault and F. Dufresne: DNA barcodes for marine biodiversity: moving fast forward. *Diversity*, **2**, 450–472 (2010).

Russo, C.A., N. Takezaki and M. Nei: Efficiencies of different genes and different tree-building methods in recovering a known vertebrate phylogeny. *Mol. Biol. Evol.,* **13**, 525-536 (1996).

Saker, M.M., S.S. Youssef, N.A. Abdallah and H.S. Bashandy: Genetic analysis of some Egyptian rice genotypes using RAPD, SSR and AFLP. *Afr. J. Biotechnol.,* **4**, 882-890 (2005).

Sankarperumal, G. and T.J. Pandian: Larval abundance of *Chironomus circumdatus* in relation to biotic and abiotic factors. *Hydrobiologia*, **246**, 205-212 (1992).

Semagn, K., A. Bjørnstad and M.N. Ndjiondjop: An overview of molecular markermethods for plants. *Afr. J. Biotechnol.,* **5**, 2540–2568 (2006).

Sharley, D.J., V. Pettigrove and Y.M. Parson: Molecular identification of *Chironomus* spp. (Diptera) for biomonitoring of aquatic ecosystems. *Aus. J. Entomol.,* **43**, 359-365 (2004).

Souza, S.G.H., V. Carpentieri-Pípolo, C.F.Ruas and V.P. Carvalho: Comparative analysis of genetic diversity among the maize inbred lines (*Zea mays* L.) obtained by RAPD and SSR markers. *Braz. Arch. Biol. Technol.,* **51**, 183-192 (2008).

Taylor, H.R. and W.E. Harris: An emergent science on the brink of irrelevance: a review of the past 8 years of DNA barcoding. *Mol. Ecol. Resour.,* **12**, 377–388(2012).

Welsh, J. and M. McClelland: Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.,* **18**, 7213–7218 (1990).

Williams, J.G.K., A.N. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey: DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.,* **18**, 6531–6535 (1990).

Williams, J.G.K., M.K. Hanafey, J.A. Rafalski and S.V. Tingey: Genetic analysis using random amplified polymorphic DNA markers. In: Recombinant DNA methodology (Ed.: R. Wu). **Vol. II**., pp. 849–883 (1995).

Yoo, H.S., J.Y. Eah, J.S. Kim, Y.J. Kim, M.S. Min, W.K. Paek, H. Lee and C.B. Kim: DNA barcoding in Korean birds. *Mol. Cells,* **22**, 323–327 (2006).