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

Adile Sari¹, Mustafa Duran¹*, Fevzi Bardakci²

¹ Pamukkale University, Faculty of Arts & Sciences, Department of Biology, 20070 Denizli, Turkey; E-mail: mduran@pau.edu.tr
² Adnan Menderes University, Faculty of Arts & Sciences, Department of Biology, 09010 Aydin, Turkey

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 Grønseth 2008). Phenotypic variation in pigmentation, another important character for distinguishing both Cricotopus species and Orthocladius species, can be highly variable (Grensen 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 cytotaxonomic 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, Kikkadze 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 Greens 2008).

Molecular-based approaches, such as DNA barcoding, are being used to supplement traditional taxonomic methods of species identification (Hibert 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 (Hibert et al. 2004a, Hogg and Hibert 2004, Ball et al. 2005, Monaghan et al. 2005, Smith et al. 2005, Hijibabaee 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 Greens 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, Stoekkle 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 Tschechkin 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′-TTTCTACAATCATAAGATATTGG-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.
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\[5′-\text{TAAACTTCAGGGTGACCAAAAAATCA-3′}\]  
(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 MgCl₂, 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 RAXMLGUI11.0 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 Greens (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 Greens (2008) and Ekrem et al. (2010).
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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 Aygırcıldır Lakes and Eğirdir-Beyesir 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 interspecific 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 Greensens (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 Greensens (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 Greensens (2008) found 1.99% for C. bicinctus, 4.60% and 0.48% with and without one individual for C. triannulatus.

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 Greensens (2008) and Ekrem et al. (2010).
and 17.94% for *P. rufiventris*. The mean interspecific divergence of all species was 14.9% in our study. However, different values of mean interspecific 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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