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Molecular Identification of Chironomid Species Based on Its-1 and Its-2 Regions of rDNA

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MOLECULAR IDENTIFICATION OF CHIRONOMID SPECIES BASED ON ITS-1 AND ITS-2 REGIONS OF rDNA

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Master of Science

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Monita Sharma ENTITLED Molecular Identification of Chironomid species based on ITS-1 and ITS-2 regions of rDNA BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Of all major aquatic invertebrate groups, members of family Chironomidae are most abundant and show a wide range of habitat preferences. The importance of correct identification of Chironomids has been realized in many bioassessment studies mainly because of their worldwide distribution, substrate specificities and predictable responses to various pollutants in the water sources. This study establishes that the sequence data from the Intergenic Spacer Regions (ITS) of ribosomal DNA could be used as molecular markers to distinguish between different Chironomidae species and also to identify them. The need to use molecular approaches, to identify various Chironomidae species, comes from the fact that the rate of misidentifications is fairly high when morphological features are used. A difference of six nucleotides in the sequence data of *Chironomus tentans* from North America and Europe suggest a low intraspecific variation. A detailed analysis of the ITS-1 and ITS-2 sequence data from seven

new species of Chironomids (*Thienemanniella xena, Xylatopus par, Tribelos fuscicorne, Robackia demeijerei, Tribelos jucundum, Polypedilum aviceps and Chironomus tentans*) along with 15 species obtained from Genbank considered in this study shows a high amount of interspecific variations and also that the European species tend to cluster close to each other when compared to North American ones. The high bootstrap values and short intercluster branches, depicted in the phylogram, might suggest presence of various clusters and rapid divergence of species, respectively within the genus Chironomus. Such phylogenetic analysis could also provide more information on the genetic relatedness among different species.

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I foremost want to thank my advisor for his support in the project design and technical assistance. Dr. Krane has always encouraged me to attend various state level meetings to learn about the ongoing research in various scientific fields. I also want to thank my committee members, Dr. Smith and Dr. Vadeboncoeur, for their advice. I also want to acknowledge my lab mates – Chad Ferguson and Joe Bartoszek, who were always ready to help if needed. I am grateful for the quality education I received at Wright State University and I hope to take my experience and go on to further successful research.

Introduction

A wide range of organisms belonging to diverse taxonomic groups is known to inhabit the sediment layer of water bodies. These organisms, also known as meiobenthos, (small benthic invertebrates that live in both marine and fresh water environments)include nematodes, crustaceans, annelids and also larval stages of organisms that become larger adult such as Chironomids (Markman *et al* 2005). The importance of many of these macroinvertebrates (aquatic invertebrates which inhabit a river channel, pond, lake, wetland or ocean) has been realized for bioassessment studies for years. More often biological monitoring is based on observing the response of organisms to changes in their environment (Carew *et al* 2003). This involves appointing a reference site and then comparing population structure and composition from other sites to the reference (Bailey 2001). For such studies an organism with a wide range of habitat preferences is required so that various sites could be compared to one another.

Chironomids or non-biting midges are known to inhabit a wide range of habitats (Entrekin *et al* 2006). Even the dry and hot African environment is known to be home for a Chironomidae species, *Polypedilum vanderplanki* (Hinton

1960). With an estimate of over 10,000 species worldwide (Cranston 1995), and more than 2,000 species in North-America (Hutchinson 1993), they make good candidates for biomonitoring. The relative immobility of the larval forms of Chironomids as compared to the winged adults, adds to the advantages of using Chironomids for bioassessment (William 1974).

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 (Little and Smol 2000), phosphorous and chlorophyll a concentration (Woodward and Shulmeister 2006 and Langdon *et al* 2006), presence of various metals (Gray 1996) and amount of organic content in these water bodies (Entrekin *et al* 2006).

For instance, the relative abundance of *Microspectra type* is known to decrease with the decrease in the amount of dissolved oxygen (Little and Smol 2000). On the other hand, a decrease in dissolved oxygen leads to an increase in the populations of *Chironomus* taxa (Little and Smol 2000). The abundance and composition of many Chironomid

species changes with the concentration of chlorophyll a in the lake (Woodward and Shulmeister 2006).

Altitude, temperature and lake productivity could also govern the community structure and composition of Chironomidae at a particular water source specifically lakes (Woodward and Shulmeister 2006, Bigler ***et al*** 2006, Saether 1975). For instance, Chironomid species like ***Cladopelma curtivalva, Cricotopus zealandicus, Cricotopus aucklanden-sis,*** and ***Polypedilum*** are most commonly found in warm waters all over the world (Walker ***et al.*** 1991; Larocque ***et al.*** 2001).

Also, sometimes the type of Chironomid community present at a particular site could predict the presence of a particular substrate. For instance, presence of ***Cricotopus bicinctus*** could be an indication of high levels of inorganic contaminants whereas ***Dicrotendipes nervosus*** is present where there is abundant decomposable organic matter (Simpson and Bode 1980). This makes Chironomids well-suited for habitat assessment. Also the presence of Chironomids in the most pristine and the most impacted habitats (DeShon 1995) may make them key indicator taxa for biological monitoring of aquatic environments (Sæther 1979).

Table 1: The major subdivisions of the Chironomidae together with the typical habitats in which they are found (Williams & Feltmate, 1992).

Subfamily	Tribe	Habitat
Tanypodinae	Coelotanypodini	littoral zone of ponds & lakes (lentic)
	Macropelopiini	streams & rivers (lotic); some lentic littoral & profundal
	Natarsiini	fast-flowing waters
	Pentaneurini	fast-flowing waters; lentic littoral; a few hygropetric
	Tanypodini	lentic littoral
Podonominae	Boreochlini	fast-flowing waters; lentic littoral; esp. cold waters
	Podonomini	fast-flowing, cold waters
Diamesinae	Boreoheptagyini	cold, fast streams
	Diamesini	fast-flowing, cold waters; springs
	Protanypini	profundal zone of lakes
Orthocladiinae	Clunionini	marine, rocky shores
	Corynoneurini	lotic fast & slow water; lentic littoral
	Metriocnemini	wide range of lentic & lotic habitats, including springs, pitcherplants, dung, interstitial, marine intertidal & semi-terrestrial
	Orthocladiini	wide range of lentic & lotic habitats, including marine intertidal
Chironominae	Chironomini	lentic, littoral/profundal; slow lotic; especially on sandy substrates & associated with aquatic macrophytes
	Tanytarsini	lotic fast & slow water; lentic littoral; occasionally in brackish water

The predictable response of populations of certain Chironomidae species to different levels of a variety of pollutants has resulted in the use of larval Chironomids in bio-assessment studies dealing with water quality (DeShon 1995 and Sæther 1979). For instance, the exposure of *Chironomus tentans* to different pollutants can have a substantial effect on their mentum teeth (Bird 1997). Similar studies have also been done on *Chironomus riparus* because of the ease of rearing the larvae of this species (MacDonald and Taylor 2006).

Table 2 shows the various Chironomidae taxa along with their pollution tolerances and habitat preferences (DeShon 1995). It is evident from table 2 that most of the taxa described in the table have tolerance values considerably higher than 12, which means that most of them have distinct habitat preferences.

In one study the abundance of various tribes of Chironomidae was monitored for a long period of time based on the levels of organic matter or biomass (Entrekin *et al* 2006). This study showed that different tribes have different tolerance level for the presence or absence of organic matter. For instance, removal of organic matter

from a stream dominated by different species of the tribe Tanytarsini, lead to an 85% decrease in abundance of the tribe (Entrekin *et al* 2006). Also, within a tribe, different genera can have different habitat preferences and pollution tolerance levels (Deshon 1995). For instance, as shown in table 2, *Polypedilum (U.) flavum* and *Polypedilum (P.) illinoense* belong to the same tribe but the tolerance value of *Polypedilum (U.) flavum* is much higher than the latter indicating that an environment with a variety of substrates is more likely to harbour *Polypedilum (P.) illinoense* rather than *Polypedilum (U.) flavum* (Deshon 1995).

The importance of Chironomids has also been realized by the United States Environmental Protection Agency (EPA) for evaluating water quality. The Invertebrate Community Index (ICI) (Ohio Environmental Protection Agency, 1987 and 1989) developed by the biologists at the EPA has been used for years for analysis of aquatic integrity. One of the metrices, included in this index, relies upon percent Tribe Tanytarsini Midge composition.

Table 2: Tolerance values for 18 common Great Lakes Chironomid taxa derived using the Ohio EPA Invertebrate Community Index (ICI) (Ohio Environmental Protection Agency, 1987 and 1989) weighted by abundance data and averaged (N ≥ 5). Comments are after DeShon 1995.*TV = Tolerance Value, where ≥ 46 = Intolerant, 45 - 36 = Moderately Intolerant, 35 - 26 Facultative, 25 - 22 Moderately Tolerant, 21 - 13 Tolerant, ≤ 12 = Very Tolerant

Taxon	*TV	Comments
Tanypodinae		
Ablabesmyia mallochi	30.1	species very common; lakes, ponds and swamps, also large shallow streams
Hayesomyia senata	32.2	throughout continental U.S., most often in rivers
Labrundinia pilosella	41.5	herbaceous marshes, ponds, lakes and slower portions of streams and rivers
Nilotanypus fimbriatus	43.2	clean, relatively shallow sandy streams, also large coastal plain rivers; some populations are pollution intolerant
Orthocladiinae		
Corynoneura celeripes	45.9	pollution sensitive; streams and rivers (26)
Corynoneura lobata	40.0	-----
Nanocladius (N.) distinctus	23.1	tolerant of high levels of nutrients; lakes, rivers, and streams
Rheocricotopus (Psilocricotopus) robacki	37.1	species often abundant in many lotic systems
Thienemanniella xena	38.2	-----
Chironominae		
Tribe Chironomini		
Dicrotendipes neomodestus	32.7	common species; rivers and streams; tolerant of high nutrients/organic wastes
Dicrotendipes lucifer	21.9	species tolerant of organic wastes
Dicrotendipes simpsoni	15.8	species normally associated with high nutrient levels or low dissolved oxygen
Parachironomus frequens	36.9	-----
Paratendipes albimanus	34.0	genus occurs in a variety of habitats
Phaenosectra flavipes	25.8	genus usually occurs in streams
Polypedilum (U.) flavum	38.6	genus is found in a wide range of habitats under a variety of environmental conditions
Polypedilum (P.) illinoense	18.4	species occurs under a wide range of conditions, including high organic loading and low dissolved oxygen
Tribe Tanytarsini		
Sublettea coffmani	47.0	genus found in lotic habitats

Another such index is the Hilsenhoff Biotic Index, (HBI) (Hilsenhoff 1987), which is scored on the basis of the tolerance of selected macroinvertebrates to organic pollution. While calculating HBI, all the individuals from one taxon are multiplied to their respective pollution tolerance values, these products are then summed and divided by the total number of individuals in the sample. The index value is then rated from 0-10, a high value means high levels of organic pollution and a low level indicates the presence of intolerant species or good quality of water source (Hilsenhoff 1987). Such indices are based on various mathematical models that involve correct counting of species under consideration. In order to get the correct number for the metrics, accurate identification of organisms is required (Newburn and Krane 2002). For instance, in case of HBI, wrong identification of individual samples could lead to a wrong index scoring which could in turn give an inaccurate assessment of the water source under consideration (Hilsenhoff 1987).

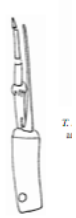
Although there is much evidence that Chironomids can be excellent biological indicators, identification to species level is frequently not possible. Even after investing time and expertise in the manual identifications, the rate of misidentifications is fairly high ranging somewhere

between 7.5-9% (Carew *et al* 2003). The often-minute dimensions of the subtle features needed to discriminate between the different species contribute significantly to the rate of misidentifications.

Anatomical features such as labial plates, mandibles and antennae are required for identifications and according to Epler (Epler 2001) an average of 6-60% species are misidentified in similar studies. Table 3 shows 4 taxa included in this study and some of the morphological features that are used to identify them. Because of the difficulties in manual identification of Chironomids, many species of Chironomids still remain unidentified to date. For instance, *Macropelopia, Procladius and Zayrelimyia* are some of the genera that are yet to be identified to species level (Boggero *et al* 2006). Also it has been realized that species belonging to genus *Thienemanniella* are generally difficult to identify due to the structural similarities (Epler 2001).

Morphologically similar and closely related species such as *Chironomus tentans* and *Chironomus pallidivittatus*

Table 3. Four taxa considered in this study along with their habitat preferences and morphological features used to identify them (Ohio Environmental Protection Agency, Technical Report, 1991 and Epler 2001)

Taxa	Comments	Antenna	Mentum and Mandible
T. fuscicorne	common on the Coastal Plain, often found in association with *T. jucundum*, lentic, indicator of slack water conditions		
T. jucundum	common on the Coastal Plain, lentic, indicator of slack water conditions		
R. demeijerei	Larvae are found in sandy substrata of streams and rivers.	-	-
P. aviceps	Common in stream and river, commonly cold water, indicator of clean water conditions. frequently been misidentified as *P. convictum*		

(Degelmann *et al* 1978) are especially a challenge because of the lack of good and complete identification keys. Most of the identification keys are based on the 4th instar stage and are specific to the region native to the expert who works with Chironomids. For instance, identification keys such as 'British non-biting midges (Diptera, Chironomidae)' (Edwards 1929) feature Chironomid species found only in United kingdom, 'Identification Manual for the Larval Chironomidae (Diptera) of North and South Carolina' (Epler 2001) describes species that inhabit the eastern part of United States of America, and 'The Genera of Larval Midges of Canada-Diptera: Chironomidae' (Oliver and Roussel 1983) could only be used to identify species native to Canada.

Above all the presence of xenobiotics in the sediment layer (Meregalli *et al* 2002) is known to cause mouthpart deformities (Vermeulen 1995) in the immature stages of Chironomids. For instance one study involving *Chironomus tentans* showed that exposure of this Chironomid to different substrates could have substantial effect on the mentum teeth (Bird 1997). Figure 1 shows the mouthpart deformities found in *Polypedilum* larvae present polluted habitats (MacDonald and Taylor 2006). It has also been shown that different

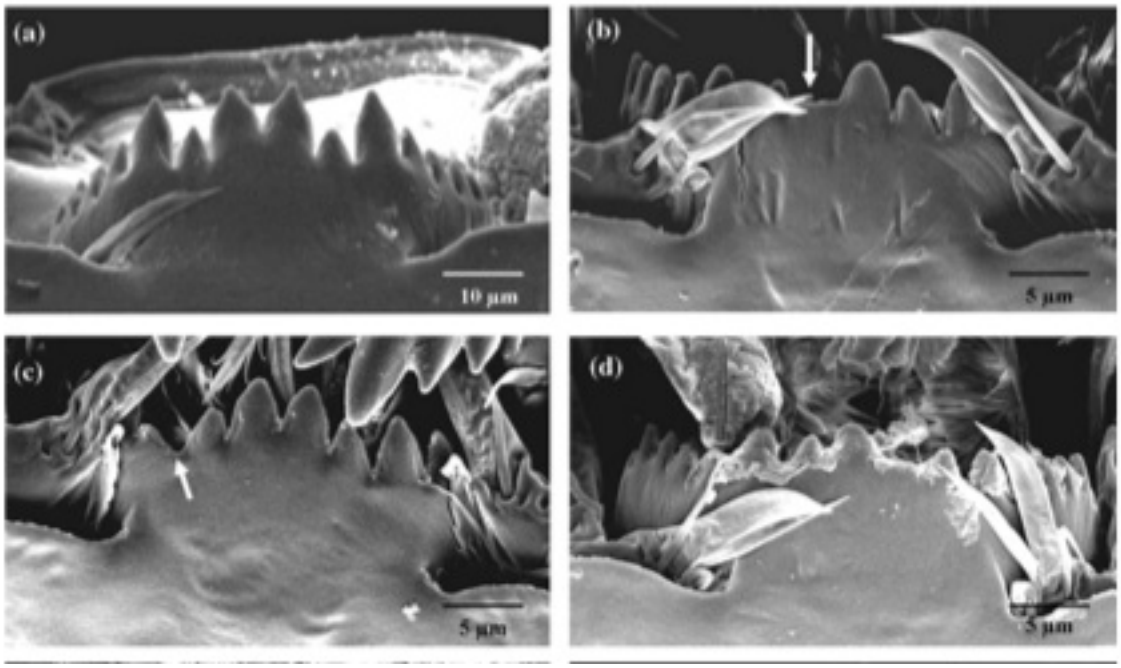


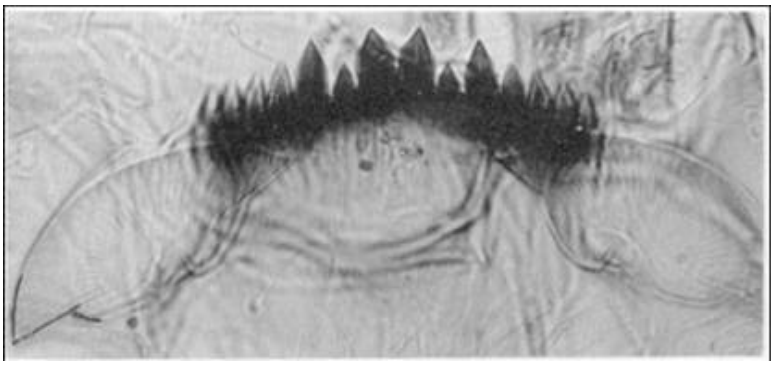
Figure 1: Mouthpart deformities found in Polypedilum larvae present in polluted water bodies (MacDonald and Taylor 2006).

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 (MacDonald and Taylor 2006). The occurrence of mouthpart deformities was found to be near 15% in case of ***Dicrotendipes*** and ***Polypedilum*** and 2.4% for that of ***Orthocladius*** (MacDonald and Taylor 2006).

Even when there are no deformities some species are so similar to each other that an expert eye can easily miss the difference. For example, Figure 2 shows the labial plates of ***Polypedilum illinosense*** and ***Polypedilum convictum.*** The only difference between the two of them is a slight change in the shape of teeth (Simpson and Bode 1980), which could easily be missed. Also, the small size of the ***Orthocladine*** and ***Diamesine*** larvae (Mason 1975) could make the manual identification process a little more difficult.

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 plumosus*** until now on the basis of morphology but a study based on karyotype structure and chromosomal polymorphism lead to differentiation of these two species

A.



B.

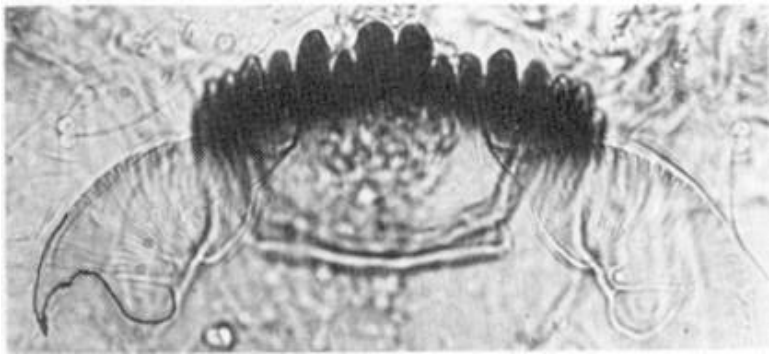


Figure 2: Morphological features are slightly different between Chironomidae species. 1 (A) the labial plate of ***Polypedilum illinosense*** 1(B) The labial plate of ***Polypedilum convictum*** (Simpson and Bode 1980).

(Kiknadze *et al* 2005). 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 (Carew 2003) 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.

Chironomid species have been analyzed phylogenetically on the basis of cytological characteristics as well as other genetic markers such as globin 2b gene (Guryev et al 2000). Figure 3 depicts the phylogenetic tree generated, for 23 species belonging to genus Chironomus, on the basis of gb2b gene data set.

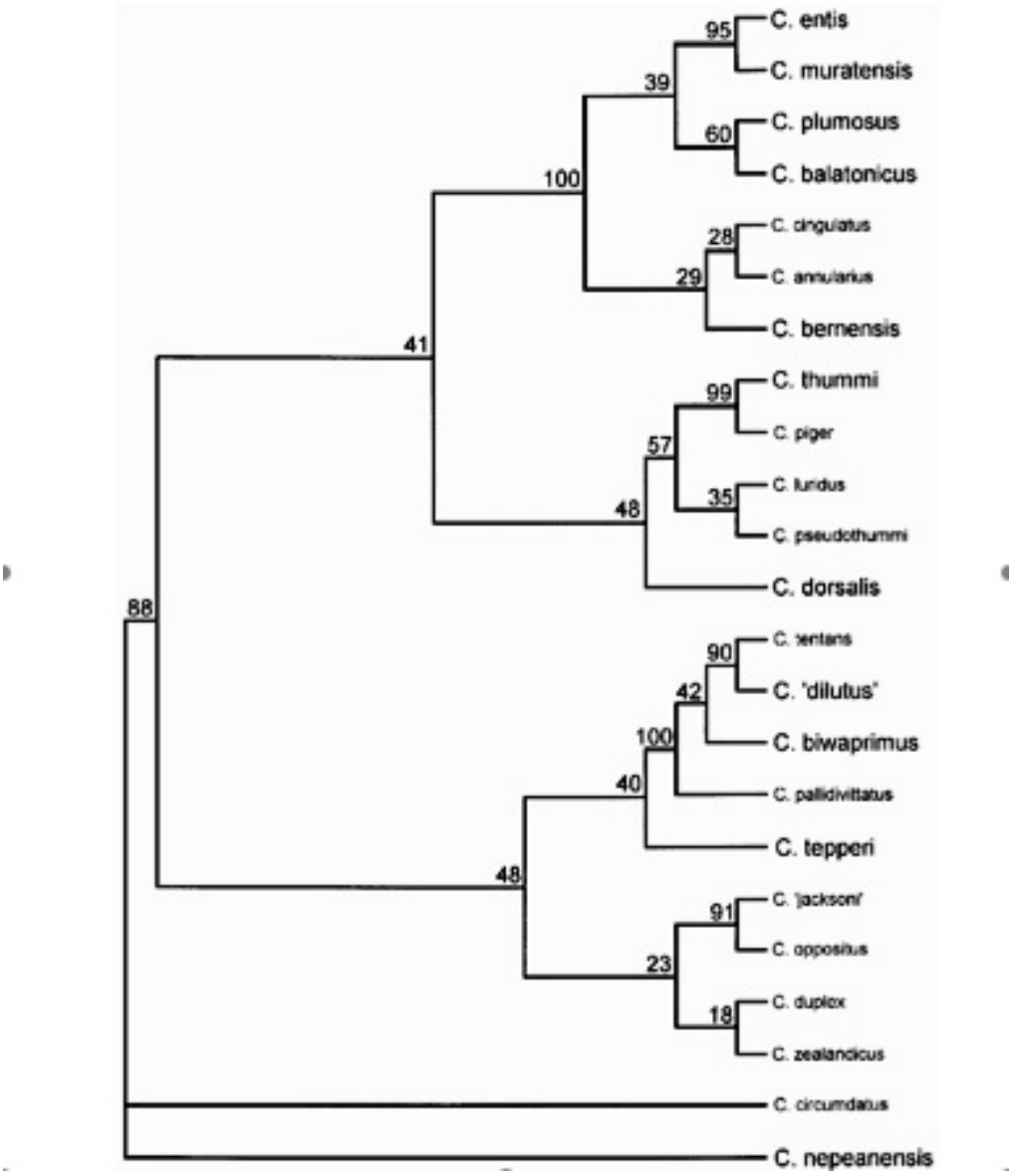


Figure 3: Phylogenetic tree of 23 ***Chironomus*** species based on gb2b gene data set (Guryev ***et al*** 2000).

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. The ITS (Intergenic Transcribed Spacer) region between the rRNA encoding regions within eukaryotic genomes correspond to just such locus (Marçon *et al* 1999 and Kocher *et al* 1989). The structural features of rRNA have been used to redefine the universal phylogenetic tree which divides the living systems into bacteria, archaebacteria and eukaryotes (Woese 1977). Sequence data along with the structural features of rRNA could even be more useful in solving the question of genetic relatedness among different species (Coleman 1997). Two internal transcribed spacer regions separate the conserved 18S, 5.8S and 28S genes as depicted in Figure 4(Hillis and Dixon 1991). The intraspecific homogeneity (Guryev *et al* 2000), interspecific divergence (Musters *et al* 1990) and availability of highly conserved sequences flanking the variable regions, makes the ITS sequences excellent marker for species identification and phylogenetic inferences in closely related species.

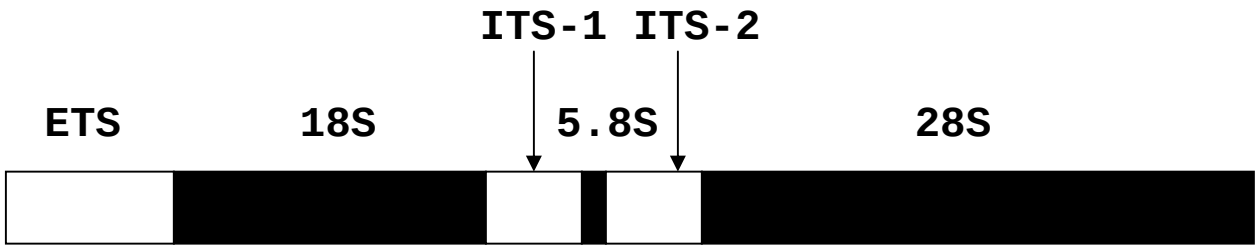


Figure 4: A diagram of rRNA encoding regions of a eukaryote showing the two intergenic spacer regions (ITS-1 and ITS-2) separating the conserved 18S, 5.8s and 28s regions. An external transcribed spacer (ETS) region is located upstream from the 18S gene (Hollis and Dixon 1991).

The fewer functional and selection constraints on the non-coding regions such as the ITS regions make them more useful for phylogenetic analysis as compared to protein coding regions such as Cytochrome oxidase gene which is highly conserved(McDonnell *et al* 2000).

The highly conserved regions of ribosomal DNA can be used to construct universal primers that can be used with a variety of different species (Hillis and Dixon 1991).

Another big advantage of using ribosomal RNA genes for such a study is that they are abundant in the nucleus (Markmann and Tautz 2005). The presence of an estimated number of 100-240 copies of rRNA genes on each sex chromosome of *Drosophila melanogaster* (Lyckegaard and Clark 1991) gives an insight into the extent of rDNA availability in the cells. Large copy number of rDNA is necessary because it cannot be amplified as per the organism's requirements unlike protein coding genes (Prokopowich 2003). The homogenization of rRNA nucleotide sequence could be attributed to the mechanisms which effect concerted evolution. These mechanisms include gene conversion, unequal crossing over or a combination of both (Michelson 1983). Unequal crossing over occurs during meiosis or during germ line mitosis when chromosomes

carrying closely linked homologous genes mispair, cross over and yield one chromosome with increased number of genes as compared to the other chromosome. Figure 5 shows how unequal crossing over leads to fixation of gene and also generation of gene families. The sequence homogeneity in a multigene family like rDNA depends on two factors, gene fixation rate and gene mutation rate. The shorter the gene fixation time in comparison to gene mutation time, the homogeneous is the sequence as is indicated in case of ribosomal DNA. On the other hand if the gene mutation time is shorter than the gene fixation time, heterogeneous multigene family will result (Hood ***et al*** 1975).

Analysis of sequence data from the ITS-2 region of rDNA from ***Anopheles flavirostris*** (Ludlow) (Diptera : Culicidae) collected from 35 different sites in Phillipines and comparision with ***Anopheles flavirostris*** of Indonesian origin revealed a sequence variation of just one base pair (Torres 2006). Analysis of the ITS-1 seuquence data from eight species of biting midge Culicoides, including samples of Culicoides impunctatus belonging to four geographically distinct locations suggest homogeneity in this gene sequence (Ritchie ***et al*** 2004). Absence of any intraspecific variation in the rDNA sequence of malaria vector ***Anopheles minimus*** collected from extreme North of

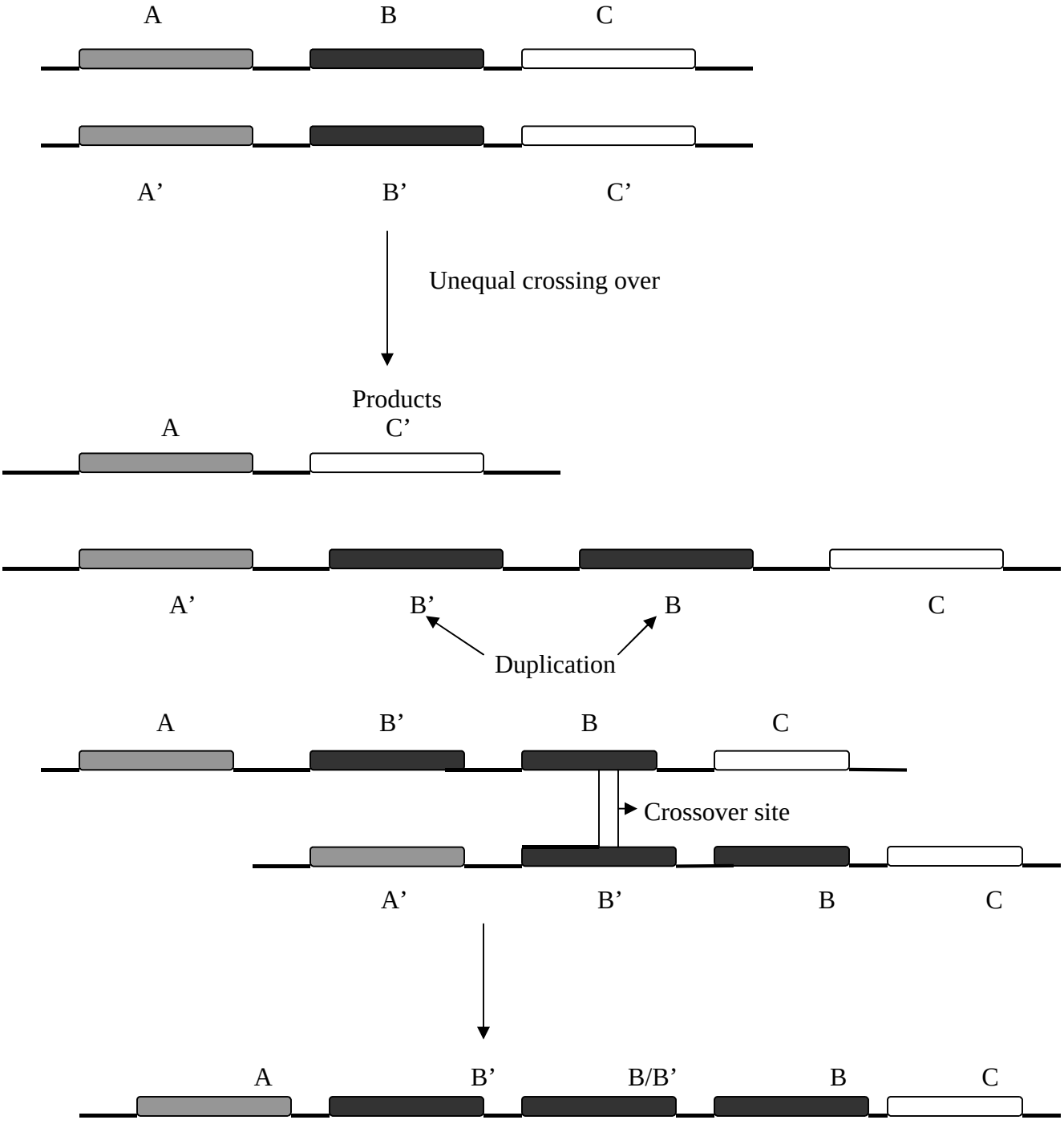


Figure 5: An illustration of unequal crossing over event leading to gene fixation.

Vietnam, Northern Laos to Central Vietnam and Northwest Cambodia (Phuc 2003), further confirms the use of sequence data from rDNA as a diagnostic tool for a particular taxon.

Although low or no intraspecific variation in rDNA sequence appears to be a norm, variation within individuals belonging to the same species is known. Analysis of the ITS-1 region of 7 species of crayfish reveals considerable amount of intraspecific variability (Harris ***et al*** 2000). A similar study involving tiger beetles, ***Cicindela dorsalis*** also reveals intraspecific variations (Vogler ***et al*** 1994). Such studies might indicate that rDNA is not a suitable marker for species differentiation but while making identifications based on sequence data the extent of intraspecific and interspecific variation is an important factor. For instance, in case of Parasitoid ***Ageniaspis*** the level of interspecific varaiation overrides the extent of intraspecific variation showing that rDNA is an appropriate marker for species differentiation (Juan 2002). A study involving genetic analysis of ***Malassezia*** isolates from dogs reveals that among the group of three genetic markers, Large subunit of rDNA, ITS-1, and chitin synthase 2 gene, the ITS-1 region showed the lowest percentage of intraspecific variation and a highest percentage of interspecific variation (Cafarchia ***et al*** 2007).

The ITS regions have successfully been used previously in identifying mislabeled cultures of green flagellates (Coleman *et al* 1997). Sequence data from the ITS region of forty different species of green flagellates was analyzed in order to find the closest genetic relative of *Chlamydomonas reinhardtii* and to identify the phylogenetic positions of the species with respect to each other (Coleman *et al* 1997). ITS region has previously been used to identify morphologically similar species of parasites (Zarlenga *et al* 1998). For instance, amplification of ITS-1 region proved to be a faster way as compared to manual identification to differentiate the eggs of *Ostertagia ostertagi* from other nematode genera. Universal primers have the potential to generate PCR bands that could identify *Ostertagia ostertagi* DNA from a mixture of DNA populations. These primers that are used to amplify the ITS-1 region and a part of 5.8S region generate 1011bp fragment in case of *Ostertagia ostertagi* and that of 600bp in case of *Haemonchus contortus, Cooperia oncophora* and *Oesophagostomum radiatum* (Zarlenga *et al* 1998). The same technique has been proved to be very beneficial in detecting interspecific variation within the genus *Ostertagia* (Zarlenga *et al* 1998).

Sequence data from the ITS region of various strains of *Saccharomyces* has been very beneficial to winemaking and brewing industries (Josepa *et al* 2000). The difficulty in identifying different strains of *Saccharomyces* on the basis of phenotypic characters makes the molecular techniques more promising (Josepa *et al* 2000). Some of the soil microbial communities have also been characterized using rDNA amplification (Hunt 2004). Sequence data from the 18S region of 28 different soil fungal communities has been used in a study in order to differentiate them from one another and also to find out the genetic relatedness among them (Hunt 2004).

The sequence data from the ITS-1 and ITS-2 regions of the rDNA has been proved especially beneficial in case of morphologically indistinguishable closely related species (Zhu *et al* 2006). One such case is that of *Contracaecum rudolphii A* and *Contracaecum rudolphii B* (Zhu *et al* 2006). These two species are morphologically indistinguishable and the only way of differentiating them from one another is through molecular techniques.

Molecular techniques could also be used as a supplement in distinguishing closely related species after they have been microscopically identified. For example, *Metachela* and *Neoplasta* (Diptera: Empididae: Hemerodromiinae) could

be differentiated using rDNA fragments combined with morphological identifications (Macdonald and HarKrider 2000).

Improvements in the ability to resolve and objectively distinguish Chironomid species have the potential to affirm present taxonomic status or clarify taxonomic ambiguities (Linevich 1963) and are likely to lead to the description of new species, potentially allowing even greater discrimination of Chironomids as ecoindicators.

Unlike manual identifications, molecular techniques require only a small amount of tissue from any part of the body. DNA could be extracted from living, dead, and even preserved tissue (Jackson *et al* 1991, Cooper 1994). The low cost and high accuracy makes these techniques feasible in large scale bioassessment projects. Above all, the interpretation of molecular sequence data requires little training compared to the time needed to train taxonomists (Carew *et al* 2003).

The analysis of molecular sequence data generally involves phylogenetic analysis. Two methods, Character-based and distance based, could be used to generate the phylogenetic trees from a data set. Neither of the two methods guarantees a true phylogenetic tree that can describe genetic relatedness among the sequences in the

data set (Krane and Raymer 2003). To come up with a reliable phylogenetic tree, the aligned sequences should be analysed based on fundamentally different distance and parsimony based methods. Of all the distance based methods Unweighted-Pair-Group Method with Arithmetic Mean (UPGMA) is the oldest and the simplest for tree construction. Parsimony forms the very basis of character based methods and abides by two main principles that is, mutations are rare events and only those relationships are correct that invoke fewest number of mutations.

This study hypothesizes that the sequence data from the ITS region can help distinguish between closely related Chironomid species in a faster and more efficient way as compared to the manual identification. A separate but related hypothesis is that intraspecific variation is much lower and can be distinguished from interspecific variation. It also anticipates that molecular DNA-based techniques could also be very helpful in predicting and or confirming genetic relatedness between Chironomid species. This could also aid in the identification of various genera that still remain identified till date.

Methods

Samples

Thirty *Chironomus tentans* samples were obtained from three different geographical locations within the USA: Ohio, Colorado and New Hampshire. These samples were provided by three independent commercial suppliers. All these samples belonged to the laboratory populations and were reared in the laboratory conditions for over 10 years so it was not possible to obtain the information about the exact location from where the starter cultures were collected. The samples obtained from Aquatic research organisms (ARO), New Hampshire, were reared in the laboratory since more than 15 years (Stan Sinitski, President, ARO) and were originally obtained from Columbia.

The identified Chironomid larval samples from different Chironomidae species, used in this project were provided by John H. Epler (Ph.D. Aquatic Entomologist) and Mike Bolton (Environmental Specialist 2, Ohio Environmental Protection Agent). The samples, *Tribelos fuscicorne, Robackia demeijerei, Tribelos jucundum, Polypedilum aviceps,* were collected from South Eastern parts of United States and (*Thienemanniella xena, Xylatopus par, Chironomus tentans* were obtained from Ohio. The samples were stored in 95% ethanol until DNA extraction.

DNA extraction

DNA was extracted from individual organisms through the use of QIAamp DNA Mini Kit for tissues (Qiagen Catalogue Number 51304). Instructions of the manufacturer were followed without alterations.

PCR was performed using specific primers designed from the conserved 18S and 28S subunits of rDNA of ***Chironomus tentans*** from Genbank (Accession number X00212). 18S primer sequence, 5'- GAT GTT CTG GGC GGC ACG CG -3', and 28S primer sequence, 5'- TTG GTT TCT TTT CCT CCC CT- 3', were used. Both primers were used in 20 pmol/uL concentrations. PCR was carried out in 25 uL volumes using 12.5 uL of Hotstar Master mix (Qiagen), 1 uL of each primer, 50 ng of template and 8.5 uL of water. A negative control with no template was used to rule out any contamination in the reaction mixture and genomic DNA extracted from ***Chironomus tentans*** was used as a positive control.

Reactions were carried out on a MJ Research Thermocycler Model PTC-150 under the following conditions: 95° C, 15 minutes; followed by 35 cycles of 95° C, 1 minute (denaturation); 63.5° C, 1 minute (annealing); 72° C, 1 minute (extension), 72^{0} C for 10 minutes and then 4^{0}(incubation) until gel was run.

Gel Electrophoresis

Gel Electrophoresis was performed with 1.5% agarose gels [Agarose DNA grade (High Melting), Fisher Scientific] prestained with 0.5 uL/100 mL of Ethidium bromide (10mg/mL). Gels were run at 100V using 0.5X TAE buffer [Prepared by mixing 10mM (1ml of 1M stock) Tris-HCl, 1mM (200 uL of 0.5 stock) EDTA and ddH20, pH 7.5] at room temperature (Figure 4-Figure 8). 0.5 M Stock solution of EDTA was prepared by adding 93.05 g of EDTA in 350 mL of ddH2O and 1.0 M Tris stock was prepared by adding 60.57 g of Tris in 350 mL of ddH_2O (Sambrook et al, 1989).

Restriction Digestion

After a single amplification product was confirmed, restriction digests, of four species (*Chironomus tentans, Thienemanniella xena, Hayesomyia senata* and *Xylatopus par*), using *Hinf*I and *Rsa*I restriction enzymes, were carried out using buffers provided by the supplier (Gibco). Presence and absence of fragments resulting from changes in recognition sites were noted. Restriction digestion was carried out at 37° C for 6 hours. [3.5 uL ddH2O, 5 uL purified PCR product, 1 uL buffer, and 0.5 uL enzyme (10U/uL)].

Gel Extraction

Gel extraction of the PCR product was performed using QIAquick Gel Extraction Kit (Qiagen). All manufacturer

instructions were followed with one exception: DNA was eluted in water instead of the Elution buffer (Buffer AE) provided with the kit. This was done in order to fulfill the sample requirements for sequencing.

DNA Sequencing

After Gel Extraction, purified PCR product was directly sent for sequencing. All the procedures were performed at least twice independently on each individual specimen of each species in order to minimize the risk of sequencing error. Two individuals of each species were sequenced to support the sequence data. Thirty individuals belonging to *Chironomus tentans* species were sequenced from three different geographical regions in order to look for intraspecific variation.

Sequence Data

4Peaks- software version 1.6 (1.6) was used to view the sequence data. Sequence data for 15 species (*Dicrotendipes fumidus, Glyptotendipes pallens, Glyptotendipes barbipes, Glyptotendipes salinus, Chironomus aprilinus, Chironomus luridus, Chironomus pseudothummi, Chironomus nuditarsis, Chironomus plumosus, Chironomus melanotus, Chironomus cingulatus, Chironomus thummi piger, Chironomus duplex, Chironomus pallidivittatus* and *Chironomus tentans*) were obtained from Genbank. Multiple sequence alignments with

hierarchical clustering, for 21 species, were generated with the help of the computer program Multalin version 5.4.1 (Corpet 1988). Phylogenetic analyses were carried out by use of PAUP software (Swofford, 1990). The file format used by PAUP software was generated using ClustalX software (Thompson 1997). A heuristic search was completed in order to get the phylogram. Sequence data from rDNA of *Drosophila melanogaster* was used as an outgroup to root the phylogenetic tree. For bootstrap analysis, the parameter that retained groups only with frequency greater than 50% was chosen. Gaps were treated as missing while generating the phylogenetic trees. The option of displaying the best trees only was chosen. The number of constant, parsimony uninformative and parsimony informative sites was also determined by getting the phylogenetic tree scores using PAUP. An Unweighted-Pair-Group-Method with Arithmetic Mean approach (UPGMA) was used to measure genetic distance between all taxa considered. The seven novel sequences will be submitted to Genbank.

The sequence data from species belonging to different genera was used to calculate the inter- and intrageneric difference. The comparison of the ITS-1 and ITS-2 sequences from *Glyptotendipes salinus* and *Chironomus tentans* showed a difference of 109 bps (number sites where differences could

have been found was 268) and 160 bps (number of sites where differences could have been found was 393) base pairs respectively. Similar comparisions were done with all the species analyzed in this study and all of them showed a 40% variability among members of different genera.

Intrageneric comparisions yielded a difference of 20% among members belonging to the same genus. For instance, ***Glyptotendipes salinus*** and ***Glyptotendipes pallens*** differ by 83 bps (number of sites where differences could have been observed was 279) in their ITS-1 region and by 94 bps (number of sites where differences could have been observed was 394) in their ITS-2 region.

Results

Experiments performed to check intraspecific variability.

To test the hypothesis intraspecific variation is much lower and can be distinguished from interspecific variation multiple individuals of ***Chironomus tentans*** belonging to a variety of geographical locations were analysed. The PCR of all thirty ***Chironomus tentans*** samples, obtained from three different geographical locations, yielded products of same size on the gel (Figures 4 to 6).

Sequence data, from thirty North American ***Chironomus tentans*** individuals, was aligned with one of the European ***Chironomus tentans*** sequence data, obtained from Genbank to check for intraspecific variations. The European ***Chironomus tentans*** showed variations at six different places in the sequence (Appendix A). No nucleotide variations were observed in the sequences obtained from the North American ***Chironomus tentans***. These results support the idea of gene homogenization that occurs in multigene families.

Experiments performed to check interspecific variability among Chironomids

PCR amplification of Chironomid species, using primers specific to conserved 18S and 28S regions, generated amplification products of distinctive lengths (Figures 7

and 8). All species were easily amplified and reproducible. The primers designed from *Chironomus tentans* rDNA sequence data were used to obtain the sequence data from the Chironomidae species analyzed in this study. The PCR conditions for all the species were the same. The sequence data ranged in length from 1012 to 1241 bps for the species that were analyzed in this study (*Xylatopus par: 1012, Robackia demeijerei: 1083, Tribelos fuscicorne: 1098, Polypedilum aviceps: 1111 Thienemanniella xena: 1146, Tribelos jucundum: 1149, Chironomus tentans: 1241).*

Hinf1 and *RsaI* digests generated distinctive Restriction Fragment Length Polymorphisms (RFLPs) between the four tested Chironomid species- *Chironomus tentans, Thienemanniella xena, Hayesomyia senata and Xylatopus par* (Figures 9 and 10). All four species produced bands of characteristic sizes after enzyme digestion of PCR products.

A multiple alignment was generated for 21 species (Appendix B). Two pairs of morphologically closely related species (Degelmann 1979) were also aligned in order to analyze the extent of difference between them (Appendix C and Appendix D). The sequence data of these four species was obtained from Genbank. *Chironomus tentans* and *Chironomus pallidivittatus,* showed 22 variations in the

sequence data. Another pair of closely related species that was analyzed was ***Chironomus thummi*** and ***Chironomus melanotus.*** This pair showed 228 variations in the sequence data.

Data analysis using distance based methods

A UPGMA statistical method (unweighted-pair-group-method) was used to measure genetic distance between all taxa considered. The distance between taxa is represented by the number of nonmatching nucleotides divided by the total number of sites where matches could be found. Table-4 shows the distance matrix generated for 22 species.

Taxa separated by the smallest distance in the matrix were ***Chironomus tentans*** and ***Chironomus pallidivittatus,*** d= 0.007. Taxa separated by the largest distance were ***Tribelos fuscicorne and Glyptotendipes pallens,*** d=0.507. The value of 'd' in table 4 was converted to percentage by multiplying it by 100 so as to get an idea about how different genera are related to each other. These values of d were then used to calculate the standard deviation for each of the genera. These calculations were done for all the genera included in this study, Genus ***Chironomus (10);*** Genus ***Glyptotendipes (3);*** and Genus ***Tribelos*** (2); Genus ***Dicrotendipes***(1); Genus ***Thienemanniella*** (1); Genus ***Polypedilum*** (1); Genus ***Robackia*** (1) and Genus ***Hayesomyia***

(1). Percentage of intrageneric variations could not be calculated for the genera with just one member but those genera were included in the calculations performed for computing intergeneric variations. The percentage of intergeneric difference in the ITS-1 and ITS-2 regions of most of the species was 33±7% while the intrageneric percentage was found to be 14±0.8% in case of Genus ***Chironomus***, 13±3% in case of ***Glyptotendipes*** and 12% in case of Genus ***Tribelos***. The exception to this trend was found in case of very closely related species, ***C.tentans*** and C. ***pallidivittatus***, where the percent difference was found to be 0.7%.

Distance matrix was also generated for 9 species belonging to genus ***Chironomus*** using the sequence data from globin gene obtained from genbank. Table 5 shows the pairwise differences between 9 species belonging to genus ***Chironomus*** based on the globin gene sequence data.

Data analysis using character based methods

Using PAUP, a phylogram of all twenty-one Chironomid species used in this study was constructed (Figure 11B). PAUP is a software that implements the parsimony approach in order to infer phylogenetic relationships. Biological parsimony is based on the assumption that mutations are

very rare events and under the light of this genetic relatedness is computed. *Drosophila melanogaster* was used as an outgroup (a specie that is known to be more distantly related to each of the remaining species than they are to each other). The outgroup was used to root the evolutionary tree. A heuristic search was completed in order to get the phylogram. In case of multiple alignments that are greater than twenty sequences deep, like the one in this study, algorithms that might not always find the most parsimonious tree must be employed (Krane and Raymer 2003). This is because the number of trees that could possibly describe relationships between small number of data sets becomes too large with the addition of a few taxa to the data set. For instance, the number of rooted trees that can describe the relationship among 15 datasets is 213,458,046,767,875. This number becomes 8,200,794,532,637,891,559,375 for a data set of 20. Heuristic method is one such method that deals with the impossibility of examining even a small fraction of the astronomical number of alternative rooted trees for deep alignments by making changes in the first tree instead of generating each alternative tree branch by branch (Krane and Raymer 2003).

Parsimony analysis was also performed to get the bootstrap values for associations between the twenty one Chironomidae species analyzed in this study (Figure 12). This analysis revealed relatedness with bootstrap values exceeding 50 percent among the twenty one Chironomidae species considered in this project. Phylogram with all the chironomid species, within genus Chironomus, was also constructed in order to analyze the genetic relatedness between the species within the genus (Figure 11B). The parsimony analysis using PAUP software revealed that out of all the nucleotides 759 characters were constant, 453 were variable or parsimony uninformative and 447 were parsimony informative.

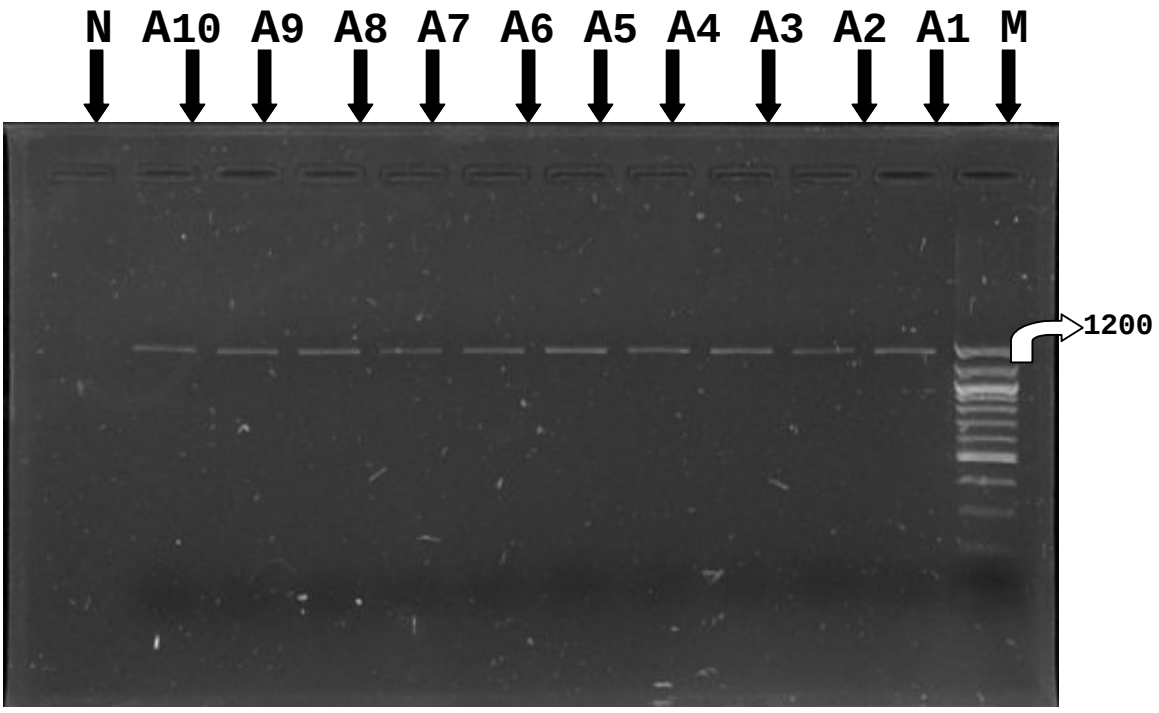


Figure 6: PCR products from the rDNA ITS-1 and ITS-2 region of ***Chironomus tentans*** species obtained from Ohio. Lane M– Size marker, Lanes A1 to A10 – PCR products from 10 different individuals of ***Chironomus tentans*** species, Lane N – Negative control. 4 uL of each PCR product was loaded on the gel.

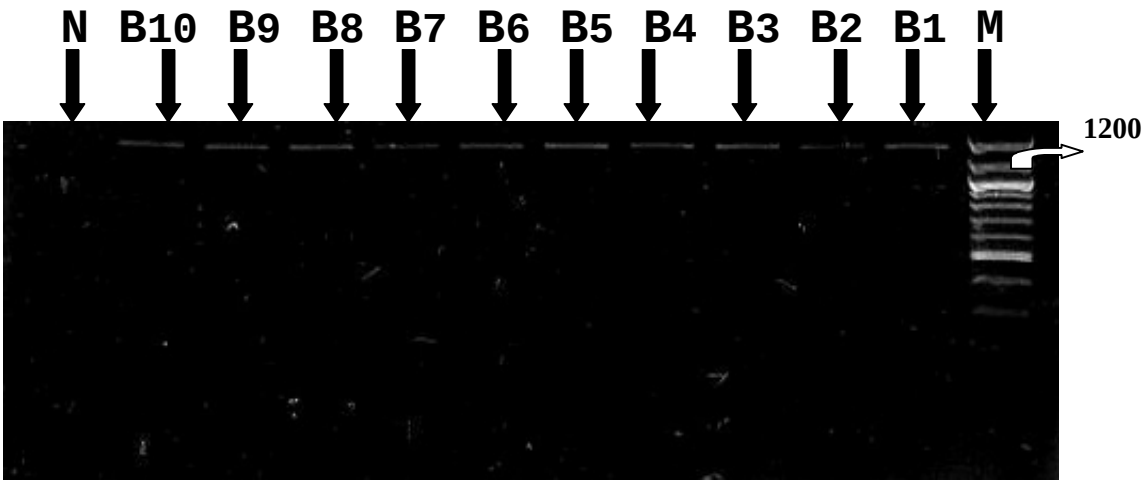


Figure 7: PCR products from the rDNA ITS-1 and ITS-2 region of ***Chironomus tentans*** species obtained from Colorado. Lane M – Size marker, Lanes B1 to B10 – PCR products from 10 different individuals of ***Chironomus tentans*** species, Lane N – Negative control. 4 uL of each PCR product was loaded on the gel.

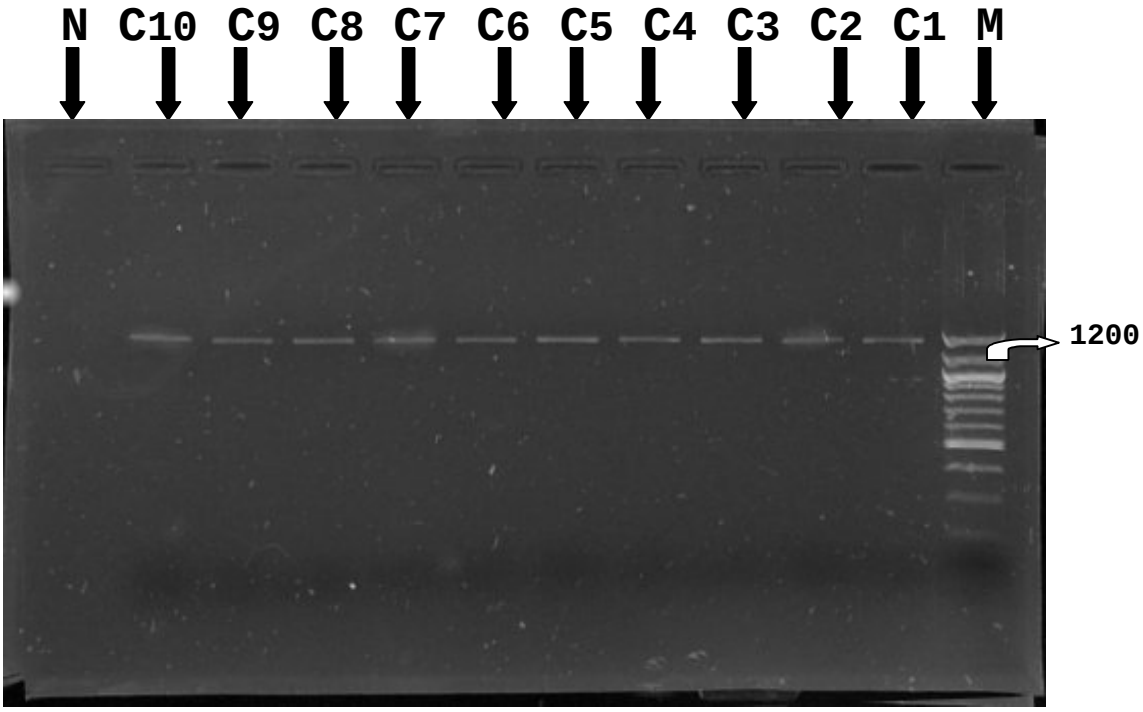


Figure 8: PCR products from the rDNA ITS-1 and ITS-2 region of ***Chironomus tentans*** species obtained from New Hampshire. Lane M– Size marker, Lanes C1 to C10 – PCR products from 10 different individuals of ***Chironomus tentans*** species, Lane N – Negative control. 4 uL of each PCR product was loaded on the gel.

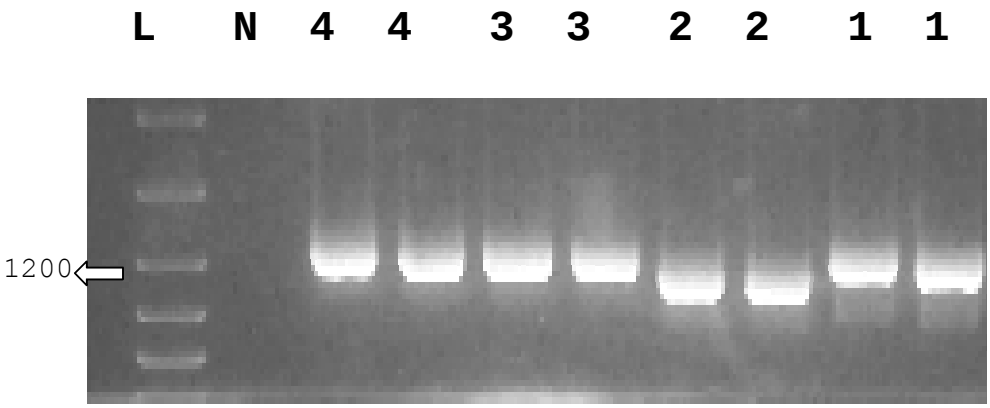


Figure 9: PCR products from the rDNA ITS-1 and ITS-2 region of four Chironomid species. Lane L – Size marker, Lanes 1,1 - *Chironomus tentans,* Lanes 2,2 – *Thienemanniella xena*, Lanes 3,3 – *Hayesomyia senata,* Lanes 4,4 – *Xylatopus par*, Lane N – Negative control.

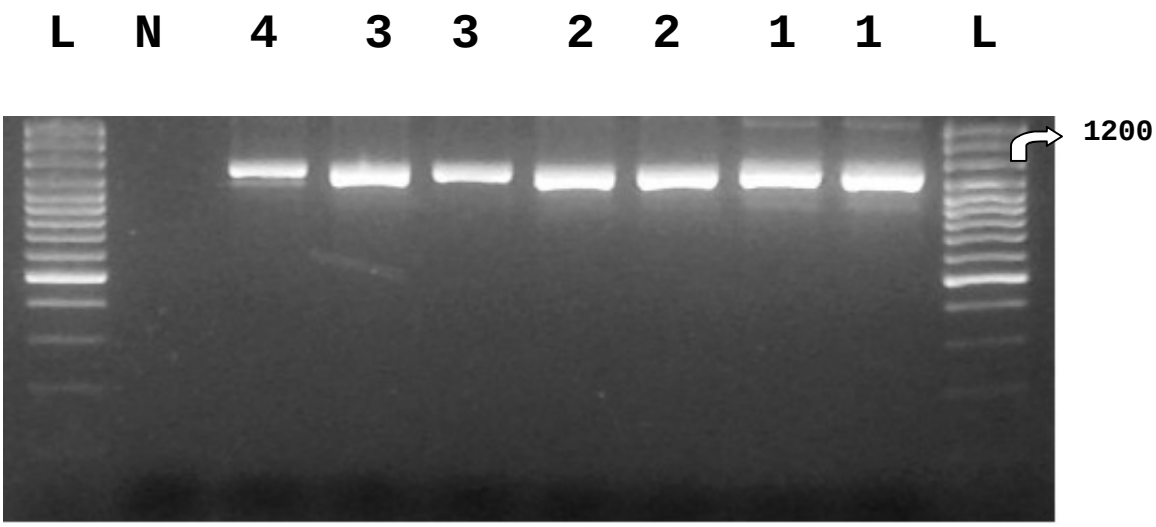


Figure 10: PCR products from the rDNA ITS-1 and ITS-2 region of four Chironomid species. Lane L – 100bp DNA Ladder, Lanes 1,1 ***Tribelos fuscicorne***, , Lanes 2,2 – ***Robackia demeijerei,*** Lanes 3,3 – ***Tribelos jucundum,*** Lane 4 ***Chironomus tentans,*** Lane N – Negative control.

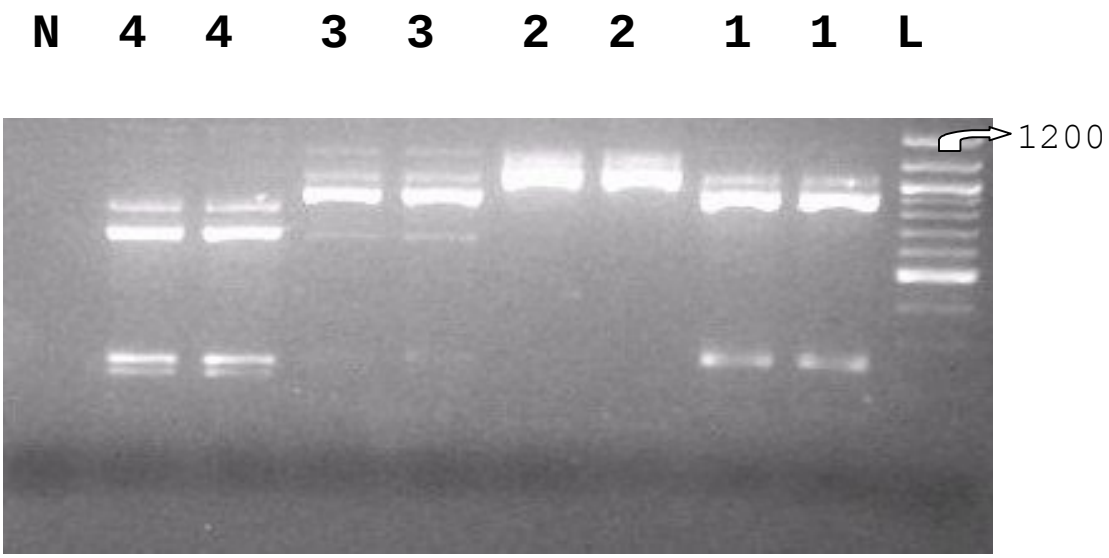


Figure 11: RFLPs (Restriction fragment length polymorphisms) of the rDNA ITS-1 and ITS-2 region generated by *Hinf*I for Chironomid species. Lane L – 100bp ladder, Lanes 1,1 - *Chironomus tentans,* Lanes 2,2 – *Thienemanniella xena,* Lanes 3,3 – *Hayesomyia senata,* Lanes 4,4 – *Xylatopus par,* Lane N – Negative control.

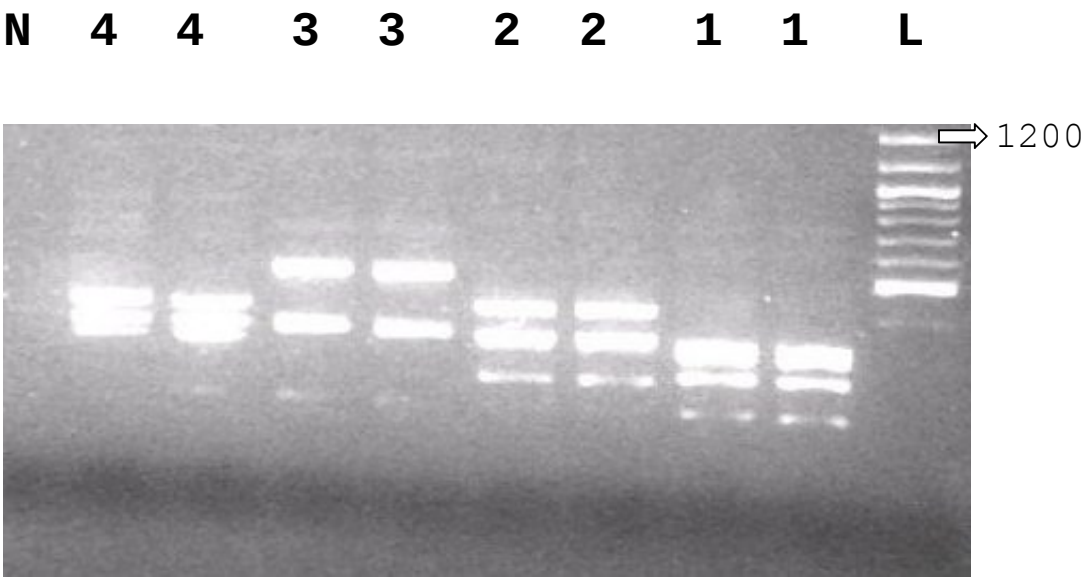


Figure 12: RFLPs (Restriction fragment length polymorphisms) of the rDNA ITS-1 and ITS-2 region generated by ***Rsa***I for Chironomid species. Lane L – Size marker, Lanes 1,1 - ***Chironomus tentans,*** Lanes 2,2 – ***Thienemanniella xena,*** Lanes 3,3 – ***Hayesomyia senata,*** Lanes 4,4 – ***Xylatopus par,*** Lane N – Negative control.

Table 4: UPGMA distance matrix. The distance (d) between taxa is represented by the number of nonmatching nucleotides divided by the total number of sites where matches could be found.

		1	2	3	4	5	6	7
1	*C. tentans* (EU)	-						
2	*C. tentans* (NA)	0.00485	-					
3	*C. duplex*	0.14340	0.14291	-				
4	*C. pseudothummi*	0.12720	0.12694	0.16007	-			
5	*C. aprilinus*	0.14634	0.15111	0.18804	0.09449	-		
6	*C. luridus*	0.13229	0.13804	0.16855	0.07326	0.09424	-	
7	*C. thummi piger*	0.14888	0.15125	0.17019	0.11879	0.14737	0.11651	-
8	*C. cingulatus*	0.16560	0.16806	0.19207	0.14742	0.16885	0.15054	0.16214
9	*C. melanotus*	0.15990	0.16229	0.17484	0.13677	0.16827	0.14362	0.16453
10	*C. plumosus*	0.15905	0.16627	0.18981	0.16012	0.17624	0.15841	0.17571
11	*C. nuditarsis*	0.17249	0.17352	0.20105	0.16150	0.17636	0.16569	0.18784
12	*G. salinus*	0.25053	0.25086	0.24702	0.23167	0.25159	0.22094	0.25173
13	*G. barbipes*	0.24707	0.24574	0.24375	0.23536	0.25212	0.22476	0.25387
14	*G. pallens*	0.26152	0.26768	0.26846	0.23493	0.24833	0.23445	0.27185
15	*D. fumidus*	0.21032	0.21514	0.23352	0.21232	0.22220	0.22015	0.24998
16	*T. jucundum*	0.29180	0.36867	0.36488	0.35320	0.36931	0.35167	0.37348
17	*T. xena*	0.29967	0.34190	0.35469	0.34653	0.35399	0.33906	0.35124
18	*P. aviceps*	0.31695	0.38867	0.38109	0.39363	0.40371	0.39216	0.39825
19	*R. demeijerei*	0.27528	0.34773	0.35439	0.34250	0.34835	0.33246	0.35302
20	*H. senata*	0.31846	0.36720	0.36859	0.36678	0.37988	0.37254	0.38294
21	*T. fuscicorne*	0.37597	0.47122	0.46443	0.44155	0.44795	0.45264	0.46429
22	*C. pallidivittatus*	0.01870	0.03209	0.15712	0.13737	0.16372	0.14735	0.16394
23	*D. melanogaster*	0.57786	0.59111	0.60868	0.59903	0.59950	0.58961	0.60180

Table 4: **UPGMA distance matrix**(contd.)

		8	9	10	11	12	13	14
8	*C. cingulatus*	-						
9	*C. melanotus*	0.03205	-					
10	*C. plumosus*	0.09175	0.08135	-				
11	*C. nuditarsis*	0.08823	0.08203	0.06261	-			
12	*G. salinus*	0.25255	0.24474	0.26006	0.26460	-		
13	*G. barbipes*	0.24254	0.24003	0.25253	0.25699	0.03437	-	
14	*G. pallens*	0.26570	0.25961	0.25613	0.27033	0.13301	0.13036	-
15	*D. fumidus*	0.22153	0.21310	0.22301	0.21901	0.24060	0.23820	0.23487
16	*T. jucundum*	0.35018	0.34584	0.36674	0.37125	0.35583	0.35958	0.36398
17	*T. xena*	0.34713	0.33820	0.36309	0.35813	0.35777	0.35534	0.36168
18	*P. aviceps*	0.39807	0.38459	0.39379	0.39950	0.43373	0.43229	0.43288
19	*R. demeijerei*	0.35770	0.35231	0.35459	0.36006	0.35308	0.35713	0.35971
20	*H. senata*	0.36422	0.35248	0.36527	0.36411	0.38059	0.38221	0.38205
21	*T. fuscicorne*	0.45674	0.45198	0.46639	0.45531	0.45373	0.45470	0.46470
22	*C. pallidivittatus*	0.17858	0.16843	0.17288	0.18450	0.25892	0.25640	0.26392
23	*D. melanogaster*	0.58781	0.58345	0.58641	0.59711	0.63204	0.63501	0.62421

		15	16	17	18	19	20	21
15	*D. fumidus*	-						
16	*T. jucundum*	0.33158	-					
17	*T. xena*	0.34359	0.34985	-				
18	*P. aviceps*	0.37753	0.37499	0.39873	-			
19	*R. demeijerei*	0.32508	0.34545	0.36660	0.33137	-		
20	*H. senata*	0.33890	0.35555	0.36979	0.36565	0.33506	-	
21	*T. fuscicorne*	0.44158	0.12785	0.43757	0.40718	0.40152	0.42320	-
22	C. pallidivittatus	0.21744	0.30105	0.31390	0.32340	0.28592	0.32808	0.37574
23	*D. melanogaster*	0.59909	0.62210	0.62889	0.62121	0.59764	0.62035	0.63381

		22	23
22	C. pallidivittatus	-	
23	D. melanogaster	0.57480	-

Table 5: UPGMA distance matrix of 9 Chironomidae species generated using gb2b gene sequence data.

		1	2	3	4	5	6	7
1	*C.luridus*	-						
2	*C.pseudothummi*	0.12477	-					
3	*C.thummi*	0.11886	0.13939	-				
4	*C.palidivittatus*	0.17822	0.20263	0.17715	-			
5	*C.duplex*	0.13279	0.15778	0.13975	0.12255	-		
6	*C.tentans*	0.26005	0.26173	0.26085	0.22389	0.23790	-	
7	*C.cingulatus*	0.25561	0.26725	0.27028	0.22584	0.23330	0.33602	-
8	*C.plumosus*	0.39145	0.41697	0.41018	0.41550	0.42196	0.45625	0.39121
9	*C.nepeanensis*	0.29476	0.24292	0.23790	0.22554	0.14420	0.30626	0.31036

		8	9
8	*C.plumosus*	-	
9	*C.nepeanensis*	0.51453	-

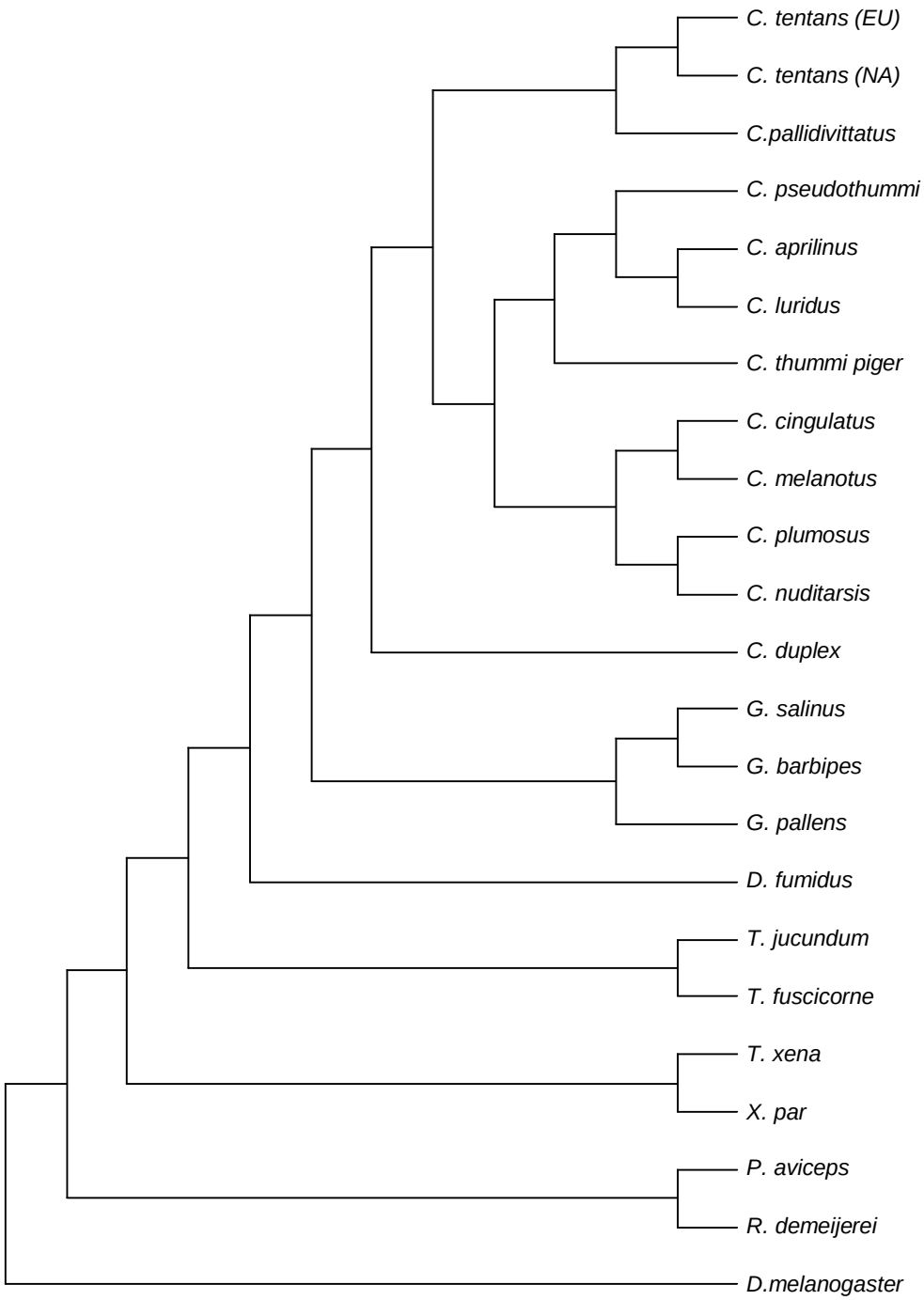


Figure 13A: Cladogram for 22 chironomid species. PAUP analysis was used to construct this tree

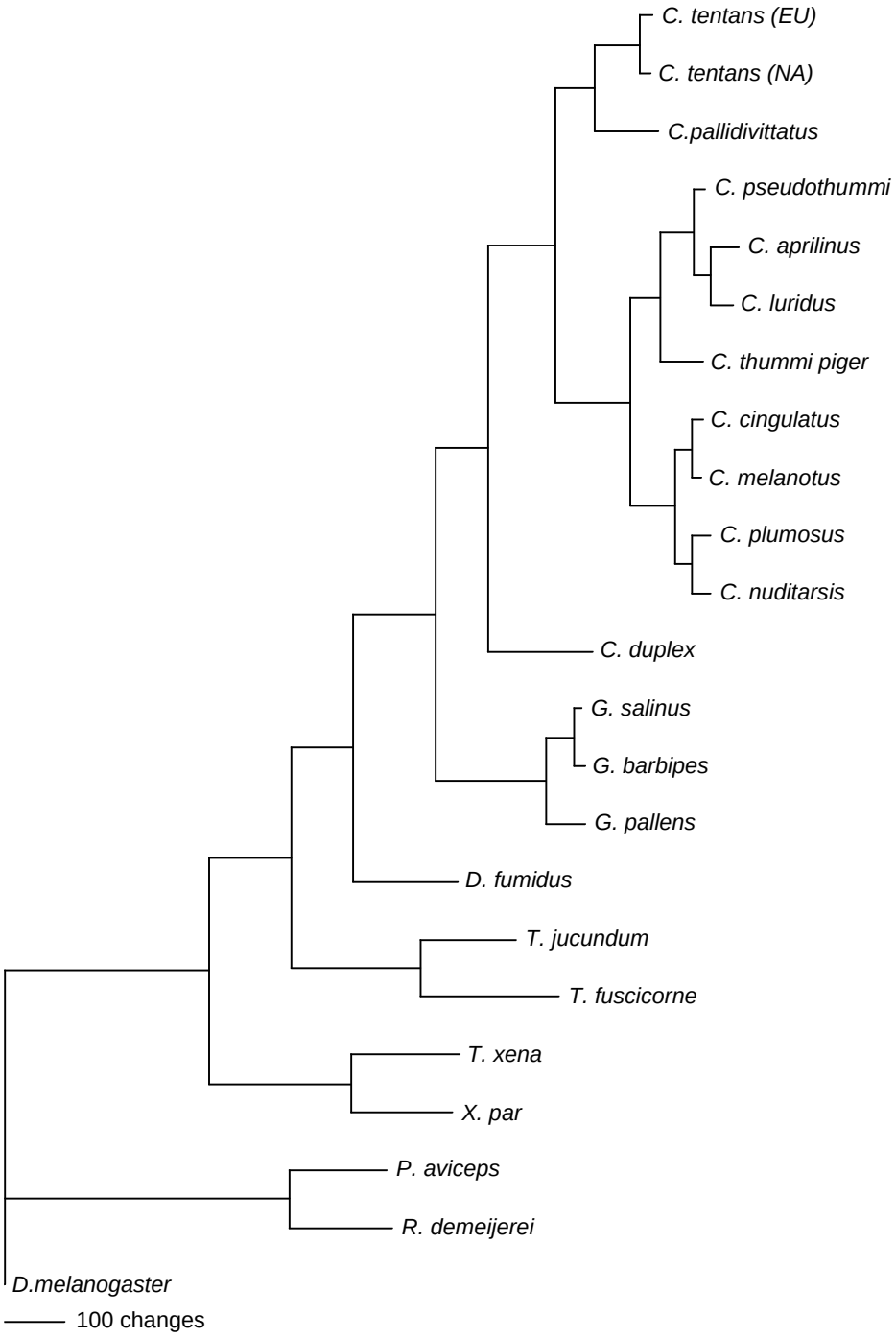


Figure 13B: A Phylogram of 22 Chironomidae species. PAUP analysis was used to construct this tree.

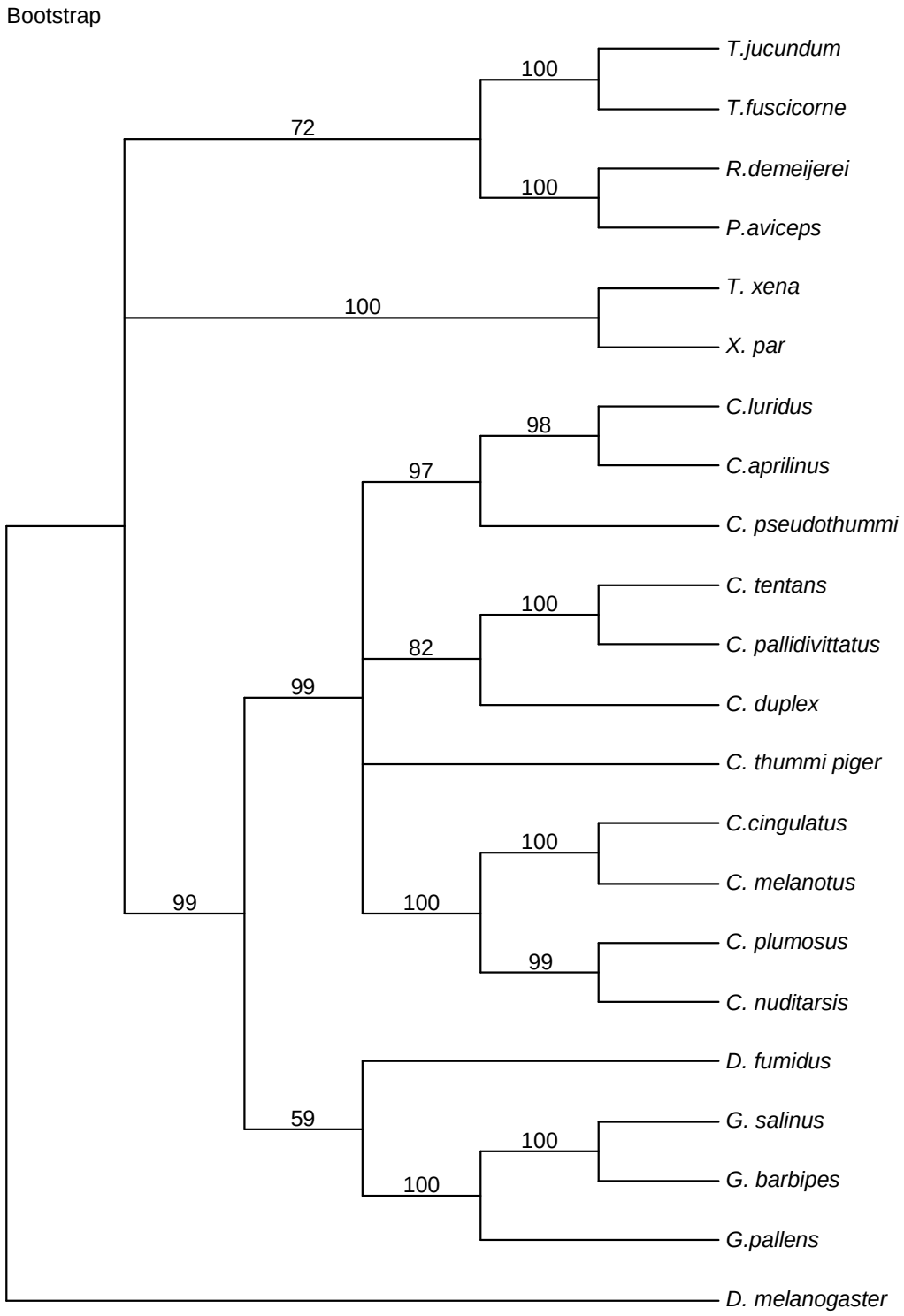


Figure 14: A Cladogram of Chironomidae species showing the bootstrap values at the nodes.

Discussion and Conclusion

This study establishes that molecular techniques are well suited for identifying Chironomids to the species level. Use of rDNA ITS-1 and ITS-2 sequence data has significant advantages over microscopic approaches for species identification. Direct sequencing of the PCR products, makes these techniques fast and reliable relative to conventional identification using slide mounting.

This research tests several hypotheses. The first hypothesis was that intraspecific variation between the rDNA nucleotide sequences of the individuals belonging to the same species but collected from different geographical regions would be much less than that seen between different Chironomidae species. A total of thirty ***Chironomus tentans*** individuals obtained from three different geographical regions (Colorado, New Hampshire, and Ohio) were analyzed at the level of their ITS-1 and ITS-2 sequences to test this hypothesis. Comparison of the PCR amplification products of each of the thirty ***Chironomus tentans*** species revealed no detectable size variation among them (Figures 6 to 8). Alignment of the 1248 base pairs of sequence information obtained for each of the individual thirty ***C. tentans*** samples also revealed no sequence variation. This

absolute invariability of rDNA sequence among different individuals of same species could be the result of mechanisms that lead to concerted evolution within arrays of tandemly arrayed genes such as gene conversion and/or unequal crossing over (Michelson 1983).

Variation in the ITS-1 and ITS-2 regions of ***C. tentans*** were only observed when sequence data from a European ***Chironomus tentans***, obtained from Genbank (Accession number X99212) was aligned with that of the North American samples sequenced in this study. European ***Chironomus tentans*** has a total of only three differences in its ITS–1 region (where there were 254 sites at which differences could have been observed in the sequence data that was available) and an additional three nucleotides in the ITS-2 region (where there were 370 sites at which differences could have been observed in the sequence data that was available) of the rDNA relative to that of the North American individuals (Appendix 1). This observation is consistent with many other examples of organisms exhibiting low intraspecific sequence variation of rDNA (Phuc 2003, Ritchie ***et al*** 2004, Torres 2006).

A second hypothesis was that the sequence data from the ITS-1 and ITS-2 regions of rDNA could be used to distinguish between different Chironomidae species. PCR

amplification products from all seven species analyzed in this study had distinctly different sizes when examined with gel electrophoresis (Figures 9 and 10).

A multiple alignment of the sequence information obtained from the seven species sequenced in this study and the additional fifteen species obtained from Genbank shows an appreciable amount of sequence variation in the ITS-1 and ITS-2 regions while the 18S and 28S regions are very well conserved (Appendix 2). The most closely related pair of Chironomid species (Degelmann 1979), ***Chironomus tentans*** and ***Chironomus pallidivittatus***, examined in this analysis had a total of 22 differences at the level of their ITS-1 and ITS-2 sequences (across 624 positions at which differences could have been observed). This variation has been found to be approximately four times the amount of intraspecific variations found between the European and North American representatives of the ***Chironomus tentans*** species. This result suggests that intraspecific variation of rDNA regions is much less than interspecific variation.

Table 4 shows the distance matrix generated for 21 species. The distance, d, is calculated by dividing the number of non-matching nucleotides by the total number of sites where matches could have been observed. Taxa separated by the smallest distance (d) in the matrix were

Chironomus tentans and *Chironomus pallidivittatus,* d= 0.007. Taxa separated by the largest distance were *Tribelos fuscicorne and Glyptotendipes pallens,* d=0.507. This distance gives an idea about the genetic relatedness between the species. The species pairs with the greatest values for d are likely have shared a common ancestor the least recently compared to species pairs that have a smaller value for 'd'. 8 Chironomidae genera, belonging to 2 subfamilies, Chironominae and Tanypodinae have been represented in the data analyzed in this study: Genus *Chironomus (10);* Genus *Glyptotendipes (3);* and Genus *Tribelos* (2); Genus *Dicrotendipes*(1); Genus *Thienemanniella* (1); Genus *Polypedilum* (1); Genus *Robackia* (1) and Genus *Hayesomyia* (1). The percentage of intergeneric difference in the ITS-1 and ITS-2 regions of most of the species was 33±7% while the intrageneric percentage was found to be 14±0.8% in case of Genus *Chironomus*, 13±3% in case of *Glyptotendipes* and 12% in case of Genus *Tribelos.* The exception to this trend was found in case of very closely related species, *C. tentans* and C. *pallidivittatus*, where the percent difference was found to be 1.8%. This percentage was calculated by converting the values of'd', in table 4, into percentage. No such trend was observed

when the distance matrix generated based on gb2b gene data was analyzed (Table 5).

Parsimony analyses also reveal several associations with bootstrap values exceeding 50% among the twenty-one Chironomidae species analyzed in this study (Figure 14). The species have been clustered together on the basis of their nucleotide sequence variations. For instance, ***Chironomus tentans*** and ***Chironomus pallidivittatus*** have the greatest sequence similarity so they have been placed very close to each other in the phylogram (Figure 14). The Genus groupings proposed in this study correspond to the ones proposed on the basis of gb2b gene (Figure 3). For instance, ***C. tentans, C. pallidivittatus and C. duplex*** have been placed in the same cluster in both the trees and show a bootstrap value of 82 in the rDNA tree and 100 in gb2b tree. ***C. luridus*** and ***C. pseudothummi*** have been grouped together and have a bootstrap value of 97 and 57 in rDNA tree and gb2b tree. ***C. cingulatus*** and ***C. plumosus*** show close associations in both phylograms.

The phylogram generated based on the sequence data from ITS-1 and ITS-2 regions of the twenty one Chironomidae species analyzed in this study suggest that the European species are more closely related to one another as compared to the North American species analyzed in this study

(Figure 14). Not much is known about the time of separation of lineages of Chironomids but the short intercluster branches, between the Chironomidae species analyzed in this study, observed in the phylogram (Figure 13B) suggests that there has either been significant gene flow between North American and European populations of Chironomids or that they have accumulated substitutions at a very low rate since they have been geographically separated.

Comparison of the phylogenetic tree, generated in this study, with that of the phylogram generated based on the gb2b gene (Figure 3) (Guryev et al 2000) reveals many similarities in terms of how various taxa have been grouped together. For instance, ***Chironomus tentans*** and ***Chironomus pallidivittatus*** have been placed in the same cluster in both the trees. ***Chironomus luridus, Chironomus pseudothummi*** and ***Chironomus thummi piger,*** all group together in one cluster in both the trees. ***Chironomus cingulatus*** and ***Chironomus plumosus*** show close associations in both phylograms. The Chironomidae species common in both the trees show similar taxonomic relatedness confirming the robustness of molecular techniques for such a study. These concordances also suggest that the rDNA

sequence information in this study have been useful in generating species trees as opposed to just gene trees.

This study is a step towards building a database of ITS-1 and ITS-2 sequence data from all Chironomus species. The availability of the nucleotide sequence data could prove to be very beneficial for bioassessment studies involving identification of thousands of samples. This kind of an approach to identify Chironomids based on their rDNA sequence data could make the process of species identification fast and accurate. This could mean processing a large number of samples in a short period of time and then comparing the sequence data to the database for species identifications. This has been demonstrated in this study when sequence data from thirty individual specimens of ***Chironomus tentans*** was compared to the Genbank entry of ***Chironomus tentans*** in order to confirm the species.

Future research could be directed towards analysis of different chironomidae species with worldwide distribution in order to see how the percentage of intaspecific and interspecific variation differs when samples from different continents are analysed. The question of whether or not habitat preference is governed by genetic makeup could be

answered by broadening the prospect of such a study to the species present around the globe.

Appendix A – Sequence alignment of rDNA for North American Chironomus ***tentans*** A1 and ***Chironomus tentans*** from Europe EU. 18S region spans from 1-354 bp, ITS-1 region spans from 355-605 bp, 5.8S region spans from 606-801 bp, ITS-2 region spans from 802-1175 bp, and 28S region spans from 1176-1248 bp. The primer sequences have been underlined.

```
    1                                                   50
EU  GATGTTCTGG GCGGCACGCG AGTTACAATG AAGCTGACAA CGTGTTACCT
A1  .......... .......... .......... .......... ..........

    51                                                 100
EU  TATCCGAGAG GATTGGGAAA TCACTTAGCC AGCTTCCTAG TTGGGATTGT
A1  .......... .......... .......... .......... ..........

    101                                                150
EU  GGACTGAAAA AGTTCACATG AACCAGGAAC TCCTAGTAAG TGTGAGTCAC
A1  .......... .......... .......... .......... ..........

    151                                                200
EU  TAGCTTGCAT TGATTACGAC CCTGATCTTT GTACACACCG CCCGTCGCTA
A1  .......... .......... .......... .......... ..........

    201                                                250
EU  TTACCGACGA ATTATTTAGT GAGATCTCTG GAGGTAAACA TTGCGGTGCC
A1  .......... .......... .......... .......... ..........

    251                                                300
EU  TCGGTATCGC GATTGCTTTT GCCAAAGTTG ATCAAACTTG ATGATTTGGA
A1  .......... .......... .......... .......... ..........
```

```
    301                                                 350
EU  GGAAATAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC
A1  .......... .......... .......... .......... ..........

    351                                                 400
EU  ATTAATGTAT GTTTTGCACA CGCATTTATG CTCTTTCATC TTGTTTTTTT
A1  .......... .......... .......... .......... .........A

    401                                                 450
EU  ATGGGGTGAG AATTATTAAT TAAAATCCTA GGTACTAGAA TTGCGATATG
A1  .......... .......... .......... .......... ..........

    451                                                 500
EU  TGTGCGATTA ATGTCGTACA CATGTTGTTG GTTTTATAAA GGGCTTCGCC
A1  ...A...... .......... .......... .......... ..........

    501                                                 550
EU  TAGGTATATT TTACTTTTTA TGCCAAAAAA CATAAAAAAA AATAAAATTG
A1  ..C....... .......... .......... .......... ..........

    551                                                 600
EU  TCGTTGTGAT TATAATAAAC AGTTTTTTCG ATAAGAAAAA ATGAATAAAC
A1  .......... .......... .......... .......... ..........

    601                                                 650
EU  AAAAACTTAA CCCTAGACAG GGGATCACTT GGCTCATGGG TCGATGAAGA
A1  .......... .......... .......... .......... ..........

    651                                                 700
EU  CCGCAGCAAA CTGCGCGTCG CCATGTGAAC TGCAGGACAC ATGATCATTG
A1  .......... .......... .......... .......... ..........
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    701                                                  750
EU  ACATGTTGAA CGCATATTGC GCCTTATACA TTTGGTTCTC TTTATAATAT
A1  .......... .......... .......... .......... ..........

    751                                                  800
EU  ACACAAAATT TATAATGTGG AACTGTATAA GGTACATATG GTTGAGTGTC
A1  .......... .......... .......... .......... ..........

    801                                                  850
EU  GTAATTTCAT ATGATTACAA CTATAAGTAT CTATCGCACA CATAGTGTTG
A1  .......... .......... .......... .......... ..........

    851                                                  900
EU  TTATAGTACA TAATAGAGTG TCATCAAAGC CGTCTCACCT CAAAGATTGA
A1  .......... .......... .......... .......... ..........

    901                                                  950
EU  TTTCTGCGCG GTGTGACGAT TTATGACTAA AATTCTAATC TAATGTCAGT
A1  .......... .......... .......... ...C...... ..........

    951                                                 1000
EU  TTACGCCTAT TTTTAAATAA ATGGGGGGAA GAGTGAAAAA TTCAAAATTC
A1  .......... .......... .......... .......... ..........

    1001                                                1050
EU  GCACATATAT GTGATGAATC TTGTGAGTCT ATTCTCTCTG GCGCTAACTT
A1  .......... ..-....T.. .......... .......... ..........

    1051                                                1100
EU  TACATATATA TATAATGTCT CGTTAGTTGC TCCTGATTTA TCCGCATGTG
A1  .......... .......... .......... .......... ..........
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    1101                                                 1150
EU  AATAACGATT TTGAGATAAA ATCATTCTTT CAAATGTACT ACTGAAGTAA
A1  .......... .......... .......... .......... ..........

    1151                                                 1200
EU  AAAAGTAAAA AAAAAAAAAA GACAATTTCG CGACCTCAAC TCATGTGAGA
A1  .......... .......... .......... .......... ..........

    1201                                               1248
EU  CTACCCCCTG AATTTAAGCA TATTAATTAG GGGAGGAAAA GAAACCAA
A1  .......... .......... .......... .......... ........
```

Appendix B – Sequence alignment of 18S to 28S subunit of rDNA for all species analyzed in this study. The primer sequences have been underlined.

```
                                1                                                  50
T. fuscicorne                   .......... .......... ...ACAAATG AAGCTGAGAA CGTGTTACCT
T. jucundum                     .......... .......... ...ACAAATG AAGCTGACAA CGTGTTACCT
T. xena                         .......... .......... ...ACAAATG CTGTCATAAG CGTGTTCCCT
P. aviceps                      .......... .......... ...ACAACCG TAGCTGACAA CGTGTCACCT
R. demeijerei                   .......... .......... ...ACAACTG AAGCTGACAA CGTGTTACTT
X. par                          .......... .......... ...ACAAATG AAGC.ATAAA CGTGCTACCT
C. tentans                      GATGTTCTGG GCGGCACGCG AGTACAAATG AAGCTGACAA CGTGTTACCT
C. pallidivittatus              .......... .......... .......... .......... ..........
C. duplex                       .......... .......... .......... .......... ..........
C. thummi piger                 .......... .......... .......... .......... ..........
C. cingulatus                   .......... .......... .......... .......... ..........
C. melanotus                    .......... .......... .......... .......... ..........
C. plumosus                     .......... .......... .......... .......... ..........
C. nuditarsis                   .......... .......... .......... .......... ..........
C. pseudothummi                 .......... .......... .......... .......... ..........
C. luridus                      .......... .......... .......... .......... ..........
C. aprilinus                    .......... .......... .......... .......... ..........
G. salinus                      .......... .......... .......... .......... ..........
G. barbipes                     .......... .......... .......... .......... ..........
G. pallens                      .......... .......... .......... .......... ..........
D. fumidus                      .......... .......... .......... .......... ..........
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```
                      51                                                  100
     T. fuscicorne    TATCCGAGAG GATTGGGAAA TCACTTAGCC AGCTTCCTAG TTGGGATTGT
       T. jucundum    TATCCGAGAG GATTGGGAAA TCACTTAGCC AGCTTCCTAG TTGGGATTGT
           T. xena    TATCCGAGAG GATTGGGTAA TCACTCAAAC GACTTCATAG TTGGGATTAT
        P. aviceps    TATCCGAGAG GATTGGGAAA TCACTCAGCC AGCTTCTTAG TTGGGATTGT
     R. demeijerei    TATCCGAGAG GATAGGGAAA TCACTCAGCC AGCTTCCTAG TTGGGATTGT
           X. par     TATCTGAAAG GATTGGGAAA TCACTGAACC GGCTCCATAG TTGGGATTGT
        C. tentans    TATCCGAGAG GATTGGGAAA TCACTTAGCC AGCTTCCTAG TTGGGATTGT
  C. pallidivittatus  .......... .......... .......... .......... ..........
          C. duplex   .......... .......... .......... .......... ..........
    C. thummi piger   .......... .......... .......... .......... ..........
      C. cingulatus   .......... .......... .......... .......... ..........
       C. melanotus   .......... .......... .......... .......... ..........
        C. plumosus   .......... .......... .......... .......... ..........
      C. nuditarsis   .......... .......... .......... .......... ..........
    C. pseudothummi   .......... .......... .......... .......... ..........
        C. luridus    .......... .......... .......... .......... ..........
      C. aprilinus    .......... .......... .......... .......... ..........
        G. salinus    .......... .......... .......... .......... ..........
       G. barbipes    .......... .......... .......... .......... ..........
        G. pallens    .......... .......... .......... .......... ..........
        D. fumidus    .......... .......... .......... .......... ..........
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                      101                                                 150
      T. fuscicorne   GGACTGAAAA AGTTCACATG AACCAGGAAC TCC.AGTAAG TGTGAGTCAC
        T. jucundum   GGACTGAAAA AGTTCACATG AACCAGGAAC TCCTAGTAAG TGTGAGTCAC
            T. xena   GGACTGTAAA AGTTCATATG AACTAGGAAT TGCTTGTAAG TGTGAGTCAC
         P. aviceps   GGACTGAAAA AGTTCACATG AACTATGAAC TCCTAGTAAG TGCGAGTCAC
       R. demeijerei  GGACTGACAA AGTTCACATG AACCAGGAAC TCCTAGTAAG TGTGAGTCAC
             X. par   GGACTGAAAA AGTTCACATA AACCATGAAT CTCTAGTAAG CGCGAGTCAC
          C. tentans  GGACTGAAAA AGTTCACATG AACCAGGAAC TCCTAGTAAG TGTGAGTCAC
  C. pallidivittatus  .......... .......... .......... .......... ..........
         C. duplex    .......... .......... .......... .......... ..........
    C. thummi piger   .......... .......... .......... .......... ..........
      C. cingulatus   .......... .......... .......... .......... ..........
       C. melanotus   .......... .......... .......... .......... ..........
       C. plumosus    .......... .......... .......... .......... ..........
      C. nuditarsis   .......... .......... .......... .......... ..........
    C. pseudothummi   .......... .......... .......... .......... ..........
        C. luridus    .......... .......... .......... .......... ..........
      C. aprilinus    .......... .......... .......... .......... ..........
        G. salinus    .......... .......... .......... .......... ..........
       G. barbipes    .......... .......... .......... .......... ..........
        G. pallens    .......... .......... .......... .......... ..........
        D. fumidus    .......... .......... .......... .......... ..........
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                      151                                                 200
      T. fuscicorne   TAGCTTGCAT TGATAATGAC TCTGATCTTT GTCCACACCG CCCGTCGCTA
        T. jucundum   TAGCTTGCAT TGATTACGAC CCTGATCTTT GTACACACCG CCCGTCGCTA
            T. xena   TAGCTTGCAT TGAATAAGTC CCTGATCTTT GTACACACCG CCCGTCGCTA
       P. aviceps     TAGCTTGCAT TGATTACGAC CCTGATCTTT GTACACACCG CCCGTCGCTA
      R. demeijerei   TAGCTTGCAT TGATTACGAC CCTGATCTTT GTACACACCG CCCGTCGCTA
            X. par    CAGCTTGTGT CGAATACATT TCTGCTCTTT GTACACACCG CCCGTCTCTA
       C. tentans     TAGCTTGCAT TGATTACGAC CCTGATCTTT GTACACACCG CCCGTCGCTA
C. pallidivittatus    .......... .......... .......... .......... ..........
         C. duplex    .......... .......... .......... .......... ..........
   C. thummi piger    .......... .......... .......... .......... ..........
     C. cingulatus    .......... .......... .......... .......... ..........
      C. melanotus    .......... .......... .......... .......... ..........
       C. plumosus    .......... .......... .......... .......... ..........
     C. nuditarsis    .......... .......... .......... .......... ..........
   C. pseudothummi    .......... .......... .......... .......... ..........
       C. luridus     .......... .......... .......... .......... ..........
      C. aprilinus    .......... .......... .......... .......... ..........
        G. salinus    .......... .......... .......... .......... ..........
       G. barbipes    .......... .......... .......... .......... ..........
        G. pallens    .......... .......... .......... .......... ..........
        D. fumidus    .......... .......... .......... .......... ..........
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```
                      201                                                 250
      T. fuscicorne   TTACCGACCA ATTATTTAGT GAGATCTCTG GAGGTGAACA TTGCGATATT
        T. jucundum   TTACCGACGA ATTATTTAGT GAGATCTCTG GAGGTGAACA TTGCGATATT
            T. xena   GTACCGACGA GTTATTTAGT GAGATCTTTG GAGATGGACA TTGTGATGGA
         P. aviceps   TTACCGACGA ATTATTTAGT GAGATCTCTG GAGGTGAGCG TTGCGATGT.
      R. demeijerei   TTACCGACGA ATTATTTAGT GAGATCTCTG GAGGTAAACA TTGCGATATC
             X. par   CTAACGATGG ATTATTTAGT GAGATCTCTG GAGGTGAACC TTGTGCTGTT
         C. tentans   TTACCGACGA ATTATTTAGT GAGATCTCTG GAGGTAAACA TTGCGGTGCC
  C. pallidivittatus   .......... .......... .......... .......... .......GCC
        C. duplex      .......... .......... .......... .......... .........C
    C. thummi piger    .......... .......... .......... .......... .........CC
      C. cingulatus    .......... .......... .......... .......... .........C
       C. melanotus    .......... .......... .......... .......... .........C
        C. plumosus    .......... .......... .......... .......... .........C
      C. nuditarsis    .......... .......... .......... .......... .........C
    C. pseudothummi    .......... .......... .......... .......... .........C
        C. luridus     .......... .......... .......... .......... .........C
       C. aprilinus    .......... .......... .......... .......... .........C
         G. salinus    .......... .......... .......... .......... .........T
        G. barbipes    .......... .......... .......... .......... .........C
         G. pallens    .......... .......... .......... .......... .........C
         D. fumidus    .......... .......... .......... .......... .........C
```

```
                      251                                                 300
     T. fuscicorne    GCTGTATCGA TCAGTGTTTT CCCCAAAATT TATCGATATT GATGCTTTGG
       T. jucundum    TCGGTATTGC G.ATTGCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
           T. xena    CTTGTTCATT ACGATTGTTC CGTCAAAGTT GATCAAACTT GATGATTTGG
      P. aviceps      TCGGCATTGC GATT.GTTTT CGCCAAAGTT GATCAACCTT GATGATTGGG
     R. demeijerei    TCGGTATTGC GATTTGATTT TGCCAAAGTT GATCAAACTT GATGATTTGG
           X. par     CGGTCATTGC GATTATCTTT TGCCATAGTT GGCCAATGTT GATGATTTGG
      C. tentans      TCGGTATCGC GATT.GCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
C. pallidivittatus    TCGGTATCGC GATT.GCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
        C. duplex     TCGGTATTGC GATT.GCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
   C. thummi piger    TCGGTGTCAC GATT.GCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
     C. cingulatus    TCGGTATTGC GATT.GCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
      C. melanotus    TCGGTATTGC GATT.GCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
       C. plumosus    TCGGTATTAC GATT.GCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
     C. nuditarsis    TCGGTATTAC GATT.GCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
   C. pseudothummi    TCGGTATCAC GATT.GCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
       C. luridus     TCGGTATCAC GATT.GCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
      C. aprilinus    TCGGTATCAC GATT.GCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
       G. salinus     TCGGTATTGC GATT.GCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
      G. barbipes     TCGGTATTGC GATT.GCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
       G. pallens     TTGGTATTGC GATT.GCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
       D. fumidus     TCGGTATTTC GATT.GCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
```

```
                    301                                                 350
      T. fuscicorne AGGAAATAAA AATCGCGACA AGGTTTCC.G TAGGTGAACC CGAA.GAAGG
        T. jucundum AGGAAATAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GAAGG
            T. xena AGGAACTAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GAAGG
         P. aviceps AGGAAATCAA CGTCGTAACA CGGCTTCCGT TAGGTGACCC TGCG.GAACG
       R. demeijerei AGGAAATACA AGTCGTAACA AGGTTTCCCG TAGGTTAACC TGCTCGAAGG
             X. par AGGCAAAAAA AGTCGTTACA TGGTTTCCGG TAGGTGACCC TGCGCGGGGG
        C. tentans AGGAAATAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GA.GG
  C. pallidivittatus AGGAAATAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GAAGG
          C. duplex AGGAAATAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GAAGG
     C. thummi piger AGGAAATAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GAAGG
       C. cingulatus AGGAAATAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GAAGG
        C. melanotus AGGAAATAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GAAGG
         C. plumosus AGGAAATAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GAAGG
       C. nuditarsis AGGAAATAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GAAGG
     C. pseudothummi AGGAAATAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GAAGG
         C. luridus AGGAAATAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GAAGG
       C. aprilinus AGGAAATAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GAAGG
         G. salinus AGGAAATAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GAAGG
       G. barbipes AGGAAATAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GAAGG
         G. pallens AGGAAATAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GAAGG
         D. fumidus AGGAAATAAA AGTCGTAACA AGGTTTCC.G TAGGTGAACC TGCG.GAAGG
```

```
                      351                                                 400
     T. fuscicorne    TTCGTTCACA GGTATC.CGC CCCCCGCTGG CTGCAGCGAT CTTGGTCA..
       T. jucundum    ATCATTAATG TATATAACTC TTATATATTA CTTTTATTAT ATCAATAAAT
           T. xena    ATCATTAAAA TTCAT....T TTTTAATGTC TAAATGCTTA CATCTCACA.
        P. aviceps    ATCATAAATG .......ATT TAATTTCATC ..TCTATGTA TGCCT.....
     R. demeijerei    ATCCTTACTG TATA...... CAACAGTGAG CCGTTATATG TATACGT...
            X. par    ATCACTA.TG .......... .CAAAGCAAG GTTTCCATGT GGGAGGGG..
        C. tentans    ATCATTAATG TATGTTTTGC ...ACACGCA ..TTTATGCT CTTTCA....
C. pallidivittatus    ATCATTAATG TATGTTTTGC ...ACACGCA ..TTTATGCT CTTTCA....
         C. duplex    ATCATTAATG TGTA...... ....TAACCA .TTATATGCT CTTTCACA..
   C. thummi piger    ATCATTAATG TATATTATAT ..CATACACA ..TTTATGCT CTTTCACC..
      C. cingulatus    ATCATTAATG TATGTTTTGC .....ACAAC .ATTTATGCT CTTTCA....
       C. melanotus    ATCATTAATG TATG..TTTC G...CACAAC ATTTTATGCT CTTTCA....
        C. plumosus    ATCATTAATG TATG..TCTC .GTACACAAC ATTTATGCGT CCTTCA....
      C. nuditarsis    ATCATTAATG TATGGTGTTT CAAACACAAC ATTTATGCGT CCTTCG....
   C. pseudothummi    ATCATTAATG TATG..TTTC .....ACAAA CATTTATGCT CCTTTCACA.
        C. luridus    ATCATTAATG TATT..AATC .TTAAACACA ATTTATGCTC TCTTCACA..
      C. aprilinus    ATCATTAATG TAAG..TTAC .ATACATACA TTCATGCTCT CTTTCACA..
        G. salinus    ATCATTAATG TATA..TCAT .TTACATTAT ATGATATGGG CTTTTATA..
      G. barbipes     ATCATTAATG TATA..TCAT .TTACGTTAT ATGATATGGG CTTTTATA..
        G. pallens    ATCATTAATG TATATTTTAT TATATGCTTT CATATGATGG CTTTTATG..
        D. fumidus    ATCATTAACG TATATAATTT ........CA TTATATGCT. CTTGTTTTTG
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                    401                                                 450
T. fuscicorne       CCTACGGAGG GTTCCAGCCA CTGATGGGAG GGTTGGTTCG ATTC......
T. jucundum         CCTAGGTACT AAAATGGCAA AGTATTAATA TATTGGGGGG TGCT......
T. xena             ACTTGTTGTG TTA.......  TGTATGAAGG GAAATTGGAA AAAC......
P. aviceps          CTCGGGCTGC CCTG...GTG CCGGCGTCA. ...GTTTC.. ..........
R. demeijerei       CCCGCTTTTC GCTG...CTG CGTTCTTAT. ...GTTTC.. ..........
X. par              GGAGCNGAGC GCGCAC.GTG G......... GGAACACACA CGAG......
C. tentans          TCTTGTTTTT TTATGGGGTG AGAA.TTATT A......ATT AA........
C. pallidivittatus  TCTTGTTTTT ..ATGGGGTG AGAA.TTATT A......ATT AA........
C. duplex           TCTTGTTTTT TTCACGAGTG AGAAATTATT ......TATA TATA......
C. thummi piger     CTTTGTTGTT .......... .......... GTGGTTTATG ACAA......
C. cingulatus       TCTTGTTTAT GTGTGAGATG TGGGGATA.. ......GAGG ACA.......
C. melanotus        TCTTGTT... ......GATG TGGGGATA.. ......GAGA ACA.......
C. plumosus         TCTTGT.... ....GAGATG TTGGTGTTTT TGGTGGGAGG ATATATGAT.
C. nuditarsis       TCTTGTTTAT ..AAGAGATG TTGGTGTTTT TTTGGGGAGA ACCTA.....
C. pseudothummi     CTTTGTTTTC ACACAAATGG GGTGA..... ..GATGTATT TTA.......
C. luridus          CTTTGTTTT. .......TTG GGTGA..... ..GATATTTA TTA.......
C. aprilinus        CTTTGTTTAT TTTGTGGAGT GTAGTAGAAG TTGATATATA TTTAATATCA
G. salinus          CATTCTATAT GTGTGTATAA AAGTTTGTGT GTGGTTTGAA ATAAATA..A
G. barbipes         CATATTCTAT GTGT..ATAA AAGCTTGTGT GTGGTTTGAA ATAAACA..A
G. pallens          CTTTAAGTA. GTGT....AA AAGTTTTGGT G.GTTTTGAA ATATT.....
D. fumidus          TCCTCTCCTA .......... .......... .......... ..........
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                    451                                                 500
      T. fuscicorne .....GCAAT CCTAGGTACT AGAATTGCGA TAACG..CAG .CTT......
        T. jucundum ......AAAA CCTTGGTACT AAGGAAACAT TACTCTTTTA TGCCTT....
            T. xena ......ACAT CCTTGGTACT AGGACTGCGA AATTGTGTAT ...TTCAAT.
         P. aviceps ......GGGT CCGGGGTTTT AGAAG.GCGT AATCGGTA.. ..........
      R. demeijerei ......TGCT CCTTGGTACT AGAATTCCCG ACTTTG.... ..........
             X. par ......GGAG CCAGGGTACT AGAGCTGCCA GATCT..CCG ..........
        C. tentans .......AAT CCTAGGTACT AGAATTGCGA TATGTGTGCG ATTA...ATG
C. pallidivittatus .......AAT CCTAGGTACT AGAATTGCGA TATGTGTGCG ATTA...ATG
          C. duplex .......AAT CCTAGGTACT AGAATTGCGA TTTGTGTGT. .......ACA
   C. thummi piger .......AAT CCTAGGTACT AGAATTGCGA TACGCGCACG CGTC...ATG
      C. cingulatus .......AAT CCTAGGTACT AGAATTGCGA TATGTG.TTG TGTT...CAC
       C. melanotus .......AAT CCTAGGTACT AGAATTGCGA TATGTG.TTG TGTT...CAC
        C. plumosus ...TATAAAT CCTAGGTACT AGAATTGCGA TATGTGCTTG TGTGTCAAAC
      C. nuditarsis .......AAT CCTAGGTACT AGAATTGTGA TATGCGTGTT TA........
   C. pseudothummi TACAACAAAT CCTAGGTACT AGAATTGCGA TACGTGTTTA CA........
        C. luridus AAC.......  CCTATGTACT AGAATTGCGA TGCGTGTGCA AGCA......
      C. aprilinus TACACTAAAT CCTAGGTACT AGAATTGCGA TACGTGTGCG CGCAT..ATG
        G. salinus ATTTGTAAAT CCTAGGTACT AGAATTGCGA TATGCATCAT ..........
      G. barbipes ATTTGTAAAT CCTAGGTACT AGAATTGCGA TATGCATCTT ..........
        G. pallens GTGTGTAAAT CCTAGGTACT AGAATTGCGA TATGCAA.GT ..........
        D. fumidus ......AAAT CCTAGGTACT AGAATTGCGA TATTCACGCA CTCTT..TTG
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                    501                                                 550
T. fuscicorne       GGCCGACCCG GTTGTTATTT GTTGCATGGC GTTAGCTCAC GGCTACCTTG
T. jucundum         AAACACGCCT GTTGTGGGTT TTTTAAAAAT ATTCGGCTAG TATTTTTTCC
T. xena             AATATATAAA GTTGTTGGTG TCTTAAAGGC CCTTCCCCAG AGGTATTCC.
P. aviceps          GAAAGCAGGC GTGGTGGATT GTAAGCAGGT ATCCGCAGCA AGG.....AG
R. demeijerei       AGCCGC.GGC GTGTGTGGCT GTATATAGGA CTTCGCCACG ........CG
X. par              GGAAGGGCAC GTTGTTGGTT TTAGATAAGG GTTTACACGC AAG......G
C. tentans          TCGTACACAT GTTGTTGGTT TTATAAAGGG CTTCGCCTAG GTATATTTTA
C. pallidivittatus  TCGTACACAT GTTGTTGGTT TTATAAAGGG CTTCGCCTAG GTATATTTTA
C. duplex           ..TCACACAT ATTGTTGGTT TTATAAAGGG CTTCGCCTAG GTAAACTTTA
C. thummi piger     .CGTGTGTGT ATTGTTGGTT TTATAAAGGG CTTCGCCTAG GTAAACTT.G
C. cingulatus       ACGCACACAT GTTGTTGGTT TTATAAAGGG CTTCGCCTAG GTAAACTT..
C. melanotus        ACGCACACAT GTTGTTGGTT TTATAAAGGG CTTCGCCTAG GTATAAACTT
C. plumosus         GCGCACACAT GTTGTTGGTT TTATAAAGGG CTTCGCCTAG GTAA.CTT..
C. nuditarsis       ...CACACAT GTTGTTGGTT TTATAAAGGG CTTCGCCTAG GTAA.CTT..
C. pseudothummi     .......CGT GTTGTTGGTT TTATAAAGGG CTTCGCCTAG GTAAACTTAC
C. luridus          .......CGC GTTGTTGGTT TTATAAAGGG CTTCGCCTAT GTAATCTTAC
C. aprilinus        TGCACGACGT GTTGTTGGTT TTATAAAGGG CTTCGCCTAG GTAATCTTAC
G. salinus          ..GTGTGCAT GTTGTTGGTT TTATAAAGAC TTTCGCCTAG GTAATCATTA
G. barbipes         ..GTATGTAT GTTGTTGGTT TTATAAAGAC TTTCGCCTAG GTAATCATTA
G. pallens          ..AAATGTAT GTTGTTAGTT TTATAAAGAC TTTCGCCTAG GTAATAATTG
D. fumidus          TTGCATGCAT GTTGTTGGTT TTATAAAGGG CTTCGCCTAN GTATACTTT.
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                      551                                                600
T. fuscicorne         CCGGGACCCT GCCGTGGGTG TGCAAACGAC TTTTTCGGCA TCGTTGTTAC
T. jucundum           CCCCAAGTTT TCCCGCGGGG GGAAAAACAT TTTTTTTTTT TCGCGCATAA
T. xena               CCATAAATTT TTT......G GGTCCAACAC TGGCGTATTA AGTGTTTC..
P. aviceps            GCAGGTGTAC ACAAGTGGAG CTAGGTGGTC AATGCATACC CGGCAATCCG
R. demeijerei         ACGGGTG... AGAGAGG... .......... .........C CG........
X. par                CCGGGGCACG AGCGAGAANG GGGCGAGCTT .........A GGTGGT....
C. tentans            CT.TTTTATG CCAAAAAA.. .CATAAAAAA AAATAAAATT .GTCGTTGT.
C. pallidivittatus    CT.TTTTATG CCAAAAAAAA ACATAAAAAA AAAAATAAAA TTGTCGTTGT
C. duplex             CT.TTTTATG CTTTCATAAA ACAAAAAAAA AAAAAAAAGA CGTTGT....
C. thummi piger       CT.TATTTTT TTTATGCCAA ACACATAATG ATGATATATA TGACGTTGT.
C. cingulatus         AC.TCTTTCT TTTATGCTAA ACACATAATT TAGAGA.... CGTTGT....
C. melanotus          AC.TCTTTCT TTTATGCTAA ACACATATTA GAGA...... CGTTGT....
C. plumosus           AC.TCTTTCT TTTATGCTAA ACACATATAA AGA....... CGTTGT....
C. nuditarsis         AC.TCTTTCT TTTATGCTAA ACACATATAA AGA....... CGTTGT....
C. pseudothummi       T........  TTTATGCCAA ACACATAATG AT........ .GTTGT....
C. luridus            TT........ TTTATGCCAA ACACATAATA ATATTGA... ..........
C. aprilinus          TT.CTTTTTT TTTATGCCAA ACACATAATT TATAAAAATT GA........
G. salinus            CT.TTTTATG CTTATGAAAT ATATACCTTT TATAAGAGTC ATGATATGTA
G. barbipes           CT.TTTTATG CTTATAAAAT ..ATACCTTT TATAAGAGTC ATGATGTATG
G. pallens            CTGTTTTATT CTTATAAATT ....GCCATT TATAAGTGTT GTGATATGGA
D. fumidus            ....TTACTT TTCATGCTTA .......... TTGAAAA... ..CCGTTGGG
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                      601                                                 650
     T. fuscicorne    AACCAACCAG TCGTGGCAAG CCTTCTAGCA AGCGACCCTA CCATGC....
       T. jucundum    AACCCCCCCT TGGGGGGGAA TTTTAAAGGA GTTATTTTTA ATTTAT....
           T. xena    ATGTTGGGAT TCTGATTGGA TATAAAAGGA AAAATTTATT AAAAAC....
        P. aviceps    AGGCGGGGGG TAATTCCATC TTCGGTATGG GCGAAGTGAT AACCCC....
     R. demeijerei    TAGAAGAGTG TGTATGGCCG AAAAATGAGA TGTGTGAACC C.........
             X. par   GTACGGCCGG TGGCAACAAT AATGAACCGC CGGGTGGGGG CACCGC....
        C. tentans    GATTATAATA AACAGTTTTT TCGATAAGAA AAAATGAATA AACAAAAA..
  C. pallidivittatus  GATTATAACA AACAGTTTTT TCGATAAGAA AAAATGAATA AACAAAAA..
          C. duplex   GATTATCCAT AAAGAATTT. TCGA.AAGAA AAGAAAAATA ACTAAAA...
    C. thummi piger   GATTTATTGT AATTGATTT. TCGA.AAGAA AAAAAAA... ACTTAAA...
      C. cingulatus   GATTGTATGG TTTATTATTT TCTTA.GTAA AAATAAACAC ACAAGA....
       C. melanotus   GATTGTATGG TTTATTATTT TTCTTA.GTA AAAATAAACA AAC.......
        C. plumosus   GATTTATACA GTTTATTATT TTTCTTATGT AAAAATAAAC ACAAAA....
      C. nuditarsis   GATTTGTGTA CGGTTTATTA TTTTTCTTAG TAAAAATAA. ACATAAAAA.
    C. pseudothummi   GATT...... ..GTAAAAAA ATTTTCGAAA GAAAATAAAA AACTTAAA..
        C. luridus    CGTT...... ..GTGATTTC AAATAAAATA AAAAAAAAAA AACTAAAA..
       C. aprilinus   CGTT...... ..GTGATTTT TAAGAAAAGA AAAAAATAAA ATAAAA....
        G. salinus    AAATTAGA.. TTATTGTGTA TGCAATAATT TAGAAGAAAA AAAAAATAAA
       G. barbipes    AAAATAAA.. TTATTGCGTA TGCGATAATT TAGAAGAAAA AAACTAAA..
         G. pallens   GGTATATT.. ATTGTGTGTG TCTAGTACAT GACAATAATT AAGAAATTGA
         D. fumidus   GATA...... ..TAGTAATG TAATAAACTA GTAATAATAA ..........
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                      651                                                 700
      T. fuscicorne  ...CTCCGG CTCAAGCAGG GA.CACGTAG GTTGGGTGGG TCGCTGAGGC
        T. jucundum  ..TTTCACC CTCAAACGGG GATCCCCTGG GTTGGGGGGG TGGATGA...
            T. xena  ...TACTCC CTGGCCAGGG GATCCACTAG GCTTCATGGG TCGATGA...
         P. aviceps  ..TTTAACC CTAGACAAGG GGAGCAGTGG GAGCCATGGG TCGATTC...
       R. demeijerei  ...TAGACC C......GGG GGATCACCTG GGTCAATGG. CCGAGAA...
             X. par  ..CTAGG.. C.....AGGG GGATCAGCTG GCTTCAAGGC TCGATCAAAG
         C. tentans  ..CTTAACC CTAGACAGGG GATCACTTGG GCTCATGGG. TCGATGA...
   C. pallidivittatus  ..CTTAACC CTAGACAGGG GATCACTTGG CT.CATGGG. TCGATGA...
          C. duplex  ..CTTAACC CTAGACAGGG GATCACTTGG GCT.CATGGG TCGATGA...
     C. thummi piger  ..CTTAACC CTAGACAGGG GATCACTTGG CTCATGGG.. TCGATGA...
       C. cingulatus  .TCTTAACC CTAGACAGGG GATCACTTGG CTCATGGG.. TCGATGA...
        C. melanotus  ...TTAACC CTAGACAGGG GATCACTTGG CTCATGGG.. TCGATGA...
         C. plumosus  ..CTTAACC CTAGACGGGG GATCACTTGG CTCATGGG.. TCGATGA...
       C. nuditarsis  ..CTTAACC CTAGACAGGG GATCACTTGG CTCATGGG.. TCGATGA...
     C. pseudothummi  ..CTTAACC CTAGACAGGG GATCACTTGG CTCATGGG.. TCGATGA...
         C. luridus  ..CTTAACC CTAGACAGGG GATCACTTGG CTCATGGG.. TCGATGA...
        C. aprilinus  ..CTTAACC CTAGACAGGG GATCACTTGG CTCATGGG.. TCGATGA...
         G. salinus  .TTTTAACC CTAGACAGAG GATCACTTGG CTCATGGG.. TCGATGA...
        G. barbipes  .TTTTAACC CTAGACAGGG GATCACTTGG GCTCATGGG. TCGATGA...
         G. pallens  .TTTTAACC CTAGACTGTG GATCACTTGG CTCATGGG.. TCGATGA...
         D. fumidus  ..TTTAACC CTAGACAGGN GATCACTTGG CTCATGGG.. TCGATGA...
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                      701                                                 750
     T. fuscicorne    AGAGCCCCAG CCCGCCGAGC GTTCCCA.TG TTAAGTCCAG GACGC.ATGA
       T. jucundum    AAACCCGCAC CCAGGGGGGC GTCCCCA.TG TGTGCTGCAG GAAAC.ATGA
           T. xena    AGACACCCAC CAAATGGGGC GTCGCCA.TG TGAATTGCAG AACACTATGA
      P. aviceps      AAACACCCCC AAAGGGGGCC GCCGCCAATG TGAGCTGCAG GAACACAGGA
     R. demeijerei    GAACACGCCA GAATTGGGGG ...GGCAATA TGAGCGGCAG GACACCATGA
           X. par     AGACGGCGCA AAAACTGGGC GTCGCCGATG AGA.CTGCAG GGCCC.ATGA
      C. tentans      AGAC.CGCAG CAAACTGCGC GTCGCCA.TG TGAACTGCAG GACAC.ATGA
C. pallidivittatus    AAAC.CGCAG CAAACTGCGC GTCGCCA.TG TGAACTGCAG GACAC.ATGA
        C. duplex     AGAC.CGCAG CAAACTGCGC GTCGCCA.TG TGAACTGCAG GACAC.ATGA
   C. thummi piger    AGAC.CGCAG CAAACTGCGC GTCGCTA.TG TGAACTGCAG GACAC.ATGA
     C. cingulatus    AGAC.CGCAG CAAACTGCGC GTCGCCA.TG TGAACTGCAG GACAC.ATGA
      C. melanotus    AGAC.CGCAG CAAACTGCGC GTCGCCA.TG TGAACTGCAG GACAC.ATGA
       C. plumosus    AGAC.CGCAG CAAACTGCGC GTCGCCA.TG TGAACTGCAG GACAC.ATGA
     C. nuditarsis    AGAC.CGCAG CAAACTGCGC GTCGCCA.TG TGAACTGCAG GACAC.ATGA
   C. pseudothummi    AGAC.CGCAG CAAACTGCGC GTCGCCA.TG TGAACTGCAG GACAC.ATGA
       C. luridus     AGAC.CGCAG CAAACTGCGC GTCGCCA.TG TGAACTGCAG GACAC.ATGA
      C. aprilinus    AGAC.CGCAG CAAACTGCGC GTCGCCA.TG TGAACTGCAG GACAC.ATGA
       G. salinus     AGAC.CGCAG CAAACTGCGC GTCGCCG.TG TGAACTGCAG GACAC.ATGA
      G. barbipes     AGAC.CGCAG CAAACTGCGC GTCGCCG.TG TGAACTGCAG GACAC.ATGA
       G. pallens     AGAC.CGCAG CAAACTGCGC GTCGCCG.TG TGAACTGCAG GACAC.ATGA
       D. fumidus     AGAC.CGCAG CAAACTGCGC GTCGCCA.TG .GAACTGCAG GACAC.ATGA
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                      751                                                 800
     T. fuscicorne  .CCATGGTCA CGTGGAGCGC ATGTCGGCGC CATATAACAT CTGGGTC...
       T. jucundum  .TCATTAACA TGTTGGACGC ATATG.GCGC CAAAAA.CAG GTGGATC...
           T. xena  .TCATTGACA AGTTGAACGC ATATG.GCAC CTTATA.CAT TTGGTTT...
        P. aviceps  TCCATGGACA AGTTGAACGC ATAATGGCGG CATTGTACAA TATGGAT...
     R. demeijerei  GTCATTGACA TGTGGAACGC AGAGT.GCGC GCTTAACCAT TTGGGTC...
            X. par  .TTATCGACA TGTTGAG.GC ATATT.GCGC CGTATA.CAT TTGGTTC...
       C. tentans   .TCATTGACA TGTTGAACGC ATATT.GCGC CTTATA.CAT TTGGTTC...
C. pallidivittatus  .TCATTGACA TGTTGAACGC ATATT.GCGC CTTATA.CAT TTGGTTC...
         C. duplex  .TCATTGACA TGTTGAACGC ATATT.GCGC CTTATA.CAT TTGGTTC...
  C. thummi piger   .TCATTGACA TGTTGAACGC ATATT.GCGC CTTATA.CAT TTGGTTC...
     C. cingulatus  .TCATTGACA TGTTGAACGC ATATT.GCGC CTTATA.CAT TTGGTTC...
      C. melanotus  .TCATTGACA TGTTGAACGC ATATT.GCGC CTTATA.CAT TTGGTTC...
       C. plumosus  .TCATTGACA TGTTGAACGC ATATT.GCGC CTTATA.CAT TTGGTTC...
     C. nuditarsis  .TCATTGACA TGTTGAACGC ATATT.GCGC CTTATA.CAT TTGGTTC...
  C. pseudothummi   .TCATTGACA TGTTGAACGC ATATT.GCGC CTTATA.CAT TTGGTTC...
       C. luridus   .TCATTGACA TGTTGAACGC ATATT.GCGC CTTATA.CAT TTGGTTC...
     C. aprilinus   .TCATTGACA TGTTGAACGC ATATT.GCGC CTTATA.CAT TTGGTTC...
       G. salinus   .TCATTGACA TGTTGAACGC ATATT.GCGC CTTATA.CAT TTGGTTC...
     G. barbipes    .TCATTGACA TGTTGAACGC ATATT.GCGC CTTATA.CAT TTGGTTC...
       G. pallens   .TCATTGACA TGTTGAACGC ATATT.GCGC CTTTTA.CAT TTGGTTC...
       D. fumidus   .TCATTGACA CGTTGAACGC ATATT.GCGC CTTTATACAT TTGGTTC...
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                        801                                                 850
      T. fuscicorne  ACCTGAGCAT ATGGTGC...  .GTGTTGTCG ......GATC AGTA......
        T. jucundum  ACA.. TAAT GTTTTCGTAA  GTTATTAGGA ......GGGC TGTA......
            T. xena  CATTTATAGC ACTAACATG.  TTTTTTCACA ......GAAC TGTA......
         P. aviceps  TCTGGCATCC AT.CTCG...  .TGGGAGTGG .....GGAAC TGTA......
      R. demeijerei  TCTCT.TAAT TTACATGA..  ..TTATGCAT .....GGGAC TGTA......
             X. par  TGTGACTCTA .AACAAG...  ......TTGT ...TAGGAAC TGTA......
        C. tentans   TCTTTATAAT ATACACAAAA  TTTATAATGT .....GGAAC TGTA......
C. pallidivittatus   TCTTTATAAT ATACACAAAA  TTTATAATGT .....GGAAC TGTA......
          C. duplex  TCTTTATAAT TAACACAAT.  TTTATAATGT .....GGAAC TGTA......
   C. thummi piger   TCTTTATAAT GTACACAAAA  ATTTTTATAA ..TGTGGGAC TGTA......
      C. cingulatus  TCTTTATAAT GT.CACA...  TTTATAATGT .....GGAAC TGTA......
       C. melanotus  TCTTTATAAT GTACACA...  TTTATAATGT .....GGAAC TGTA......
       C. plumosus   TCTTTATAAT GTACAC....  TTTATAATGT .....GGGAC TGTA......
      C. nuditarsis  TCTTTATAAT GTACACAC..  TTTATAATGT .....GGGAC TGTA......
C. pseudothummi      TCTTTATAAT GTACACAATA  TTTATAATGT .....GGGAC TGTA......
        C. luridus   TCTTTATAAT GTACACAATT  TATTTATAAT ...GTGGGAC TGTA......
      C. aprilinus   TCTTTATAAT GTACACACAA  TAATATTTAT AATGTGGGAC TGTA......
        G. salinus   TCTTTAAAAG GAA.......  .......... .......AAC TGTA......
      G. barbipes    TCTTTAAAAG GAA.......  .......... .......AAC TGTA......
        G. pallens   TCTTTAAAAG GAA.......  .......... .......AAC TGTA......
        D. fumidus   TC......TC GTT.......  .......... .....GGAAC TGTA......
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                      851                                                 900
     T. fuscicorne    TGGGGAGCCT GATGGTTCAG TGCCGTAATT TCGACCGAT. .CCTCAGTAA
       T. jucundum    TAAGGAACAT .ATGGTGGGG TGTGGTAACT TCATTCAA.. .CTTCAATAA
           T. xena    TAAGGTACAT .AGGGTTGAG GGTCGTAATT TCAAT.GCA. .AAGGAACTA
      P. aviceps      CAAGGGTACA TATGGT.GAG TGTCGTAATT TCATTCACTT CAACCTGTAA
     R. demeijerei    TAAGG.TACA TATGGTTGAG TGTCGTGATT TCTTACAATT TCAATTACAA
           X. par     TAAGCCACAT ATGGTTGAGG TGTCGTAATT TCATTGAATT TGAATTATAA
       C. tentans     TAAGGTACAT .ATGGTTGAG TGTCGTAATT TCATATGATT ACAACTATAA
C. pallidivittatus    TAAGGTACAT .ATGGTTGAG TGTCGTAATT TCATATGATT ACAACTATAA
         C. duplex    TAAGGTACAT .ATGGTTGAG TGTCGTAATT TCATATGATT ACAATTATAA
   C. thummi piger    TAAGGTACAT .ATGGTTGAG TGTCGTAATT TCATATGATT GCAACTATCA
     C. cingulatus    TAAGGTACAT .ATGGTTGAG TGTCGTAATT TCATATGATT AAAACTATCA
      C. melanotus    TAAGGTACAT .ATGGTTGAG TGTCGTAATT TCATATGATT AAAACTATAA
       C. plumosus    TAAGGTACAT .ATGGTTGAG TGTCGTAATT TCATATGATT TAAACTATAA
     C. nuditarsis    TAAGGTACAT .ATGGTTGAG TGTCGTAATT TCATATGATT AAAACTATAA
   C. pseudothummi    TAAGGTACAT .ATGGTTGAG TGTCGTAATT TCATATGATT ACAACTATCA
       C. luridus     TAAGGTACAT .ATGGTTGAG TGTCGTAATT TCATATGATT GCAACTACAA
     C. aprilinus     TAAGGTACAT .ATGGTTGAG TGTCGTAATT TCATATGATT GCAACTATCA
       G. salinus     TAAGGTACAT .ATGGTTGAG TGTCGTAATT TCATTAAATT TCAACTACAA
      G. barbipes     TAAGGTACAT .ATGGTTGAG TGTCGTAATT TCATTAAATT TCAACTACAA
       G. pallens     AAAGGTACAT .ATGGTTGAG TGTCGTAATT TCATTAAATT TCAACTACAA
       D. fumidus     TAAAGTACAT .ATGGTTGAG TGTCGTAATT TCATCAAATT TCAACTACAA
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                        901                                                 950
     T. fuscicorne      GACCCGACGG TTCAGGCTA. .......... ..CGGGCGTG CCATGCTGCC
       T. jucundum      TAAACAATGT TTCTATATA. .......... ...TGGCATA TAATATGGTC
           T. xena      TAAACAGGTT AACATTTAA. .......... ..CATGTAGA ATATAGTGTC
        P. aviceps      GCGAACGGGC ACGCGTTCG. .......... ..TTCGCACA ATAGAATGTC
     R. demeijerei      GTATCCC..C ACCCGT..G. .......... ..GATGCACA ATAAAATGTC
             X. par     GACATTAT.. TATTATGAG. .......... ..ATCCATGA TGATGATGTA
        C. tentans      GTATCTATCG CACACATAGT GTTGTT.  . ATAGTACATA ATAGAGTGTC
C. pallidivittatus      GTATCTATCG CACACATAGT GT.GTT . . ATAATACATA ATAGAGTGTC
          C. duplex     GTAGTGAGAT CTCTCTCTCT GTAGTATATC CCATTACACA ATAGAGTGTC
   C. thummi piger      GTATATAATA AAAATATATT .......... AT.ATGCATA ATAGAGTGTC
       C. cingulatus    GCGT.T.GTG TTGTTAAACA CACACAC... ACAGCGCATA ATAGAGTGTC
        C. melanotus    GCGT.T.GTT GTTTGTTACA CACACAC... ACAGCGCATA ATAGAGTGTC
         C. plumosus    GCGTGT.GTA CACTCACTTT TGTGTGT... ATAGCGCATA ATAGAGTGTC
       C. nuditarsis    GCGTTT.TTA TGTGTACACA CGT....... ATAGCGCATA ATAGAGTGTC
   C. pseudothummi      GTATTGTATG TGTCTACACA TAC....... ACAGTACATA ATAGAGTGTC
         C. luridus     GTATTGTGT. ....TACAC. .......... ACAGTACACA ATAGAGTGTC
       C. aprilinus     GTATTGTG.. ......CACA C......... ACAGTACACA ATAGAGTGTC
         G. salinus     GTATCATTTG ATATATAT.. .......... ..GATACACA ATAGAATGTC
       G. barbipes      GTATCATTTG ATATAT.... .......... ..GATACACA ATAGAATGTC
         G. pallens     GTATTGAACG CCATTGTG.. .......... ..TGTGTGTG TGTGTGTTTG
         D. fumidus     GTGTGCGACG TTCCAGTCA. .......... ..CGCGCAAA ATATAGTGTC
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                        951                                                1000
     T. fuscicorne      ATACCAGCCT TCCAGCG.GG CGCA..... .......... ..GTATGGAT
       T. jucundum      ATAAGAATAT TCGTGGG.GA ATGTA..... .......... ..TTATGAAT
           T. xena      ATTAAAGATT ACTTTCCTTT ATCGGA.... .......... .TAAGTAAAT
         P. aviceps     ATCAAAGC.A CCGCTCTCTC GTCACGAGC. .......... TGGCCGGTAT
       R. demeijerei    ATTAAAGCTA TTGCGTGTAT GCATATATGC .......ATG CCTCAATAA
            X. par      TAACTGAATG CCATTAAAGC CTATCCACTT .......GTT GAGTATAGAT
         C. tentans     ATCAAAGCCG TCTCACCTCA AAGATTGATT TCTGCGCG.. GTGTGACGAT
  C. pallidivittatus    ATCAAAGCCG TCTCGCCTCA AAGATTGATT TCTGCGCGGT GTGTGACGAT
          C. duplex     ATCAAAGCCG CCGTCCGCGT ATGTG.GAT. .......... ...GGGCGAT
     C. thummi piger    ATCAAAGCCG TCGTCTTACC .......... .......... ..GCGACGAT
       C. cingulatus    ATTAAAGCCG TCGCTGCTGC TACCTAGTAG .......... TGGTGACGAT
        C. melanotus    ATTAAAGCCG TCGCTGCTAC TTAGTAG... .......... TGGTGATGAT
         C. plumosus    ATTAAAGCCG TCTCTCCATT GCTACTTGTA ..GCAGTGTG TTGTGATGAT
       C. nuditarsis    TTTAAAGCCA TCTCGTTGCT GCTACTTGTA .GTGGTGGTG GTGTGATGAT
     C. pseudothummi    ATTAAAGCTG TCGAGCATCA TATTCTCGTA ......TGTG .CGTGACAAT
         C. luridus     ATCAAAGCCG TCGTCTCACA .......... .......... ..GCGACGAT
       C. aprilinus     ATCAAAGCCG TCACACCAAG .......... .......... .TGCGACGAT
         G. salinus     ATTAAAGCTA TCCTCTCATA TATGTATATA .......... TGATGATAAT
        G. barbipes     ATTAAAGCTA TCCTCTCATA TATACAATAT .......... .GATGATAAT
         G. pallens     GTACAAAATA GAGTGTCAGT AAAGCTATCA ......ATTG TGATGATAAT
         D. fumidus     ATTAAAGATG TCTCCTCTGA .......... .......... ...TGGCAAT
```

```
                      1001                                                1050
T. fuscicorne         TTATGACTAA AATGCTTAT. T.AA.TGTCC GTTTA.ACGC CACCATTTC.
T. jucundum           TTATGACTAA AATTCTAAA. T.AA.TGTCA GTTTA.ACGC CTTTATATT.
T. xena               TTAGGACTAA GATACTAAT. T.AAATGCCA GTTTG.TCGC CAATCTTAT.
P. aviceps            TTATGACTAA AATTCTGAT. T.AAATGTCA GTTTA.CCGT CTGGATAAG.
R. demeijerei         TTATGACTAA AATTCTAAAG TCAAATGTCA GTTTA.TTGC CTTGATATA.
X. par                TTATGGCTAA GGTTCTTTA. ....TTGTCA GTTTG.TCGC CTCATATTC.
C. tentans            TTATGACTAA AATTCTAATC T.AA.TGTCA GTTT..ACGC CTATTTTT..
C. pallidivittatus    TTATGACTAA AATCCTAATC T.AA.TGTCA GTTT..ACGC CTATTTTT..
C. duplex             TTATGACTAA AATGCTAATC T.AAATGTCA GTTT..ACGC CTATTTTT..
C. thummi piger       TTATGACTAA AATGCTAATC T.AA.TGTCA GTTAC.ACGC CTATTTTT..
C. cingulatus         TTATGACTAA AATGCTAATC T.AA.TGTCA GTTT..ACGC CTATTTTT..
C. melanotus          TTATGACTAA AATGCTAATC T.AA.TGTCA GTTT..ACGC CTATTTTT..
C. plumosus           TTATGACTAA AATGCTAATC T.AA.TGTCA GTTT..ACGC CTATTTTT..
C. nuditarsis         TTATGACTAA AATGCTAATC T.GA.TGTCA GTTT..ACGC CTATTTTT..
C. pseudothummi       TTATGACTAA AATGCTAATC T.AA.TGTCA GTTT..ACGC CTATTTTT..
C. luridus            TTATGACTAA AATGCTAATC T.AAATGTCA GTTAC.ACGC CTATTTTT..
C. aprilinus          TTATGACTAA AATGCTAATC T.AA.TGTCA GTTTATACGC CTATTTTATC
G. salinus            ATATGACTAA AATTCTGAT. T.AA.TGTCA GTTT..ACGC CACTTTTTCT
G. barbipes           TTATGACTAA AATTCTGAT. T.AA.TGTCA GTTT..ACGC CACTTATTCT
G. pallens            TTATGACTAA AATTCTGAT. T.AA.TGTCA GTTT..ACGC CACTT....T
D. fumidus            TTATGACTAA AATTCTGAG. T.TA.TGTCA GTTT..ACGC CTTTAT....
```

```
                      1051                                                1100
T. fuscicorne  .......... .GGGAGCGGC ACGGAGAAAA GGCTCCTAAC AGCT......
T. jucundum    .......... .GGAAGGGAA AGGAAGAAAT GTGTACGAAC AGACA.....
T. xena        .......... .GATAGATAC T...ATATAT G.CTATAAAT TCATT.....
P. aviceps     .......... ACGCGATGAA CACACGCTCG TGTACGTATC GTCG......
R. demeijerei  .......... TGAATAATGT GTTATATAAA GGACCTGATT TTCT......
X. par         .......... .GTTTGATTC ATTCATTTAT GAAAGGAAAA AAAGA.....
C. tentans     AAATAAAT.. .GGGGGG..A AGAGTGAAAA AT.TCAAAAT TCG.......
C. pallidivittatus AAATAAAT.. .GGGGGG..A AGAGTGAAAA AT.TCAAAAT TCG.......
C. duplex      AAATAAAT.. .GGGGGAGAA AGAGTGAAAA CT.TCAAAAT TCG.......
C. thummi piger AAATAAAT.. .GGGGGG..A AGAGTGAAAA CT.TCAAAAA ATTCGAGCGC
C. cingulatus  AAGTAAAT.. .GGGGGG..A AGAGTGAAAA AAATCAAAAT TCG....CAC
C. melanotus   AAGTAAAT.. .GGGGGG..A AGAGTGAAAA AA.TAAAAAT TCG....CAC
C. plumosus    AAGTAAAT.. .GGGGGG..A AGAGTGAAAA AAATCAAAAT TCGTA..CAC
C. nuditarsis  AAGTAAAT.. .GGGGGG..A AGAGTGAAAA AA.TCAAAAT TCG....CAC
C. pseudothummi AAATAAAT.. .GGGGGG..A AGAGTGAAAA CT.TCAAAAT TCGCG....C
C. luridus     AAATAAAT.. .GGGGGG..G AGAGTGAAAA GT.TCAAAAT TCGGG....C
C. aprilinus   AAATAAAT.. .GGGGGG..A AGAGTGAAAA CT.TCATTAT TCGCGTGCAC
G. salinus     TGCTCTCTCT TAACTGATTG AGTGAGATAG GAGGGAAGAA TATGA.....
G. barbipes    TGCTCTCTCT TAACCGATTG AGTGAGAAAG GAGGGAAGAA TATGA.....
G. pallens     TACTCTC... .AAGTGTGTG AGAGAGAATG GAGGGAAGAA TATGG.....
D. fumidus     .AATGAA... .GGGAGG... AATCTGAAAA GGTTCAATTC ATTCA.....
```

```
                    1101                                                1150
T. fuscicorne       .......... ..CTCTCTT. TTGTTTGG.. .......... ..........
T. jucundum         .......... .......... ....CTTATT TTT.TAGATT .TTGGG....
T. xena             .......... .......... ........TA TTCATAGGAA TATTTATG..
P. aviceps          .......... .......... ........CT GTC....... ...TCTCTCC
R. demeijerei       .......... .TTATAT... .......... ........AA AGTTATTTT.
X. par              .......... .......... .......... ....... .. ..........
C. tentans          ........CA CATATATGTG ATG.AATCTT GTGA.GTCT. ATTC.TCTCT
C. pallidivittatus  ........CA CATATATGTG ATG.AATCTT GTGA.GTCT. ATTC.TCTCT
C. duplex           ........CT CGCATGTACT ATATGTATGT GTGATATG.. AGTC.TCTCT
C. thummi piger     GCACTGTGCA CGAGTCTCTT GTG.AGTATT TTCATTGAAA ATTC.TCTCT
C. cingulatus       ATTCACGTGA TGAATATTGA GTG.TTTCTT TTCATTGAAA AGTCCTCTCT
C. melanotus        ATACACGTGA TGAATATTGA GTG.TTTCTT TTCATTGAAA AGTCCTCTCT
C. plumosus         ATACATGTGA TGAATATATT GAGTTTTCTT TTCATTGAAA AGTCCTCTCT
C. nuditarsis       ATACATGTGA TGAATACATT GAGCTTTCTT TTCATTGAAA AGTCCTCTCT
C. pseudothummi     ACACACTGCA CGAGTCTTGT GAT...TATT TTCATTTGAA AATTCTCTCT
C. luridus          ACACACTGCA CGAGTCTTGT GAG...TATT TTCATTGAA. AATTCTCTCT
C. aprilinus        ACACACTGCA CGAGTCTCGT GAG..TAATT TTCATTGAA. AATTCTCTCT
G. salinus          .......AAA TGAGTTCATA AT...TCGTT TTCAATAGA. AATTCTCTTT
G. barbipes         .......AAA TGAGTTCATA AT...TCGTT TTCAATAGA. AATTCTCTTT
G. pallens          .......AAA TGAGTTCATA AT...TCGTT TTCAATAGA. AATTTCTCTT
D. fumidus          .......... .......... .........  CACATGATGA ATATCTCTTT
```

```
                      1151                                                1200
   T. fuscicorne      GGCGCTATCT CTACA..... ..........  .......... ....CTCTAA
     T. jucundum      GGCGCTAACT CTACG..... ..........  .......... ...CAG.TAT
         T. xena      GGCGTCAACT ATACG..... ..........  .......... ....TATTAA
      P. aviceps      GACGCTAACT TTACA..... ..........  .......... ..TCA..CGC
    R. demeijerei     GGCGCTAACT TTACA..... ..........  .......... ..GAC..CTC
           X. par     .GCGCTAACT TTACG.... ..........  .......... ...ATTTTTAC
      C. tentans      GGCGCTAACT TTACA..... ..........  .......... .TATATATAT
C. pallidivittatus    GGCGCTAACT TTACA..... ..........  .......... .TATATATAT
        C. duplex     GGCGCTAACT TTACA..... ..........  .......... ...CGTT...
   C. thummi piger    GGCGCTAACT TTACA..... ..........  .......... .TGATTTAAT
     C. cingulatus    GGCGCTAACT TTACAGACGC GCGCTTACAC  ACACTTGTGT GTGTGTATAT
      C. melanotus    GGCGCTAACT TTACAGTCAC GC..TTAC..  ACACTTGTGT GTGTTTGCAT
       C. plumosus    GGCGCTAACT TTACAAAAA. .....TATAT  ACCTTTGTGT GT..ATATAT
     C. nuditarsis    GGCGCTAACT TTACAAAAA. .....TATAT  ACCTTCGTGT GTATATGTTA
   C. pseudothummi    GGCGCTAACT TTACA..... ..........  .......... .......TA.
       C. luridus     GGCGCTAACT TTACA..... ..........  .......... .......TAA
     C. aprilinus     GGCGCTAACT TTACA..... ..........  .......... .TACATACAC
       G. salinus     GGCGCTAACT TTACA..... ..........  .......... ..ACATTCAT
      G. barbipes     GGCGCTAACT TTACA..... ..........  .......... ..ACATTTAT
       G. pallens     GGCGCTAACT TTACA..... ..........  .......... ....TGTAAA
       D. fumidus     GGCGCCAACT TTACA..... ..........  .......... ...TATGCA.
```

```
                        1201                                                1250
     T. fuscicorne      TTTGAT.GT. .GTTGTGATG .......... TCGTCCCATG CGAGGAG...
       T. jucundum      TTGTAT.GT. TGGTTGAATG .......... TCGTCAAAGT TTCTTCATAT
           T. xena      TTTATG..TT AGTTTGGATG TTATAATATA TCCAGAATAA TGATGAA...
        P. aviceps      ATGTA...AC GGTTTGGTTG TCACGGTGAG AGCAGCAG.. ..........
     R. demeijerei      GTGTATATAA TGTTTAGTTG TCACAAATAA TGGAGAA... .AATCAC...
            X. par      AATTTATTAA AGTTTAGTTG TATCTTTTA. .......... .TCTAAT...
        C. tentans      A....ATGTC TCGTTAGTTG CTCCT..GAT TTATCCGCAT GTGAATAAC.
  C. pallidivittatus    ATATAATGTC TCGTTAGTTG CTCCT..GAT TTATCCGCAT GTGAATAAC.
          C. duplex     .....GTGTC TCGTTAGTTG CTCCT..GAC TCGTTGACGT TGATTTTGA.
    C. thummi piger     TTT..GTGTC TCGTTGGTTG CTCCCTGGAC TCGTTGGTGT TTGCAATTC.
      C. cingulatus     GCATGGTATG TTGTTAGTTG CACTTGATTC ATCACAATA. .ACTGTTT..
       C. melanotus     G..TGGTATG TTGTTAGTTG CACTTGATTC ATCACAAAAC TACTGTAT..
        C. plumosus     GTATATGTCA T.GTTAGTTG CAGCTTATTC AGCACGAAA. TACTGTGTGT
      C. nuditarsis     GTAATATATA T.GTTAGTTG CAGGTTATTC AGCACGAAA. AACTGTGTAT
    C. pseudothummi     ..TATGTGTC TCGTTAGTTG CTCCTGATTC TCGTTGT... ..TGCT....
        C. luridus      ..TTTGTGTG TCGTTAGTTG CTCCTGATTC ..GTTGT... ..TGCT....
      C. aprilinus      ATTATATGTC TCGTTGGTTG CTTCCGATTC TCATTGTGCT TGTGTT....
        G. salinus      AT.GTGTATT ATGTTAGTTG CCGATAAAAA ATTTCATTAT TGATATAAC.
      G. barbipes       ATTGTGTATT ATGTTAGTTG CCGATAAAAA ATTTCATTAT TGATATAAC.
        G. pallens      CATATGTATG GTGCTAGTTG CCGAAATAAA ATTTCATTAT TGATATGGC.
        D. fumidus      ....TGTATA TAATTGGTTG CCAAAAAATT CGTCGCTAAA ATGTGTGT..
```

```
                          1251                                                1300
      T. fuscicorne  ......GTTA AGGAGACTT. ........CT CTCTTTTCTT ..TAATGTAG
        T. jucundum  ......GTTA TA.AGACAT. .......CAT TCTTTTTTTC ..AAAT..AG
            T. xena  TTATTGAAAG AGTATATATA .GAG...... ........CC ACC..AGTAG
         P. aviceps  ......CAAC ACTATAC... ........GC CGCTGT.GTG .ATTTAGTCT
       R. demeijerei ......CAAT ATAATAC... ......TAAT ..ATATTGTA TAGAAGGAAA
             X. par  ......AAAT GTATGG.... AGAGAATAAT AGC....... AAGAATATT.
        C. tentans   ......GATT TTGAGAT... ......AAAT .CATTCTTTC ..AAATGT..
C. pallidivittatus   ......GATT TTGAAAT... .....AAAAT  CATTCTTTC ..AAATGT..
          C. duplex  ......GATA GTAAAAA... .......GTA GTTCTTTCTC TAATGTTTAT
    C. thummi piger  ......GATT TTGAGAACAA CAAAAGAGTA GTTCTTCCTA ATGTGTGTAT
      C. cingulatus  ......GTGA GTAACGATTT TGAGAAAAAT TCATACTTTC ..TAATGT..
       C. melanotus  ......GTGA GTAACGATTT TGAGAAAAAG TCATTCTTTC ..TAATGT..
        C. plumosus  ATATGTGTGG GTAACGATTT TGAGAAAAAG AGTCATTCTT TCTAATGT..
      C. nuditarsis  ......GTGG GCAACGATTT TGAGAAAAAG ..TCATTCTT TCTAATGT..
   C. pseudothummi   ......GTAC GATTTTGTA. ....AGAAAA AGTAATTCTT TCTAATGTA.
        C. luridus   ......GTAC GCGATTTTG. ....AGAAAC AGTAGTTCTT TCCAATGTGT
      C. aprilinus   ......GTCC ACGGATTTTG GAGTAGAAAA AGTAATTCTT TCTAATGTGT
        G. salinus   ......GATT TTGGACATT. ....AAAAAA ATGTATTCTT ..TAATGTA.
       G. barbipes   ......GATT TTGGACATT. ....GAAAA. .TATATTCTT ..TAATGTA.
        G. pallens   ......GATT TTGAACAAA. ....AAA... ...TATTCTT TAATGTGTA.
        D. fumidus   ......GAGT GTGATAGAN. .GCCGAAGTA GAGTTTCTCA TAAGTAGT..
```

```
                    1301                                                1350
T. fuscicorne       ATACTGACGC A.AATTTTAT GTGAGTATAT ATATAATAT. ..........
T. jucundum         CTT.TGAAGC A.AAAA..CA GAGCTGATGA AAA...GTGT ..........
T. xena             CAAGAAAAAA C.TTCGTAAT CATCATATCT ...TGTGTGT GA........
P. aviceps          .AAACTGTGG .TATAGTAAT .....GGCGG AGTTAATAT. ..........
R. demeijerei       GACTGTATGA TGTAGA.TGT AAACGAAGTC ATAGC..... ..........
X. par              GATTGACAGC AGAAAAAACA AAACAGAAGT AATATAT... ..........
C. tentans          ..ACTACTGA AGTAAAAAAG .TAAAAAAAA AAAAAA.... GACAA ....
C. pallidivittatus  ..ACTACTGA AGTAAAGAAG .TAAAAAAAA AAA.......  GACAA ....
C. duplex           ..AATACTGA AGTAATTTTT .GATATACAT ATTAAA.... GACAA ....
C. thummi piger     CCAATACTGA AGTAAATATT ATATATATAT ATATATATGT GTATATAAGA
C. cingulatus       GTAATACTGA AG.....TGT ATAAATGGA. ATATAATAGA GAGA...CGA
C. melanotus        ..ACTACTGA .......GGT ATAAATGG.. ATATAATATA GAGAGA.CGA
C. plumosus         ..ACTACTGA GGATATATGA ATATATGAAT ATATAATATA GAGAG..CGA
C. nuditarsis       ..ACTACTGA AGGTGTAT.A TTACATGGAT ATGTAATAGA GAGAGAGAGA
C. pseudothummi     .CCTA.CTGA AGTAAATAAA AGATAAAAAA AAAATTAA.. GACAA.....
C. luridus          TCTAG.CTGA AGTAAAAAAA AAATAAGAAA AAATTTAA.. GACGA.....
C. aprilinus        ACCCGACTGA AGTGTA...G TAATAGAAGA AAAAAAAA.. GACGA.....
G. salinus          ..AATTGTAT CACATATAT. ATAAATATAA ACGAGAAAA. GAAAT.....
G. barbipes         ..AATTGTAT CAAACATATT ATAATAATAA ACGAGAAAA. GAAAT.....
G. pallens          ..AATTGTGT CA........ ........AA AAGCAAAAG. GAAAT.....
D. fumidus          ACTGTAGCGA GTTATGATAA TAATAGAAAA AAAAAATA.. GA........
```

```
                    1351                                                1400
T. fuscicorne       .......... ......TACG CGACC.TCAA CTCATGTGTG ACTACCCCCT
T. jucundum         .......... ......TACG CGACC.TCAA CTCATGTGTG ACTACCCCCC
T. xena             .......... ......TGAG CGACC.TCAA CTCATGTGTG ACTACCCCCT
P. aviceps          .......... ......CACG CGACC.TCAA CTCATGTGTG ACTACCCCCC
R. demeijerei       .......... ......TTCG CGACC.TCAA CTCATGTGTG ACTACCCCC.
X. par              .......... ......TAAG CGACCCTCAA CTCATGTGTG ACTACCCCCC
C. tentans          .......... .....TTTCG CGACC.TCAA CTCATGTGAG ACTACCCCCT
C. pallidivittatus  .......... .....TTTCG CGACC.TCAA CTCATGTGAG ACTACCCCCT
C. duplex           .......... .....TTTCG CGACC.TCAA CTCATGTGTG ACTACCCCCT
C. thummi piger     TGAAAGACGA CGACAATTCG CGACC.TCAA CTCATGTGTG ACTACCCCCT
C. cingulatus       .......... .....TTTCG CGACC.TCAA CTCATGTGTG ACTACCCCCT
C. melanotus        .......... .....TTTCG CGACC.TCAA CTCATGTGTG ACTACCCCCT
C. plumosus         .......... .....TTTCG CGACC.TCAA CTCATGTGTG ACTACCCCCT
C. nuditarsis       .......... .....TTTCG CGACC.TCAA CTCATGTGTG ACTACCCCCT
C. pseudothummi     .......... ......TTCG CGACC.TCAA CTCATGTGTG ACTACCCCCT
C. luridus          .......... ....TTCTCG CGACC.TCAA CTCATGTGTG ACTACCCCCT
C. aprilinus        .......... ......TTCG CGACC.TCAA CTCATGTGTG ACTACCCCCT
G. salinus          .......... .....TTTCG CGACC.TCAA CTCATGTGTG ACTACCCCCT
G. barbipes         .......... .....TTTCG CGACC.TCAA CTCATGTGTG ACTACCCCCT
G. pallens          .......... ....TTATCG CGACC.TCAA CTCATGTGTG ACTACCCCCT
D. fumidus          .......... ......ATCG CGACC.TCAA CTCATGTGTG ACTACCCCCT
```

```
                    1401                                          1480
      T. fuscicorne GATCTTA... ......... ......... .........
        T. jucundum TGATT..... ......... ......... .........
            T. xena GAATT..... ......... ......... .........
          P. aviceps TGATT..... ......... ......... .........
       R. demeijerei TGATT..... ......... ......... .........
             X. par TGATT..... ......... ......... .........
          C. tentans GAATTTAAGC ATATTAATTA GGGGAGGAAA AGAAACCAAC
C. pallidivittatus GAAT..... ......... ......... .........
         C. duplex GAATT..... ......... ......... .........
    C. thummi piger GAAT..... ......... ......... .........
      C. cingulatus GAAT..... ......... ......... .........
       C. melanotus GAATT..... ......... ......... .........
        C. plumosus GAAT..... ......... ......... .........
      C. nuditarsis GAAT..... ......... ......... .........
    C. pseudothummi GAATT..... ......... ......... .........
        C. luridus GAATT..... ......... ......... .........
       C. aprilinus GAATT..... ......... ......... .........
         G. salinus GAATT..... ......... ......... .........
        G. barbipes GAATT..... ......... ......... .........
         G. pallens AAATTT.... ......... ......... .........
         D. fumidus GAATT..... ......... ......... .........
```

Appendix C1 – Sequence alignment of ITS-1 region of two closely related Chironomid species.

```
                        1                                                  50
C. pallidivittatus      ATGTATGTTT TGCACACGCA TTTATGCTCT TTCATCTTGT TTTT--ATGG
C. tentans              .......... .......... .......... .......... ....TT....

                        51                                                 100
C. pallidivittatus      GGTGAGAATT ATTAATTAAA ATCCTAGGTA CTAGAATTGC GATATGTGTG
C. tentans              .......... .......... .......... .......... ..........

                        101                                                150
C. pallidivittatus      CGATTAATGT CGTACACATG TTGTTGGTTT TATAAAGGGC TTCGCCTAGG
C. tentans              .......... .......... .......... .......... ..........
```

```
                    151                                                 200
C. pallidivittatus  TATATTTTAC TTTTTATGCC AAAAAAAAAC ATAAAAAAAA AAATAAAATT
       C. tentans   .......... .......... ......---. .......... .--.......

                    201                                                 250
C. pallidivittatus  GTCGTTGTGA TTATAACAAA CAGTTTTTTC GATAAGAAAA AATGAATAAA
       C. tentans   .......... ......T... .......... .......... ..........

                   251
C. pallidivittatus  CAAAAACTT
       C. tentans   .........
```

Appendix C2 – Sequence alignment of ITS-2 region of two closely related ***Chironomidae*** species.

```
                        1                                                  50
C. pallidivittatus      ATTTCATATG ATTACAACTA TAAGTATCTA TCGCACACAT AGTGT-GTTA
C. tentans              .......... .......... .......... .......... .....T....
                        51                                                 100
C. pallidivittatus      TAATACATAA TAGAGTGTCA TCAAAGCCGT CTCGCCTCAA AGATTGATTT
C. tentans              ..G....... .......... .......... ...A...... ..........
                        101                                                150
C. pallidivittatus      CTGCGCGGTG TGTGACGATT TATGACTAAA ATCCTAATCT AATGTCAGTT
C. tentans              .......... ..--...... .......... ..T....... ..........
                        151                                                200
C. pallidivittatus      TACGCCTATT TTTAAATAAA TGGGGGGAAG AGTGAAAAAT TCAAAATTCG
C. tentans              .......... .......... .......... .......... ..........
```

```
                    201                                                 250
C. pallidivittatus  CACATATATG TGATGAATCT TGTGAGTCTA TTCTCTCTGG CGCTAACTTT
       C. tentans   .......... .......... .......... .......... ..........
                    251                                                 300
C. pallidivittatus  ACATATATAT ATATATAATG TCTCGTTAGT TGCTCCTGAT TTATCCGCAT
       C. tentans   .......... ...----... .......... .......... ..........
                    301                                                 350
C. pallidivittatus  GTGAATAACG ATTTTGAAAT AAAATCATTC TTTCAAATGT ACTACTGAAG
       C. tentans   .......... .......G.. .......... .......... ..........
                         351                    376
C. pallidivittatus  TAAAGAAGTA AAAAAAAAAA GACA
       C. tentans   ....A..... .......... A.AGAC
```

Appendix C3. Sequence alignment of two closely related Chironomid species. 18S subunit spans from 1-107; ITS-1 region spans from bp 108-364; 5.8 region spans from bp 365-487; ITS-2 region spans from 560-932; and 28S region spans from bp 933-978.

```
                        1                                                  50
C. pallidivittatus      GCCTCGGTAT CGCGATTGCT TTTGCCAAAG TTGATCAAAC TTGATGATTT
C. tentans               ......... ......... ......... ......... .........
                        51                                                 100
C. pallidivittatus      GGAGGAAATA AAAGTCGTAA CAAGGTTTCC GTAGGTGAAC CTGCGGAAGG
C. tentans              ......... ......... ......... ......... .......-..
                        101                                                150
C. pallidivittatus      ATCATTAATG TATGTTTTGC ACACGCATTT ATGCTCTTTC ATCTTGTTTT
C. tentans              ......... ......... ......... ......... .........
                        151                                                200
C. pallidivittatus      T--ATGGGGT GAGAATTATT AATTAAAATC CTAGGTACTA GAATTGCGAT
C. tentans              .TT..... ......... ......... ......... .........
```

```
                    201                                                 250
C. pallidivittatus  ATGTGTGCGA TTAATGTCGT ACACATGTTG TTGGTTTTAT AAAGGGCTTC
      C. tentans    .......... .......... .......... .......... ..........
                    251                                                 300
C. pallidivittatus  GCCTAGGTAT ATTTTACTTT TTATGCCAAA AAAAAACATA AAAAAAAAAA
      C. tentans    .......... .......... .......... ...---.... ........--
                    301                                                 350
C. pallidivittatus  TAAAATTGTC GTTGTGATTA TAACAAACAG TTTTTTCGAT AAGAAAAAAT
      C. tentans    .......... .......... ...T...... .......... ..........
                    351                                                 400
C. pallidivittatus  GAATAAACAA AAACTTAACC CTAGACAGGG GATCACTTGG CTCATGGGTC
      C. tentans    .......... .......... .......... .......... ..........
                    401                                                 450
C. pallidivittatus  GATGAAAACC GCAGCAAACT GCGCGTCGCC ATGTGAACTG CAGGACACAT
      C. tentans    ......G... .......... .......... .......... ..........
```

```
                    451                                                 500
C. pallidivittatus  GATCATTGAC ATGTTGAACG CATATTGCGC CTTATACATT TGGTTCTCTT
      C. tentans    .......... .......... .......... .......... ..........
                    501                                                 550
C. pallidivittatus  TATAATATAC ACAAAATTTA TAATGTGGAA CTGTATAAGG TACATATGGT
      C. tentans    .......... .......... .......... .......... ..........
                    551                                                 600
C. pallidivittatus  TGAGTGTCGT AATTTCATAT GATTACAACT ATAAGTATCT ATCGCACACA
      C. tentans    .......... .......... .......... .......... ..........

                    601                                                 650
C. pallidivittatus  TAGTGT-GTT ATAATACATA ATAGAGTGTC ATCAAAGCCG TCTCGCCTCA
      C. tentans    ......T... ...G...... .......... .......... ....A.....
```

```
                    651                                                 700
C. pallidivittatus  AAGATTGATT TCTGCGCGGT GTGTGACGAT TTATGACTAA AATCCTAATC
      C. tentans    .......... .......... ...--..... .......... ...T......
                    701                                                 750
C. pallidivittatus  TAATGTCAGT TTACGCCTAT TTTTAAATAA ATGGGGGGAA GAGTGAAAAA
      C. tentans    .......... .......... .......... .......... ..........

                    751                                                 800
C. pallidivittatus  TTCAAAATTC GCACATATAT GTGATGAATC TTGTGAGTCT ATTCTCTCTG
      C. tentans    .......... .......... .......... .......... ..........

                    801                                                 850
C. pallidivittatus  GCGCTAACTT TACATATATA TATATATAAT GTCTCGTTAG TTGCTCCTGA
      C. tentans    .......... .......... ....----.. .......... ..........
```

```
                    851                                                900
C. pallidivittatus  TTTATCCGCA TGTGAATAAC GATTTTGAAA TAAAATCATT CTTTCAAATG
       C. tentans   .......... .......... .........G. .......... ..........

                    901                                                950
C. pallidivittatus  TACTACTGAA GTAAAGAAGT AAAAAAAAAA A---GACAAT TTCGCGACCT
       C. tentans   .......... .....A.... .......... .AAA...... ..........

                      951                          978
C. pallidivittatus  CAACTCATGT GAGACTACCC CCTGAAT
       C. tentans   .......... .......... .......T
```

Appendix D – Sequence alignment of two closely related Chironomid species. 18S subunit spans from 1-105; ITS-1 region spans from bp 106-337; 5.8 region spans from bp 338-460; ITS-2 region spans from 530-935; and 28S region spans from bp 936-980.

```
           1                                                   50
C.melanotus  CTCGGTATTG CGATTGCTTT TGCCAAAGTT GATCAAACTT GATGATTTGG
  C.thummi   ......G.CA .......... .......... .......... ..........

           51                                                 100
C.melanotus  AGGAAATAAA AGTCGTAACA AGGTTTCCGT AGGTGAACCT GCGGAAGGAT
  C.thummi   .......... .......... .......... .......... ..........

           101                                                150
C.melanotus  CATTAATGTA TGTT-TCGCA CAACATTTTA TGCTCTTTCA -----TCTTG
  C.thummi   .......... .A..A.AT.. T.CACA.... .......... CACTT.G...

           151                                                200
C.melanotus  TTGATGTGG- ---GGATAGA GAACAAATCC TAGGTACTAG AATTGCGATA
  C.thummi   ...T...T.T TGT..T.TAT ..CA...... .......... ........A.

           201                                                250
C.melanotus  TGTGTTGTGT TCACACGCAC ACATGTTGTT GGTTTTATAA AGGGCTTCGC
  C.thummi   C.C.CGCGCG ...TG..TGT GTG.A..... .......... ..........
```

```
            251                                                  300
C.melanotus  CTAGGTATAA ACTTACTCTT TCTTTTATGC TAAACACATA TTAGA-----
   C.thummi  ........--. ....G..TA. .T........ C......... A..ATAATAA

            301                                                  350
C.melanotus  ---------- -GACGTTGTG ATTGTATGGT TTATTATTTT TCTTAGTAAA
   C.thummi  TAATATTATA T......... ...TAT..-- .A...GA... ..GA.AG...

            351                                                  400
C.melanotus  AATAAACAAA ---------- CTTAACCCTA GACAGGGGAT CACTTGGCTC
   C.thummi  ..A....... AAAACTTAAA .......... .......... ..........

            401                                                  450
C.melanotus  ATGGGTCGAT GAAGACCGCA GCAAACTGCG CGTCGCCATG TGAACTGCAG
   C.thummi  .......... .......... .......... ......T... ..........

            451                                                  500
C.melanotus  GACACATGAT CATTGACATG TTGAACGCAT ATTGCGCCTT ATACATTTGG
   C.thummi  .......... .......... .......... .......... ..........

            501                                                  550
C.melanotus  TTCTCTTTAT AATGTACACA ------TTTA TAATGTGGAA CTGTATAAGG
   C.thummi  .......... .......... AAAATT.... ........G. ..........
```

```
           551                                                  600
C.melanotus  TACATATGGT TGAGTGTCGT AATTTCATAT GATTAAAACT ATAAGCGTTG
   C.thummi  .......... .......... .......... ....GC.... ..C..-----


           601                                                  650
C.melanotus  TTGTTTGTTA CACACACACA CAGCGCATAA TAGAGTGTCA TTAAAGCCGT
   C.thummi  -.A.A.AA.. A...T.T.TT ATAT...... .......... .C........

           651                                                  700
C.melanotus  CGCTGCTACT TAGTAGTGGT GATGATTTAT GACTAAAATG CTAATCTAAT
   C.thummi  ..-----T.. ..CC----.C ..C....... .......... ..........

           701                                                  750
C.melanotus  GTCAGTTT-A CGCCTATTTT TAAGTAAATG GGGGGAAGAG TGAAAAAAT-
   C.thummi  .......AC. .......... ...A...... .......... ......CT.C

           751                                                  800
C.melanotus  -AAAAATTCG CACATACAC- GTG-ATGAAT ATTGAGTGTT TCTTTTCATT
   C.thummi  A......... AG.GCG...T ...C.C..G. C.CTT...AG .A........

           801                                                  850
C.melanotus  GAAAAGTCCT CTCTGGCGCT AACTTTACA- -GTCACGCTT ACACACTTGT
   C.thummi  .....-.T.. .......... .G.......T GA.TTAAT.. TGTGT..C..

           851                                                  900
C.melanotus  GTGTGTTTGC ATGTGGTATG TTGTTAGTTG CACTTGATTC ATCACAAAAC
   C.thummi  TG..TGC.C. C..-.ACTC. ...G.GT... ..A..CGA.T T.G.--G...
```

```
           901                                                  950
C.melanotus  TACTGTATGT GAGTAACGAT TTTGAGAAAA AGTCATTCTT TCTAATGTAC
   C.thummi  A..AAA.--- .....GTTC. .CCT.ATGTG T..ATCCAA. A..G.A...A


           951                                                 1000
C.melanotus  TACTGAGGTA TAAATGGATA TAATATAGAG AGACGATTTC GCGACCTCAA
   C.thummi  ATA.T.TA.. ...GAT..A. G.CG.----- C...A...-. ..........

           1001                                                1050
C.melanotus  CTCATGTGTG ACTACCCCCT GAATTTAAGC ATATTAATTA GGGGAGGAAA
   C.thummi  .......... .......... .......... .......... ..........

           1051                                                1100
C.melanotus  AGAAACCAAC AGGGATTCCC TTAGTAGTGG CGAACGAAAC GGGATCAGCC
   C.thummi  .......... .......... .......... .......... ..........


           1101               1121
C.melanotus  CATCACGTAG GATCATAGGC T
   C.thummi  .......... .......... .
```

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