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Discrimination of Orthocladiinae Species (Diptera: Chironomidae) by using *cytochrome c oxidase subunit I*

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Abstract: Chironomidae family (Diptera) is more than 120 million years old and has undergone extensive adaptive radiation to occupy a wider range of microhabitats than any other aquatic insect group at present. Chironomidae are often the most abundant group of insects in freshwater environments worldwide. It includes over 10 000 species, distributed from the tropics to the Arctic in lakes, streams and puddles. Unfortunately, the larval stage of Chironomidae, commonly collected in aquatic sampling surveys, possesses relatively few morphological characteristics useful for their identification. Molecular approaches are now being used for identification and taxonomic resolution in many animal taxa including chironomids. In this study, mitochondrial gene, *cytochrome c oxidase subunit I*, has been used as phylogenetic marker in Orthocladiinae species collected from 12 lakes of Turkey. Neighbour-joining, maximum parsimony and maximum likelihood analysis have been used to identify the relationship between species. According to phylogenetic analysis *Cricotopus bicintus*, *Cricotopus sylvestris*, *Cricotopus flavocinctus*, *Cricotopus patens*, *Rheocricotopus atripes* and genus *Psectrocladius* have been formed monophyletic groups.

Key words: *cytochrome c oxidase subunit I*, DNA barcoding, Orthocladiinae, *Cricotopus*, *Psectrocladius*, Turkey

Introduction

Chironomidae (Diptera) is one of the most species rich and widely distributed insect families. Chironomids are found on all continents and in virtually all habitats from the tropics to the Arctic in lakes, streams and puddles, and are important elements of both terrestrial and aquatic ecosystems (Ekrem and Willassen 2004).

The adopted identification of chironomids is mainly based on morphological characters of larvae and imago, as it is of many other groups of insects. However, the larval stage of chironomids, commonly collected in aquatic sampling surveys, possesses relatively few morphological characteristics useful for identification (Sharley *et al*. 2004). For example, the identification of *Cricotopus*, *Orthocladius* and *Paratrichocladius* larvae is extremely difficult and depends on minute structures of the head capsule, e.g. the labral setae and premandibles (Epler 2001). Mature fourth instar larvae are required for effective use of larval keys; however, wear and damage of mouthpart structures may confuse identification (Sinclair and Gresens 2008). Phenotypic variation in pigmentation, another important character for distinguishing both *Cricotopus* species and *Orthocladius* species, can be highly variable (Gresens *et al*. 2007). The morphological differences between closely related species are often subtle, and information from more than one life stage as well as data on behaviour and ecology frequently are of great help in species delimitation. Some cytological methods, including a study of the combination of chromosome arms (cytocomplexes) and comparison of the band patterns of

polytene chromosomes, were recently suggested for chironomid taxonomy and systematic (Michailova 1985, Kiknadze *et al*. 1996). In additional to these characters DNA barcodes have been shown to provide a set of useful signs for species identification, together with morphological or cytogenetic data (Martin 1979, Michailova 1989). They can be used to better understand taxonomic boundaries in Chironomidae (Ekrem *et al*. 2007, Pfenninger *et al*. 2007, Sinclair and Gresens 2008).

Molecular-based approaches, such as DNA barcoding, are being used to supplement traditional taxonomic methods of species identification (Hebert *et al*. 2003, Savolainen *et al*. 2005, Witt *et al*. 2006). It represents a shift from the near-exclusive reliance on morphological characters for the identification and detection of species to an approach that includes molecular characters in species discrimination (Ekrem *et al.* 2010). DNA barcoding relies on sequence variation in short fragments of DNA to serve as a unique species identifier. Variation in the sequence of the mitochondrial gene, *cytochrome c oxidase subunit I* (*COI*), has proven informative for many animal taxa, including insects (Hebert *et al*. 2004a, Hogg and Hebert 2004, Ball *et al*. 2005, Monaghan *et al*. 2005, Smith *et al*. 2005, Hajibabaei *et al*. 2006, Meier *et al*. 2006). Numerous studies have established its effectiveness in species identification in various animal groups (Golding *et al*. 2009), including the family Chironomidae (Carew *et al*. 2005, 2007, Ekrem *et al*. 2007, Pfenninger *et al*. 2007, Sinclair and Gresens 2008). An acknowledged advantage of DNA barcoding is the possibility to easily associate different life stages of the same species (Blaxter 2004, Ekrem *et al*. 2007, Stoeckle 2003). This is particularly valuable to the study of organisms with morphologically inseparable immature stages, and of taxa which are difficult to rear, such as many freshwater insects adapted to cold, ultra-oligotrophic or other special habitats that are hard to imitate in the laboratory. Several studies have demonstrated the usefulness of DNA barcodes to associate life stages in practice (Caterino and Tishechkin 2006, Pegg *et al*. 2006, Zhou *et al*. 2009); Carew *et al*. (2005) and Ekrem *et al*. (2007) have found that partial *COI* gene sequences can be used to link different life stages of the same species in Chironomidae.

In this study, we tested the efficacy of DNA barcoding for the identification of species from subfamily Orthocladiinae collected from 12 lakes in Turkey. We compared *COI* sequence data with identifications based on the morphological characteristics of the larvae to create DNA sequence profiles specific to each species.

Material and Methods

Larval Chironomids were collected from 12 lakes of Turkey (Fig. 1). Larvae were collected during autumn, summer and spring 2009 and 2010. Collected larvae were kept in 96% ethanol until identification and total DNA isolation. For morphological identification, bodies of larvae were characterized in terms of segment number, existence of ventral and lateral gills and morphological characters of gills etc. Then, head capsules were examined and these head capsule slides were mounted in Euparal (Brooks *et al*. 2007). Species identifications were made using the taxonomic keys of Hirvenoja (1973), Cranston (1979), Cranston *et al*. (1983), Moller Pillot (1984) and Schmid (1986) where some species have presented in a groupe.

Total DNA was extracted from body of larvae according to the Hillis and Moritz (1990). Larval bodies were air dried then homogenized in extraction buffer (500 µL STE, 12.5 µL Proteinaz K (19.6 mg mL^{-1}, 25 µL 10% SDS) and incubated for 4 h at 55 °C. After incubation, phenol-chloroform method was applied and DNA was precipitated with the addition of 3 M NaAc and 100% ice-cold ethanol. Then, the DNA pellet was washed with 70% ethanol, air-dried and resuspended in 100 µL TE buffer.

A 653-bp fragment of *COI* mitochondrial gene was amplified with the primer pair 911 (5′-TTTCTACAAATCATAAAGATATTGG-3′) and 912

Fig. 1. Geographical locations of study sites. Letters in brackets indicate sample codes of lakes, and the numbers show the number of sampling stations.

Table 1. List of species of which *COI* sequences were used in neighbour-joining, maximum parsimony and maximum likelihood trees and their accession numbers in GenBank with references.

Species	Accession Number	Reference
Drosophila melanogaster voucher TDWG0805	HQ979116	
Cricotopus sylvestris	DQ865184	Sinclair and Gresens (2008)
Cricotopus trifascia	DQ865182	Sinclair and Gresens (2008)
Cricotopus bicinctus	DQ865175	Sinclair and Gresens (2008)
Cricotopus triannulatus	DQ865174	Sinclair and Gresens (2008)
Cricotopus tristis isolate ar6	DQ865173	Sinclair and Gresens (2008)
Cricotopus tristis isolate ar21	HQ865181	Sinclair and Gresens (2008)
Orthocladius robacki	DQ865178	Sinclair and Gresens (2008)
Orthocladius dorenus	DQ865176	Sinclair and Gresens (2008)
Orthocladius oliveri	DQ865177	Sinclair and Gresens (2008)
Orthocladius nigritus	DQ865179	Sinclair and Gresens (2008)
Rheocricotopus effusus voucher SOE409	HQ105342	Ekrem *et al*. (2010)
Rheocricotopus effusus voucher SOE396	HM406100	Ekrem *et al*. (2010)
Rheocricotopus effusus voucher SOE474	HM406119	Ekrem *et al*. (2010)
Rheocricotopus atripes voucher SOE259	HQ105328	Ekrem *et al*. (2010)
Rheocricotopus atripes voucher SOE134	HQ105325	Ekrem *et al*. (2010)

(5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al*. 1994). DNA was amplified in the following 25 µL reaction containing 2.5 µL of 10X PCR buffer, 1.5 mM $MgCl_2$, 300 µM dNTP, 0.4 µM of each primer, 5 units of *Taq* polymerase and template DNA. PCR were performed in 53 °C annealing temperature. PCR products were verified by electrophoresis on a 1% agarose gel with ethidium bromide and sequenced in both directions using the forward and reverse primers. Some of *COI* sequences were obtained from GenBank, and their accession numbers were given in Table 1.

Nucleotide sequences of 512-bp were aligned by eye using MEGA 5Beta#7 and BioEdit. Genetic distances were calculated using Kimura-2-parameter (K2P) distance model (Kimura 1980). Neighbor-joining (NJ) and maximum parsimony (MP) trees of the K2P distances were created in PAUP *4.0b10 (Licence code; ADU B418788) (Swafford 2002). Bootstrap analysis was performed with 1000 replicates. Intraspecific and interspecific sequence divergence based on K2P distances were calculated for all species, and mean intraspecific and interspecific K2P divergences were calculated from the pairwise comparisons within each species and genus. Maximum likelihood (ML) analyses were performed in RAXMLGUI1.0 by using GTRGAMMA model with 1000 bootstrap replicates. *Drosophila melanogaster* was used as outgroup to root the phylogenetic trees.

Results and Discussion

A total of 69 larval orthoclads were analysed, and 11 *Cricotopus*, 4 *Orthocladius*, 2 *Psectrocladius*, 2 *Rheocricotopus*, 1 *Paratrichocladius* and 1 *Eukiefferiella* species were identified. Mitochondrial gene, *cytochrome c oxidase subunit I* (*COI*) fragment, has been used as a phylogenetic marker to identify phylogenetic relationships among species.

Molecular classifications of *C. bicinctus*, *C. sylvestris*, *C. flavocinctus*, *C. patens*, *R. atripes*, *Ps. barbimanus* and *Ps. limbatellus* species produced monophyletic groups in phylogenetic analysis of NJ, MP and ML (Fig. 2, 3 and 4, respectively). Generic monophyly of *Psectrocladius* was well supported in NJ, MP and ML trees. Furthermore, *R. effusus* together with *R. atripes* formed a monophyletic group in ML tree while they formed to distinctly separate genetic groups in NJ and MP trees. *P. barbimanus* and *P. limbatellus* formed a clade with 61%, 58% and 85% bootstrap support in NJ, MP and ML trees, respectively. However, *C. flavocinctus* and *C. patens* formed a clade with 100% support in all trees.

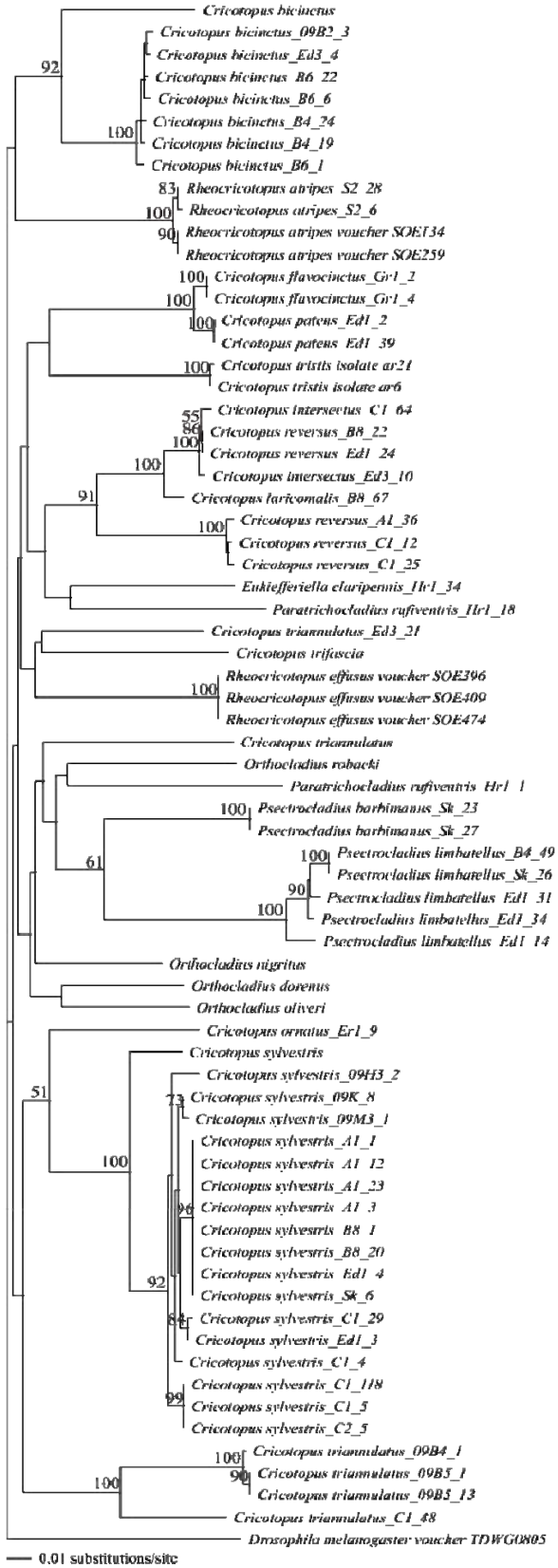

Fig. 2. NJ tree of *COI* sequence divergences (K2P) in Orthocladiinae species from Turkey. Numbers at nodes indicate bootstrap scores after 1000 replicates. The species with only species name or voucher/isolate code were obtained from Sinclair and Gresens (2008) and Ekrem *et al*. (2010).

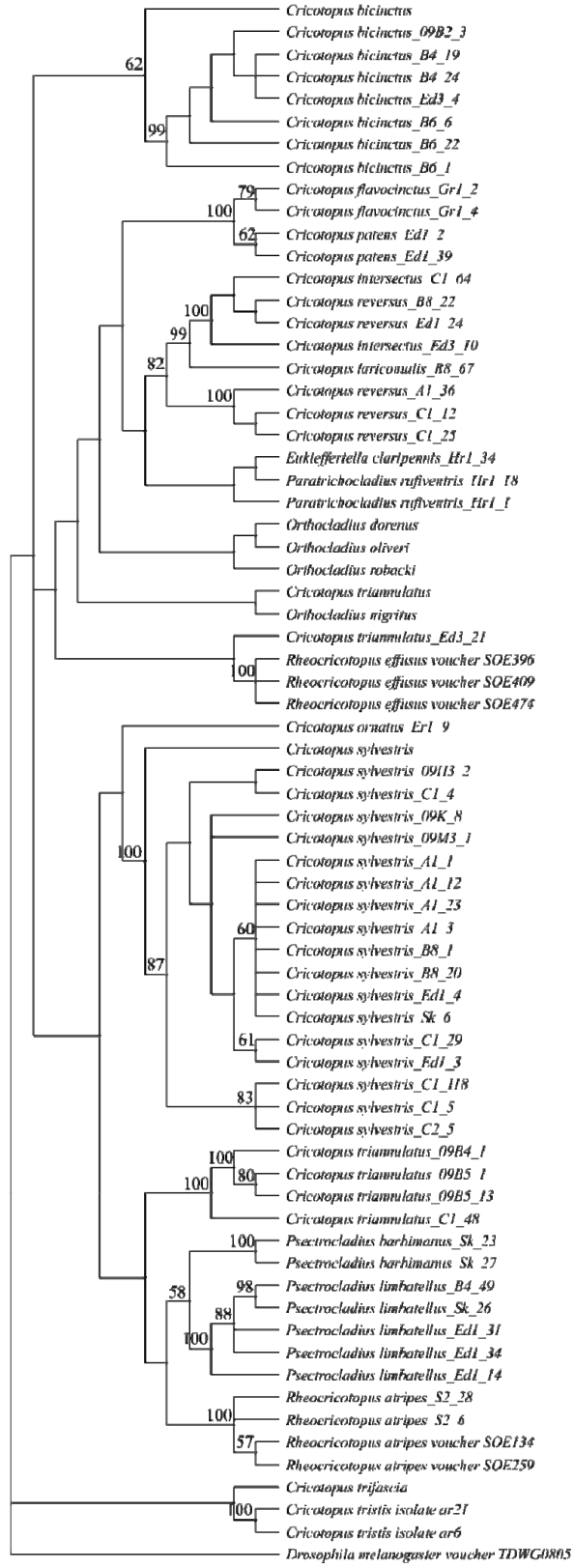

Fig. 3. 50% majority-rule consensus MP tree of 69 Orthocladiinae species based on *COI* sequences. Numbers at nodes indicate bootstrap scores after 1000 replicates. The species with only species name or voucher/isolate code were obtained from Sinclair and Gresens (2008) and Ekrem *et al*. (2010).

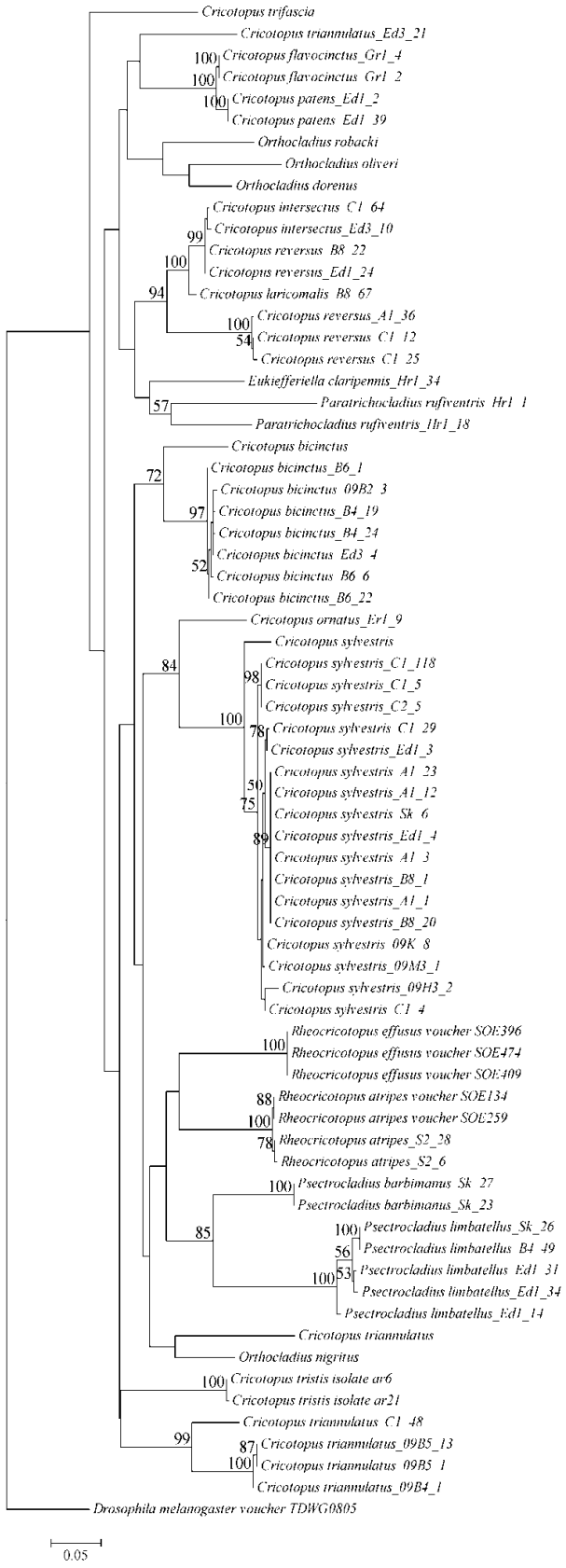

Fig. 4. ML tree of *COI* sequences in Orthocladiinae species from Turkey. Numbers at nodes indicate bootstrap scores after 1000 replicates. The species with only species name or voucher/isolate code were obtained from Sinclair and Gresens (2008) and Ekrem *et al*. (2010).

In NJ, MP and ML trees, *C. sylvestris* was separated from *C. ornatus*, and they two formed a clade with 51% and 84% bootstrap support in NJ and ML trees, respectively and also in MP tree. *C. reversus*, *C. intersectus* and *C. laricomalis* from same subgenus (*Isocladius*) were placed in a group in all trees, but individuals of *C. reversus* collected from Aygır-Çıldır Lakes and Eğirdir-Beyşehir Lakes (Fig. 1) were separated from each other.

The mean intraspecific K2P nucleotide divergences for *Cricotopus*, *Paratrichocladius*, *Psectrocladius* and *Rheocricotopus* species were given in Table 2. The interspecific K2P nucleotide divergences varied from 0 to 18.8% (mean 12.8%) within *Cricotopus*, from 10.5 to 13.2% (mean 12.3%) within *Orthocladius*, from 0 to 15.8% (mean 8.1%) within *Psectrocladius* and from 0 to 15.2% (mean 8.7%) within *Rheocricotopus*. The mean interspesific divergence was 14.9% for all species studied.

Of 512 sites in *COI* alignment of 70 taxa, there were 298 constant sites, 26 variable sites and 188 parsimony-informative sites. The consistency index was 0.320, retention and homoplasy indexes were 0.761 and 0.680, respectively.

Our results suggest that using the *COI* gene in DNA barcoding can be helpful in identifying *Cricotopus*, *Psectrocladius* and *Rheocricotopus* species. Phylogenetic analysis of *C. bicinctus*, *C. sylvestris*, *C. flavocinctus*, *C. patens*, *R. atripes*, *P. barbimanus* and *P. limbatellus* produced monophyletic groups in all trees. However, this *COI* sequence was not adequate in all cases. Sinclair and Gresens (2008) used *COI* gene for discrimination and phylogenetic analysis of *Cricotopus* species. Their MP and NJ trees produced monophyletic groups in *C. bicinctus*, *C. sylvestris* and *C. tremulus* species with *Orthocladius nigritus*, *O. oliveri* and *O. robacki*. Guryev *et al.* (2001), Carew *et al*. (2005), Ekrem *et al*. (2007) and (2010) studied on different Chironomid species, and they used mitochondrial *COI* gene as a phylogenetic marker in their studies.

The mean intraspecific nucleotide divergences reported in present study can be compared with the values from Sinclair and Gresens (2008). We found 2.7% nucleotide divergence for *C. bicinctus*, 1.4% for *C. sylvestris*, 12.4% and 5% with and without two individuals for *C. triannulatus* and 16.9% for *P. rufiventris* while Sinclair and Gresens (2008) found 1.99% for *C. bicinctus*, 4.60% and 0.48% with and without one individual for *C. triannulatus*

Table 2. Mean and range of intraspecific Kimura-2-parameter nucleotide divergences for *Cricotopus*, *Paratrichocladius*, *Psectrocladius* and *Rheocricotopus* species.

Species	Number of specimens	Mean (%)	Range (%)
Cricotopus bicinctus	8	2.7	0.2-9.6
Cricotopus flavocinctus	2	0	-
Cricotopus intersectus	2	0.6	-
Cricotopus patens	2	0	-
Cricotopus reversus	5	6.1	0-9.9
Cricotopus sylvestris	18	1.4	0-4.9
Cricotopus triannulatus	6	12.4	0-18.1
	4 (without Ed3_21 and the one from GenBank)	5.0	0-10.0
Cricotopus tristis	2	0.2	-
Paratrichocladius rufiventris	2	16.9	-
Psectrocladius barbimanus	2	0	-
Psectrocladius limbatellus	5	1.6	0-2.8
Rheocricotopus atripes	4	0.4	0-0.6
Rheocricotopus effusus	3	0	-

and 17.94% for *P. rufiventris*. The mean interspesific divergence of all species was 14.9% in our study. However, different values of mean interspesific divergence were reported in previous studies. Mean interspecific divergences of 4.41-6.02% (Hajibabaei *et al*. 2006) and 9.38% (Wiemers and Fiedler 2007) in Lepidopterans; 7.93% (Hebert *et al*. 2004b) in birds; 5.78% (Smith *et al*. 2006) in parasitoid flies; 18.1% in mayflies (Ball *et al*. 2005); and 16.2% (Ekrem *et al*. 2007) in Chironomidae have been reported. These values clearly show that a single threshold value for identification of species is not possible, and more studies will be needed to determine the best method for making these kinds of decisions.

A common conclusion of studies on chironomid taxa is that *COI* sequences work well for species identification in the majority of cases. In some cases, because only *COI* sequences may not be useful for identifying the species accurately, it is recommended that additional nuclear markers should be used. The use of nuclear markers may provide more accurate results.

DNA barcoding has proven extremely useful for identifying organisms to species level and resolving taxonomic conflicts. Recently, these tools have been applied to the Chironomidae (Carew *et al*. 2005, Ekrem *et al*. 2007, Pfenninger *et al.* 2007, Sinclair and Gresens 2008). Sequence data from the mitochondrial gene *COI* has been successful in monophyletic classifications that are largely congruent with morphological species in the genus *Chironomus* (Sharley *et al.* 2004, Carew *et al*. 2005), in the subfamily Orthocladiinae (Sinclair and Gresens 2008) and a large number of *Tanytarsini* genera (Carew *et al.* 2007, Ekrem *et al.* 2007). There are not many studies on subfamily Orthocladiinae with application of mitochondrial *COI* sequence. Also, there is no molecular study not only on the subfamily Orthocladiinae but also on the family Chironomidae in Turkey. Therefore, our study is novel in that extends application of *COI* sequence analysis to six genera in the subfamily Orthocladiinae (*Cricotopus*, *Orthocladius*, *Psectrocladius*, *Rheocricotopus*, *Paratrichocladius* and *Eukiefferiella*), which have been difficult to discriminate in larval and even pupal life stages.

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References

Ball S. L., P. D. N. Hebert, S. K. Burian and J. M. Webb 2005. Biological identifications of mayflies (Ephemeroptera) using DNA barcodes. – *Journal of the North American benthological society*, **24**: 508-524.

Blaxter M. L. 2004. The promise of a DNA taxonomy. – *Philosophical transactions of the royal society of London*, *Biological sciences*, Series B, **359**: 669-679.

Carew M. E., V. Pettigrove and A. A. Hoffmann 2005. The utility of DNA markers in classical taxonomy: using *cytochrome oxidase I* markers to differentiate Australian Cladopelma (Diptera: Chironomidae) midges. – *Annals of the entomological society of America*, **98**: 587-594.

Carew M. E., V. Pettigrove, R. L. Cox and A. A. Hoffmann 2007. DNA identification of urban Tanytarsini chironomids (Diptera: Chironomidae). – *Journal of the North American benthological society*, **26**: 587-600.

Caterino M. S., A. K. Tishechkin 2006. DNA identification and morphological description of the first confirmed larvae of Hetaeriinae (Coleoptera: Histeridae). – *Systematic entomology*, **31**: 405-418.

Cranston P. S. 1979. The biosystematics of British aquatic larval Orthocladiinae (Diptera: Chironomidae), PhD Thesis, London University, London.

Cranston P. S., D. R. Oliver and O. A. Saether 1983. The larvae of Orthocladiinae (Diptera: Chironomidae) of the Holarctic region-keys and diagnoses. – *Entomologica scandinavica supplement*, **19**: 149-291.

Ekrem T., E. Willassen 2004. Exploring Tanytarsini relationships (Diptera: Chironomidae) using mitochondrial *COII* gene sequences. – *Insect systematic and evolution*, **35**: 263-276.

Ekrem T., E. Willassen and E. Stur 2007. A comprehensive DNA sequence library is essential for identification with DNA barcodes. – *Molecular phylogenetics and evolution*, **43**: 530-542.

Ekrem T., E. Stur and P. D. N. Hebert 2010. Females do count: Documenting Chironomidae (Diptera) species diversity using DNA barcoding. – *Organisms diversity and evolution*, **10**: 397-408.

Epler J.H. 2001. Identification manual for the larval Chironomidae (Diptera) of North and South Carolina. North Carolina Department of Environmental and Natural Resources; Division of Water Quality, Special Publication SJ2001-SP13.

Folmer O., M. Black, W. Hoeh, R. Lutz and R. Vrijenhoek 1994. DNA primers for amplification of mitochondrial *cytochrome c oxidase subunit I* from diverse Metazoan invertebrates. – *Molecular marine biology and biotechnology*, **3**: 294-299.

Golding G. B., R. Hanner and P. D. N. Hebert 2009. Preface. Special issue on DNA barcoding. – *Molecular ecology resources*, **9**: 4-6.

Gresens S.E., K. T. Belt, J. A. Tang, D. C. Gwinn and P. A. Banks 2007. Temporal and spatial responses of Chironomidae (Diptera) and other benthic invertebrates to urban stormwater runoff. – *Hydrobiologia*, **575**: 173-190.

Guryev V., I. Makarevitch, A. Blinov and J. Martin 2001. Phylogeny of the genus *Chironomus* (Diptera) inferred from DNA sequences of mitochondrial *cytochrome b and cytochrome oxidase I*. – *Molecular phylogenetics and evolution*, **19**: 9-21.

Hajibabaei M., D. H. Janzen, J. M. Burns, W. Hallwachs and P. D. N. Hebert 2006. DNA barcodes distinguish species of tropical Lepidoptera. – *Proceedings of the national academy of science*, **103**: 968-977.

Hebert P. D. N., A. Cywinska, S. L. Ball and J. R. deWaard 2003. Biological identification through DNA barcodes. – *Proceedings of the royal society of London*, Series B, **270**: 313-321.

Hebert P. D. N., E. H. Penton, J. M. Burns, D. H. Janzen and W. Hallwachs 2004a. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. – *Proceedings of the national academy of science*, **101**: 14812-14817.

Hebert P. D. N., M. Y. Stoeckle, T. S. Zemlak and C. M. Francis 2004b. Identification of birds through DNA barcodes. – *Public library of science*, Biology **2**: 1657-1663.

Hillis D. M., C. Moritz 1990. Molecular systematics. – In: Sinauer (Eds.): Sunderland. MA, **16**: 588 p.

Hirvenoja M. 1973. Revision der gattung *Cricotopus* van der Wulp und ihrer verwandten (Diptera: Chironomidae). – *Annales zoologici fennici*, **10**: 1-363.

Hogg I.D., P. D. N. Hebert 2004. Biological identification of springtails (Hexapoda: Collembola) from the Canadian Arctic, using mitochondrial DNA barcodes. – *Canadian journal of zoology*, **82**: 749-754.

Kiknadze I. I., M. G. Butler, K. G. Aimanova, L. I. Gunderina and K. Cooper 1996. Geographic variation in polytene chromosome banding pattern of the Holarctic midge *Chironomus* (*Camptochironomus*) *tentans* (Fabricius). – *Canadian journal of zoology*, **74**: 171-191.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. – *Journal of molecular evolution*, **16**: 111-120.

Martin J. 1979. Chromosomes as tools in taxonomy and phylogeny of Chironomidae (Diptera). -*Entomologica scandinavica supplement*, **10**: 67-74.

Meier R., K. Shiyang, G. Vaidya and P. K. L. Ng 2006. DNA barcoding and taxonomy in Diptera: A tale of high intraspecific variability and low identification success. – *Systematic biology*, **55**: 715-728.

Michailova P. V. 1985. Tendencies in the karyotype evolution of species of the family Chironomidae (Diptera). – *Acta zoological Bulgarica*, **26**: 3-22.

Moller Pillot H. K. M. 1984. Nederlandse faunistische mededelingen 1B. De larven der Nederlandse Chironomidae (Diptera) (Orthocladiinae sensu lato). Nederland. 188 p.

Monaghan M. T., M. Balke, T. R. Gregory and A. P. Vogler 2005. DNA-based species delineation in tropical beetles using mitochondrial and nuclear markers. – *Philosophical transactions of the royal society of London*, Series B, **360**: 1925-1933.

Pegg G. G., B. Sinclair, L. Briskey and W. J. Aspden 2006. MtDNA barcode identification of fish larvae in the southern Great Barrier Reef, Australia. -In: Olivar M. P. and J. J. Govoni (Eds.): Recent advances in the study of fish eggs and larvae. Barcelona. – *Scientia Marina*, 7-12.

Pfenninger M., C. Nowak, C. Kley, D. Steinke and B. Streit 2007. Utility of DNA taxonomy and barcoding for the

inference of larval community structure in morphologically cryptic *Chironomus* (Diptera) species. – *Molecular ecology*, **16**: 1957-1968.

Savolainen V., R. S. Cowan, A. P. Vogler, G. K. Roderick and R. Lane 2005. Towards writing the encyclopedia of life: an introduction to DNA barcoding. – *Philosophical transactions of the royal society*, Series B, **360**: 1805-1811.

Schmid P. E. 1986. The larvae of Chironomids. The composition of species in the mountain brook 'Oberer Seebach'. – *Jahresbericht der biologischen station lunz*, **9**: 66-241.

Sharley D. J., V. Pettigrew and Y. V. Parsons 2004. Molecular identification of *Chironomus* spp. (Diptera) for biomonitoring of aquatic ecosystems. – *Australian journal of entomology*, **43**: 359-365.

Sinclair, C. S., S. E. Gresens 2008. Discrimination of *Cricotopus* species (Diptera: Chironomidae) by DNA barcoding. – *Bulletin of entomological research*, **98**: 555-563.

Smith M. A., B. L. Fisher and P. D. N. Hebert 2005. DNA barcoding for effective diversity assessment of a hyperdiverse arthropod group: the ants of Madagascar. – *Philosophical transactions of the royal society of London*, Series B, **360**: 1825-1834.

Smith M. A., N. E. Woodley, D. H. Janzen, W. Hallwachs and P. D. N. Hebert 2006. DNA barcodes reveal cryptic hostspecificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). – *Proceedings of the national academy of science*, **103**: 3657-3662.

Stoeckle M. 2003. Taxonomy, DNA, and the barcode of life. -*Bioscience*, **53**: 796-797.

Swofford D. L. 2002. PAUP *. Phylogenetic analysis using parsimony (*and Other Methods), Version 4.0b10, Sunderland, Massachusetts, Sinauer Associates.

Witt J. D. S., D. Threloff and P. D. N. Hebert 2006. DNA barcoding reveals extraordinary cryptic diversity in an amphipod genus: implications for desert spring conservation. – *Molecular ecology*, **15**: 3073-3082.

Wiemers M., K. Fiedler 2007. Does the DNA barcoding gap exist? A case study in blue butterflies (Lepidoptera: Lycaenidae). – *Frontiers in zoology*, **4**: 8.

Zhou X., S. Adamowicz, L. Jacobus, R. E. DeWalt and P. Hebert 2009. Towards a comprehensive barcode library for arctic life Ephemeroptera, Plecoptera, and Trichoptera of Churchill, Manitoba, Canada. – *Frontiers in zoology*, **6** (30): 1-9.