DISSERTATION

PHYLOGENY OF SELECTED SPECIES OF SUBGENUS CULEX MOSQUITOES (DIPTERA: CULICIDAE) FROM THE U.S., PUERTO RICO, AND GUATEMALA, BASED ON NUCLEOTIDE SEQUENCES FROM THREE GENES

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY STEPHEN DEUS ENTITLED PHYLOGENY OF SELECTED SPECIES OF SUBGENUS *CULEX* MOSQUITOES (DIPTERA: CULICIDAE) FROM THE U.S., PUERTO RICO, AND GUATEMALA, BASED ON NUCLEOTIDE SEQUENCES FROM THREE GENES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

PHYLOGENY OF SELECTED SPECIES OF SUBGENUS *CULEX* MOSQUITOES (DIPTERA: CULICIDAE) FROM THE U.S., PUERTO RICO, AND GUATEMALA, BASED ON NUCLEOTIDE SEQUENCES FROM THREE GENES

Phylogeny was inferred for subgenus *Culex* mosquitoes from the United States, Guatemala and Puerto Rico based on aligned nucleotide sequences from three genetic regions: 705 base pairs (bp) of the mitochondrial cytochrome oxidase subunit II (*COII*) gene, a region of ribosomal DNA containing internal transcribed spacers (*ITS*) 1 and 2, and 850 bp of the nuclear protein-coding *white* gene. Each gene was evaluated for its utility in phylogenetic inference, as were four methods of phylogenetic analysis: neighbor-joining distance, parsimony, maximum likelihood, and Bayesian analysis. A simultaneous analysis of the *COII* and *ITS* data was performed using the Bayesian method. The phylogeny challenges the monophyly of subgenus *Culex* and helps clarify the phylogenetic positions of subgenus *Phenacomyia* and genus *Deinocerites* relative to subgenus *Culex*.

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CHAPTER 1: Introduction

The research described here proposes a molecular phylogeny of *Culex* (*Culex*) mosquitoes (Diptera: Culicidae) collected from regions of North and Central America and the Caribbean. Characters used in the analysis were aligned nucleotide sequences from regions of three distinct genes. The three genes used were: (1) a cloned portion of nuclear ribosomal DNA (rDNA) of an average of 1,250 base pairs (bp) in length that includes the two internal transcribed spacer (*ITS*) regions and the region encoding the 5.8S ribosomal subunit, (2) an 850 bp region of the nuclear *white* gene, and (3) a 705 bp region of the mitochondrial cytochrome oxidase subunit II (*COII*) gene. The ingroup for phylogenetic analyses consisted of nucleotide sequences from 19 taxa from the mosquito subgenus *Culex*. In most analyses, the outgroup taxa consist of six species of the subgenus *Melanoconion*. Additional outgroup taxa were included in some analyses when relevant nucleotide sequence data was available from Genbank. The species *Culex lactator* Dyar and Knab 1906 is included as part of the ingroup despite its designation as a member of the subgenus *Phenacomyia* (Harbach and Peyton 1992) so that the validity of this subgeneric designation might be tested. The species *Deinocerites cancer* Theobald is also treated as an ingroup taxon despite its membership in the genus *Deinocerites* in order to test assertions that the genus *Deinocerites* belongs in a natural assemblage within the genus *Culex* (Mallampalli 1995; Navarro and Liria 2000). All specimens used are reared from field collected larvae unless otherwise noted. Phylogenetic analyses are performed using the PAUP* v4.0b10 (Swofford 2003), Mr.

Bayes v3.1.2 (Huelsenbeck *et al.* 2001, Ronquist and Huelsenbeck. 2003) and Modeltest v3.7 (Posada and Crandall 1998) software packages as described herein.

This dissertation is presented in two chapters. The present chapter serves as an introduction to concepts that are important in subsequent chapters, including a detailed history of systematics of the genus *Culex,* and provides an overview of the research presented herein. Chapter 2 presents the details of deducing a phylogeny of subgenus *Culex* using the *COII, ITS,* and *white* genes and proposes a phylogeny based on the combination of sequence data from the first two genes.

The study of phylogeny is the inference of the evolutionary history of any taxon of biological entities. Phylogenetic studies describe the hierarchical relationships among taxa and place them into an evolutionary context. Thus, any such study assumes that the diversity of life observed today falls into hierarchical categorizations and that this hierarchy can be explained by descent with modification. Such modification might arise through natural selection or through stochastic events. The concept of natural selection has been scrutinized as the field of biological study has grown and changed, allowing explanations of life's diversity to be formed. The New Synthesis, or Neo-Darwinian era of the early to mid twentieth century joined evolutionary theory with the freshly rediscovered work of Mendel and its suggestion of a heritable genetic mode of inheritance. Subsequently, the field of molecular biology provided the capability to study heritable molecules as characters for inferring the phylogeny of taxa. Barring some unforeseen discovery, it is believed that the most fundamental of these characters is the sequence of nucleotides in a specimen's DNA. Phylogenetic studies can impact the taxonomic classification of organisms, can inform our knowledge of common biological processes among related organisms, and can also help us to answer questions of phylogeography.

The present study is confined to a single subgenus of mosquitoes, subgenus *Culex*, collected within the United States, Puerto Rico, and Guatemala. A number of factors make subgenus *Culex* an appealing taxon for this study. Members of this subgenus are vectors of human filariasis (W.H.O. 1992) and arthropod-borne viruses (arboviruses) (Karabatsos 1985; Service 2001), including West Nile virus (Sardelis *et al.* 2001; Turell *et al.* 2005; Nasci *et al.* 2001) and St. Louis encephalitis virus (Mitchell *et al.* 1980). Recent studies bring into question certain issues that are relevant to the subgenus *Culex*, such as the phylogenetic position of subgenus *Culex* relative to the coastal crab-hole breeding genus *Deinocerites* (Mallampalli 1995; Navarro and Liria 2000) or to the subgenus *Phenacomyia* (Harbach and Peyton 1992). A few current studies exist that elucidate the internal phylogeny of subgenus *Culex* at the species level (Miller *et al.* 1996; Shepard *et al.* 2006), but most studies examined only a few species to represent the subgenus and tended to be concerned with determining deeper relationships (Navarro and Liria 2000; Miller *et al.* 1997; Besansky and Fahey 1997).

The geographical regions covered (North and Central America and the Caribbean) were appropriate for the study because they included tropical, subtropical, and temperate zones inhabited by subgenus *Culex*. The subgenus *Culex* is distributed worldwide, yet this study examines only species found within a portion of the total distribution of the subgenus. It is not determined whether species confined to this distribution form a natural clade. The study area covered a large enough range in latitude and ecological diversity to allow for phylogeographical conclusions to be drawn.

The results of this research should assist systematists and vector biologists whose work concerns the Culicidae in general and the subgenus *Culex* in particular. The first aim of this research is to evaluate the utility of the three genes being studied for their ability to resolve species-level relationships within subgenus *Culex.* The nucleotide sequences must contain sufficient polymorphisms to allow distinction among the closely related taxa being studied, without introducing too much homoplasious data. A character state is homoplasious if it has arisen in multiple taxa by means other than inheritance from a common ancestor. Since they are not the result of inheritance, homoplasious data can suggest an incorrect phylogeny. The reason behind selecting three disparate genes is the hope of minimizing any linkage effects between genes that might result in a false appearance of congruence between the different data sets. The study of each individual gene can produce only a phylogeny of that particular gene. It is important to consider that any given gene tree does not necessarily reflect the true species phylogeny. A number of factors can result in incongruence between a gene tree and the true species phylogeny, such as incomplete lineage sorting at the time of speciation, or introgressive hybridization (Avise 2004; Graur and Li 2000). It is also important to consider that data sets from different genes may be incongruent because of effects caused by different modes of inheritance or different evolutionary processes affecting the different data sets (Bull *et al.* 1993). Certainly, the inheritance of mtDNA from maternal cytoplasm differs from the inheritance of nuclear genes and this difference may affect the compatibility of the data sets (Moore 1995). The species phylogeny is a history of speciation events, which do not necessarily correspond with the history of the fixation of genetic characters; thus, the study of each gene can only be said to produce an estimated phylogeny.

Congruence among the results derived from the distinct genes should therefore estimate the true species phylogeny. The second aim of this research is to evaluate the different methods of phylogenetic analysis being used. These methods are: neighbor-joining distance, parsimony, maximum-likelihood, and Bayesian analysis, each of which is described later in this chapter. It is of interest to determine whether these methods recover consistent phylogenies and whether that phylogeny is consistent with previously proposed relationships. The main goal of this research is to propose a phylogeny of subgenus *Culex* from the studied region that utilizes the favored genetic data and methods of analysis. This proposed phylogeny is compared with previously published hypotheses and interpreted with regard to morphology, geography, and vector biology.

Importance of this study

A preponderance of phylogenetic studies of the Culicidae performed to date study relationships within the subfamily Anophelinae, which contains the medically important genus *Anopheles*, or within the large tribe Aedini, which contains many nuisance mosquitoes and the medically important genus *Aedes*. By contrast, significantly less attention has been paid to relationships within the tribe Culicini. This tribe contains the genera *Culex, Deinocerites,* and *Galindomyia*, the largest of which is the genus *Culex.* The ~800 species of the genus *Culex* are subdivided into 24 subgenera worldwide, including the subgenus *Culex,* which is the focus of this study. A hallmark of subgeneric rank is that the morphological character states that distinguish subgenera are often only apparent at certain life stages. The subgenus *Culex* contains >200 species worldwide, 22 of which have been reported from the United States, Puerto Rico, and Guatemala. The vector status of some subgenus *Culex* mosquitoes is mentioned above. The potential for the spread of disease by these mosquitoes has resulted in much alpha-taxonomy in the form of new species descriptions. A wealth of new species descriptions resulted from investigations by entomologists in Central America during construction of the Panama Canal. Regrettably, little work has been done on the beta-taxonomy, or the uncovering of a hierarchy of natural relationships within this subgenus.

It is hoped that the phylogenetic inferences made herein will be useful to systematists and medical entomologists whose work concerns the family Culicidae. At its most basic level, this research helps to fill in the gaps of the phylogenetic history of subgenus *Culex* in the study regions at the species level. Certain conclusions impact current or proposed schemes of classification and should be of special interest to mosquito systematists. For example, it is of interest to determine whether or not the phylogeny proposed herein is consistent with previously published phylogenies of the internal relationships of subgenus *Culex* that were based either on morphology (Navarro and Liria 2000) or on molecules (Miller *et al.* 1996; Shepard 2006).

As with most contemporary phylogenetic studies, this research utilizes the concept of cladistics as introduced by Hennig (1966). The great advantage of a cladistic analysis is its ability to objectively test for monophyly, or to determine whether or not a group has arisen as a natural clade. The results of this research should help to determine the validity of certain taxonomic schemes. This research tests the monophyly of the subgenus *Culex*, which has previously been treated as a monophyletic group without rigorous testing of this hypothesis. This research tests whether members of the recently created subgenus *Phenacomyia* actually arose from within subgenus *Culex* or are indeed a distinct lineage. Also, this research tests the hypothesis that *Deinocerites,* which is believed to have arisen from genus *Culex* (Navarro and Liria, 2000; Mallampalli, 1995) might belong to subgenus *Culex.*

This research should also help in answering questions regarding the phylogeography of the subgenus in North and Central America and the Caribbean. The science of phylogeography seeks to make sense of phylogenetic hypotheses in light of geographical structures and events. Belkin (1962) proposed that the geography of the "American Mediterranean", which is a combination of the Gulf of Mexico and the Caribbean Sea, impacted speciation events in the subgenus *Culex.* The abundance of species belonging to subgenus *Culex* that are found in and around the Caribbean lends credence to Belkin's proposal that this region served as a center of new species radiation for the subgenus. Certain clades that are proposed in this study are isolated from other species by land or water barriers or by great geographical distances and changes in latitude. These issues are addressed more closely as part of the discussion of the proposed phylogenies that follow.

Conclusions drawn by this research should be of interest to medical entomologists who study members of the subgenus *Culex* as disease vectors. The proposed phylogeny may assist in predicting vector species that have thus far gone undetected. Closely related species are likely to share similar physiological characteristics, such as vector competence for a given arbovirus. A species sharing a recent common ancestor with known vector species might be a competent vector as well and should be noted for further study. Of course, other factors such as the ecology or abundance of a mosquito will affect its vector potential and these factors should also be considered before drawing conclusions about the importance of a mosquito species as a disease vector. Finally, it is of interest to determine whether vector competence for certain pathogenic agents has evolved independently in multiple lineages within the subgenus *Culex*.

General methodology

This section is intended to give a general overview of the methodology used to conduct this research. A detailed description of procedures can be found in the Experimental Procedures section of the chapter that follows.

Mosquitoes

Whenever practical, mosquito specimens were collected in the field as larvae and individually reared at a field station to the adult stage so that fourth instar and pupal exuviae could be preserved and associated with their appropriate adult body as described by Belkin *et al.* (1965). Species identifications were conducted based mainly upon fourth instar larval morphology using appropriate keys for the collection locality. The genitalia of adult males that were selected for further study were removed and slide mounted so that the genitalia slide could be used as a voucher for species identification before the remainder of the body was used for DNA extraction. The male genitalia were also used to confirm species identification, particularly when identification to species was difficult due to cryptic morphology of the adult stage or due to the poor condition of larval exuviae. Some specimens were shipped as larvae to the Centers for Disease Control (CDC) Division of Vector-Bourne Infectious Disease (DVBID), where individual rearings were conducted in the insectary. Molecular data obtained from Genbank was also used to supplement the data obtained from reared specimens.

Molecular genetic markers

The three genes were chosen because they contained an appropriate mix of conservative regions, necessary for PCR priming sites, and regions with interspecific variation, necessary to provide phylogenetic signal. The structure and function of each of these genes is described in further detail in the chapter that follows.

The nucleotide sequences obtained from each gene were derived from cloned molecules grown in a single colony of *Escherichia coli* bacteria that had been transformed with a plasmid containing the desired molecule. The Invitrogen TOPO TA cloning plasmid that was used provided additional 5' and 3' priming sites for sequencing so that sequence data had more overlap and redundancy, since each gene was sequenced at least 2 times in each direction, starting from different priming sites. The cloning procedure helped to amplify and purify the desired molecule so that there was a sufficient amount of purified product for multiple sequencing efforts. An additional advantage to cloning was that the transformed bacteria containing the molecule of interest could be easily stored for later growth, if necessary, by saving a glycerol stock of the bacterial colony at ultra-low temperature.

Phylogenetic analysis

Character states used for phylogenetic analysis were the nucleotide bases at aligned sites among each species in the study. Nucleotide sequences were examined from each of the three genes individually, and the sequences from *COII* and *ITS* were also combined and analyzed together in a simultaneous analysis.

The PAUP* v4.0b10 (Swofford 2003) computer software package was used to perform three different types of analyses. The neighbor-joining (NJ) distance method was used to quickly create phylogenetic trees based upon a distance matrix that measures genetic distance between taxa and joins the most similar taxa together. Although several different distance metrics were tried, which corrected for multiple mutations, the trees presented here used uncorrected distances. Although different distance metrics were tested initially, they tended to yield the same results as the simpler, uncorrected distance, possibly because the species are so recently diverged that multiple mutations have not had time to accumulate. The parsimony method was also used, which selects phylogenetic trees that can be explained by the fewest number of evolutionary changes. The maximum likelihood (ML) method, which selects the phylogenetic tree that is the most likely to result from the data and a statistical model of nucleotide transformation, was the third method used. The computer program Modeltest v3.7 (Posada and Crandall 1998) was used to determine the best model to use with the ML method without overparameterizing the analysis. The computer program Mr. Bayes v3.1.2 (Huelsenbeck *et al.* 2001, Ronquist and Huelsenbeck. 2003) was used for a fourth method, Bayesian analysis. While Bayesian analysis has existed as a method of statistical analysis for centuries, its use in phylogenetic analysis was not possible until computers became powerful enough to handle the large amounts of data and parameters used for the analysis. Like ML analysis, Bayesian analysis of phylogenetic data uses a likelihood function, but differs in its methodology. While ML analysis scores the likelihood of heuristicly identified trees, given the data, Bayesian analysis scores the likelihood of the data, given a tree identified by a Markov-chain Monte Carlo exploration of possible trees. Using Bayesian analysis, a tree is ultimately selected after the exploration of tree space appears to have stabilized around a set of trees for which the data have atrong likelihood.

Morphological versus molecular methods

Traditional phylogenetic hypotheses of the Culicidae tend to be those that were derived by studying morphological characters. Early work on mosquito phylogenetics predates the discovery of DNA and the invention of techniques in molecular biology that followed. The advent of techniques in molecular biology has provided the researcher with a wealth of characters for phylogenetic study, and advances in computer science have provided the means for analyzing this abundance of data. However, molecular data is not necessarily an improvement over morphological data when used for phylogenetic analysis. The addition of more characters is often accompanied by the addition of more homoplasious characters, which can tend to negate any potential advantage. Where molecular means truly excel is in their ability to detect differences between closely related taxa. Noncoding regions or first and third codon positions of some protein coding regions were found in this research to contain sufficient polymorphism to distinguish among most of the closely related subgeneric species.

Previously published phylogenetic hypotheses of taxa within the Culicidae exist based upon morphological and/or molecular data. The morphology-based studies tend to examine deep relationships. An early attempt at providing an evolution-based classification of the Culicidae using morphological characters was performed by Ross

(1951). More recent phylogenetic studies based upon mosquito morphology have studied relationships at the levels of family (Harbach and Kitching 1998), subfamily (Sallum *et al.* 2000; Harbach and Kitching 2005), tribe (Reinert *et al.* 2004), and subgenus (Navarro and Liria 2000; Reinert *et al.* 2006). Molecule-based studies examine different levels of relationships, depending upon the molecule used. The nuclear protein-coding *white* gene was used to determine relationships at the family level (Besansky and Fahey 1997), and additional nuclear genes were used to determine relationships among subgenera within genus *Anopheles* (Krzywinski *et al.* 2001). The 18S subunit of ribosomal DNA was used to study relationships at the levels of order (Friedrich and Tautz 1997), infraorder (Miller *et al.* 1997; Pawlowski *et al.* 1996), genus and subgenus (Shepard 2005). The noncoding *ITS* regions of rDNA have been used to examine very low-level relationships, including distinctions among members of the *Cx. pipiens* complex (Miller *et al.* 1996). One study (Garros *et al.* 2005b) combined morphological and molecular information to assess the relationship of two species groups within the genus *Anopheles*.

Definition of terms

It is appropriate at this time to define certain terms as they will be used in this dissertation. This is necessary because certain of these terms are treated as synonyms in the current literature or have differences of meaning that are subtle. These terms are: systematics, taxonomy (including alpha-, beta-, and gamma-taxonomy), nomenclature, classification, phylogenetics, and cladistics.

Systematics is the study of the diversity of life and the relationships among groups of living things. The definition of this term is somewhat loose because this is an umbrella term that describes a collection of several more distinct fields of study. Each of the terms that are defined in the following paragraphs describes a field of study that comes under this umbrella term.

Taxonomy is an area of work within systematics. In biology, the term taxonomy refers to the naming of organisms. As shall be discussed shortly, even the naming of organisms, when done according to the rules of nomenclature, makes certain conjectures about an organism's relation to other living things. So, the term taxonomy is more appropriately defined in a broader sense to include both the naming of organisms as well as the investigation into that organism's relationship to other living things. In order to clarify the meaning of this term, it is often subdivided into alpha-, beta-, and gamma-taxonomy (Mayr, 1982).

The term alpha-taxonomy refers solely to the describing and naming of species, and is thus synonymous with the strict definition of taxonomy. The discovery of a new species is acknowledged by the scientific community when a species description is written and published and the species is given a binomial Latin name according to the rules of nomenclature. These rules are currently described for animal taxa in the International Code of Zoological Nomenclature (The Code), maintained by the International Commission on Zoological Nomenclature (ICZN, 1999). The description of a new species implies that the described taxon fits the definition of a distinct species under one of many species concepts (Wheeler and Meier 2000; Coyne and Orr 2004). Thus, a practitioner of alpha-taxonomy needs a working knowledge of species concepts, an understanding of The Code, and proficiency in collecting and preserving specimens that serve as type specimens and vouchers for the newly described species.

The term beta-taxonomy refers to the study of the relationships among described species and the placement of those species into a hierarchical scheme. This can refer to the recognition of deeper relationships among related species. In recent decades, molecular characters have been used either to detect relationships that might have gone undetected using only morphological characters or to test the validity of relationships previously established by morphological data. Although alpha- and beta-taxonomy are distinct disciplines, they often intersect because newly discovered relationships, or new interpretations of relationships, are often followed by nomenclatorial changes.

The term gamma-taxonomy refers to the study of population genetics, speciation, and evolution. These are the processes that have resulted in the hierarchical organization of biological organisms that is observed today and that is utilized explicitly in beta taxonomy. Mayr (1982) does not consider gamma-taxonomy to truly be taxonomy, because it does not involve the naming of organisms. However, the importance of gamma taxonomy to an overall understanding of biological processes, including evolution, should not be overlooked.

The term nomenclature, like alpha-taxonomy, refers to the naming of taxa, but is a bit more specific with regard to proper adherence to The Code. The publication of the 10th Edition of Systema Naturae by Linnaeus (1758) establishes the now familiar requirement that species be given a Latin binomial. The nomenclatorial process must adhere to rules published in the most recent edition of The Code (ICZN, 1999).

The term classification combines nomenclature and beta-taxonomy. The science of classification is concerned with assuring that the name given to a species reflects its assumed position in a hierarchical scheme. Although nomenclature is not the primary concern of classification, the establishment of a *classification* scheme does impact a taxon's name, while the establishment of a relationship through *beta-taxonomy* does not presuppose a nomenclatorial change.

The term phylogenetics, like gamma-taxonomy, refers to the uncovering of relationships among taxa, with the added element that these relationships are believed to have arisen through descent with modification. The mechanism of this modification may or may not be the natural selection described by Darwin (1859) and Wallace (1870). The modifications could have arisen, for example, through the random fixation of neutral mutations (Kimura 1968), a mechanism believed to be quite common at the molecular level. The important point is that the hierarchical relationship arose because of changes that accumulated over time and that were passed down from ancestor to descendant.

Finally, the term cladistics refers to the method developed by Hennig (1966) for determining phylogenetic relationships. Cladistics is not the only method that can be used in phylogenetics. Alternative methods include phenetics (or "numerical taxonomy") and phyletics (or "intuitive systematics"). The hallmark of cladistics is that taxa sharing a recent common ancestor can be identified by the presence of synapomorphies (or, shared derived characters) inherited from the common ancestor that are not present in more distant relatives. The methodology of cladistics as it is used in parsimony analysis is well described in Kitching *et al.* (1998).

Importance of systematics

Systematic research has far-reaching effects that impact other branches of biological science. Early systematic work was concerned mainly with species description. With few exceptions, the ideology of early systematists harkens back to the Aristotelian concept of unchanging, typological species. A few early systematists, most famously Jean-Baptiste de Lamarck, recognized a plasticity of species morphology over time, but had difficulty explaining it. It was not until Darwin published his thoughts on natural selection (1859) that a plausible mechanism for evolution was believed to have been found, sparking research into what would become phylogenetics.

Approaches to systematics have changed through time and continue to change. The dawn of cladistic theory changed the way phylogenetic relationships among species were detected. The growth of computer power has allowed for the invention of very complex phylogenetic analyses that search among staggering numbers of hypothetical relationships to find the most likely one. Even the fundamental science of species discovery has been shaken by the emergence of new technology. For example, new species complexes in the mosquito genus *Anopheles* have been discovered based on polytene chromosome banding patterns (Munstermann 1997; Norris 2002). This brings into question what characters are admissible for a species description. The recent effort to "bar code" all species by keeping a computer database of regions of their mtDNA sequence data is likely to revolutionize the way species information is stored and accessed and the way species are identified from the field (Savolainen *et al.* 2005; Wheeler, 2004). In recent decades, a whole new branch of systematics, known as population genetics, has used molecular markers to study relationships among individuals within a species (Hartl and Clark 2006).

Systematic research affects many branches of science. The rules of nomenclature are in place to aid communication among biologists, who trust that a single Latin binomial refers to the same species worldwide. In today's age of huge scientific literature databases, the Latin binomial serves as a useful keyword for narrowing electronic literature searches. Systematic studies also aid in the preservation of endangered species by alerting us to dwindling species counts in certain biota or to population bottlenecks that can limit genetic diversity and thus limit the ability of organisms to survive adverse environmental conditions. Systematic studies help channel the flow of funds for research institutions. Public attention tends to focus on the charismatic and aesthetically pleasing taxa, but researchers must consider much more when selecting taxa to study. By having a working knowledge of the relationships among species, the research findings from one species might be found to be applicable to a related species.

Systematics studies can have a direct effect on the field of vector biology by directing appropriate attention and funds where they are most effective. The nomenclatorial aspect of systematics is crucial for effective communication among entomologists, vector biologists, and mosquito control workers who might be interested in the recent findings on a particular species. One element of systematics is to record the known distribution of species. By learning of these distributions, researchers or vector control officials are better informed about which species might be found in their geographic region and about the ecology of those species. For example, vector control officials might adjust the time of day or night at which they spray adulticide based on reports of the mosquito species known in their region coupled with literature reporting the flight times for the known species (e.g. Savage *et al.* 2006). Also, species that are reported in large numbers or in close proximity to disease reservoirs might be treated as having a higher vector potential. Information on the relationships among species can also impact work in vector biology. As mentioned earlier, species that are found to share a recent common ancestor with a known vector species deserve close study since they have the potential of being vectors themselves. Additionally, closely related species might be effectively controlled by a single vector control strategy.

The systematics of species complexes has had a crucial effect on vector biology research. Morphologically similar cryptic species complexes were discovered in the genus *Anopheles*, some members of which vector human malaria parasites. Examples include members of the *Anopheles maculipennis* and *Anopheles gambiae* complexes which are known to have different distributions, host preferences, pesticide resistances, or larval habitats (see Service 2001). Systematic studies have uncovered the presence of morphologically identical *Anopheles* species with very different vectorial capacity, have recorded their different distributions, and have reported techniques for genetic distinction among them (Collins and Paskewitz 1996; Munstermann 1997).

History of *Culex* systematics

The next several pages recount events that have lead to the current state of systematics in the genus *Culex*. Particular attention is given to issues of classification and rearrangements of this classification over time. Attention is also given to the larger subgenera within genus *Culex*, their importance to biologists and the reasons for forming or rearranging these groups. Much reference is made to Groups, Subgroups, Series, and Sections that have been proposed by mosquito systematists. While these categories are not taxonomic ranks that are codified by the ICZN, and do not imply phylogenetic relationships, they are useful means of categorization that aid workers in discussing grossly similar species together. Only the Family, Genus, and Species ranks are regulated by the ICZN. Over time different systematists have used different informal categories to organize the species being studied without implying formal modifications to the codified taxonomic ranks.

Family Culicidae

The mosquitoes consist of as many as 3200 described species currently classified in the Family Culicidae, Order Diptera (Service, 2001). Synapomorphies of the family Culicidae include the conspicuous proboscis with biting mouthparts and the presence of scales on the wing veins (Service 2001). The systematics of the Culicidae in the eighteenth century is reviewed by Knight (1972) in an account which touches upon the personal lives of these early mosquito workers. The genus name *Culex* is used by Linnaeus (1758) in his tenth edition of *Systema Naturae*. His Latin description of the genus describes the presence of biting mouthparts with fine stylets contained within a flexible proboscis (translation from Snow 2001a). Two species described in this early work by Linnaeus, *Culex pipiens* L. 1758 and *Culex bifurcatus* L. 1758, are still considered by current systematists to be among the Culicidae. The specific name *pipiens* is a reference to the high pitched "piping" sound made by the flying adult (Snow 2001b). The type specimen of *Cx. pipiens* used by Linnaeus was examined by Harbach *et al.* (1985) and found to be a badly damaged specimen belonging to the genus *Aedes*. A neotype (a subsequent description of a specimen used to serve as the concept of the species, ICZN 1999) conforming to the current concept of *Cx. pipiens* has since been designated (Harbach *et al.*, 1985). The *Cx. bifurcatus* described by Linnaeus has been synonymized under *Cx. pipiens* (Edwards, 1932). All mosquitoes described in the eighteenth century were placed in the genus *Culex*, as no other genera were proposed until Meigen designated the genera *Aedes* and *Anopheles* in 1818.

The genus name *Culex*, Latin for gnat, midge, or mosquito, forms the root for the family name Culicidae. The earliest attempt to organize an inner classification for these insects is found in a monograph by Theobald (1910). An early revision of the family by Edwards (1921) divides the tribes and genera of mosquitoes into two series: Series A consists mainly of brightly ornamented diurnal species (species in the tribes Megarhini, now Toxorhynchitini, and Sabethini), while Series B generally consists of less ornamented species and includes most species of medical and pest importance (Dyar and Shannon, 1924). The current state of classification of the Culicidae is based upon the foundation laid out by Edwards (1932), which supplanted the earlier classification schemes. Important revisions have been made to this classification scheme, which are described below.

The most obvious difference between the work of Edwards (1932) and the current state of mosquito systematics is in the sheer number of described species, which has more than doubled. In a 1990 memorial to John Belkin, Zavortink (1990) claims that mosquito taxonomy is still mired in the definition, or alpha-taxonomy, stage and that only 25-50% of mosquito species have been described. Another obvious change to mosquito classification since the scheme of Edwards (1932) has arisen because of changes in the concept of the deeper relationships. Early workers (Edwards, 1932; Belkin, 1962) included dixid and chaoborid midges among the Culicidae. Stone (1957) placed these midges into their own families and designated the Culicidae as the "true mosquitoes" (subfamily Culicinae of Edwards, 1932). This concept required shifts in subfamily and tribal designations, which are reflected in "A Catalog of the Mosquitoes of the World", which consists of Knight and Stone (1977) and Stone *et al.* (1959) with additional supplements by Stone (1961, 1963, 1967) and Ward (1984, 1992).

The web site of the Walter Reed Biosystematics Unit Systematic Catalog of Culicidae (http://www.mosquitocatalog.org/main.asp) provides an on-line resource for mosquito systematists and aspires to keep its catalog current as accepted classifications change. The family Culicidae as it is now defined is believed to be a natural, monophyletic group (Wood and Borkent 1989) and is divided into three subfamilies: Anophelinae, Toxorhynchitinae, and Culicinae. However, Harbach and Kitching (1998), in their phylogenetic study using 74 morphological characters from all life stages, find no evidence for the subfamily status of Toxorhynchitinae and suggest that these mosquitoes be given tribal status as tribe Toxorhynchitini under subfamily Culicinae.

The largest of the subfamilies is the Culicinae, which contained 2925 species as of the writing of Service in 1993. Knight and Stone (1977) divide the large subfamily Culicinae into two tribes: Culicini and Sabethini. When the current subfamilies Anophelinae, Toxorhynchitinae, and Culicinae had been given tribal status, the Sabethini were treated at an equal rank with these other three groups (Edwards 1932); however, this decision seems to have been made for purposes of identification keys and not as part of a natural classification. In contrast to the two-tribe system of the Culicinae, many workers today still accept the tribal designations of Belkin (1962), such that the Culicinae are divided into ten tribes: Aedeomyiini, Aedini, Culicini, Culisetini, Ficalbini, Hodgesiini, Mansoniini, Orthopodomyiini, Sabethini, and Uranotaeniini. The genus *Culex* is placed, along with the genera *Deinocerites* and *Galindomyia*, in the tribe Culicini in the ten-tribe system. This tribe is currently often considered to include the genus *Lutzia,* which was resurrected from being a subgenus of *Culex* by Tanaka (2003). Both Mattingly (1969) and Service (1993) retain the two-tribe system of Stone *et al.* (1959) and divide the Culicini into four groups, which are of ecological significance but are not phylogenetic or taxonomic groups. The informal groups of Mattingly (1969) and Service (1993) are (1) the Aedine genera, (2) the quasi-Sabethine genera, (3) vegetation-seeking genera, and (4) miscellaneous genera. The genera *Culex*, *Culiseta*, *Deinocerites*, *Galindomyia*, and *Orthopodomyia* are placed among the miscellaneous genera.

A number of other informal groups have been used in classifying the inner relationships of the Culicidae. Dyar and Shannon (1924) divide their Culicini, which is roughly today's Culicinae, into the *Aedes* Group and the *Culex* Group, with some intermediates, based upon the pointed or blunt shape of the female abdomen. The tribe Culicini of Edwards (1932) is equivalent to today's Culicinae and was divided by Edwards into five groups: the *Sabethes* Group, the *Uranotaenia* Group, the *Theobaldia* (now *Culiseta*) Group, the *Aedes* Group, and the *Culex* Group. Edwards (1932) bases these groups mainly on chaetotaxy, wing venation, and the male genitalia; yet, he allows that these adult characters are not always strong enough to provide the necessary distinction and that larval and pupal characters as well as some knowledge of life history are needed.

Genus Culex

The genus *Culex* contains 779 described species distributed worldwide, making this the second largest genus of the Culicidae, next to *Aedes* (Knight and Stone 1977). Today's classification divides the genus *Culex* into 24 subgenera. The hemisphere of distribution and the species counts for these subgenera are summarized in Table 1.1. The status of the subgeneric classifications under genus *Culex* is currently in a state of flux. Tanaka (2003) suggests that these subgenera be reevaluated and that some may be sufficiently morphologically distinct to warrant their elevation to generic status. Tanaka (2004), in a study of mosquito pupae in Japan, has recently resurrected two Old World subgenera from synonymy with subgenus *Culex.* These recently resurrected subgenera are *Sirivanakarnius* Bohart and *Oculiomyia* Theobald, which have been included in Table 1.1. The subgenus *Lutzia* is not included in Table 1.1 since this group was elevated to genus by Tanaka (2003), but will be discussed among the "Selected subgenera of Culex" as is explained below. Subgenera with a worldwide distribution are *Neoculex* Dyar, and *Culex* Linnaeus. The former subgenus *Lutzia* also has a worldwide distribution. Subgenera restricted to the Old World are *Acalleomyia* Leicester, *Acallyntrum* Stone and Penn, *Afroculex* Danilov, *Barraudius* Edwards, *Culiciomyia* Theobald, *Eumelanomyia* Theobald, *Kitzmilleria* Danilov, *Lasiosiphon* Kirkpatrick, *Lophoceraomyia* Theobald, *Maillotia* Theobald, and the two recently resurrected subgenera *Oculeomyia* and *Sirivanakarnius*. Subgenera restricted to the New World are *Aedinus* Lutz, *Allimanta* Casal and Garcia, *Anoedioporpa* Dyar, *Belkinomyia* Adames and Galindo, *Carrollia* Lutz, *Melanoconion* Theobald, *Micraedes* Coquillett, *Microculex* Theobald, *Phenacomyia* Harbach and Peyton, and *Tinolestes* Coquillet. While most of the subgenera contain fewer than ten species, and seven of them contain only a single species, there are five subgenera that contain more than 50 species. These large subgenera are *Culex* (239 species), *Culiciomyia* (55 species), *Eumelanomyia* (78 species), *Lophoceramyia* (113 species), and *Melanoconion* (156 species). Phylogenetic relationships among seven New World subgenera were studied by Navarro and Liria (2000).

Selected subgenera of Culex

This section provides a brief examination of the bionomics and taxonomic history of selected subgenera of *Culex*. These subgenera have been selected because of their large species count numbers, their importance for entomologists and vector biologists, and/or their taxonomic history.

Edwards (1932) subdivides the large Old World subgenus *Culiciomyia* into two "slightly different" groups based mainly on presence/absence of pleural scales and abdominal bands in the adult. Group A (the *fragilis* or *Culiciomyia* Group) is found in the Oriental, Indomalayan, and Australasian regions, while the more primitive Group B (the *nebulosus* or *Pectinopalpus* Group) is found only in the Ethiopian region. Members of this subgenus are not noted as disease vectors, nor are they significant pests.

The large Old World subgenus *Eumelanomyia* is one of the largest of the *Culex* subgenera despite the fact that its distribution is mostly restricted to the Oriental and Ethiopian regions. *Eumelanomyia* has been treated as a genus (Theobald, 1909). Early confusion about this group seems to revolve around different interpretations of the affinities between the two subgenera *Eumelanomyia* and *Neoculex*, as well as the two former subgenera *Mochthogenes* and *Protomelanoconion*. Edwards (1932) treats *Eumelanomyia* as one of three groups within his subgenus *Neoculex*. Later work by Edwards (1941) still uses *Eumelanomyia* as a group under subgenus *Neoculex*, only this time it is one of five groups placed in this subgenus. Sirivanakarn (1971) graphically reviews these rearrangements and provides his own scheme, based mainly on male genitalia, in which he resurrects *Eumelanomyia* as a subgenus of *Culex*, where it remains today. The group *Mochthogenes* was previously a subgenus (Edwards 1930, 1932), but is used as one of four groups by Sirivanakarn (1971) under his resurrected subgenus *Eumelanomyia*. Some difficulties in this group may arise due to the fact that many of the descriptive works examined only adult males; the females and larvae of most species were not studied intensely until Sirivanakarn (1972) collected Asian specimens as part of his evidence for resurrecting the subgenus *Eumelanomyia*.

The subgenus *Neoculex*, with 24 species currently described worldwide, is similar in appearance to subgenus *Culex*, thus the name, but is separated from the *Culex* by Dyar (1905) based on male genitalia. Adult specimens can be distinguished from subgenus *Culex* quite easily because the pale bands of the abdominal terga are basal in *Culex* and apical in *Neoculex*. The subgenus is large enough to have been the subject of several different internal organization schemes. Many of these rearrangements seem to have been the result of different interpretations of the affinity between *Neoculex* and *Eumelanomyia* and are described above. Edwards (1932) divides the subgenus into three groups, all of which were previously treated as genera. These are the *Neoculex* Group, the *Eumelanomyia* Group, and the *Protomelanoconion* Group. This classification by Edwards (1932) has received criticism (Belkin, 1962; Mattingly and Marks, 1955;

Sirivanakarn, 1971) mainly because of the use of weak characters of the male phallosome, which make the scheme questionable from the standpoint of cladistics. Later work by Edwards (1942) recognizes five groups instead of three. A sixth group is added by King and Hoogstraal (1947), although this is done with apparent uncertainty. Sirivanakarn (1971) considers the shape of the phallosome, as well as the spicules of the proctiger, when he proposes resurrection of the subgenera *Maillotia* and *Eumelanomyia* from synonymy with *Neoculex* and treats the former subgenus *Mochthogenes* as a species group under *Eumelanomyia*. To make an etymological note, the subgenus name *Maillotia* is Latin for "swaddling clothes", which presumably refers to the broad scales on the head and thorax (Snow 2001c). The end result of Sirivanakarn's (1971) work with *Neoculex* is the splitting of the former subgenus *Neoculex* into three subgenera, these being *Neoculex*, *Maillotia*, and *Eumelanomyia*, and changing the subgenus *Mochthogenes* to a species group under the newly elevated *Eumelanomyia*. The *Neoculex* of Sirivanakarn (1971) consists of most of the mosquitoes formerly placed in subgenus *Mochthogenes* as well as some already placed in *Neoculex*. Sirivanakarn (1971) also divides each of the four subgenera into three or four species groups.

Members of the large Old World subgenus *Lophoceraomyia* in New Guinea and the Bismarck Archipelago were reviewed by Sirivanakarn (1968). This work makes reference to ongoing difficulties in the systematics of this complex group, as discussed by Belkin (1962) and Colless (1965). Edwards (1932) recognized three groups within the subgenus, which he later (1934) amended to account for the new classification of pitcher-plant breeding species of *Lophoceraomyia*. Belkin (1962) was impressed by the "great development" and "intense speciation" of these mosquitoes in the Southern Pacific, and conjectured that the complex affinities of the group are attributable to previous interspecific hybridization events, perhaps with members of the subgenera *Neoculex*, *Culiciomyia*, or *Mochthogenes*. Colless (1965) uses a different internal classification of *Lophoceraomyia* by recognizing only two groups, the *fraudatrix* Group and the *mammilifier* Group, with the *mammilifier* Group being divided into two subgroups. Colless (1965) also makes note of four groups of sibling species, similar in adult morphology but different in larval habitat, and contemplates the possibility of studying modes of speciation among them. Sirivanakarn's work (1968) retains the two groups of Colless (1965), except that the *mammilifier* Group is divided into three subgroups instead of two.

The medically important large New World subgenus *Melanoconion* has been subdivided into three Sections. Sirivanakarn (1982) reviews the history of the systematics of this group and proposes his own classification scheme, which implements the three Sections: Ocellatus Section, Spissipes Section, and Melanoconion Section (in keeping with conventions used in Sirivanakarn [1982], the typeset and capitalization depart from the convention used in the remainder of this dissertation). The subgenus *Melanoconion* was originally a genus, proposed by Theobald (1903), with six species. Soon, however, Dyar and Knab (1906) synonymized the genus *Melanoconion* under *Culex*. The name was not used even as a species group by Howard *et al.* (1915). A later revision of the *Culex* by Dyar (1918), focusing upon male genitalia, uses *Melanoconion* as a subgenus within *Culex*. In a later treatment of American mosquitoes, Dyar (1928) recognizes only three subgenera of *Culex*, these being *Culex*, *Melanoconion* and *Mochlostyrax*. Sirivanakarn considers the subgeneric status of *Melanoconion* to have stabilized when Rozeboom and Komp (1950) synonymize *Mochlostyrax* under *Melanoconion.* An attempt was made by Foote (1954) to keep *Melanoconion* and *Mochlostyrax* as separate subgenera, and this separation appears in the mosquito catalog of Stone, Knight, and Starcke (1959). More recent editions of the catalog (Knight and Stone, 1977) follow the recommendations of Rozeboom and Komp (1950) as well as Belkin (1968) in synonymizing *Mochlostyrax* under *Melanoconion.* Sallum and Forattini (1996) use the most recent classification scheme of Sirivanakarn (1982) in updating identification keys for the medically important Spissipes Section. Members of the Spissipes Section of subgenus *Melanoconion* are the principal vectors of enzootic Venezuelan Equine Encephalitis Virus transmission, yet they have not been implicated in epizootic transmission of this virus (Service, 2001). Pecor *et al.* (1992) remove members of the Ocellatus Section from subgenus *Melanoconion*, leaving these mosquitoes currently with uncertain subgeneric status.

The genus *Lutzia* Theobald 1903 was treated as a subgenus of *Culex* when Edwards (1932) expanded his interpretation of the genus *Culex* to include the former genera *Lutzia* and *Carrollia*. Ever since this re-classification, *Carrollia* remains a subgenus. *Lutzia* remained a subgenus of *Culex* until Tanaka (2003) proposed its resurrection as a genus, containing three subgenera of its own (these subgenera being *Lutzia* Theobald 1903, and two new ones, *Insulalutzia* Tanaka 2003, and *Metalutzia* Tanaka 2003). Much of the current literature continues to treat *Lutzia* as a subgenus of *Culex*; therefore, it is worth discussing the use of *Lutzia* as a subgenus.

Edwards (1932) divides the subgenus *Lutzia* into two groups, the New World *Lutzia* Group and the Old World *Jamesia* Group. Strong characters distinguish between the two groups, which may explain why the *Jamesia* Group was interpreted as a new genus by Christophers (1906). Most workers report difficulty in distinguishing among species within these groups, although Bram (1967b) testifies to the utility of the phallosome for this purpose. Belkin (1962) concurs with the classification of Edwards (1932), adding that *Lutzia* appears to be an ancient lineage, with affinities to the subgenera *Culex* and *Culiciomyia*. The *Lutzia* mosquitoes are of biological interest because their larvae are predatory on other mosquito larvae and have modified mouthparts to aid this predation (Service 1993; Carpenter and LaCasse 1955). Evidence supporting the elevation of *Lutzia* to generic rank is provided by Navarro and Liria (2000), who conclude that *Lutzia* forms a distinct basal clade within the Neotropical Culicini. Of course, the study of Navarro and Liria (2000) is based entirely on larval mouth parts, which are certain to be unique amongst the *Lutzia* for purposes of predation.

Subgenus Culex

The subgenus *Culex* is the focus of this research and thus deserves added attention in this section on systematic history. Table 1.2 lists the species of subgenus *Culex* with reported distributions within the study area and lists the countries from which each has been reported according to published literature as of August 2008. Edwards (1932) proposed the subgenus *Culex* based almost entirely upon adult morphology. In Edwards's original description, the most useful characters for distinguishing this subgenus from other subgenera are characters of the male genitalia. The paraproct is crowned by a dense tuft of hair or spines at the tip and frequently has a strong basal arm. The importance of these synapomorphies in defining the subgenus is supported by Bram

(1967a). Edwards also describes basal and apical rings on the tarsi, but states that the tarsi can be black in some species. This difference forms the major distinction between the two groups discussed below.

The medically important, world-wide distributed subgenus *Culex* is further divided into groups and series by Edwards (1932). Edwards divides the subgenus *Culex* into two groups, calling them Group A and Group B. Group A is more usefully referred to as the *lasioconops,* or more commonly *sitiens* Group, and its distribution is restricted to the Old World. Group B is also called the *pipiens* Group and consists of New World species, as well as some species with a world wide distribution. Edwards further divides the *sitiens* Group into four Series: the *bitaeniorhynchus* Series (12 species), the *gelidus* Series (four species), the *sitiens* Series (11 species), and the *mimeticus* Series (3 species). These series are defined largely, but not exclusively, upon ornamentation of the mesonotum (dorsal sclerites of the mesothorax). The *pipiens* Group is also divided into four series, with a fifth series left ill-defined. The four series of the *pipiens* Group are the *tarsalis* Series (23 species), the *salinarius-apicinus* Series (18 species), the *pipiens* Series (21 species), and the *decens* Series (15 species). The fifth series of the *pipiens* Group consists of 12 species, mostly of Oriental or Australasian distribution. Edwards distinguishes among series belonging to the *pipiens* Group largely based upon geographic distribution and the presence or absence of rings on the hind tarsi. Barraud (1934) concurs with the presence of two main groups within subgenus *Culex* based upon distribution and the presence/absence of tarsal rings, a banded proboscis, and a mesepimeral bristle (a stiff hair on the posterior half of the mesopleuron). The predominantly Oriental species studied by Barraud (1934) lack a mesepimeral bristle and have banded tarsi and probosci, thus conforming to the *sitiens* Group. The strong morphological characters of the two groups are useful to medical entomologists in the Oriental region for distinguishing potential Japanese encephalitis vectors (belonging to *sitiens* Group) from potential filariasis vectors (belonging to the *pipiens* Group).

Sirivanakarn (1976) utilizes a slightly different internal arrangement of the subgenus *Culex* in his work with 42 Oriental species. Again, the two main groups of Edwards, the *Cx. sitiens* Group and the *Cx. pipiens* Group, are agreed upon by Sirivanakarn, but the subdivision of each group is different. While Edwards (1932) divides the *Cx. sitiens* Group into the four series mentioned above, and Bram (1967a) divides the *Cx. sitiens* Group into two subgroups (*Cx. vishnui* and *Cx. sitiens*), Sirivanakarn divides the *Cx. sitiens* Group into six subgroups: the *Cx. gelidus* Subgroup, the *Cx. bitaeniorhynchus* Subgroup, the *Cx. sitiens* Subgroup, the *Cx. vishnui* Subgroup, the *Cx. barraudi* Subgroup, and the *Cx. mimeticus* Subgroup. Of these subgroups, the *Cx. barraudi* Subgroup is a novel classification in Sirivanakarn (1976) and the *Cx. vishnui* Subgroup is based upon the classifications of Bram (1967a). Sirivanakarn's four divisions of the *Cx. pipiens* Group are quite novel and consist of the *Cx. pipiens* Subgroup, the *Cx. trifilatus* Subgroup, the *Cx. theileri* Subgroup, and the *Cx. univittatus* Subgroup.

The subgenus *Culex* includes members of the widely studied, yet taxonomically difficult *Cx. pipiens* complex. The *Cx. pipiens* complex consists of a number of taxa that are morphologically identical, or nearly so. Many workers disagree on whether members of this complex should be given full species status or should be treated as subsepecies of *Cx. pipiens* (Mattingly *et al.*, 1951; Barr, 1982). The complex includes six taxa, which are usually named in recent literature as follows: *Cx. pipiens*, *Culex quinquefasciatus, Culex pipiens* form *molestus, Culex pipiens pallens, Culex globocoxitus,* and *Culex pipiens australicus*. It should be noted that forms are not codified by the ICZN, and so *Cx. pipiens* form *molestus* is not recognized as a distinct taxon under the Code. The two most common members of the *Cx. pipiens* complex are *Cx. pipiens*, which has a worldwide distribution in temperate zones, and *Cx. quinquefasciatus*, which has a worldwide distribution in tropical and subtropical zones. The two taxa are sympatric where their ecosystems overlap, and they are believed to hybridize in these zones (Barr, 1957). Such a zone of hybridization is believed to exist in North America between the latitudes 36 and 39 degrees North (Barr 1957), with some exceptions, notably those discovered in California (Tabachnik and Powell 1983; Urbanelli *et al.* 1997). Outside of the zone of hybridization, biological factors distinguish between the two taxa. *Culex pipiens* is ornithophilic, diapauses in winter as an adult female, and is eurygamous (requires large open spaces to breed.) *Culex quinquefasciatus* will bite man more readily than *Cx. pipiens*, is incapable of winter diapause, is stenogamous (capable of breeding in confined spaces), and is capable of autogeny. *Culex pipiens* form *molestus* shares behavioral characteristics with *Cx. quinquefasciatus*, but is found in temperate zones, where it inhabits warm underground shelters, such as subway stations. The name *molestus* refers to the voracious feeding habit of this form. The *molestus* form is a particularly notorious feeder on humans in the Underground subway terminals of England. Fonseca *et al.* (2004) attribute the rapid transmission of West Nile Virus in North America on the existence of hybrids between the ornithophilic *Cx. pipiens* and its mammophilic form *molestus*. *Culex pipiens pallens* is reported in some accounts to be a hybrid between *Cx. pipiens* and *Cx. quinquefasciatus*, while most Japanese publications treat *pallens* as a species. The distribution of the lesser studied taxa *Cx. australicus* and *Cx. globocoxitus* is confined to Australia.

Current state of subgenus *Culex* phylogeny

This is the first study to examine subgenus *Culex* phylogeny with a comprehensive selection of species and multiple molecular sources of data. Previous phylogenetic studies involving subgenus *Culex* investigate the phylogeny of deeper relationships and so only a few taxa from subgenus *Culex* were used to represent the subgenus in these studies. Navarro and Liria (2000) examined four species from the subgenus in their morphological study, which examined the relationship among seven *Culex* subgenera. Shepard *et al.* (2006) examined six taxa in subgenus *Culex*, and Miller *et al.* (1996) examined ten taxa in subgenus *Culex*, seven of which are distributed in the region studied here.

Because no previous phylogenetic studies include a sufficient number of subgenus *Culex* species, there is currently no scientifically established phylogeny of many of the species included in this study. This can cause difficulty in evaluating the results of this study through comparison with previous studies. One way in which the trees presented here can be evaluated is by examining the strength of their statistical values (likelihood, bootstrap value, or posterior probability value). The results can also be evaluated with regard to the phylogenetic positions of a few species that this study has in common with previous studies. For example, the basal position of the *Cx. pipiens* complex relative to New World subgenus *Culex* is well established by all three previous studies mentioned above. Additionally, Miller *et al.* (1996) find a (*Cx. restuans* + (*Cx. salinarius* + *Cx. erythrothorax*)) relationship and since the current study includes these three species, the previously proposed relationship can be used as a framework for evaluating the position of these three species among a larger pool of taxa.

Other methods were needed to evaluate the phylogenetic position of taxa that are not included in previous studies. Early culicidologists did not propose phylogenetic trees with the rigor of cladistic analysis, yet they did propose intuitive classifications based on gross morphology and distribution, such as the classification schemes of Edwards (1932) and Sirivanakarn (1976) discussed above. Carpenter and LaCasse (1955) arrange the genera and subgenera of their *Mosquitoes of North America* according to a natural classification based on that of Edwards (1932), although the species are listed alphabetically. Additionally, Belkin (1962) frequently notes the morphological and ecological similarities among species and speculates on their close affinities. The trees produced by the current study were compared with these previous, more intuitive, concepts, keeping in mind that the previous concepts were not phylogenetic hypotheses. Finally, the current trees were evaluated with regard to certain morphological features, such as the male genitalia and the larval chaetotaxy, which have been used as morphological characters for phylogenetic studies in other mosquitoes (Harbach and Kitching, 2005; Reinert *et al.,* 2004; Garros *et al.,* 2005b).

CHAPTER 2: Phylogeny of selected species of subgenus *Culex* mosquitoes (Diptera: Culicidae) from the U.S., Puerto Rico, and Guatemala, based on nucleotide sequences from three genes

Introduction

The purpose of this research was to test the monophyly of subgenus *Culex,* and to determine its phylogenetic position relative to the subgenus *Phenacomyia* and the genus *Deinocerites*. As subsidiary objectives, the utility of each of three molecular data sets and of each of four phylogenetic methods was evaluated. This research proposes a phylogeny of select species of subgenus *Culex* mosquitoes (Diptera: Culicidae) collected in North and Central America and the Caribbean. Separate gene phylogenies are derived from aligned nucleotide sequences of parts of three genes: the cytochrome oxidase subunit II (*COII*) mitochondrial gene, a region including the internal transcribed spacers (*ITS*) 1 and 2 of nuclear ribosomal DNA, and the nuclear protein-coding *white* gene. The nucleotide sequence data from the *COII* and *ITS* genes were combined to allow a simultaneous analysis of the two data sets.

The subgenus *Phenacomyia* and the genus *Deinocerites* were treated as ingroup taxa to objectively determine their phylogenetic positions relative to subgenus *Culex.* Subgenus *Phenacomyia* was created based on morphological features at all life stages (Harbach and Peyton, 1995) and contains species that were formerly placed within subgenus *Culex.* It has been speculated that *Deinocerites* arose from genus *Culex* stock

(Mallampalli, 1995; Navarro and Liria, 2000), yet it is currently undetermined which subgenus within *Culex* is most closely related to *Deinocerites*.

The purpose of previous phylogenetic studies involving subgenus *Culex* has been to elucidate the phylogeny of deeper relationships and so only a few species from subgenus *Culex* were used to represent the subgenus. Navarro and Liria (2000) examined four species from the subgenus in their morphological study. Shepard *et al.* (2006) examined six taxa in subgenus *Culex*, and Miller *et al.* (1996) examined ten taxa in subgenus *Culex*, seven of which are distributed in the region studied here. The study described here examined 17 species from the subgenus *Culex*.

The COII *gene*

No previous studies have used mitochondrial DNA to examine species-level relationships within subgenus *Culex*. Because of their rapid rate of evolution, mitochondrial genes tend to be polymorphic among and within species. A 705 base pair (bp) region of *COII* was used. This mitochondrial gene encodes a subunit of the cytochrome c oxidase enzyme, which is bound to the inner mitochondrial membrane and functions as the terminal enzyme in the respiratory chain (Gennis and Ferguson-Miller 1995). The subunit rests on the external side of the mitochondrial membrane where the copper at its center receives electrons from cytochrome c so that they may be passed on to the catalytic subunit I. In animals, thirteen subunits comprise the cytochrome c oxidase enzyme, three of which are coded for in the mitochondrial genome (including subunit II) and ten of which are coded for in the nuclear genome. This gene is highly conserved among all animals (Schmidt *et al.* 2001), presumably because of its crucial function in ATP production. The *COII* gene is found between genes for tRNA^{Leu} and tRNA^{Lyc}, which provide the 5' and 3' annealing sites for the polymerase chain reaction (PCR) primers used here.

Mitochondrial DNA (mtDNA) provides a good source of phylogenetic signal because of its maternal inheritance and rapid rate of evolution. Differences in the restriction fragment length polymorphism (RFLP) profiles of cryptic *Anopheles* species (e.g. Mitchell *et al.* 1992) demonstrate that even very closely related mosquito species can have detectable differences in their mtDNA. The different mutation rates at different codon positions within the *COII* gene have been found to be useful in determining relationships at different levels among the insects, although difficulty was encountered in resolving relationships among insect orders (Liu and Beckenbach 1992). Jinfu and Chaohui (2002) investigated the divergence among three mosquito genera in two subfamilies and Pruess *et al.* (2000) investigated simuliid phylogeny. Chen *et al.* (2003) use *COII* and a region of ribosomal DNA to investigate the phylogeny of *Anopheles* subgenus *Cellia* mosquitoes and to test for congruence of these two data sets.

The ITS *gene*

For the *ITS* data, a region of ribosomal DNA (rDNA) was sequenced. The region includes two noncoding regions, called internal transcribed spacers (*ITS*) 1 and 2, and the entire 5.8S ribosomal subunit gene. The 18S and 28S regions, which flank the molecule, contain the PCR priming sites used for amplification and sequencing and are excluded from analysis because these regions contain little variation. The 5.8S region is highly conserved and was useful in aligning the sequences, but was excluded from phylogenetic analysis. The entire cloned molecule is an average of 1145 bp in length with much variation among species. For brevity, the entire cloned region is often referred to here as “ITS”. The molecule exists in a tandem array in the nuclear genome and exists in many copies, each of which is referred to as a gene family member. From 500-600 copies per genome have been estimated in *Anopheles gambiae* (Collins *et al.* 1999) and 1200 copies per genome have been reported in *Aedes aegypti* (Park and Fallon 1990), although copy number can change across life stages. Gene conversion and unequal crossing over tend to conserve the nucleotide sequence among different members of this gene family. However, different sequences can be found among gene family members within the same specimen.

The rDNA in which the *ITS* regions are found encodes the RNA that, along with proteins, makes up the subunits of ribosomes. Because the end product is RNA, and not a peptide, the nucleotide sequence is not organized into codons. The coding regions are under selective constraint because the secondary structure of the ribosomal RNA is crucial to ribosomal function, and this selective constraint is evidenced by the highly conserved nature of these coding regions among all eukaryotic organisms. The gene is organized into three regions, which encode the 18S, 5.8S, and 28S ribosomal subunits. Each gene family member in the tandem array is separated by a noncoding intergenic spacer. Two additional noncoding regions, *ITS1* and *ITS2*, separate 18S from 5.8S and 5.8S from 28S, respectively. It is these two noncoding regions that are used here for phylogenetic analysis. In the living cell, these noncoding regions are cleaved from the RNA product, allowing for proper conformation of the ribosomal subunits.

The *ITS* regions lend themselves well to phylogenetic studies. The noncoding regions evolve rapidly, providing sufficient polymorphism to distinguish among very closely related species. Primers that anneal to the 18S and 28S coding regions can often be found in the literature and applied to a wide range of taxa. The coding regions have been used to investigate relationships within the infraorder Culicomorpha (Miller *et al.* 1997; Pawlowski *et al.* 1996). Relationships among anopheline species have been studied using the coding regions (Bargues *et al.* 2006) as well as the noncoding regions (Ma and Xu, 2005). Some studies use rDNA in combination with other gene regions to investigate anopheline relationships (Garros *et al.* 2005; Torres *et al.* 2006). Shepard *et al.* (2006) use the 18S region to study relationships among nine genera of mosquitoes. The noncoding *ITS* regions are used by Miller *et al* (1996) to study relationships within the genus *Culex*. Miller *et al.* (1996) were unable to distinguish among *Cx. pipiens* complex members, although this could be attributed to the fact that some specimens were collected within the hybridization zone for this complex.

The white *gene*

For the *white* data, a region of DNA was sequenced that spans most of the fourth exon and the 5' third of the fifth exon of the *white* gene, including two introns, the second of which has only been found in non-anopheline mosquitoes (Besansky and Fahey 1997).

In *Drosophila, white* is an X-linked gene that encodes a membrane-bound protein, called a traffic ATPase transporter, which binds ATP and participates in the transport of eye pigment precursor molecules into pigment cells of the eye (Ewart *et al.* 1994). Degenerate PCR primers were used to amplify and clone the orthologous gene in

Anopheles gambiae (Besanksy *et al.* 1995) and in *Aedes aegypti* (Coates *et al.* 1997).

Certain characteristics of this protein-coding gene make it attractive for phylogenetic study. The amino acid sequence can aid in the alignment process, or can be used as the character data for phylogenetic analysis. When working at the nucleotide sequence level, each of the three codon positions can be examined separately or differentially weighted. Because of the degeneracy of the genetic code, the third codon position is less constrained than the first two and more likely to retain mutations. This can lead to difficulty because multiple mutations may have occurred at the third codon position that would go undetected when examining extant species. The intron regions also provide relatively unconstrained characters, and the studied region of the *white* gene contains two introns for the ingroup taxa. The *white* gene is also of interest because it is a nuclear gene, thus not linked to the other protein-coding gene being studied.

Few current studies utilize the *white* gene for phylogenetic study of the Culicidae. Besansky and Fahey (1997) used *white* to study deep relationships among the Culicidae, examining 13 species from three subfamilies. The details of the research of Besansky and Fahey (1997) are explored in this paragraph. Nucleotide sequences of *white* were found to be very divergent at the third codon position, which raised the possibility that the phylogenetic signal could be saturated by multiple substitutions when studying such widely divergent taxa. However, use of the skewness test (Hillis and Huelsenbeck 1992), which assesses the phylogenetic signal of the data based on a distribution of tree lengths for randomly generated trees, provided no reason to exclude nucleotides in the third codon position. Codon bias was detected in this third codon position by calculating the effective number of codons (Wright 1990), which prompted the authors to speculate on the unreliability of *white* for their particular study. Many degenerate codon positions and variable sites were given zero weight when successive approximations weighting (Farris 1969) was employed. Besansky and Fahey (1997) were only able to recover the traditional phylogeny of the taxa when large parts of their data set were effectively removed from analysis by giving them zero weight. Intron data were not considered in the Besansky and Fahey (1997) study, presumably because the data were saturated with a history of mutations and difficult to align. The scope of the Besansky and Fahey (1997) study examined widely divergent species, such that the outgroup taxa were outside of the family Culicidae.

Later research (Krzywinsky *et al.* 2001) used *white* as one of four regions for inferring relationships within subfamily Anophelinae, and reported that *white* provided a useful level of phylogenetic signal at such a level of study. Of the few remaining phylogenetic studies of insects using *white,* Baker *et al.* (2001) included *white* among six gene regions used to study relationships among stalk-eyed flies (Diopsidae) and found the third codon position of *white* to be the most variable character from the three protein-coding nuclear regions studied. No previous studies have used *white* to examine relationships within a subgenus or within genus *Culex.*

Phylogenetic methods evaluated

Four different methods of phylogenetic analysis were explored here: neighbor-joining (NJ) distance, parsimony, maximum likelihood (ML), and Bayesian analysis. Each method was applied to each of the three data sets. The NJ distance method creates a phylogenetic tree based upon a distance matrix that measures genetic distance between all possible taxon pairs and joins the most similar taxa together. Several different distance metrics are available, which correct for multiple mutations at the same site; the trees presented here used uncorrected distances. The parsimony method selects phylogenetic trees that can be explained by the fewest number of evolutionary changes. The maximum likelihood (ML) method selects the phylogenetic tree that is the most likely to have occurred, given the data and a model of nucleotide transformation. Like ML analysis, Bayesian analysis of phylogenetic data uses a likelihood function, but differs in its methodology. While ML analysis scores the likelihood of heuristicly identified trees, given the data, Bayesian analysis scores the likelihood of the data, given a tree identified by a Markov-chain Monte Carlo exploration of possible trees. Bayesian analysis results in a single tree, and statistical confidence in each node of this tree is provided by posterior probability values. The methods were evaluated based upon their scientific merits as well as their ability to produce trees that score well on statistical tests when used on the particular problem at hand.

Dealing with multiple data sets

In recent years, the publication of phylogenetic research that utilizes data from multiple sources has grown in popularity. Within studies of culicid phylogeny, several researchers examined multiple genes, often from a combination of mitochondrial and nuclear sources (Chen *et al.* 2003; Garros *et al.* 2005a; Wilkerson *et al.* 2006). Examples also exist that examine morphological characters from multiple life stages (Reinert *et al.* 2004, 2006; Harbach and Kitching 2005), but few studies exist on the Culicidae that examine both molecular and morphological data (for an exception, see Garros *et al.* 2005b).

Much debate exists in the literature regarding whether different data sets should be combined or examined separately. In support of combining the data before analysis are Kluge (1989, 1998) and Miyamoto (1985), among others. However, Miyamoto openly concedes to changing his opinion and chooses that it is best not to combine data (Miyamoto and Fitch 1995), which is a viewpoint shared by de Queiroz (1993) and Marshall (1992), among others. Advocates of keeping the data separate argue that there may be legitimate biological reasons that explain the differences among data sets, and by combining the analyses, one loses the opportunity to explore the reasons for these differences. Such reasons could be incomplete lineage sorting, genetic introgression, differential base composition, or differential evolutionary rates. These factors contribute to the potential disconnect between a gene's history and the species history. On the other hand, the combination of data sets into a simultaneous analysis can have certain advantages, discussed later.

To determine whether separate analyses should be combined, a method for measuring the success of combined versus uncombined approaches is needed. The incongruence length difference test (ILD), also called the partition homogeneity test (Farris *et al.* 1994) provides a means of testing the homogeneity of different data sets, or different partitions of a single data set. Genes that support different phylogenies might have evolved under different conditions and should not be combined into a single analysis, which assumes a single evolutionary model (Bull *et al.* 1993). The ILD test calculates the length difference between the most parsimonious (MP) tree from a combined analysis and the sum of tree lengths from separate analyses. The statistical significance of the tree length from the combined analysis is determined by comparing it with a distribution of tree lengths produced through creating random partitions of the data. Combination of data sets into a simultaneous analysis is only advised when the partition homogeneity test shows significant homogeneity among data sets (Bull *et al.* 1993; Miyamoto and Fitch 1995).

The two approaches to dealing with multiple data sets are called the taxonomic congruence approach and the total evidence approach. The taxonomic congruence approach first analyzes the data separately, but then constructs a consensus tree of each of the gene trees. The taxonomic congruence approach is also called partitioned analysis. This approach is advocated by Lanyon (1993), Penny and Hendy (1986), and others. The appropriate techniques for constructing the resulting consensus of trees begin with the work of Adams (1972), who describes a consensus method that preserves all nestings present in the contributing source trees. The Adams consensus tree is conceptually different from the strict consensus tree, which collapses groups that do not appear in all contributing source trees. An additional method for constructing consensus trees is the semi-strict consensus, which preserves nestings that are not contradicted by another tree.

The total evidence approach involves taking each of the data sets (in this case, the nucleotide sequence alignments) and appending them to create a single large data set and then analyzing this data set by the phylogenetic method of choice. The total evidence approach has also been called simultaneous analysis. There are potential pitfalls to the total evidence approach. If the different data sets evolved under different conditions, combining them into a single matrix might force the investigator to assume a single model of evolution, which might fail to apply to some or all of the data. This problem has been solved in version 3 of the Mr.Bayes software package (Ronquist and Huelsenbeck 2003), which allows for different models to be applied to different partitions of the data. Yet the total evidence approach has certain advantages over taxonomic congruence. The total evidence approach has been demonstrated to uncover hidden phylogenetic signal that is otherwise not detected when data sets are analyzed separately (Barrett *et al.* 1991). As the name implies, the total evidence approach makes use of all the synapomorphies evident in the total data set and investigates the data directly, rather than assimilating summaries of previous analyses. By investigating the evidence directly, fewer *ad hoc* hypotheses are introduced. Finally, Eernisse and Kluge (1993) compared a total evidence approach and a taxonomic congruence approach when investigating amniote phylogeny and found only the total evidence approach to recover the traditional phylogeny with strong support, according to decay indeces (Bremer 1988) at each node. In the study described here, the selected data sets or trees were subjected to both the total evidence and the taxonomic congruence approaches because the superiority of either of these two approaches is still the subject of debate in the current literature (Fitzhhugh 2006).

Results

Nucleotide sequence and alignment of COII *data*

Nucleotide sequences for a 705 bp region of the *COII* gene were determined for 36 specimens from 23 species. The sequence is well conserved and does not contain introns, facilitating manual alignment. Codon positions were determined by translating the sequence with the invertebrate mitochondrial genetic code and ensuring that the amino acid sequence conformed to that published by Ho *et al.* (1995). Like most insect mitochondrial genes, *COII* has an AT-rich codon bias, explaining some lack of diversity at the nucleotide sequence level.

There were 491 invariable sites and of the remaining 214 variable sites, 66 were autapomorphies, leaving 148 parsimony-informative sites. The entire region consists of coding DNA with 145.76 synonymous sites and 559.24 nonsynonymous sites (real numbers represent an average among all sequences.) There were 32 nonsynonymous changes detected and 155 synonymous changes. The average number of nucleotide differences per synonymous site (Π_S), with the Jukes and Cantor correction for multiple hits, is 0.34618. The average number of nucleotide differences at nonsynonymous sites (Π_a), with the Jukes and Cantor correction, is 0.01913. The ratio of Π_a / Π_s is 0.05597, which is well below 1, as would be expected for a gene under a high level of functional constraint.

Phylogenetic analysis of COII *data*

The *COII* data was partitioned into its three codon positions, which served to be useful for inferring phylogeny. When the second codon position was analyzed alone (not shown) the resulting trees were characterized by very poor resolution and placement of outgroup taxa on internal nodes of the ingroup. The poor resolution suggested that the second codon position contains little phylogenetic signal, and the unusual topology suggested that its data are misleading. Because there is so little phylogenetic signal in the second codon position of *COII* (only 5 parsimony-informative sites), this position is easily swayed by a few homoplasious characters. The second codon position was excluded from all analyses based on this finding and on the finding that, of the three codon positions, only the first and third positions were congruent with each other under the ILD test.

For the NJ distance analysis of each of the three genes, different distance metrics were tested initially, but they tended to yield the same results as the simpler, uncorrected, distance, possibly because the species are so recently diverged that multiple mutations have not had time to accumulate. Also, the AT-rich bias of mitochondrial DNA would reduce the effects of multiple mutations, since the molecule has fewer bases to which it is likely to mutate.

The NJ distance analysis of *COII* (Fig. 2.1) has a minimum evolution score (sum of all branch lengths) of 1.02464. The 50% majority-rule bootstrap support for this tree resulted in a less well resolved tree (Figure 2.2). The bootstrap values indicate that a few relationships are well supported. Well supported relationships are the closeness of the *Culex pipiens* complex members, a (*Culex secutor* + (*Culex salinarius* + *Culex erythrothorax*)) clade, and a sister relationship of *Culex tarsalis* and *Culex stigmatosoma*. There is also strong support for a clade containing *Culex mollis, Culex declarator, Culex nigripalpus, Culex mollis* A, and *Culex chidesteri*, although relationships among these species are ambiguous.

Parsimony analysis of the *COII* gene resulted in 14 MP trees with a length of 540 steps and a rescaled consistency (RC) index of 0.3229. A strict consensus of all 14 MP trees is presented in Figure 2.3. This tree is well resolved considering that it is a strict consensus of 14 different trees and illustrates the areas of conflict among MP trees, which appear as polytomies. One contentious area arose in determining the relationship among *Cx. mollis, Cx. nigripalpus,* and a clade consisting of (*Cx. declarator* + (*Cx. mollis* A + *Cx. chidesteri*)). Note that the interrelationship among these taxa was poorly resolved in the NJ distance analysis as well. Ambiguity also arose regarding the relationship among *Culex janitor, Culex habilitator,* and a sister grouping of *Culex tarsalis* and *Culex stigmatosoma*. The MP trees disagree on the relationship of this group to another group consisting of a (((*Cx. salinarius* + *Cx. erythrothorax*) + *Cx. secutor*) + *Culex thriambus*) clade and to *Culex* (*Phenacomyia*) *lactator.* All MP trees place subgenus *Phenacomyia* within subgenus *Culex*, although its exact position within subgenus *Culex* is ambiguous. Finally, the MP trees place *De. cancer* as sister to the subgenus *Melanoconion* outgroup taxa and not within subgenus *Culex.*

The ML analysis used a GTR+I+G model (Tavare, 1986), which had been suggested by the hierarchical likelihood ratio test (hLRT) within Modeltest 3.7 (Posada and Crandall, 1998). After five iterations of estimating the model parameter values, a tree with negative log likelihood of 3061.81451 was found (Figure 2.4). The nodes of certain clades in this tree have been labeled A, B, C, or D to aid in later discussion. This tree shares many features with the previously mentioned analyses, especially the parsimony analysis. All three methods discussed so far agree on the close relationship among *Cx. janitor, Cx. habilitator, Cx. stigmatosoma,* and *Cx. tarsalis*, while the parsimony and ML analyses also agree that *Culex restuans* forms a sister taxon to this group, as seen in clade A. The parsimony and ML analyses also agree with the placement of *Culex interrogator* as sister taxon to the non-*Cx. pipiens* complex ingroup despite the fact that the NJ distance analysis suggests a very different position for *Cx.*

interrogator. All three analyses agree on a (*Cx. secutor* + (*Cx. salinarius* + *Cx. erythrothorax*)) clade, as shown in Clade B. The ML analysis places *De. cancer* within the *Melanoconion* outgroup, as does the NJ distance analysis. Conversely, *De. cancer* is sister to the *Melanoconion* outgroup in the parsimony tree.

Bootstrap support values for the ML tree were determined using 100 pseudoreplicates and a 50% majority rule consensus tree is presented in Figure 2.5. Poor bootstrap support values of <70% are found for some conspecific groups, such as the pair of *Cx. chidesteri* specimens and the pair of *Cx. nigripalpus* specimens. These low scores may have arisen due to sampling error because of the small number of pseudoreplicates. Only 100 pseudoreplicates were run because of the demand on microprocessor memory that is used for ML bootstrapping. Low bootstrap support is also seen for the placement of *De. cancer* at an internal node within subgenus *Melanoconion.* Clade A (Fig. 2.4) has low bootstrap support (<50%) and is collapsed in this tree. However, this clade does appear with all four methods used with the exception of the NJ distance method, which excluded *Cx. restuans* from this clade.

The Bayesian analysis used the same GTR+I+G model as did the ML analysis, with ranges for the parameter values estimated during the analysis. The Bayesian analysis was run for 6 x 10^6 generations. Stationarity of the two simultaneous runs was assessed to have been reached because the average standard deviation of split frequencies was 0.00392, the plot of generation versus log probability of the data showed no trends toward increase or decrease, and the potential scale reduction factor for each parameter was 1.00. A summary tree was created after eliminating a "burn-in" of 6000 generations (Figure 2.6).

The mean model parameter values determined by Mr. Bayes v 3.0 are consistent with the model parameters estimated for ML analysis. Both methods assume a high stationary state frequency of A and T nucleotides, and this aspect of the model is consistent with the A + T rich nature of this gene in insects (Liu and Beckenbach 1992). The model also assumes a high rate of C-T transitions. The Bayesian analysis tree is similar to the previously discussed *COII* trees, with better resolution.

The results from each of the methods of phylogenetic analysis were found to be congruent, with very few differences among them. PAUP* was used to determine a strict consensus tree of the trees produced by all four methods (Figure 2.7). Although this tree is not well resolved, it does illustrate a few points about 15the phylogeny of the *COII* gene. All phylogenetic methods agree on the presence of a (*Cx. secutor* + (*Cx. salinarius* + *Cx. erythrothorax*)) clade and on a sister relationship between *Cx. tarsalis* and *Cx. stigmatosoma*. All four methods agree on a close relationship among *Cx. mollis, Cx. nigripalpus, Cx. declarator, Cx. mollis* A, and *Cx. chidesteri*, with the latter three species forming an internal subclade. The monophyly of the *Cx. pipiens* complex is agreed upon by all four methods. All four methods place *Cx. (Phe.) lactator* on an internal branch within the subgenus *Culex,* although its exact position within subgenus *Culex* is not always the same. Finally, *De. cancer* joins the subgenus *Melanoconion* outgroup in all analyses.

A second strict consensus tree among methods was made without including the NJ distance tree (Fig. 2.8). This consensus tree illustrates the close relationship among *Cx. restuans, Cx. janitor, Cx. habilitator, Cx. stigmatosoma,* and *Cx. tarsalis* that was found in all analyses except the NJ distance analysis, which places *Cx. restuans* elsewhere. The position of *Cx. thriambus* is ambiguous in that it differs with each analysis.

Nucleotide sequence and alignment of ITS *data*

Nucleotide sequences for the ITS region were determined for 39 specimens, representing 26 species. This alignment includes the coding regions, although these regions are not used for the phylogenetic analysis. The alignment has a total length of 1500 characters. Regions that aligned ambiguously were excluded from analysis, and only well aligned regions were presumed to be homologous. Well aligned sites make up 116 characters from *ITS1* and 124 characters from *ITS2*. Within the internal transcribed spacers, 891 characters that appear to align ambiguously have been removed from the analysis, leaving 240 characters to be used for analysis. These characters contain sufficient phylogenetic signal so that they produce well resolved trees that are almost strictly bifurcating.

The number of parsimony-informative sites differs between the *ITS1* and *ITS2* regions. The total *ITS1* region has 262 and the total *ITS2* region has 325 parsimony-informative sites. When only the well-aligned regions are considered, *ITS1* has 94 parsimony-informative sites and *ITS2* has 52 parsimony-informative sites. This is far more informative than the coding regions, which contain only 12 parsimony-informative sites. Thus, nearly all of the phylogenetic signal necessary for distinguishing relationships among these taxa is found within the noncoding regions.

Phylogenetic analysis of ITS *data*

Firstly, a NJ distance tree with uncorrected distances was constructed in which both *ITS1* and *ITS2* are analyzed simultaneously (Figure 2.9). This tree has a minimum evolution score of 1.67483. Bootstrap support values were determined for the NJ distance tree by running 1000 pseudoreplicates (Figure 2.10).

Three parsimony analyses were conducted. The first analysis used both *ITS1* and *ITS2* regions, which were analyzed simultaneously. This first analysis found a single MP tree of length 3537 steps and a RC index of 0.4084 (Figure 2.11). Bootstrap support values for this analysis are shown in a separate tree (Figure 2.12). Most relationships are well supported on this tree, with the exception of the position of *Cx. salinarius* relative to a sister grouping of *Cx. erythrothorax* and *Cx. secutor,* as well as the internal arrangement of some *Cx. pipiens* complex specimens.

The second parsimony analysis used *ITS1* alone. This analysis found two MP trees of length 4245 steps and RC=0.3520 (strict consensus Fig.2.13). The only disagreement between these MP trees was in the internal relationships among the *Cx. pipiens* complex members. Bootstrap support values for this analysis are shown in Figure 2.14.

The third parsimony analysis uses *ITS2* alone. Parsimony analysis found six MP trees of length 1657 steps and RC=0.3840 (strict consensus Fig. 2.15). Bootstrap support values for this analysis are presented in Figure 2.16.

Tofi evaluate the differences between the results of parsimony analysis of *ITS1* versus *ITS2*, a strict consensus of the trees presented in Figures 2.13 and 2.15 was constructed (Figure 2.17). This tree demonstrates that three major lineages can be found within subgenus *Culex* whether analyzing *ITS1* or *ITS2*. The first of these lineages consists of an assemblage of *Cx. interrogator, Cx. chidesteri, Cx. habilitator, Cx. janitor, Cx. tarsalis, Cx. stigmatosoma, Cx. thriambus, Cx. nigripalpus,* and the subgenus *Phenacomyia*. The second lineage consists of the species *Cx. erythrothorax, Cx. secutor, Cx. salinarius, Cx. declarator, Cx. mollis, Cx. coronator,* and *Cx. restuans*. The third lineage, which is basal to the remaining ingroup taxa, contains the *Cx. pipiens* complex members. The internal relationships inside each of these major clades are poorly resolved in this consensus tree because the *ITS1* and *ITS2* regions disagree regarding some aspects of these internal relationships.

The ML analysis was run three times, once with *ITS2* alone, once with *ITS1* alone, and once with *ITS1* and *ITS2* analyzed simultaneously in the same data set. For the ML analysis of *ITS2*, Modeltest recommended the HKY + G model (Hasegawa *et al.* 1985). The HKY model assumes two nucleotide substitution types, transitions and transversions, and unequal base frequencies. The HKY+G model has one additional parameter, allowing for gamma distributed rate variation. Precise parameter values and tree maximum likelihood values settled after only two iterations of optimizing the most likely tree found by a heuristic search. The estimated transition:transversion ratio was 1.328519, and gamma distributed rates had a shape parameter (alpha) of 0.512852. The estimated base frequencies were A=0.231812, C=0.327926, and G=0.231761. The resulting tree is presented in Figure 2.18. As was seen in the parsimony analysis, this tree also finds three major lineages within subgenus *Culex,* although the exact species composition of each of these lineages differs from that of the parsimony analysis. In particular, the species *Cx. thriambus* and the subgenus *Phenacomyia* are associated with different lineages in the ML analysis compared with the parsimony analysis. Also, *Cx. tarsalis* is not placed within any of these three lineages in the ML results for *ITS2*.

For the *ITS1* data, Modeltest recommended the HKY+I+G model. This is nearly the same model as that used for *ITS2*, but with the added parameter of having a proportion of invariant sites. Parameter values for this model were refined by successive iterations in PAUP*, and were slightly different from the parameters for *ITS2*, above. The estimated transition:transversion ratio was not much different at 1.055403. However, the shape parameter was higher (2.728332) and there was also an estimated proportion of invariant sites, although this proportion was low (0.027989). The resulting ML tree from the *ITS1* data is presented here as Figure 2.19. The *ITS1* data again presents three major lineages within subgenus *Culex*. For the *ITS1* data these major lineages contain the same taxa that were found in the major lineages from the parsimony analysis of *ITS1* and *ITS2* together.

Since Modeltest suggested two similar, yet slightly different, models for *ITS1* versus *ITS2*, the hLRT test was performed to determine whether the two different models performed significantly differently on the same data set. The hLRT was performed as described by Posada and Crandall (2001), which requires that the likelihoods be calculated for the same data under two different models, one of which is a special case of the other. The data set used for the test was the combined *ITS1+ITS2* data. Under the more parameter-rich HKY+I+G model, the negative log of the maximum likelihood was 14620.68805. Under the slightly less parameter-rich HKY+G model, this same data set has a negative log maximum likelihood of 14715.25146. The test statistic is

$$\delta = 2(\ln L_1 - \ln L_2),$$

where $\ln L_1$ is the maximum likelihood under the more parameter-rich model and $\ln L_2$ is the maximum likelihood under the less parameter-rich model. The value of δ = 189.12682, which was compared with a χ^2 table with one degree of freedom, because of the one added parameter (a proportion of invariant sites) in the more complex model. The very low p value indicates that the more parameter-rich model significantly improves the maximum likelihood, more than would be expected due to chance effects from merely adding another parameter. For the third ML run, in which *ITS1* and *ITS2* are analyzed simultaneously in the same data set, the HKY+I+G model was therefore used. The resulting tree has a negative log likelihood value of 14620.68805 (Figure 2.20). This tree finds three major clades within subgenus *Culex* and the species composition of these major clades matches that found in the parsimony analyses and in the ML analysis of *ITS1* alone.

The Bayesian analysis was run a single time, using the combined *ITS1* and *ITS2* data. The general model type entered into Mr.Bayes contained 2 substitution types, a proportion of invariant sites, and gamma distributed rate variation. Bayesian analysis was run for 1 x 10^6 generations. When analysis was stopped, the standard deviation of split frequencies between the two chains was 0.002406, suggesting that the analysis had reached stationarity. After removing a "burn-in" of 1000 generations, a summary tree was produced (Figure 2.21). This tree also presents three major clades within subgenus *Culex,* with the same species composition in each major clade that was seen in the parsimony analysis and in all ML analyses except when *ITS2* was examined alone.

In order to visualize the similarity among the results of the four methods of phylogenetic analysis used here, a consensus was made among selected trees. Figure

2.22 shows a strict consensus among all four methods when *ITS1* and *ITS2* are analyzed simultaneously. This tree shows the three clades within subgenus *Culex*. This tree contains polytomies where the different methods disagree on precise branching order.

Nucleotide sequence and alignment of white *data*

Because of difficulties in the cloning and sequencing procedures, *white* nucleotide sequences were obtained for only 19 specimens, representing 18 species. *Deinocerites cancer* has a longer sequence of 912 bp due mostly to repeated A and T bases within the first intron. Two outgroup taxa that are unique to the *white* data (*Tripteroides bambusa* and *Aedes triseriatus*) were included in the alignment because of the availability of their nucleotide sequences in Genbank and because they align well with the ingroup taxa. When the non-*Culex* species were included, the alignment resulted in a matrix with 978 positions, with gaps being introduced only within the introns and at the site of a possible indel event, evidenced by 60 extra nucleotides found for *Cx. nigripalpus*.

Phylogenetic analysis of white *data*

A NJ distance tree was constructed with bootstrap support values (Figure 2.23). The bootstrap tree shows that the *Cx. pipiens* complex can be clearly separated from the remaining subgenus *Culex* taxa, but that other deep relationships are not well supported.

Early attempts at parsimony analysis were performed using the inferred amino acid sequence, but these sequences were so highly conserved that nearly the entire ingroup was represented in a polytomy (not shown). Thereafter, parsimony analysis was performed on several different partitions of the nucleotide sequence data set. When the entire alignment was used and all characters given equal weight, two MP trees were found, with length 1134 steps and CI= 0.6111, one of which is presented in Figure 2.24. The topology of this tree differs from that of the NJ distance analysis in the deeper arrangements of the major clades and in the branching order of a clade containing *Cx. habilitator, Cx. nigripalpus,* and *Cx. interrogator.* Reweighting of characters by successive approximations character weighting (Farris 1969) resulted in a grossly similar tree with a much shorter tree length (411.57310 steps) and higher CI=0.8475 (Fig. 2.25). The shorter tree length and higher CI are inevitable consequences of the reweighting procedure, which downweights homoplasious characters. The non-integer value of the tree length is also a consequence of the reweighting procedure, since the number of steps that a character can contribute to a tree's length can now be less than one. Once enough iterations have been run such that the tree topology has stabilized, it is believed that the tree better matches the true phylogeny because homoplasy (convergence, reversal, or parallelism) has been minimized (Farris 1969). Continued iterations were unnecessary in this case, since the tree topology remained stable after a single reweighting procedure.

Parsimony analysis was also conducted with the coding regions alone, resulting in 102 MP trees of length 670 and CI= 0.6537. After reweighting of characters twice by successive approximations, a single MP tree was found, with length 281.03830 and CI= 0.9086 (Figure 2.26). The topology of this tree differs from those produced by the other data sets, in that the *Cx. pipiens* complex is given a derived position. This topology is found even when additional outgroup taxa were included to ensure that the primitive state was well represented (not shown). When parsimony analysis was performed on the introns alone, which used 194 characters with 118 parsimony-informative characters, five

MP trees were found, with length 605 steps and CI= 0.6066. Reweighting by successive approximations resulted in a single MP tree being found for the intron data, with length 213.70754 steps and CI= 0.7879 (Figure 2.27). This tree recovers the basal position of the *Cx. pipiens* complex, although the remaining topology still differs from all other analyses.

Parsimony analysis was also performed on each of the three codon positions separately. Each run contained 248 characters. The first codon position had 17 parsimony-informative sites, the second codon position had only 12 parsimony-informative sites, and the third codon position had 137 parsimony-informative sites. The first codon position produced 54 MP trees on the first run, and four MP trees after reweighting of characters, with a length of 63.95238 and CI= 0.9211. A strict consensus of these four MP trees was not well resolved (Figure 2.28). The second codon position produced 40 MP trees on the first run, and 12 after reweighting characters twice, with length 36.25 steps and CI= 0.9517. These MP trees are poorly resolved, presumably because of the low number of parsimony-informative sites and are not shown here. The third codon position produced eight MP trees on the first run, with length 549 steps and CI= 0.6357. All eight trees were well resolved, compared with the first two codon positions, but they all place the *Cx. pipiens* complex in a derived position. One of these MP trees is presented in Figure 2.29. Reweighting of characters did not recover the basal position of the *Cx. pipiens* complex.

Maximum likelihood analyses were performed on each of six partitions of the data, using the models suggested by Modeltest. When the entire alignment was used, and the HKY+I+G model was optimized, a single tree was found with negative log likelihood

= 6216.72777 (Figure 2.30). This result differs from the parsimony result that used the same data and does not present the *Cx. pipiens* complex in a basal position relative to the remaining subgenus *Culex* species. When only the coding regions were used, along with optimized parameters of the K80+I+G model, a tree with negative log likelihood= 4061.01337 was found, which differs in topology from the tree that uses the entire data set, but still has the derived position for the *Cx. pipiens* complex. When using only the introns and the optimized HKY+I+G model, a tree with negative log likelihood= 1974.92947 was found, which does present the *Cx. pipiens* complex in the basal position, but it moves two outgroup taxa to an internal branch within *Culex* (Figure 2.31).

The ML analyses that excluded the third codon position or the introns did produce topologies that are somewhat consistent with other analyses and with the NJ distance and parsimony analyses of *white*. It seems likely that these third codon positions had diverged to the point at which their phylogenetic signal had become unreadable, as was suggested when Besansky and Fahey (1997) used *white* to study more distantly related taxa. Modeltest was used to suggest a model when the third codon is excluded and another when both the third codon and the introns are excluded. Modeltest suggested a HKY+I+G model when the third codon is excluded, and the resulting tree is presented as Figure 2.32. The topology of this tree approaches the results observed in earlier analyses. When both the third codon position and the introns are excluded, and the suggested HKY+G model was used, a tree with negative log likelihood = 1455.70818 was found (Figure 2.33). This tree is less well resolved than the tree that includes the introns, presumably because it is lacking the highly variable characters found in the introns. The result of excluding the third codon position and the introns was that the basal position of the *Cx. pipiens* complex was recovered, but the remaining nodes of the tree are still inconsistent with earlier results.

Bayesian analysis was performed with the third codon position and introns excluded, two substitution types, and gamma distributed rate variation. The analysis was run for 1 x 10^6 generations, at which time the standard deviation of split frequencies was 0.007364 and the potential scale reduction factor for all estimated parameters was 1.000, suggesting that the run had reached stationarity. The resulting summary tree (Fig. 2.34) was not well resolved and has a basal polytomy of four clades plus *Cx. mollis*. The separation of the tree into the five groups of the basal polytomy is supported by a posterior probability value of 0.72 and each of these groups is found in other analyses. In hopes of improving the resolution of the deeper relationships, the Bayesian analysis was run again, the conditions were the same except that this time the intron data was included and a proportion of invariant sites were assumed. This still resulted in a poorly resolved tree (Figure 2.35). The split that separated *De. cancer* from the ingroup is absent when introns are included.

Simultaneous analysis of COII *and* ITS *data by Bayesian analysis method*

The *COII* and *ITS* data were found to be congruent by the ILD test. All four methods of phylogenetic analysis were used on a simultaneous analysis of the two data sets, with comparable results in that each analysis shows the same three major clades, one of which is the *Cx. pipiens* complex. The Bayesian analysis (Figure 2.36) was preferred because of the ability to apply different models to each data partition. Bayesian analysis was run on the combined *COII* and *ITS* data for 2.1x10^6 generations, at which time the average standard deviation of split frequencies was 0.0125 and the potential scale reduction factor for each estimated parameter was at or near 1.000.

Figure 2.39 is a nucleotide alignment that shows only the parsimony-informative characters of the total evidence data. Taxa are displayed in the same order as in Figure 2.36 for quick comparison. Because this alignment does not contain the complete, intact, sequences, the various regions of each gene are not easily recognized. However, this figure is a useful tool for visualizing the many sequence variations that contribute strongly to the tree topology. Trends that distinguish the outgroup from the ingroup and the *Cx. pipiens* complex from the remaining taxa are apparent. Trends can be found that separate the other major lineages within subgenus *Culex,* but are not as readily apparent.

Discussion

Interpretation of the COII *data analyzed with ML in Figure 2.4*

The *COII* ML tree shown in Figure 2.4 is consistent with many morphological traits. The most internal node of Clade A presents a sister relationship of *Cx. tarsalis* and *Cx. stigmatosoma*, which, in the adult stage, share white tarsal rings and a white ringed proboscis as well as strong teeth on the male phallosome (Carpenter and LaCasse, 1955). However, the fourth instar larvae of these two species are not morphologically similar. The well-supported sister relationship between *Cx. salinarius* and *Cx. erythrothorax,* shown in clade B, makes sense with respect to the fourth instar larvae of these species, which have similar setal arrangements on the siphon and both have a glabrous thoracic integument. These two species also have morphologically similar adult male genitalia, with six to twelve strong teeth on the plates of the phallosome (Carpenter and LaCasse, 1955).

Clade C contains *Cx. nigripalpus,* and *Cx. chidesteri,* which are morphologically similar as adults, both having dark-scaled abdominal terga. In light of these morphological similarities, it is not surprising that clade C is found in this phylogenetic analysis; however, the internal arrangement among the species does not group the most morphologically similar species into the same lineages. It is important to note that the bootstrap support for this internal arrangement is low (Fig. 2.5).

Clade D is a polytomy consisting of all of the *Cx. pipiens* complex specimens, which are morphologically similar. These species can be reliably distinguished morphologically only by measuring the DV/D ratio of the adult male genitalia (Sundararaman 1949; Barr 1957).

Recognized vectors of Flaviviruses and Alphaviruses are scattered throughout the phylogeny and are seen within each of the major clades discussed here. Clade A contains *Cx. tarsalis*, the primary vector of Western Equine Encephalitis (WEE) virus as well as a primary vector of West Nile (WN) virus in the western U.S. Clade A also contains *Cx. stigmatosoma,* which is believed to have a role in Saint Louis Encephalitis (SLE) virus transmission, and *Cx. restuans*, an enzootic vector of WN virus. Clade B contains *Cx. salinarius,* which has a role in transmitting the Eastern Equine Encephalitis (EEE) virus (an Alphavirus) and SLE virus (a Flavivirus) as well as being a likely bridge vector of WN virus from avian cycles to mammals. Clade C contains *Cx. nigripalpus,* with demonstrated roles in EEE and SLE virus transmission. Finally, clade D contains the primary enzootic vectors of WN virus and epidemic vectors of SLE. The fact that only a portion of the subgenus *Culex* mosquitoes studied here are implicated in arbovirus transmission does not mean that the remaining species have no role in disease transmission. Many of the remaining species have not been sufficiently investigated for their potential role in arbovirus maintenance. The fact that species with demonstrated roles in arbovirus transmission are scattered throughout this subgenus *Culex* phylogeny suggests that vector competence has been gained or lost independently and that many additional species of subgenus *Culex* could have as-yet undetected roles as disease vectors.

Interpretation of ITS data analyzed by ML in Figure 2.20

A tree produced by the ML method is discussed here (Figure 2.20), which examined both *ITS1* and *ITS2.* The relationships presented in this tree are largely consistent with the other analyses. Selected clades presented in this tree have been labeled A, B, or C to aid in further discussion.

This phylogeny does not support the transfer of *Cx. lactator* from the subgenus *Culex* and into the subgenus *Phenacomyia*, as is proposed by Harbach and Peyton (1992), since it was not present *Phenacomyia* as a lineage that is distinct from subgenus *Culex.* Rather, this phylogeny places *Cx. lactator* on an internal branch of subgenus *Culex.* In this tree, *Cx. lactator* is presented as a member of Clade A. However, it should be noted that in the ML analysis of the *ITS2* data alone, *Cx. lactator* still arises from within subgenus *Culex,* but is more closely associated with the members of Clade B (Figure 2.18). The *COII* data also found that *Cx. lactator* arises from within subgenus *Culex.*

As with the *COII* data, genus *Deinocerites* does not join the subgenus *Culex*. Rather, *Deinocerites* is a sister taxon to subgenus *Melanoconion*. The present study did not include sufficient subgenera of *Culex* to determine which subgenus is most closely related to *Deinocerites*. However, the arrangement found here is consistent with Navarro and Liria (2000), who included seven subgenera of *Culex* in their study and did not find *Deinocerites* to be a close relative to subgenus *Culex*.

No morphological features, from any life stage, were identified that clearly separate Clade A from Clade B. At best, the adults of Clade A indicate a tendency toward white-banded or pale tarsi and probosci. However, as an exception, *Cx. secutor* has clearly banded tarsi, yet is a member of Clade B. Such an exception reinforce the concept that banded tarsi or probosci are not strong characters for phylogenetic inference. Some suggestion of this division into Clades A and B was also seen in the phylogenetic analysis of Miller *et al.* (1996), who did find *Cx. salinarius, Cx. erythrothorax,* and *Cx. restuans* as a distinct clade from *Cx. tarsalis* and *Cx. nigripalpus*. Clade C consists of the *Cx. pipiens* complex, which can usually be identified as adults by the curved white bands on the tergum and course golden-brown scales of the scutum. *Culex pipiens* complex members can usually also be identified as fourth instar larvae by a combination of their low siphon index and arrangement of four setal tufts on the siphon

Clade A includes the relationship ((*Cx. nigripalpus* + *Cx. stigmatosoma*) + (*Cx. interrogator* + *Cx. chidesteri*)). The sister relationship of *Cx. nigripalpus* and *Cx. stigmatosoma* is not seen in analyses of the other genes, although the two species do share a deeper relationship in many analyses. Likewise, the sister relationship of *Cx. interrogator* and *Cx. chidesteri* is not found among all analyses, but is shown in Figure

2.20. Only some general morphological similarity seems to tie these Clade A taxa together, yet the incorporation of additional taxa into future analyses may clarify these relationships. The subapical lobe of the basistyle on the male genitalia from the Clade A species is grossly similar and tends to share an arrangement of a single seta, a broad leaf, and three rods.

Of the Clade A taxa, the only ones included in the tree of Miller *et al.* (1996) are *Cx. nigripalpus* and *Cx. tarsalis*. Miller *et al.* (1996) were surprised by the apparent close relationship between *Cx. nigripalpus* and *Cx. tarsalis* on their tree, since the two species do not share much morphological similarity, and suggest that studying additional subgenus *Culex* taxa would help to resolve the relationship between these two species. Such an effort was made here, and it was found that Clade A, which includes seven species that were not studied by Miller *et al.* (1996), shows that *Cx. nigripalpus* and *Cx. tarsalis* are not closely related.

Clade B includes a relationship of (*Cx. salinarius* + (*Cx. secutor* + *Cx. erythrothorax*)). The morphological similarities between *Cx. salinarius* and *Cx. erythrothorax* are discussed above because a similar relationship was found with the *COII* data. The placement of *Cx. secutor* as sister species to *Cx. erythrothorax* instead of a (*Cx. salinarius* + *Cx. erythrothorax*) coupling in this tree differs from the *COII* results. It should be noted that the ML analysis of *ITS2* alone (Figure 2.18) retains the (*Cx. secutor* + (*Cx. salinarius* + *Cx. erythrothorax*)) clade that was seen for the *COII* data, but that all other *ITS* analyses presented here place *Cx. secutor* and *Cx. erythrothorax* as sister taxa. The hind tarsi of *Cx. secutor* adults are conspicuously banded, unlike either *Cx. salinarius* or *Cx. erythrothorax*, but this is not considered to be a strong character for phylogenetic inference (Edwards 1932). A glabrous thoracic integument is found in all three species, but this can also be found in many other species and can, thus, not be considered to be a synapomorphic character for this clade.

The basalmost species of Clade B is *Cx. restuans*. The position of *Cx. restuans* relative to *Cx. salinarius* and *Cx. erythrothorax* is consistent with Miller *et al.* (1996) who found a (*Cx. restuans* + (*Cx. salinarius* + *Cx. erythrothorax*)) relationship, but did not include five additional species found in Figure 2.20. The present study confirms the sister relationship between *Cx. salinarius* and *Cx. erythrothorax* and helps to resolve the relationship of these two species relative to *Cx. restuans* by including additional species.

Clade B shows a close relationship between *Cx. mollis* and *Cx. declarator*, which had also been seen in the *COII* analysis. The two species *Cx. declarator* and *Cx. mollis* are practically indistinguishable as adults, and the fourth instar larvae are only distinguished by Clark-Gil and Darsie (1983) using the position of the basalmost seta relative to the pectin spine.

Despite treating it as an ingroup taxon, the species *Lt. tigripes* joins the outgroup taxa. Until recently, this species was considered to be a member of the genus *Culex*, in the subgenus *Lutzia* (Tanaka, 2003). The larvae of *Lutzia* species are predacious and thus have modified larval mouthparts. It was the distinct nature of these mouthparts that prompted Tanaka to treat *Lutzia* as a distinct genus. The current study was not designed to determine the closest relative to *Lutzia* among the subgenera of *Culex*. However, previous studies have addressed this issue. Navarro and Liria (2000), who base their phylogeny on the characters of larval mouthparts, place both *Lutzia* and *Phenacomyia* on separate branches from subgenus *Culex,* although not with very strong bootstrap support.

Miller *et al.* (1996) placed *Lutzia* as sister taxon to subgenus *Culex* based on a Kimura-2-parameter-corrected NJ distance tree.

Clade C represents all of the *Cx. pipiens* complex members included in this study, which share very similar morphology. The internal arrangement of this clade is less certain than Figure 2.20 suggests. In other analyses, including bootstrap support trees, the *Cx. pipiens* complex members often appear in different arrangements or in a polytomy. The basal position of the *Cx. pipiens* complex relative to the remaining subgenus *Culex* mosquitoes complies with the concept that the *Cx. pipiens* complex mosquitoes are Old World taxa, and thus form a different lineage from the New World taxa seen in the rest of the tree.

Deinocerites cancer arises within the genus *Culex*, but never from subgenus *Culex*. This phylogenetic position is consistent with Navarro and Liria (2000), who found *De. melanophylum* to be a close relative to *Melanoconion,* but not to subgenus *Culex*. *Deinocerites* species are morphologically distinct from genus *Culex* in the fourth instar larva in that the *Deinocerites* head capsule is more circular and has a triangular pouch laterally; also the *Deinocerites* anal segment has two distinct, well separated plates instead of the completely ringed anal segment of genus *Culex*. Also unlike *Culex,* the antennae of *Deinocerites* adults have a long first flagellar segment and the male antennae are not plumose. Figure 2.20 suggests that *Deinocerites* arose from within the genus *Culex*. Although the closest subgenus to *Deinocerites* has not yet been determined, the current study excludes subgenus *Culex* as being this closest relative.

Difficulties with white *for phylogenetic inference*

Difficulties did arise in using *white* for this study. The gene posed a technical challenge in the molecular procedures. The previously published degenerate PCR primers used for amplification are non-specific, resulting in amplification of several extraneous molecules. Although the correct molecule could usually be determined based upon its molecular weight on an agarose gel, this technique was not always successful. Additional unexplored problems in the subsequent steps of the cloning process thwarted efforts to obtain *white* nucleotide sequences from several taxa. Krzywinsky *et al.* (2001) report cloning difficulties as well. This difficulty could arise because of the variable length of introns as well as the variable presence/absence of introns reported for this gene (Kryziwinsky and Besansky 2002).

Difficulties encountered by Besansky and Fahey (1997) in utilizing *white* for phylogenetic inference foreshadowed the difficulties encountered here. In order to recognize relationships among widely divergent taxa, Besansky and Fahey (1997) resorted to analyzing select regions of the gene. When select regions were also analyzed in the current study, relationships among the subgeneric taxa were poorly resolved, presumably because insufficient phylogenetic signal was found among closely related species.

A sister relationship between *Cx. thriambus* and *Cx. stigmatosoma* is found in nearly all *white* analyses, although the position of *Cx. thriambus* was ambiguous when examining *COII* or *ITS*. As adults, both species have banded tarsi, but few other obvious morphological features in common at any life stage. The two species also have overlapping distributions in the U.S., covering the western coast and most of Texas, although *Cx. thriambus* has a wider reported geographic range that includes Central

America. The monophyly of the *Cx. pipiens* complex is strongly supported and confirmed by all analyses. It is not unexpected that such a highly conserved gene as *white* would not contain sufficient polymorphism to resolve the relationship of these two close relatives. A sister relationship between *Cx. salinarius* and *Cx. restuans* is found in the *white* analyses, whereas the placement of *Cx. restuans* was difficult to determine with *COII* or *ITS*. These two species are both SLE vectors, and can be difficult to distinguish as adults when specimens are damaged, which prompted Crabtree *et al.* (1995) to design a PCR protocol to aid in identifying specimens, but if specimens are well preserved, the two species can be readily distinguished as adults or larvae. Another common feature among nearly all of the *white* analyses is a close relationship among *Cx. habilitator, Cx. nigripalpus,* and *Cx. interrogator.* These three species were also found to be closely related when using *ITS*, but not when using *COII*. No obvious morphological features unite these taxa, but they do share a gross resemblance of features on the subapical lobe of the basistyle of the male genitalia.

The *white* gene phylogeny does not agree with previously published phylogenies or with the *COII* and *ITS* results. The NJ distance and parsimony methods yielded results that are similar to each other, but that differ considerably from the ML and Bayesian results. The ML and Bayesian analysis methods produced results that were clearly at odds with traditional taxonomy, such as placing *Tripteroides* and *Aedes* species within subgenus *Culex* (Figure 2.31). The same general methodology was used in the *white* analysis that was used in the *COII* and *ITS* analyses, and yet the latter two genes produced results that were more consistent with previously published works.

The reweighting of characters by successive approximations, conducted as part of the parsimony analysis, reduced the number of MP trees. Since this reweighting procedure downweights characters based on their effect on the RC, it is effectively reducing the effect of homoplasious characters. Before reweighting, a large number of MP trees were found because of the presence of homoplasy. Attempts to reduce the weight of these homoplasious characters, often to the point of excluding them entirely, often served only to produce polytomies in the ingroup. The same approach of reweighting was used by Besansky and Fahey (1997) and successfully recovered the traditional phylogeny, but the taxa they studied were far more divergent.

The region of *white* studied is of medium length for this research, but is quite small if only the variant sites are considered (105 characters, 36 of which are parsimony-informative when the third codon position and introns are excluded). With such a small number of characters, stochastic effects such as random genetic drift could bias the analysis. The nucleotide sequence alignment is unlikely to have been flawed, since it was guided by inferred amino acid sequences, which matched very well with published sequences. The model of nucleotide transformation used for ML analysis might be suspect. Several different models were tried, as were several different partitions of the data set, yet the ML results were still either inconsistent with previous results or were poorly resolved. Bayesian analysis should overcome problems that arise from over-parameterizing the model because of the way "nuisance parameters" are marginalized. However, the Bayesian analysis tended to produce poorly resolved trees, suggesting that marginalization of the nuisance parameters only served to weaken the resolution. All of this suggests that *white* simply does not possess enough phylogenetic signal to allow resolution of relationships within subgenus *Culex*, and that what variation *white* does possess is homoplasious.

Evaluating the three genes for utility in phylogenetics of subgenus Culex

Phylogenetic analyses have been presented based on the nucleotide sequences from three different genes: *COII*, *ITS*, and *white*. These three genes were selected based on their utility in published studies as well as their independence from each other in the mosquito genome. Certain elements of each gene make it operationally independent. For example, *ITS* does not encode amino acids, and so nucleotide changes do not cause protein changes that might be acted upon by selection, as they would be for the two protein-coding genes. The *ITS* region differs from *COII* and *white* in that it exists in multiple tandem repeats, which evolve in concert, rather than in a single copy. Alternatively, *COII* is a mitochondrial gene and is thus inherited by a different mechanism than the two nuclear genes, and is processed by mitochondrial machinery, such as DNA polymerase γ, which is subject to unique base composition biases and proofreading mechanisms (Kaguni 2004). The importance of independence of data sets has particular relevance in molecular phylogenetic studies. Because of gene linkage, loci can fail to evolve independently from each other, since they assort in a nonrandom fashion during meiosis. However, the three data sets studied here are probably independent because of many of the differences mentioned.

The genes used for each data set have their own unique problems and mechanisms affecting their evolutionary paths. The *COII* gene is part of the mitochondrial genome and is thus maternally inherited through the cytoplasm. In animals, mitochondrial genes are generally characterized by rapid base substitutions, which occur at a rate that is five to ten times higher than single copy nuclear genes (Brown *et al.* 1979). The enzyme DNA polymerase γ is unique to the mitochondria and is involved in the replication, recombination, and repair of the mitochondrial genome. The rapid evolution of mitochondrial genomes is believed to be due in part to the limited DNA repair machinery of the DNA polymerase γ enzyme (Kaguni 2004). The mitochondrial genome is also subject to base composition biases that are caused by nucleotide pool imbalances found in the mitochondria (Kaguni 2004).

The mechanisms of the evolution of *ITS* are quite different from the other two genes. The *ITS* region is nuclear and is thus inherited sexually with the nuclear genome. The two internal transcribed spacers evolve very rapidly because they probably do not encode biologically functional molecules and are thus assumed to be free of any functional constraints. Because they exist in highly repeated tandem arrays, members of the *ITS* array evolve in concert, by such mechanisms as gene conversion and unequal crossing-over.

The coding regions of the *white* gene, especially the first and second codon positions, are characterized by highly conserved nucleotide sequence. This high level of conservation is due, presumably, to the fact that *white* is an active gene that encodes a functional protein. The fact that the encoded protein is a putative membrane-bound traffic ATPase transporter indicates that there would be much need for conservative sequences that encode the membrane-spanning regions, the ATP-binding region, and the substrate-binding regions. Other factors potentially affecting the evolution of *white* are the apparent indel events that have produced an erratic pattern of intron presence/absence (Krzywinski and Besansky 2002), and the X-linked inheritance of the gene.

In evaluating the three data sets, it was deemed best to discard the *white* data set as uninformative at best, or misleading at worst. Thus, the results of the *white* data set were not incorporated into the final phylogeny. At best, some of the *white* results, such as the parsimony result from analysis of the entire region (Figure 2.24) can be said to lend support to the basal furcations showing the presence of three major lineages in subgenus *Culex*. The *white* data set is very homoplasious and at times produced results that are very much at odds with traditional classification, such as placing *Aedes* and *Tripteroides* on internal branches within subgenus *Culex* (see Figure 2.31). When the effects of homoplasious characters were reduced, through reweighting characters by successive approximations, trees were poorly resolved. When the ILD test was performed, with 100 replicates, *white* failed to satisfy the null hypothesis of being congruent with either the *COII* or the *ITS* data sets (p=0.01 in both cases). Most importantly, the technical problems that arose in cloning and sequencing *white* resulted in fewer ingroup taxa being available for analysis, which limited the number of taxa that could be considered if *white* were combined with or compared to the other two genes.

Both the *COII* and *ITS* data sets have qualities making them desirable for phylogenetic inference of these mosquitoes, and so these genes were incorporated into the phylogeny proposed here. The signal/noise ratio of the *COII* data seems to have been appropriate for the taxa studied. Despite the AT-rich bias in the *COII* molecule, this property does not appear to have limited the variety of character states to the point at which phylogenetic resolution is lost. Although this is a protein-coding gene like *white,* the unique DNA replication machinery of the mitochondria could be responsible for producing this optimal level of signal. A 705 bp sequence produced well resolved trees of the relationships among these mosquitoes. The short size of the region studied and the lack of introns greatly facilitated amplification and alignment.

Many aspects of the *ITS* region studied make it another good gene for this study. The high copy number meant that relatively small quantities of extracted DNA were needed for successful amplification. Also, this region has been used previously for phylogenetic studies of a wide range of taxa, including some of the ingroup taxa studied here, thus facilitating a comparison of results with those presented elsewhere in the literature. Alignment was relatively easy, despite the inability to use inferred amino acids for guidance, and those sites that were difficult to align within the non-coding spacers were excluded from the analysis. The results from the *ITS* data set were consistent with previous studies (Miller *et al.,* 1996; Navarro and Liria, 2000; Shepard *et al.,* 2006) and shared the presence of some clades with the *COII* results.

Support for combining both the *COII* and *ITS* data sets comes from the results of the ILD test, which showed the *COII* and *ITS* data sets to be congruent with each other. That is, when the second codon is excluded from *COII* and the difficult-to-align regions are excluded from the *ITS* data, the two data sets are congruent. Any inconsistencies between the two data sets might be explained by their different histories and modes of evolution.

Evaluating the methods of phylogenetic analysis

The four different methods of analysis used tend to produce consistent, but not identical, results. Each of the four phylogenetic methods utilized in this research has its own advantages and disadvantages. Much was gained by performing all four methods, so that their results could be compared. The NJ distance technique is fast and provides a reasonable estimation of phylogenetic history based on overall similarity, since similar taxa tend to be close relatives. Bootstrap support values, using 1000 or more pseudoreplicates, can be determined very rapidly. The NJ distance trees were also useful starting trees for the heuristic searches performed while optimizing the ML parameters, but should not be treated as phylogenetic hypotheses because they lack the sound scientific foundation found in other methods. A disadvantage of the NJ distance method is that it is not character-based, and thus its phylogenetic hypotheses are not supported by cladistic theory, which uses synapomorphy in homologous characters.

The parsimony method is relatively fast, especially with few taxa. Parsimony has the advantage of being supported by sound scientific philosophy. Since it is character-based, parsimony uses cladistic theory, and since it selects the shortest tree (requiring the fewest suppositions of homoplasy) it also conforms to the principle of Occam's Razor, which assumes that the simplest explanation is often the truest. The parsimony method was helpful in elucidating the presence of homoplasious data in the *white* analysis when reweighting of data produced a reduced tree length and increased CI. One disadvantage of the parsimony method, known as long branch attraction (Bergsten 2005), refers to the tendency of rapidly evolving taxa to be inferred as close relatives, despite their true evolutionary histories. This occurs in molecular studies because there are only four possible character states and when two truly distant taxa develop the same end state, through multiple mutations, this is interpreted as a synapomorphy by parsimony analysis, and so the two taxa appear to be close relatives. Another disadvantage of the parsimony method is that it only utilizes parsimony-informative characters, not the entire data set, and it does not take advantage of well-studied models of nucleotide transformation.

The ML method, on the other hand, does make explicit use of models of nucleotide transformation, making it well suited for molecular phylogenetic studies. Another appeal of the ML method is that it makes use of the entire data set, not just the parsimony-informative sites, when it considers such things as base frequencies or the proportion of invariable sites. The ML method was useful in examining *white,* since different models were explored for several different partitions of the data in attempts to recover a reasonable tree, although one was not found. One disadvantage of the ML method is that it is very slow and computationally intensive. The use of models in the ML method can be seen as the addition of *ad hoc* hypotheses and therefore less sound under the principle of Occam's Razor.

Finally, Bayesian analysis shares the same advantages as the ML method. Additionally, the posterior probability values provided by Bayesian analysis eliminate the need to run bootstrap replicates. Also, because of the way in which Bayesian analysis estimates model parameters, the researcher need not be as exact in providing model parameter values as compared to the ML method. Bayesian analysis was advantageous in the simultaneous analysis of *COII* and *ITS* since the Mr. Bayes program allows different models to be used for different partitions of the data. A disadvantage is that Bayesian analysis is extremely slow, and there is no certain way to determine when the run is completed. A similar problem arises when running heuristic tree searches for parsimony or ML in that the researcher does not know whether the hill-climbing algorithm used to find the best tree has found merely a "local" optimum or the best, "global" optimum.

Both the ML and Bayesian analysis methods were found to be preferable for this study. Because both ML and Bayesian analysis utilize models of nucleotide transformation, the researcher is made aware of important factors in the evolution of the molecule being studied by noting which model parameters seem to be needed to produce a plausible2 phylogeny. Additionally, the two methods can be said to complement each other. While ML analysis requires specific parameter values in order to score trees during a tree search, the Bayesian analysis method estimates these parameter values, as well as posterior probability values, while simultaneously conducting the tree search. Although the time required for Bayesian analysis to reach stationarity tends to be longer than the time required to find a ML tree, the multiple replications required to provide bootstrap support values for ML cause the ML method to be much slower.

Interpretation of the total evidence tree using the COII *and* ITS *data and Bayesian analysis (Figure 2.36)*

The total evidence tree (Fig. 2.36) is better resolved than the taxonomic congruence tree (Figure 2.37), which is an Adams consensus of ML trees from the *COII* and *ITS* data. The Adams consensus method was used for taxonomic congruence because it preserves a reasonable amount of hierarchy, unlike the strict or semi-strict methods, which can produce poorly resolved consensus trees. The Adams consensus method interprets trees as a nested set of ancestor-descendant groupings, and preserves those groupings that share a common ancestor in each of the contributing trees. By virtue of this methodology, Adams consensus trees can present relationships that are not found in any of the contributing trees, which can make interpretation difficult. This taxonomic congruence tree does present a plausible species phylogeny that is consistent with previous publications, but it is poorly resolved, especially regarding deeper relationships within the subgenus. Poor resolution in an Adams consensus tree arises from disagreement on the common ancestor of nested taxa from the contributing trees. While the *COII* and *ITS* data do agree on certain relationships, trees from the two data sets disagree on the phylogenetic positions of common ancestors among these relationships.

While the total evidence and taxonomic congruence approaches both present three major lineages within subgenus *Culex,* the two approaches disagree on the species composition of these lineages. The tree produced by the total evidence approach is discussed further because this tree presents relationships that are consistent with previous findings and is better resolved than the taxonomic congruence tree. The poor resolution of the taxonomic congruence tree could be interpreted as a useful indicator of recent or rapid speciation.

The topology of the ingroup taxa on the total evidence tree is nearly identical to the ML tree from the combined *ITS1* and *ITS2* data. A few differences arise, especially among the outgroup taxa, because the *ITS* analysis includes some taxa that were not included in the *COII* data and could therefore not be included in a simultaneous analysis of the two data sets. The only notable difference between the total evidence tree and the Bayesian analysis of *ITS* is in the posterior probability values. These values are lower in the total evidence tree at certain nodes where the *COII* data disagree with the *ITS* data. The fact that the total evidence tree so closely matches the *ITS* tree reflects the fact that a majority of the characters used in the total evidence analysis come from the *ITS* nucleotide sequence and not the *COII* sequence.

Figure 2.36 presents three major lineages within subgenus *Culex,* which have been labeled A, B, and C. These are the same three lineages supported by the *ITS* data. No clear trends in morphology, distribution, or vector status correlate with the split between lineages A and B. The very low posterior probability value at the node separating lineage A from lineage B (0.63) suggests that this split is not well supported by the total evidence data. Although this split was very well supported by the *ITS* data (see Figure 2.21), the inclusion of the *COII* data greatly decreased the posterior probability value at this node. This decrease in posterior probability value seems to arise because the *COII* data does not split the topology into the same two sets of species that the *ITS* data does. So, the appearance of this split into lineages A and B seems to be a factor of the *ITS* data, but is not well supported by *COII.* In order to visually illustrate the importance of the node support values, those nodes with posterior probability values below 80% have been collapsed in Figure 2.38. This tree is not as well resolved, but illustrates the relationships that are well supported by both data sets.

The degree to which the *COII* data disagrees with the *ITS* data can be inferred from the posterior probability node values. Certain nodes are very well supported in the total evidence tree, having a posterior probability value of 1. These well supported nodes tend to be those that describe deep relationships found by both the *ITS* and *COII* data sets. One such node describes a (*Cx. salinarius* + (*Cx. secutor* + *Cx. erythrothorax*)) relationship. Although the *COII* data find a different internal relationship among these three species, *COII* does place these three species together in a monophyletic group.

Another well supported node describes a (*Cx. coronator* (*Cx. mollis* A + (*Cx. declarator* + *Cx. mollis*))) relationship. Strong support for this assemblage supports the possibility that the specimen called *Cx. mollis* A is likely to be either *Cx. mollis* or *Cx. declarator.*

One well supported group describes a relationship of ((*Cx. habilitator* +*Cx. janitor*) + (*Cx. stigmatosoma* + (*Cx. nigripalpus* + (*Cx. chidesteri* + *Cx. interrogator*)))). The distribution of some of these species is relatively limited. For example, the sister species *Cx. habilitator* and *Cx. janitor* are limited to the Caribbean. However, it is difficult to find a distributional trend in this entire group as a whole because some species, such as *Cx. nigripalpus* and *Cx. interrogator,* have wide distributions that cover most of the New World tropics and subtropics. One noted morphological trend uniting the species in this group is a gross similarity of the arrangement of appendages on the subapical lobe of the gonostylus of the adult male genitalia, which consists of an arrangement of a single seta, a broad leaf, and three rods. This group contains two demonstrated disease vectors. Both *Cx. nigripalpus* and *Cx. stigmatosoma* have roles in the transmission of St. Louis Encephalitis (SLE) virus (Service 2001).

Another well supported group contains (*Cx. coronator* + (*Cx. mollis* A + (*Cx. mollis* + *Cx. declarator*)). The spines on the apical end of the fourth instar siphon make *Cx. coronator* morphologically distinct from the other three species in this group. The mosquitoes of this group are found throughout the Neotropical region, with *Cx. coronator* recently being reported from the southern United States, as well (Varnado *et al.* 2005). This group does not contain any currently identifiead primary vectors of human disease.

A well supported group of (*Cx. salinarius* + (*Cx. erythrothorax* +*Cx. secutor*)) is found. The morphological similarities between *Cx. salinarius* and *Cx. erythrothorax* abound. The male genitalia of both species have six to twelve strong teeth on the plates of the phallosome. The fourth instar larvae of both species have a glabrous thorax, and the larval siphon has four to five pairs of irregularly placed setae. The larval siphon of *Cx. secutor*, on the other hand, is quite different, having a straight row of ten densely packed setal tufts. There are additional factors making *Cx. secutor* an unusual member of this clade. While both *Cx. erythrothorax* and *Cx. salinarius* are distributed well into the temperate latitudes of the U.S., *Cx. secutor* is limited to the Caribbean. Also, *Cx. erythrothorax* and *Cx. salinarius* are established arbovirus vectors, yet *Cx. secutor* is not currently a known arbovirus vector.

A well supported sister relationship between *Cx. thriambus* and *Cx. (Phe.) lactator* places subgenus *Phenacomyia* on a branch arising from within subgenus *Culex*. The phylogenetic position of *Phenacomyia* should be investigated further, using all three species of this subgenus (*Culex corniger, Cx. lactator,* and *Culex airozai*). The morphological features that define *Phenacomyia* (Harbach and Peyton 1992) are not present in *Cx. thriambus*. The long branch lengths separating *Phenacomyia* from *Cx. thriambus* suggest that much evolution has occurred since the two diverged and that the two taxa are not close relatives. The reported tropical and subtropical distribution range of *Cx. thriambus* in the Western hemisphere is slightly larger than that of *Phenacomyia,* which is limited to the Neotropics. In light of the current tree, the elevation of *Cx. lactator* to a new subgenus makes the subgenus *Culex* paraphyletic.

The final well supported clade, labeled as Clade C, contains members of the *Cx. pipiens* complex. This forms the most basal clade of the subgenus *Culex* taxa studied. The basal position, and the fact that these mosquitoes form their own lineage, is expected in light of suggestion that *Cx. pipiens* complex mosquitoes are an Old World taxon that was only recently introduced into the New World (Fonseca *et al* 2004). The *Cx. pipiens* complex contains additional taxa that are not included in this study. *Culex pipiens pallens* is treated by some mosquito systematists as a subspecies of *Cx. pipiens* (Tanaka *et al.* 1979), while others find evidence that it is derived from hybridization of *Cx. pipiens* and *Cx. quinquefasciatus* (Miller *et al.* 1996). Two more members of the complex, *Culex globocoxilus* and *Culex australicus,* are not distributed in the New World. The taxon *Cx. pipiens* form *molestus,* which is currently treated as a biological form of *Cx. pipiens*, can be found in temperate zones, including North America and was included in the *ITS* study because sequence data was available through Genbank. The two most common members of this complex, *Cx. pipiens* and *Cx. quinquefasciatus*, are important vectors of arboviruses and human filariasis.

Despite being treated as an ingroup taxon, *De. cancer* is placed outside of the subgenus *Culex* in every analysis. The fact that *De. cancer* arises from within subgenus *Melanoconion* in the total evidence tree might be evidence of the close affinity of *De. cancer* to this subgenus. However, additional subgenera should be studied to better determine the relation of *Deinocerites* among the subgenera of *Culex.*

With the exception of *Cx. pipiens* complex members, well established disease vectors are not necessarily closely associated on the tree topology. Other prominent disease vectors, such as *Cx. nigripalpus, Cx. salinarius,* and *Cx. tarsalis,* are found throughout the tree. Thus, no clear trend was identified that would indicate that vector competence, even for a particular pathogen, is correlated with phylogenetic position.

Conclusions

In conclusion, the *COII* and *ITS* genes were found to be effective for phylogenetic inference of subgenus *Culex* species from North and Central America and the Caribbean. The *COII* and *ITS* data were found to be congruent by the ILD test and were combined for a simultaneous analysis. Congruence between such disparate molecules may be indicative of the underlying species phylogeny. The *white* gene, on the other hand, tended to produce poorly resolved trees or trees that did not even support the monophyly of the subfamily Culicinae. Both the ML and Bayesian analysis methods were useful for molecular phylogenetic inference. The total evidence, or simultaneous analysis, approach to combining data sets was found to perform well, especially when combined with Bayesian analysis so that multiple models could be used and so that the posterior probability values of each node might be used as indicators of disagreement among the different data sets. Three major lineages were found within subgenus *Culex,* the most tribasal lineage consisting of the *Cx. pipiens* complex. However, separation of the two more derived lineages is poorly supported. The basal position of the *Cx. pipiens* complex relative to the remaining subgenus *Culex,* and *Phenacomyia,* mosquitoes is consistent with the hypothesis that the *Cx. pipiens* complex is a primitive lineage (Fonseca *et al.,* 2004). The analyses performed here were unable to distinguish between *Cx. pipiens* and *Cx. quinquefasciatus,* which is not surprising considering the nearly indistinguishable morphology of the two species, which suggests a recent divergence

Genus *Deinocerites* was found to arise from within genus *Culex,* but not from within subgenus *Culex.* This conclusion does not contradict the results of Navarro and Liria (2000), who find that *Deinocerites melanophylum* Dyar and Knab is more closely allied with a clade consisting of subgenera *Melanoconion, Microculex,* and *Anoedioporpa* than it is to subgenus *Culex*. Yet the closest *Culex* subgenus to *Deinocerites* is still undetermined.

Subgenus *Phenacomyia* was found to arise from within subgenus *Culex,* making subgenus *Culex* paraphyletic. The apparent presence of paraphyly does not invalidate the decision to place a group of species in a new subgenus if one recognizes a distinction between classification and cladistic analysis (Savage 2005). However, recent debate relevant to the issue of classification versus cladistic analysis (Black 2004) shows that the distinction between the two is not clear-cut. Further investigation into the phylogenetic history of *Phenacomyia* utilizing specimens from all three species included in the subgenus is warranted.

Species-level phylogenetic relationships within subgenus *Culex* have not been firmly established in previous studies, because previous studies examine only a few species from the subgenus (Miller *et al.,* 1996; Navarro and Liria, 2000; Shepard *et al.,* 2006). The present research contains the greatest number of subgenus *Culex* species of any phylogenetic study to date. Thus, the species-level relationships presented here could only be checked for consistency with regard to a few previously studied species, and these were found to be consistent.

Several species of subgenus *Culex* mosquitoes are medically important as disease vectors (Service 2001). Known arbovirus vectors are found throughout the lineages proposed by the trees presented here, but not in any readily discernable pattern. Vector competence cannot be said to have favored a certain lineage within the subgenus, but rather vector competence seems to have arisen more than once within the subgenus.

While it is best to focus vector control efforts on those species that have previously demonstrated roles in the transmission cycles of disease agents, it is also important to be mindful of the potential for other members of subgenus *Culex* to act as disease vectors.

Future research can expand upon the current study. Such studies might include additional species of subgenus *Culex,* including those from outside the geographic range studied here. Inclusion of the other two species of *Phenacomyia* (*Culex corniger* Theobald and *Culex airozai* Lane) would assist in determining the phylogenetic placement of this group. The closest extant relative to *Deinocerites* could not be determined in the current study, yet future studies that include several subgenera of *Culex* may clarify this issue. Finally, the current study would be improved by the addition of more characters, either morphological or from other regions of the genome.

Experimental procedures

Mosquito specimens

The sources of specimens used in this study are summarized in Table 2.1. The ingroup contained 17 species from subgenus *Culex.* These are *Culex chidesteri* Dyar, *Culex coronator* Dyar and Knab, *Culex declarator* Dyar and Knab, *Culex erythrothorax* Dyar, *Culex habilitator* Dyar and Knab, *Culex interrogator* Dyar and Knab, *Culex janitor* Theobald, *Culex mollis* Dyar and Knab, *Culex nigripalpus* Theobald, *Culex pipiens* Linnaeus, *Culex quinquefasciatus* Say, *Culex restuans* Theobald, *Culex salinarius* Coquillett, *Culex secutor* Theobald, *Culex stigmatosoma* Dyar, *Culex tarsalis* Coquillett, and *Culex thriambus* Dyar. The appropriate identification of one additional specimen, which has been called *Culex mollis* A, is undetermined, but has larval characteristics of *Cx. declarator* and male genitalia characteristics of *Cx. mollis.*

The species *Culex (Phenacomyia) lactator* Dyar and Knab and *Deinocerites cancer* Theobald are treated as ingroup taxa to test their phylogenetic positions relative to subgenus *Culex.* Outgroup taxa consisted of members of the subgenus *Melanoconion.* The *ITS* phylogeny includes additional outgroup taxa from subgenera *Micraedes* and *Neoculex* as well as genus *Lutzia.* Because of difficulties cloning and sequencing *white,* the outgroup taxa consist not of *Melanoconion* species, but of *Tripteroides bambusa* (Yamada) and *Aedes (Ohochlerotatus) triseriatus* (Say) because of the availability of these sequences from Genbank.

Specimens were field collected as larvae and individually reared to the adult stage so that larval and pupal exuviae and male genitalia could be preserved and slide mounted for use in species identification. Nucleotide sequences obtained through Genbank are designated as such on the trees.

Specimens were collected in the field by dipping for fourth instar larvae in aquatic habitats. Specimens were individually reared to the adult stage as described by Belkin *et al.* (1965) either at field stations or in the insectary at the Centers for Disease Control and Prevention (CDC) Division of Vector-Borne Infectious Disease (DVBID). Fourth instar larval and pupal exuviae were mounted on microscope slides in Euparal and dried at 60°C for six weeks. Adult male bodies to be used for subsequent molecular research were stored in 100% ethyl alcohol and stored at -20° C upon arrival at the CDC DVBID. Adult male genitalia were removed and mounted onto microscope slides in a drop of copal-phenol solution.

Specimens were identified to species based primarily on characters of the fourth instar larval exuviae. Species identifications were confirmed by considering geographic range, habitat, and adult morphology, including that of the male genitalia. North American specimens were identified using Darsie and Ward (2004), with specimens from Florida confirmed using Darsie and Morris (2000); specimens from Guatemala were identified using Clark-Gil and Darsie (1983), with confirmation from Darsie and Ward (2004) where appropriate; specimens from Puerto Rico were identified using Belkin *et al.* (1970). All identifications were verified using at least one of Bram (1967), Carpenter and LaCasse (1955), and Lane (1953). The identification of *Cx. lactator* was further confirmed using Strickman and Pratt (1989) by examining other adults reared from the same collection site as well as seven larval characters from the fourth instar larval exuvia.

DNA extraction, amplification, cloning, and sequencing

Adult bodies were triturated individually in 180-200 µl of phosphate-buffered saline (PBS). The full homogenate was used for DNA extraction using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA) following the protocol designed for extraction of DNA from insects. Extracted DNA was eluted from the membrane twice in 200 µl of nuclease-free water (Amresco, Solon, OH) and combined for a total eluate volume of 400 µl. Concentration of DNA was determined by spectrophotometer.

The region of the *COII* gene was amplified by PCR, using primers described by Ho *et al.* (1995). A 50µl reaction was prepared containing 1X of Gene Amp PCR Buffer I (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% wt:vol gelatin) (Applied Biosystems, Foster City, CA), 0.2 mM of each dNTP (Roche Diagnostics, Indianapolis, IN), 800 nM of each primer, 2.5 U of Amplitaq DNA polymerase (Applied Biosystems), and 75 ng of template DNA. Amplification used the following program: 3 min at 94° C, followed by 30 cycles of 94° C for 1 min, 48° C for 1 min, and 72° C for 1 min, and ending with 8 min at 72° C. The amplified product was purified using Qiagen's Qiaquick PCR Purification Kit (Qiagen, Valencia, CA) and eluted in 30 µl of nuclease-free water.

The desired region of the *ITS* gene was amplified by PCR, using primers described by Porter and Collins (1991). A 100µl reaction was prepared with 1X of Gene Amp PCR Buffer I (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% wt:vol gelatin) (Applied Biosystems, Foster City, CA), 1 mM of dNTP mix (Roche Diagnostics, Indianapolis, IN), 120 nM of each primer, 2.5 U of Amplitaq DNA polymerase (Applied Biosystems), and 60 ng of template DNA. Amplification was conducted under the following thermal cycler conditions: one cycle at 97°C for 4 minutes, followed by 30 cycles of 96°C for 30 seconds, 48°C for 30 seconds, and 72°C for 2 minutes, and ending with a 5 minute extension step at 72°C. The PCR product was run on a 1.8% agarose gel, buffered with 0.5X TBE to verify presence of a ~1100 bp amplicon.

Degenerate primers described by Besansky and Fahey (1997) were used for amplification of *white.* A 50 µl reaction was created with 1X of Gene Amp PCR Buffer I (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% wt:vol gelatin) (Applied Biosystems, Foster City, CA), 0.2 mM of dNTP mix (Roche Diagnostics, Indianapolis, IN), 2 µM of each primer, 2.5 U of Amplitaq DNA polymerase (Applied Biosystems), and 125 ng of template DNA. Amplification was conducted under the following thermal cycler conditions: one cycle of 96° C for 1 min, followed by 35 cycles of 93° C for 20 sec, 45° C for 1 min, and 73° C for 2 min. The *white* PCR product was run on a low melting point 1.8% agarose gel. Several bands of amplification were visible, but were distinctly separated on the gel. The appropriate amplicon was presumed based upon its size when compared with a 100 bp ladder (Invitrogen, Carlsbad, CA), since the appropriate amplicon should be ~850 bp. The selected band was cut from the gel and purified, using Qiagen's Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA). In cases wherein the selected band failed to produce the expected sequence, or when no band of appropriate size was visible, multiple bands were cut from a wider region of the lane of the gel in hopes of finding the correct amplicon.

Each molecule was next cloned with the TOPO-TA ligation vector pCR4TOPO (Invitrogen, Carlsbad, CA) used to transform One Shot Chemically Competent *Escherichia coli* (Invitrogen, Carlsbad, CA) by heat shock per the manufacturer's instructions. Colonies of the transformed bacteria were visible on tetracycline-treated LB agar plates (Invitrogen, Carlsbad, CA) after 12 hours of incubation at 37° C in a CO_2 incubator. Colonies were picked and incubated in tetracycline-treated LB broth (Invitrogen, Carlsbad, CA) for 16 hours at 37° C in a rotating incubator before treatment with the Qiagen Qiaprep Spin Miniprep Kit (Qiagen, Valencia, CA) and eluting in 50 μl of nuclease-free water. This product was dried in a vacuum centrifuge and reconstituted in 400 μl of nuclease-free water.

Molecules were sequenced, using a 3130 Genetic Analyzer and the BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, Foster City, CA). Sequencing conditions for each molecule are described below.

The *COII* molecule was sequenced three times in the forward and four times in the reverse direction, using primers M13 Forward (Invitrogen, Carlsbad, CA), the PCR primers described by Ho *et al.* (1995), CO2F419 (5'-AACCGAATTATTTTACCTT-3'), M13 Reverse (Invitrogen), T3 (Invitrogen), and CO2R567 (5'-ACCAAAGAAAAGTCCAG-3').

The *ITS* molecule was sequenced three times in the forward direction and 5 times in the reverse direction. The primers used were the PCR primers described by Porter and Collins(1991), as well as M13 Forward (Invitrogen, Carlsbad, CA), CP17F (5'-GCGCCGCGGTGTGAACTGCAGGACACATG-3'), M13 Reverse (Invitrogen), T3 (Invitrogen), CP17RECO (5'-GTGAATTCCATGTGTCCTGCAGTTCACA-3'), and 58RECO (5'-GTGAATTCTCGGTGTTCATGTGTCCTGC-3').

The *white* molecule was sequenced three times in the forward and four times in the reverse direction, using the PCR primers described by Besansky and Fahey (1997), as well as M13 Forward (Invitrogen, Carlsbad, CA), WF2 (5'-AACGTCGAGGAGCACTACTTC -3'), M13 Reverse (Invitrogen), T3 (Invitrogen), and WR3 (5'- CGCACCTTGACCAGCATC -3'). Additional sequencing runs were performed when sequencing peaks were ambiguous.

Contiguous sequences were constructed using Lasergene's Seqman II (DNA Star, Madison, WI). Substitution rates and other statistics were determined using DNAsp v 4.10.9 (Rozas and Rozas 1995), using the *Drosophila* mtDNA genetic code.
Sequence alignments were made manually, except within introns and for the entire *ITS* data set, for which ClustalW was used (Thompson *et al.* 1994). For *ITS*, a gap open penalty of 3 and gap extension penalty of 1 were used. By visual inspection, certain

regions of *ITS* were noted that appear to align well and have stretches of at least four nucleotides of similar or identical sequence. Poorly aligned regions were excluded from phylogenetic analysis of the data set.

Phylogenetic analysis

Phylogenetic analysis of the data was performed using neighbor-joining (NJ) distance, parsimony, maximum likelihood (ML), and Bayesian analysis by using PAUP* v4.0b10 for Windows (Swofford, 2003) or Mr. Bayes v3.1.2 (Huelsenbeck *et al.* 2001, Ronquist and Huelsenbeck. 2003).

Uncorrected distances were used for construction of the neighbor-joining (NJ) distance tree. Ties were encountered during distance tree construction and were broken randomly. Bootstrap analyses for distance were conducted with 1000 replicates and NJ tree searches.

For parsimony analysis, a heuristic tree search was conducted with the starting tree created by simple stepwise addition, TBR branch swapping, and the steepest descent function not in effect. Alignment gaps were treated as missing. When necessary, the maximum number of trees saved was automatically increased by 100 trees until analysis was completed. Bootstrap support values for parsimony were calculated with 1000 pseudoreplicates and the same heuristic tree search methods described above. When mentioned, reweighting of characters by successive approximations character weighting was done using the "reweight" command of PAUP*.

The ML analysis was also performed using PAUP*. Values for the likelihood model parameters were determined using the hierarchical Likelihood Ratio Test (hLRT) in Modeltest 3.7 (Posada and Crandall, 1998). Heuristic tree searches were conducted using the same methodology as that used in parsimony analysis. Bootstrap values for ML were calculated using 100 pseudoreplicates.

Bayesian analysis was performed using Mr. Bayes v3.1.2 (Huelsenbeck *et al.* 2001, Ronquist and Huelsenbeck. 2003). When Modeltest is used to suggest a model for the data set, the authors of Mr. Bayes reccommend using the same general model type for Bayesian analysis, then letting Mr. Bayes estimate the model's parameter values. The default setting of one cold chain and three heated chains, which can explore treespace faster, was used to speed the search.

In all PAUP* analyses in which *De. cancer* was treated as an ingroup taxon and subgenus *Melanoconion* comprises the outgroup, the program warned that the tree could not be rooted such that the specified outgroup is monophyletic. This warning occurred because the *De. cancer* sample always joined the *Melanoconion* outgroup. The identity of these samples as the source of the warning message was tested by temporarily removing them from the analysis, which stopped the warning. The homogeneity of partitioned data sets test was performed under the parsimony criterion with 100 repetitions of heuristic tree searching, the maximum number of saved trees kept at 100, and gaps treated as missing data.

TABLES

Table 1.1. Summary of the distribution and species counts of subgenera of *Culex*.

Distribution	Subgenus	Number of species
Worldwide	*Culex*	239
	Neoculex	24
Old World	*Acalleomyia*	1
	Acallyntrum	8
	Afroculex	1
	Barraudius	4
	Culiciomyia	55
	Eumelanomyia	78
	Kitzmilleria	1
	Lasiosiphon	1
	Lophoceraomyia	113
	Mailloitia	12
	Oculeomyia	2
	Sirivanakarnius	1
New World	*Aedinus*	4
	Allimanta	1
	Anoedioporpa	13
	Belkinomyia	1
	Carrollia	17
	Melanoconion	156
	Micraedes	8
	Microculex	36
	Phenacomyia	3
	Tinolestes	3

Table 1.2. Species of subgenus *Culex* in geographic area of interest, their type locality, and reported distribution, based on WRBU web site.

Species	**Type Locality**	**Reported Distribution**
Culex aquarius Strickman 1990	San Isidro de Coronada, Costa Rica	Costa Rica, Panama
Culex bahamensis Dyar and Knab1906	Bahamas	Bahamas, Cuba, Grand Cayman Isles, Jamaica, Puerto Rico, USA, Virgin Isles
Culex bidens Dyar 1922	Rosario, Bolivia	Argentina, Bolivia, Brazil, Mexico, Paraguay, Venezuela
Culex bonneae Dyar and Knab 1919	Compagnie des Mines d'Or, Surinam	Brazil, Colombia, Costa Rica, French Guyana, Panama, Surinam
Culex chidesteri Dyar 1921	Colon, Panama	Argentina, Brazil, Colombia, Costa Rica, Cuba, Ecuador, Jamaica, Lesser Antilles, Mexico, Panama, Paraguay, Puerto Rico, USA, Venezuela
Culex corniger Theobald 1903	Para, Brazil	Belize, Bolivia, Brazil, Colombia, Costa Rica, Cuba, Ecuador, El Salvador, French Guyana, Guadelupe, Guatemala, Guyana, Haiti, Honduras, Jamaica, Mexico, Nicaragua, Panama, Peru, Suriname, Trinidad and Tobago, Uruguay, Venezuela
Culex coronator Dyar and Knab 1906	St. Joseph, Trinidad	Argentina, Belize, Bolivia, Brazil, Colombia, Costa Rica, El Salvador, French Guyana, Guatemala, Honduras, Mexico, Nicaragua, Panama, Paraguay, Peru, Suriname, Trinidad and Tobago, southern USA, Venezuela
Culex declarator Dyar and Knab 1906	Trinidad	Belize, Bolivia, Brazil, Costa Rica, El Salvador, French Guyana, Guyana, Lesser Antilles, Mexico, Panama, Paraguay, Peru, Suriname, Trinidad and Tobago, Uruguay, USA, Venezuela
Culex delys Howard 1915	Tabernilla, Panama	Panama
Culex dohenyi Hogue 1975	Wafer Bay, Costa Rica	Costa Rica
Culex duplicator Dyar and Knab 1909	San Prancisco Mines, Dominican Republic	Dominican Republic, Haiti
Culex erythrothorax Dyar 1907	Davis Slough, CA, USA	Mexico, USA

Table 1.2. Continued.

Species	**Type Locality**	**Reported Distribution**
Culex garciai Broche 2000	Sierra Maestra, Cuba	Cuba
Culex habilitator Dyar and Knab 1906	San Domingo, Dominican Republic	Dominican Republic, Lesser Antilles, Peru, Puerto Rico, Trinidad and Tobago
Culex inflictus Theobald 1901	Grenada	Belize, Columbia, Costa Rica, Cuba, Grenada, Lesser Antilles, Mexico, Panama, Trinidad and Tobago, Venezuela
Culex interrogator Dyar and Knab 1906	Rincon Antonio, Mexico	El Salvador, Mexico, Nicaragua, Panama, USA
Culex janitor Theobald 1903	Kingston, Jamaica	Haiti, Jamaica, Puerto Rico
Culex laticlasper Galindo and Blanton 1954	Cerro Punta, Panama	Panama
Culex mollis Dyar and Knab 1906	Sangre Grande, Trinidad	Belize, Brazil, Colombia, Costa Rica, Ecuador, French Guyana, Guatemala, Guyana, Honduras, Mexico, Nicaragua, Panama, Peru, Suriname, Trinidad and Tobago, Venezuela
Culex nigripalpus Theobald 1901	St. Lucia Island, Lesser Antilles	Antilles, Barbados, Belize, Brazil, Colombia, Costa Rica, Cuba, Dominican Republic, Ecuador, El Salvador, Guatemala, Guyana, Honduras, Jamaica, Mexico, Nicaragua, Panama, Paraguay, Suriname, Trinidad and Tobago, USA, Venezuela
Culex ousqua Dyar 1918	Panama	Belize, Colombia, Costa Rica, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Panama
Culex pinarocampa Dyar and Knab 1908	Cordoba, Mexico	Costa Rica, Mexico, Panama
Culex pipiens Linnaeus 1758	Sweden	Worldwide temperate zones
Culex pseudostigmatosoma Strickman 1990	Llano Guagololo, Honduras	Honduras
Culex quinquefasciatus Say 1823	Louisiana, USA	Worldwide tropics and subtropics
Culex restuans Theobald 1901	Ontario, Canada	Canada, Mexico, USA

Table 1.2. Continued.

Species	**Type Locality**	**Reported Distribution**
Culex salinarius Coquillett 1904	New Jersey, USA	Bermuda, Canada, Mexico, USA
Culex saltanensis Dyar 1928	Camp Santa de Salta, Argentina	Argentina, Brazil, Panama, Venezuela
Culex scimitar Branch and Seabrook 1959	Hog Island, Bahamas	Bahamas
Culex secutor Theobald 1901	Cinchona, Jamaica	Jamaica, Lesser Antilles, Puerto Rico
Culex sphinx Howard 1912	Nassau, Bahamas	Bahamas, Cuba
Culex stenolepis Dyar 1908	Cordoba, Mexico	Costa Rica, Mexico
Culex stigmatosoma Dyar 1907	Pasadena, CA, USA	USA
Culex tarsalis Coquillett 1896	Argus Mtns, California, USA	Canada, Mexico, USA
Culex thriambus Dyar 1921	Kerrville, Texas, USA	Colombia, Costa Rica, Dominican Republic, Mexico, Panama, USA
Culex usquatissimus Dyar 1922	Toro Point, Panama	Colombia, Costa Rica, Ecuador, Guyana, Panama, Venezuela
Culex usquatus Dyar 1918	Panama	Argentina, Brazil, Mexico, Panama, Paraguay
Culex yojoae Strickman 1990	La Joya, Honduras	Belize, Honduras

Table 2.1. Specimens examined for the three molecular studies, with check marks (✓) indicating the studies in which they were used, their species name, unique identifier code, collection region, and collector.

Genes studied			**Species**	**Unique identifier**	**Collection region**	**Collector**
COII	**ITS**	***white***				
✓	✓		*Culex chidesteri*	GU2006.39.25	El Peten, Guatemala	H. Savage et al.
✓	✓		“	GU2006.39.26	El Peten, Guatemala	H. Savage et al.
✓			*Culex coronator*	GU2006.55.08	Izabal, Guatemala	H. Savage et al.
	✓		“	MS2004.01.01	Copiah Co., Mississippi	J. Goddard
✓	✓	✓	“	MS2004.01.03	Copiah Co., Mississippi	J. Goddard
✓			“	MS2004.01.06	Copiah Co., Mississippi	J. Goddard
✓	✓		*Culex declarator*	GU2004.41.27	Guatemala, Guatemala	H. Savage et al.
✓	✓	✓	*Culex erythrothorax*	CA2006.01.02	Kern Co., California	W. Reisen et al.
✓	✓	✓	*Culex habilitator*	PR2004.13.02.	Aguada, Puerto Rico	H. Savage and K. Bennett
✓	✓		*Culex mollis* A	GU2006.18.09	El Peten, Guatemala	H. Savage et al.
✓	✓	✓	*Culex interrogator*	GU2004.72.05	Guatemala, Guatemala	H. Savage et al.
	✓		“	GU2006.23.05	El Peten, Guatemala	H. Savage et al.
✓	✓		“	GU2006.23.23	El Peten, Guatemala	H. Savage et al.
✓	✓	✓	*Culex janitor*	PR2004.14.06	Maricao, Puerto Rico	H. Savage and K. Bennett
✓	✓	✓	*Culex mollis*	GU2004.69.28	Guatemala, Guatemala	H. Savage et al.
✓	✓	✓	*Culex nigripalpus*	FL2005.06.28	Miami-Dade Co., Florida	M. Williams
✓			“	GU2006.22.03	El Peten, Guatemala	H. Savage et al.
✓	✓	✓	*Culex pipiens*	CT2006.01.03	New Haven Co., Connecticut	T. Andreadis
		✓	“	P801c	Larimer Co., Colorado	H. Savage

Table 2.1. Continued.

Genes studied			Species	Unique identifier	Collection region	Collector
COII	ITS	*white*				
✓	✓		*Culex quinquefasciatus*	CA2004.02.24	San Diego Co., California	S. Aspen
✓			“	CA2004.07.11	Kern Co., California	S. Aspen
✓	✓	✓	“	FL2005.19.05	Monroe Co., Florida	M. Williams
✓	✓	✓	*Culex restuans*	MS2004.01.SEA1	Copiah Co., Mississippi	J. Goddard
✓	✓	✓	*Culex salinarius*	NJ2005.03.04	Bergen Co., New Jersey	A. Farajollahi
✓	✓	✓	*Culex secutor*	PR2004.97.207	Caribbean National Forest, Puerto Rico	H. Savage and K. Bennett
✓	✓	✓	*Culex stigmatosoma*	TX2003.03.68	Big Bend National Park, Texas	H. Savage and K. Bennett
✓	✓	✓	*Culex tarsalis*	CA2004.06.07	San Diego Co., California	S. Aspen
✓	✓	✓	*Culex thriambus*	GU2004.02.22	Guatemala, Guatemala	H. Savage et al.
✓	✓		*Culex (Phenacomyia) lactator*	GU2006.30.1	El Peten, Guatemala	H. Savage et al.
✓	✓		“	GU2006.47.1	El Peten, Guatemala	H. Savage et al.
	✓		*Culex (Melanoconion) dunni*	GU2006.39.03	El Peten, Guatemala	H. Savage et al.
✓			*Culex (Melanoconion) educator*	GU2006.42.14	El Peten, Guatemala	H. Savage et al.
✓			“	GU2006.43.1	El Peten, Guatemala	H. Savage et al.
✓			*Culex (Melanoconion) erraticus*	GU2006.06.01	El Peten, Guatemala	H. Savage et al.
✓	✓		“	GU2006.06.06	El Peten, Guatemala	H. Savage et al.

Table 2.1. Continued.

Genes studied			**Species**	**Unique identifier**	**Collection region**	**Collector**
COII	**ITS**	***white***				
✓			*Culex (Melanoconion) pilosus*	GU2006.41.12	El Peten, Guatemala	H. Savage et al.
✓	✓		"	GU2006.55.02	Izabal, Guatemala	H. Savage et al.
	✓		*Culex (Micraedes) antillomagnorum*	PR2004.18.03	Maricao, Puerto Rico	H. Savage and K. Bennett
✓	✓		*Deinocerites cancer*	FL2006.01.05	Indian River Co., Florida	R. Connelly
	✓		"	12310105	Monroe Co., Florida	M. Godsey et al.

FIGURES

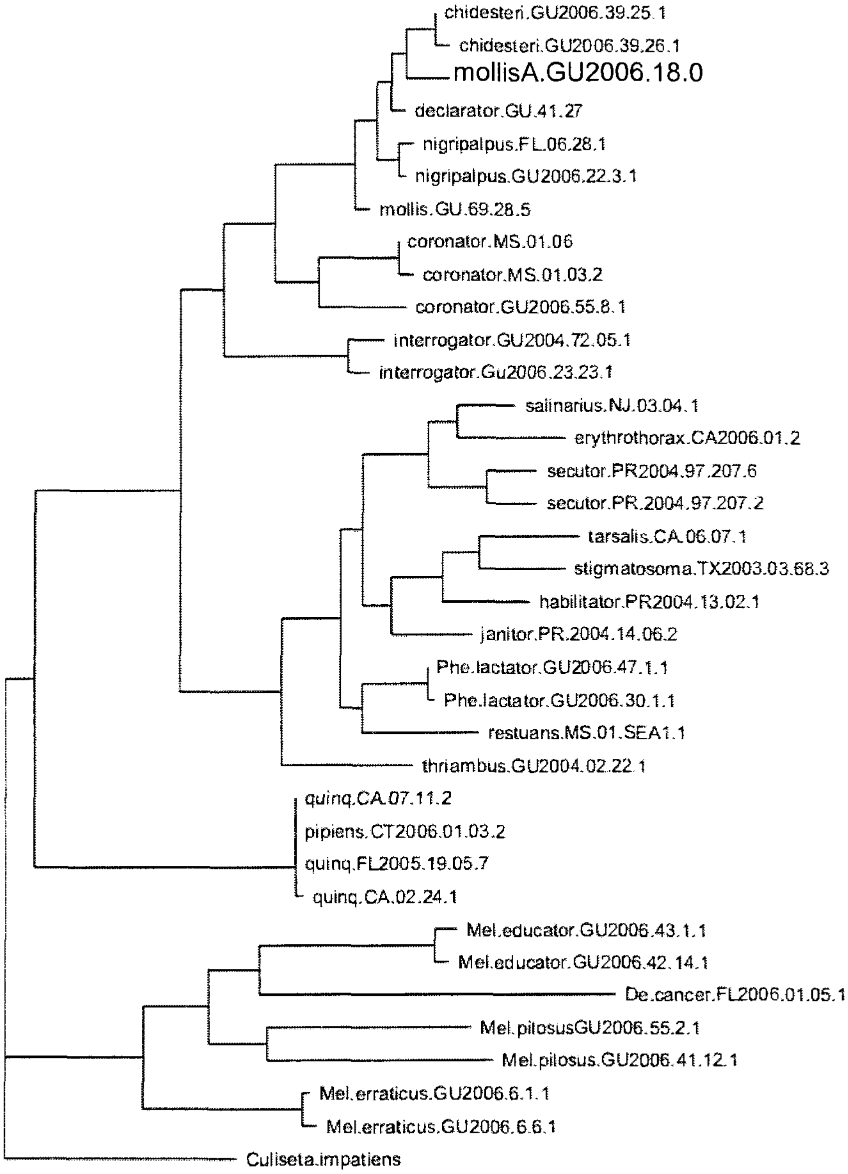

Figure 2.1. Neighbor-joining tree of first and third codon positions of *COII* data set. Minimum evolution score= 1.02464.

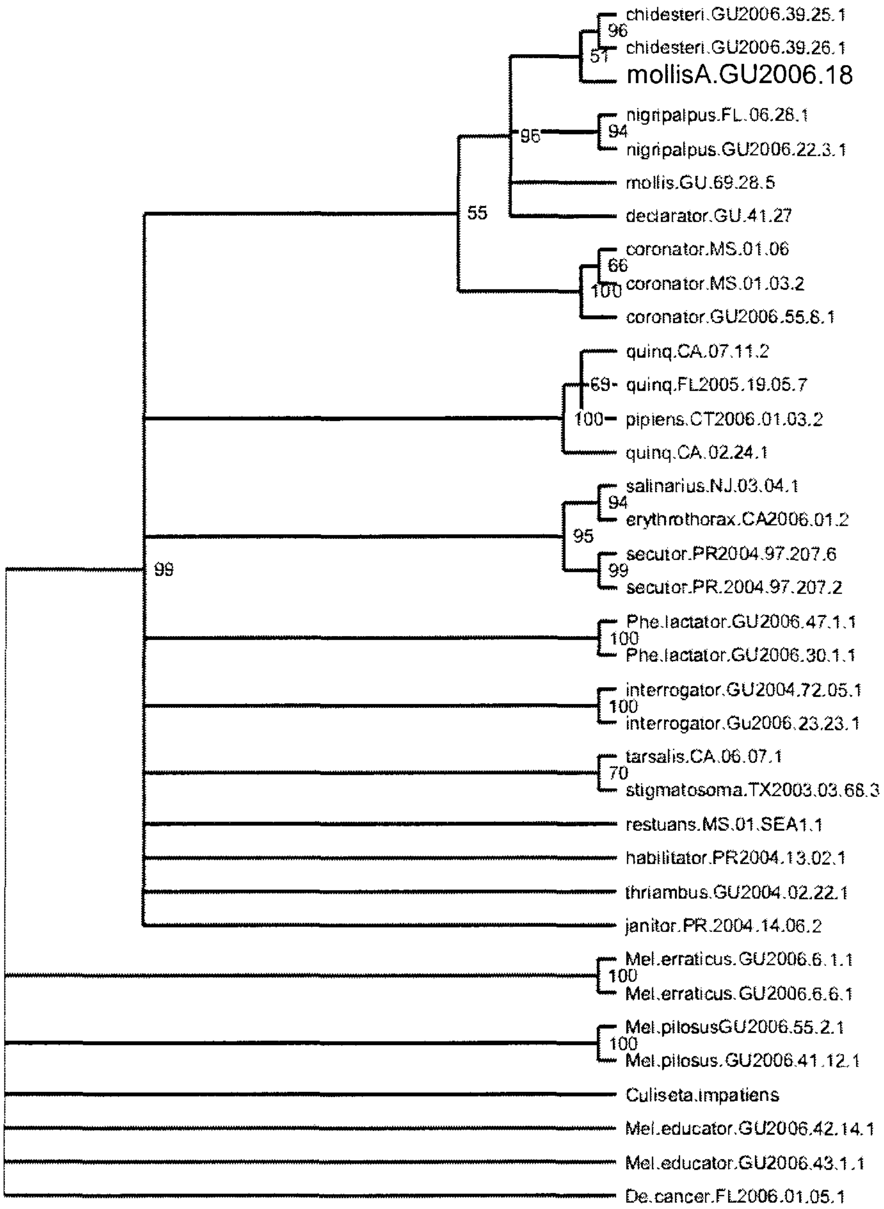

Figure 2.2. Bootstrap 50% majority rule consensus tree of first and third codons of *COII* data, with 1000 pseudoreplicates. Node labels indicate bootstrap percent values.

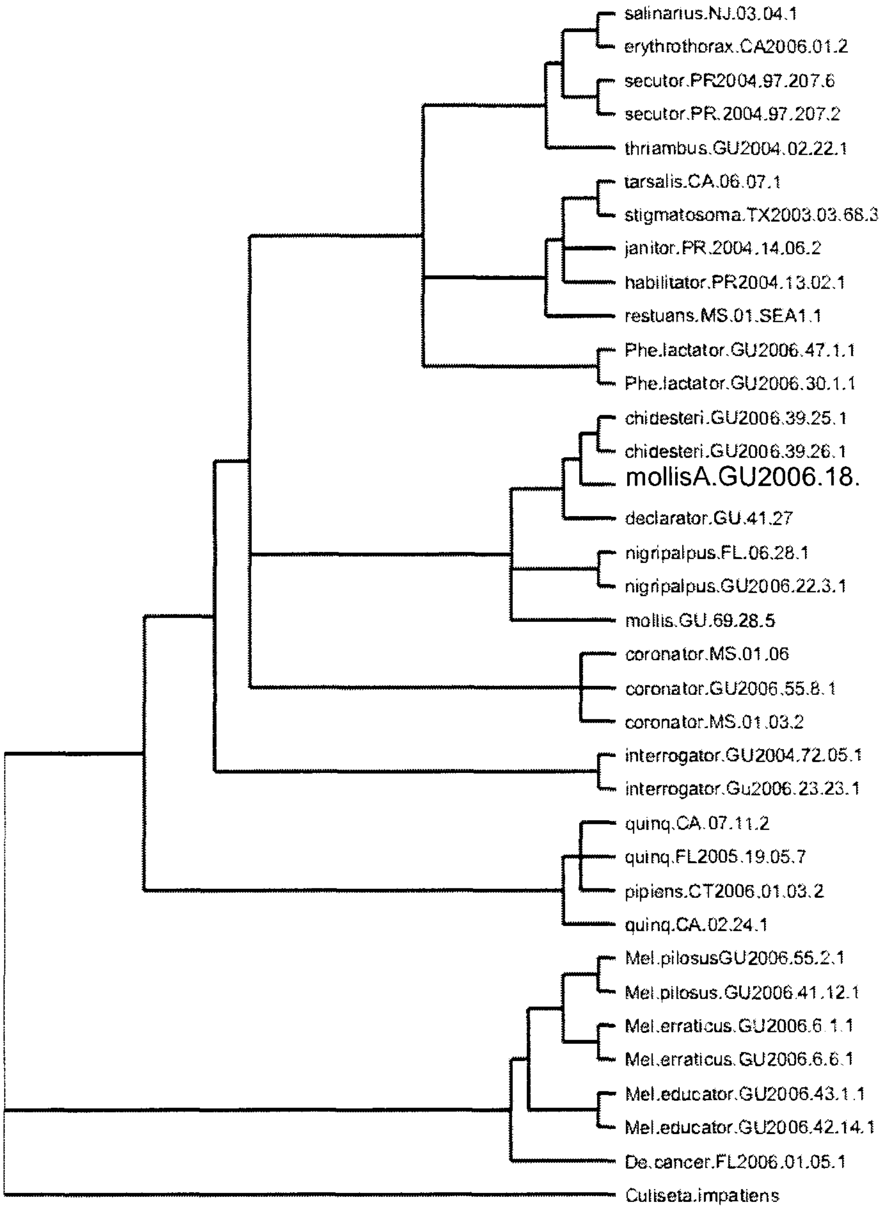

Figure 2.3. Strict consensus of 14 most parsimonious trees found for *COII,* with the second codon position excluded.

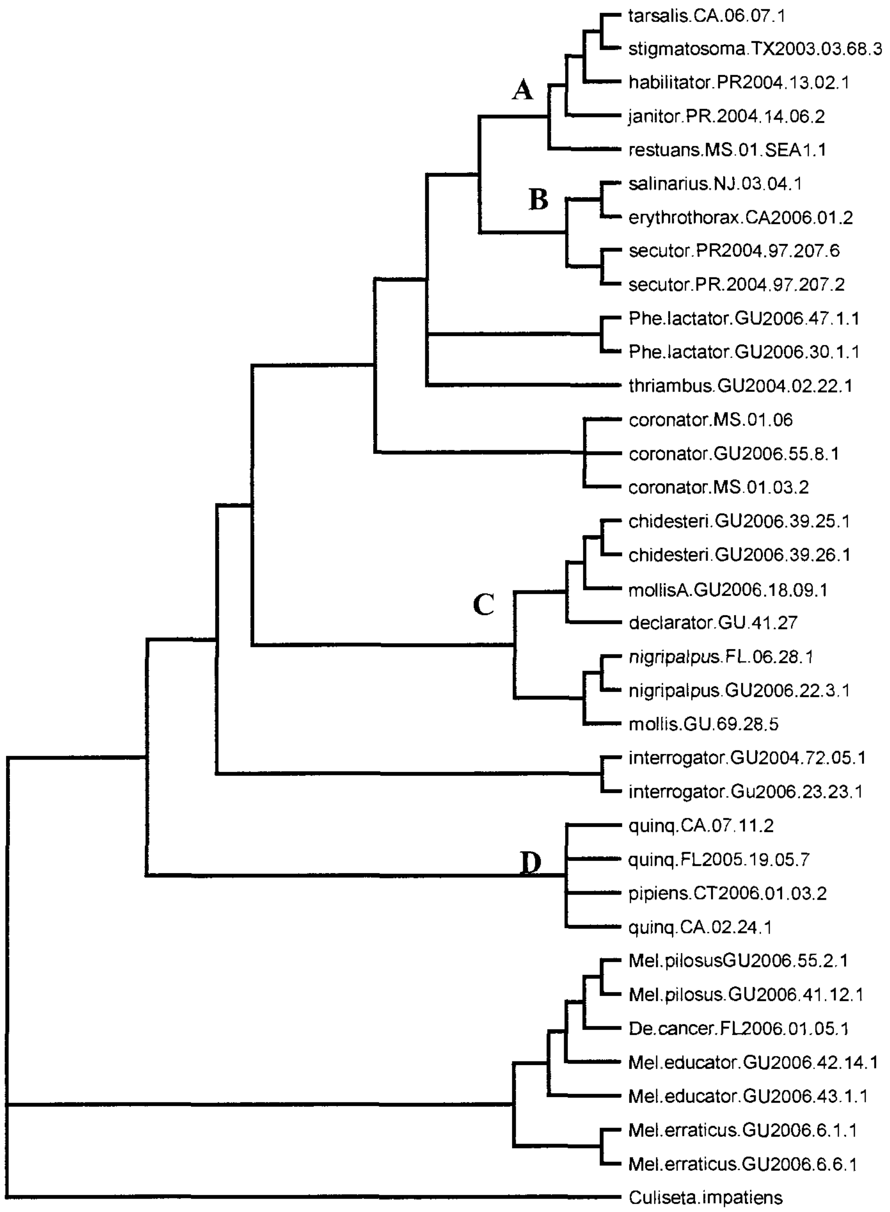

Figure 2.4. Maximum likelihood tree of first and third codon positions of *COII* data. Negative log likelihood = 3061.81451.

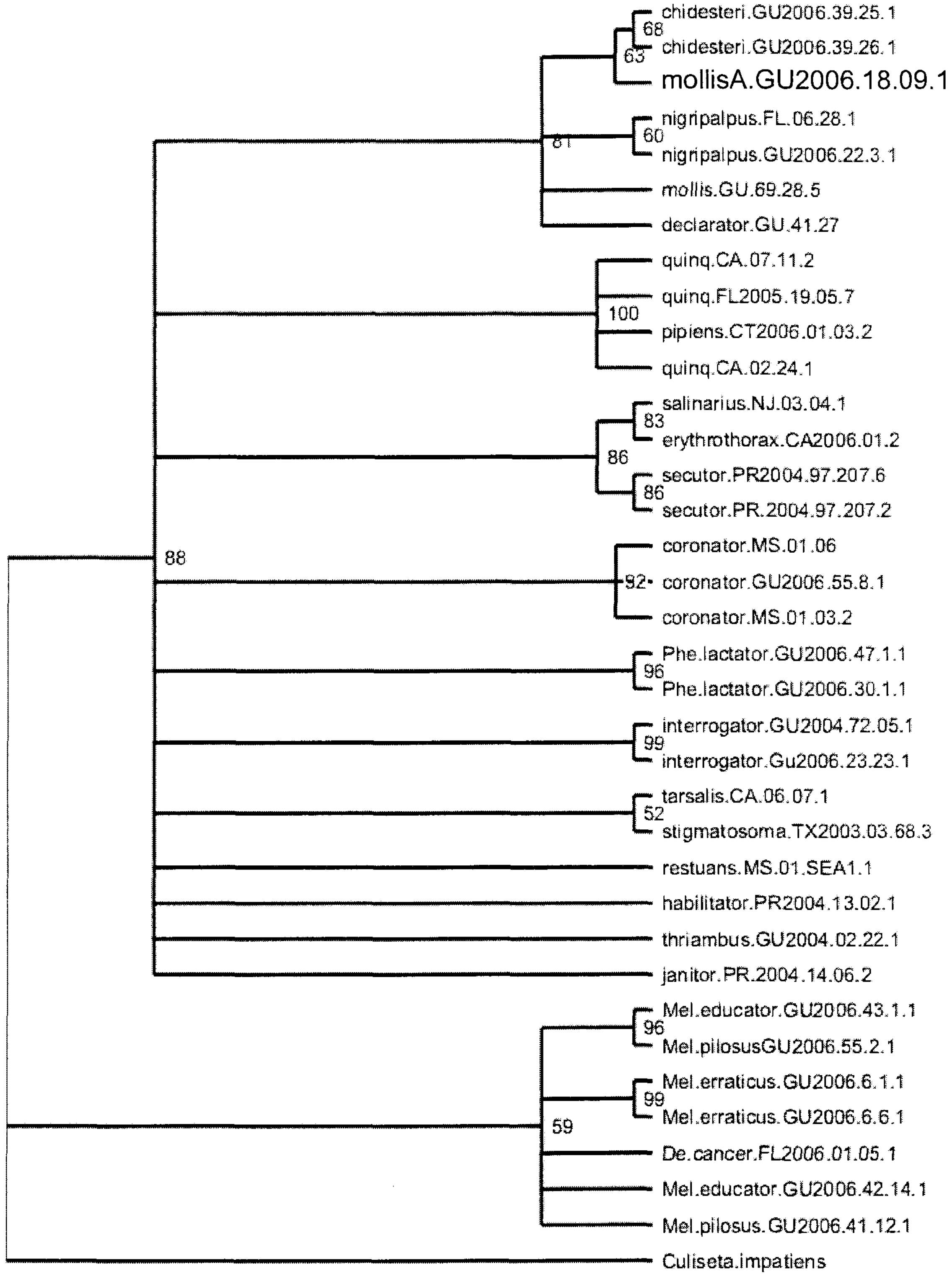

Figure 2.5. Bootstrap support values of ML tree presented in Figure 2.4, presented as node labels. This is a 50% majority rule consensus tree of 100 pseudoreplicates.

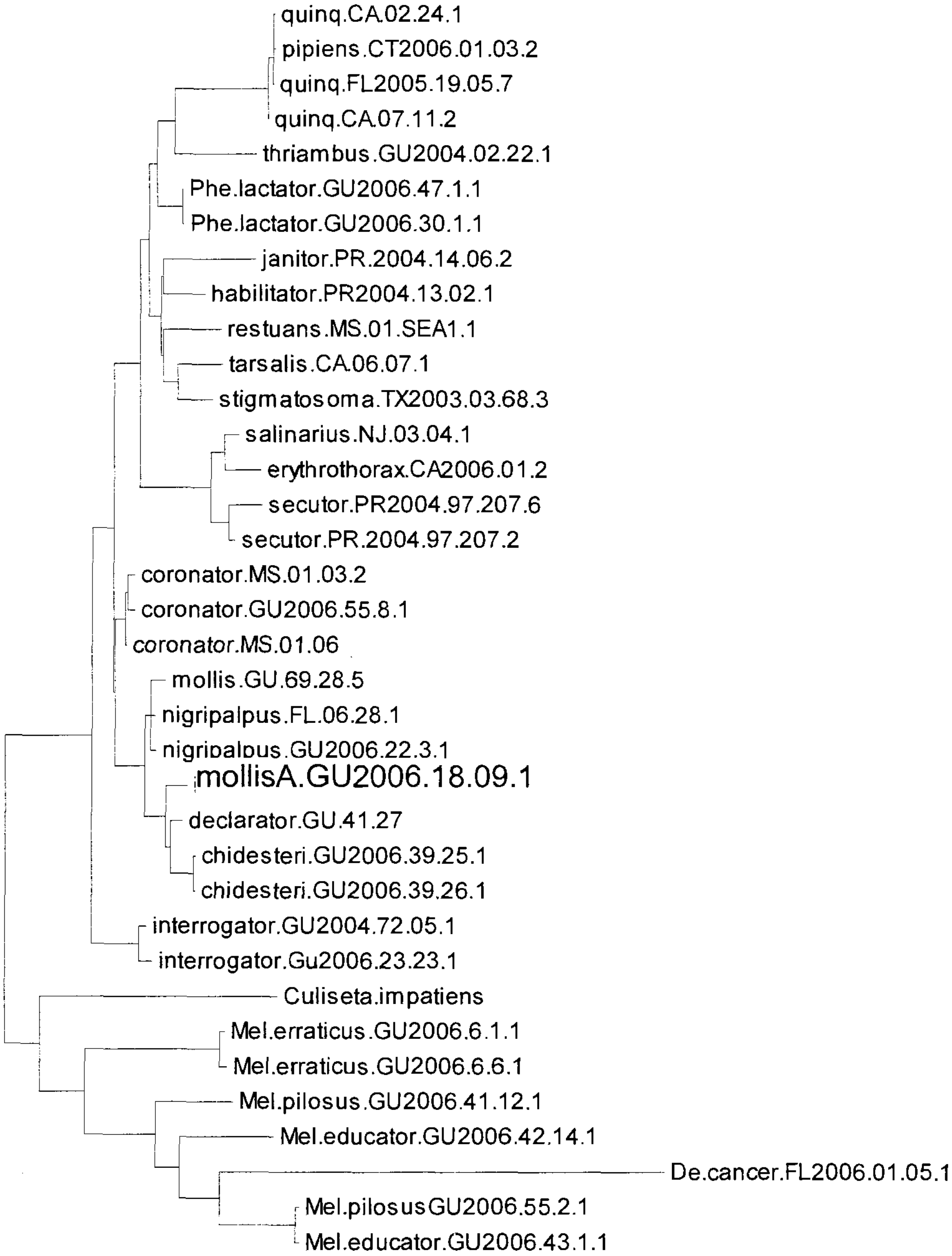

Figure 2.6. Bayesian analysis tree produced from *COII* data. Node labels represent posterior probability of each clade.

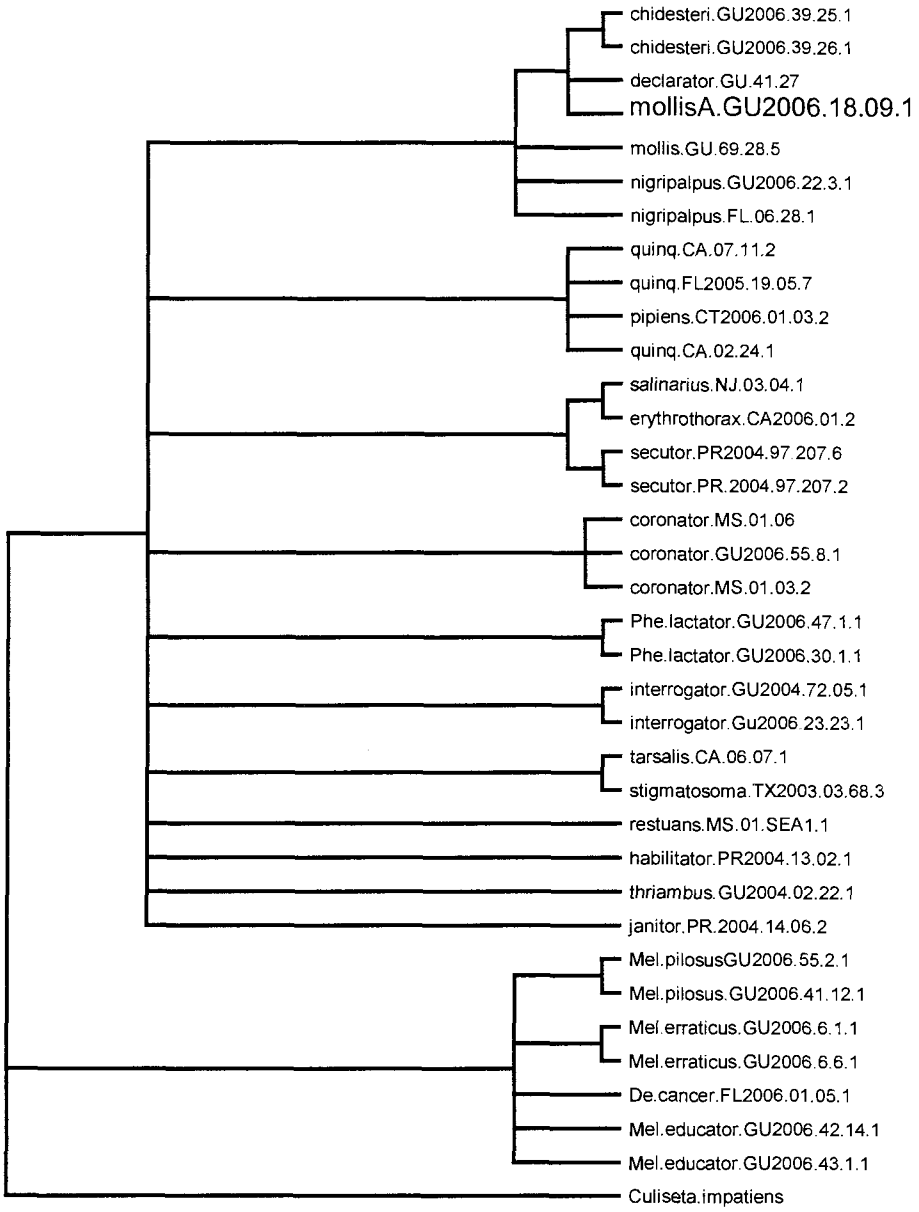

Figure 2.7. Strict consensus of neighbor-joining, parsimony, maximum likelihood, and Bayesian analysis trees for *COII* data.

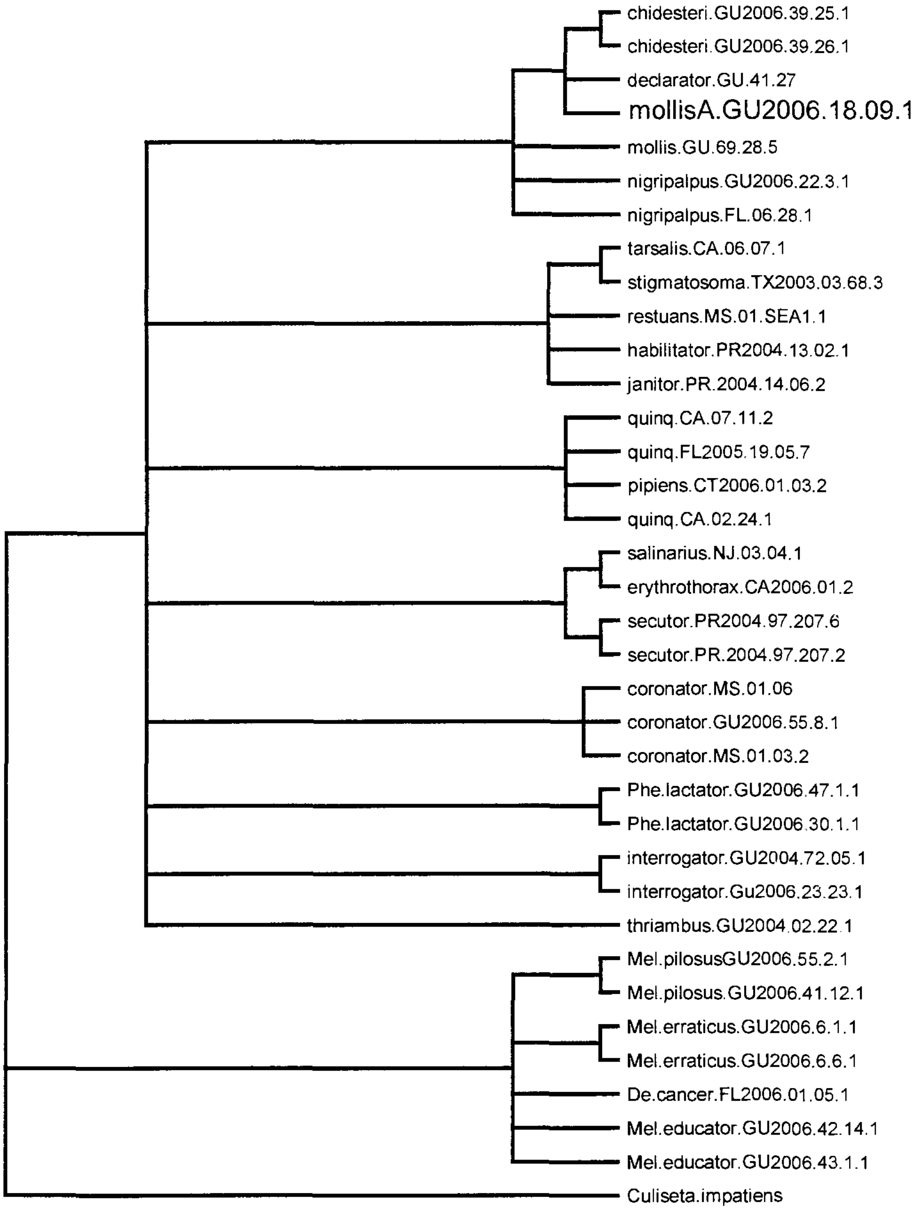

Figure 2.8. Strict consensus of parsimony, maximum likelihood, and Bayesian analyses of *COII* data.

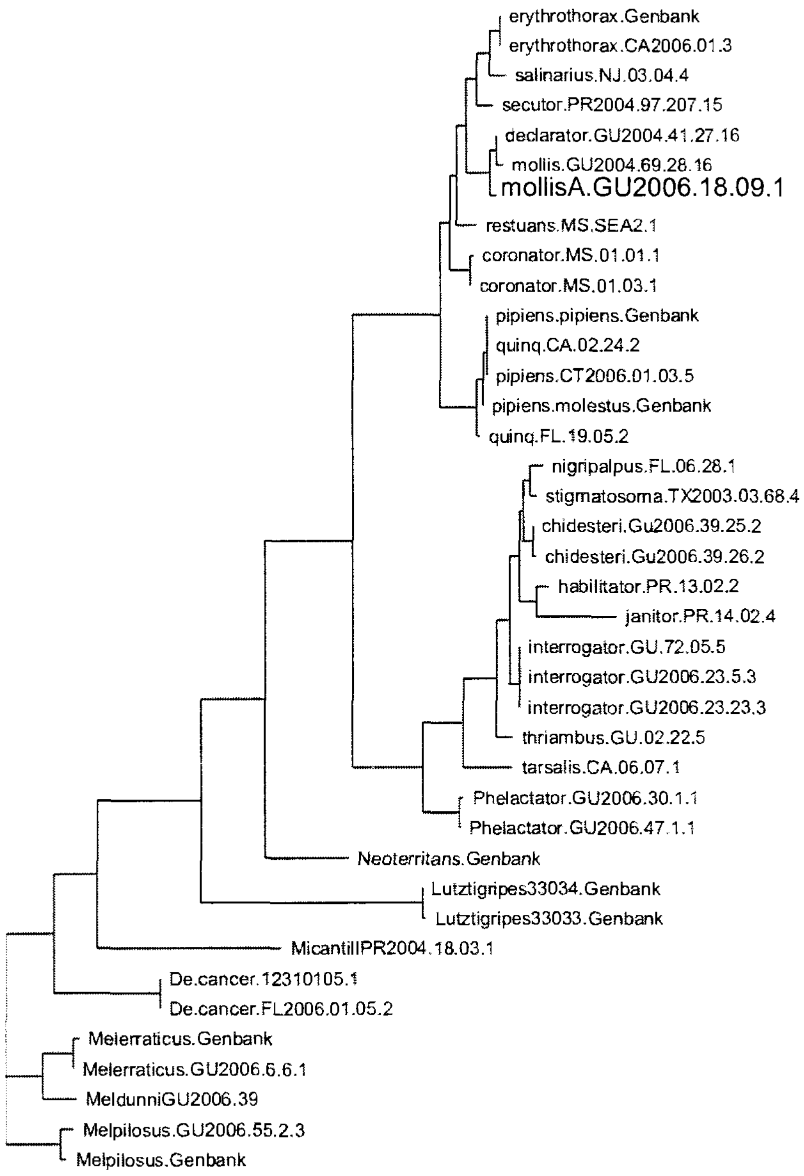

Figure 2.9. Neighbor-joining tree of *ITS* data.

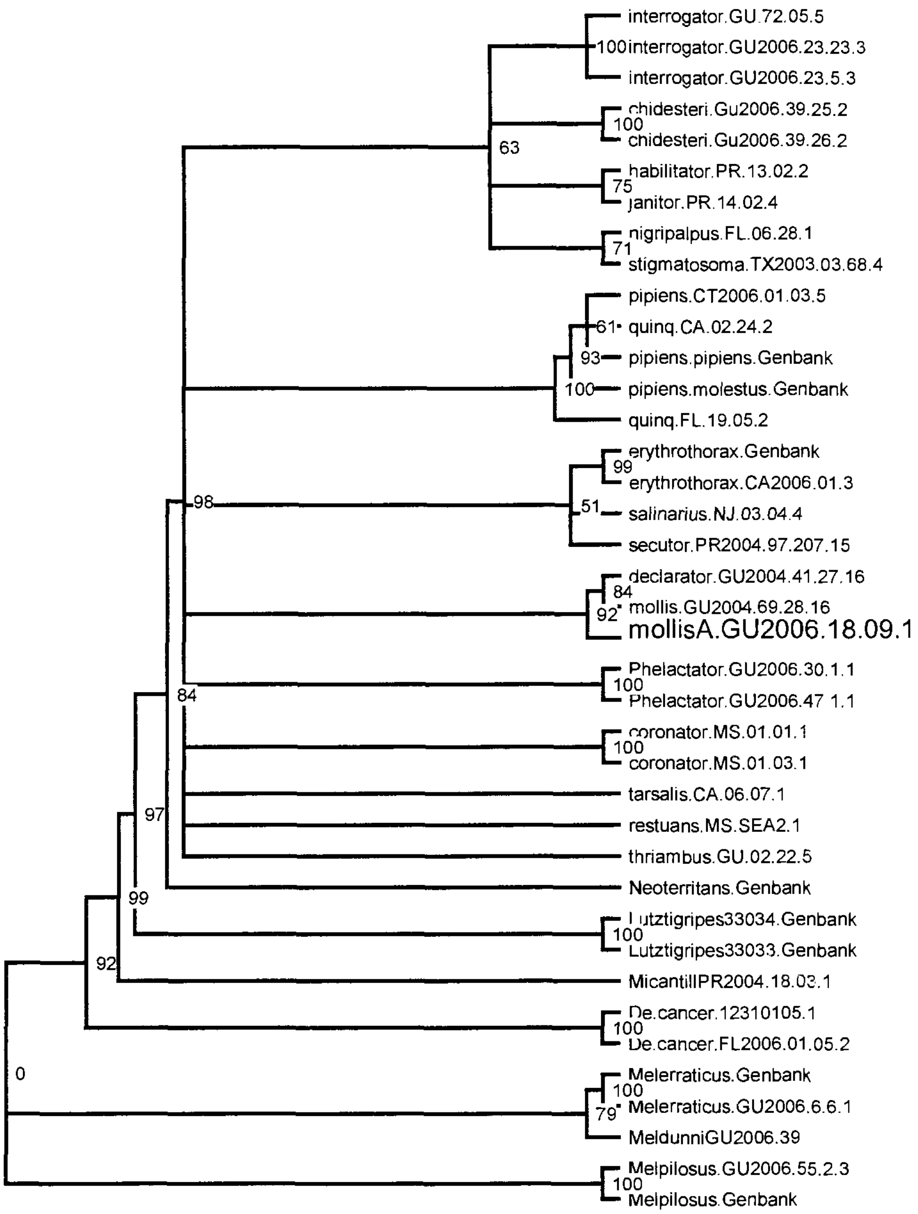

Figure 2.10. 50% majority-rule bootstrap consensus tree, from 1000 bootstrap replicates of NJ analysis of *ITS* data. Node labels represent percent bootstrap support.

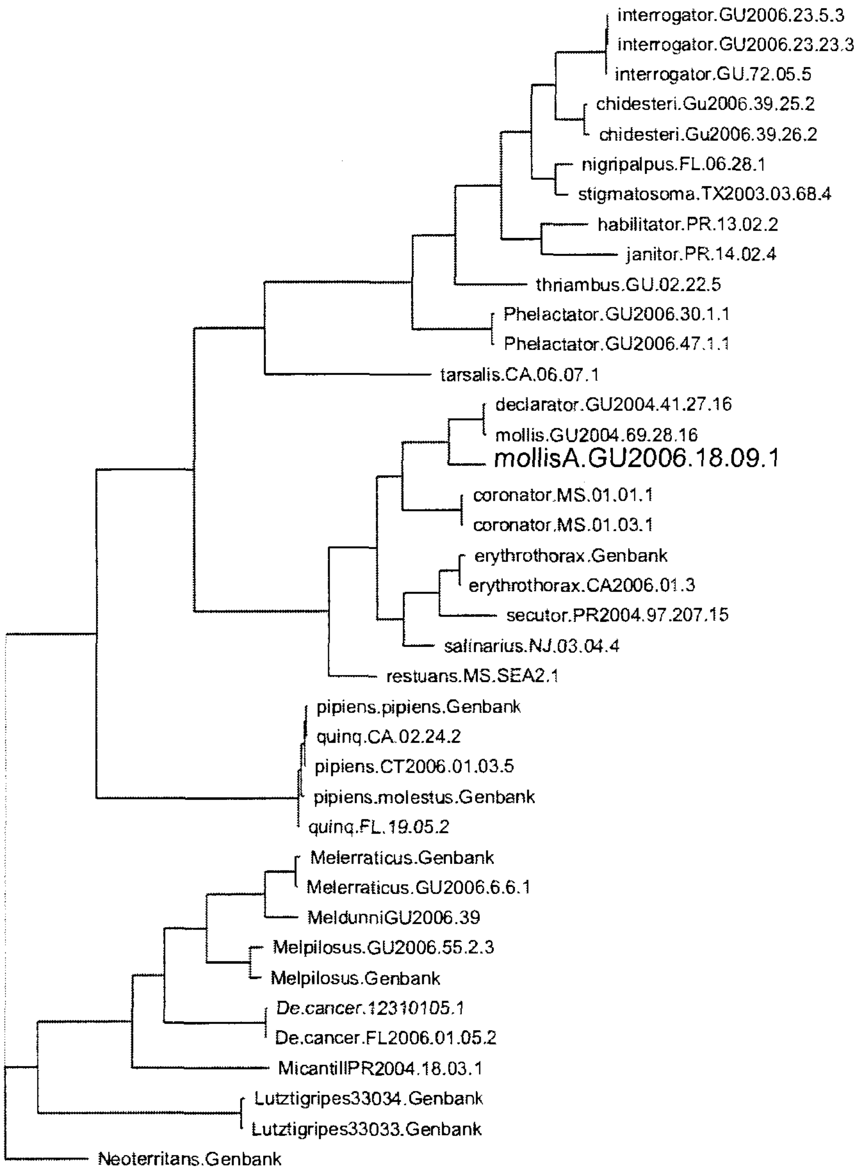

Figure 2.11. Most-parsimonious tree determined from *ITS1* and *ITS2* analyzed simultaneously. The length of the MP tree is 3537 steps and the rescaled consistency index = 0.4084.

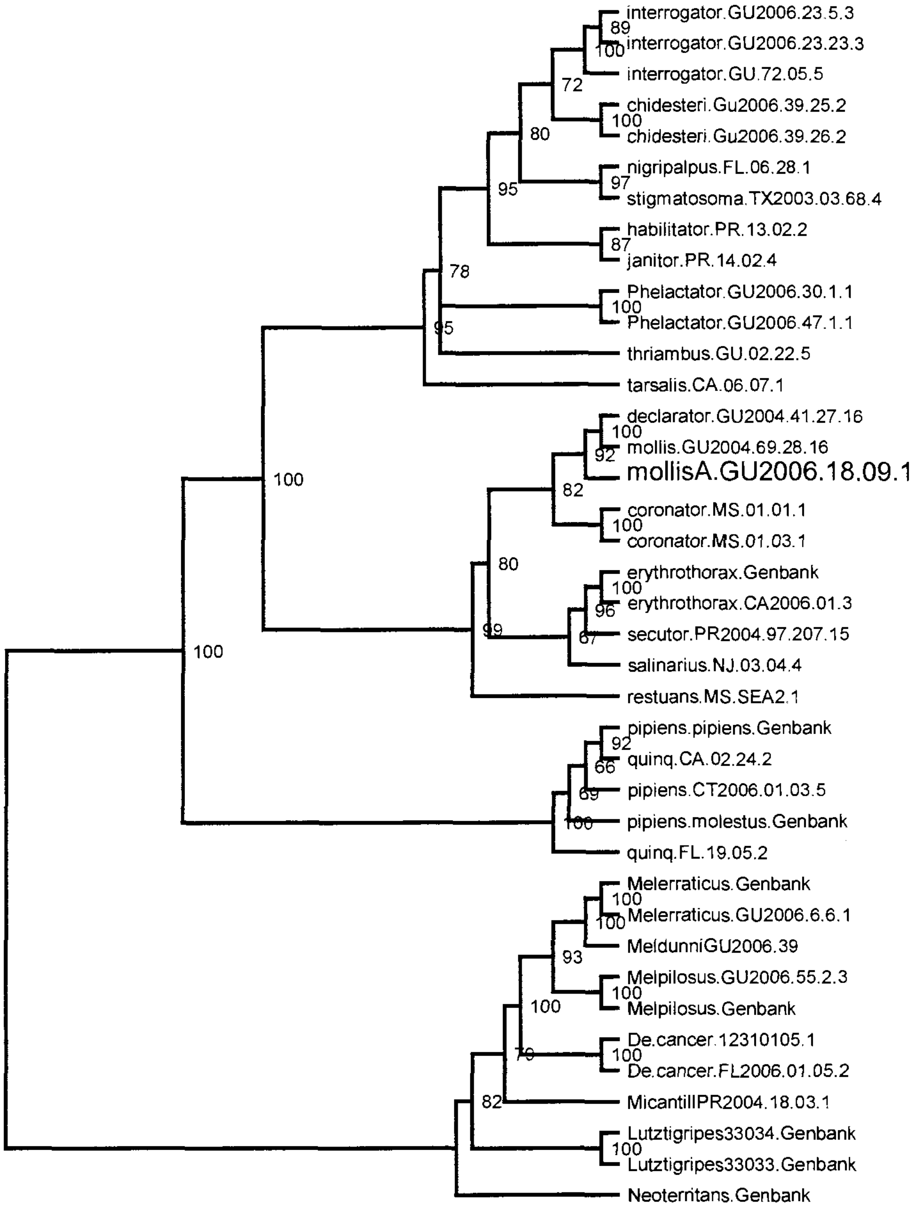

Figure 2.12. 50% majority-rule bootstrap support tree for parsimony analysis of *ITS1* and *ITS2* combined. Support values were calculated by running 1000 pseudoreplicates.

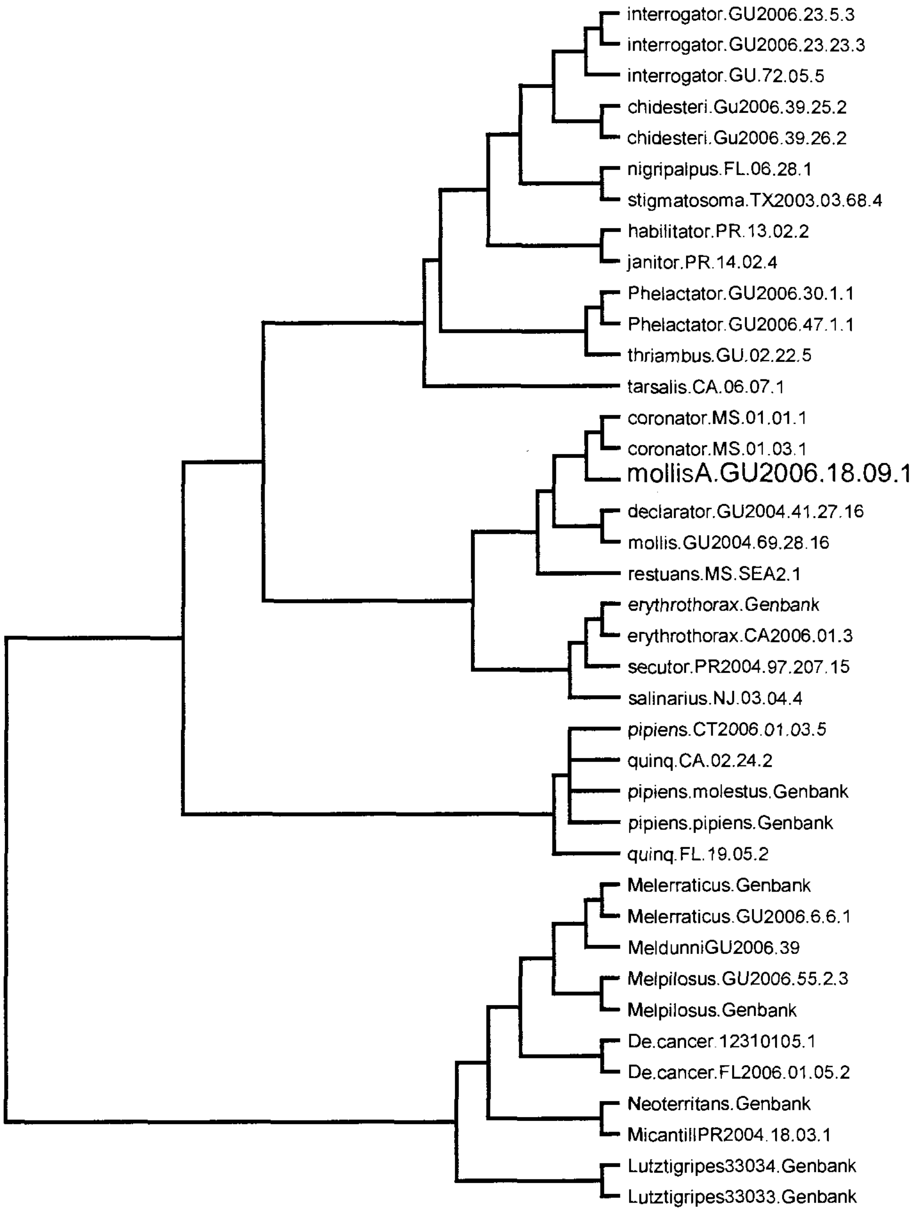

Figure 2.13. Strict consensus of two MP trees from parsimony analysis of *ITS1* data. Length= 4245 steps, RC=0.3520.

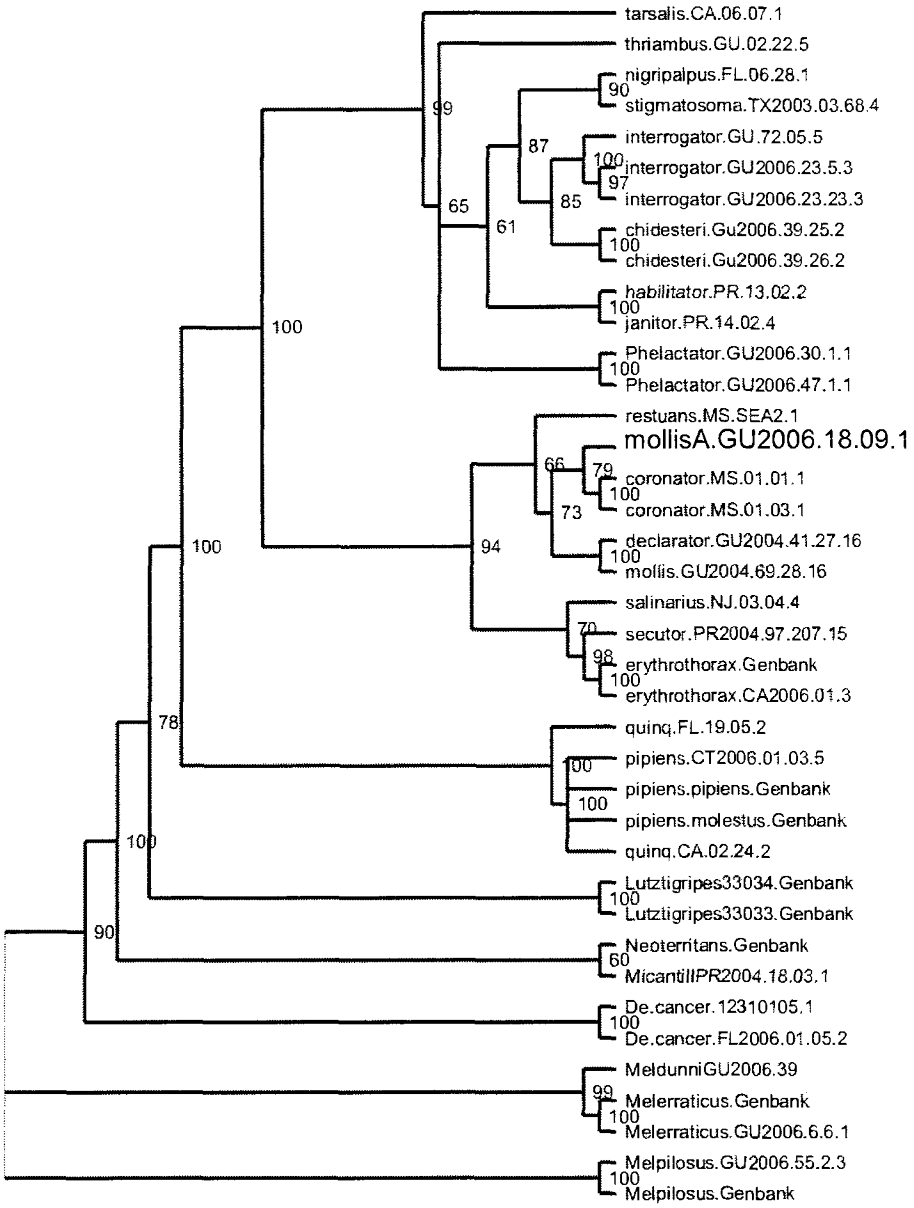

Figure 2.14. 50% majority-rule bootstrap support tree of parsimony analysis for *ITS1* data. Support values were calculated by running 1000 pseudoreplicates.

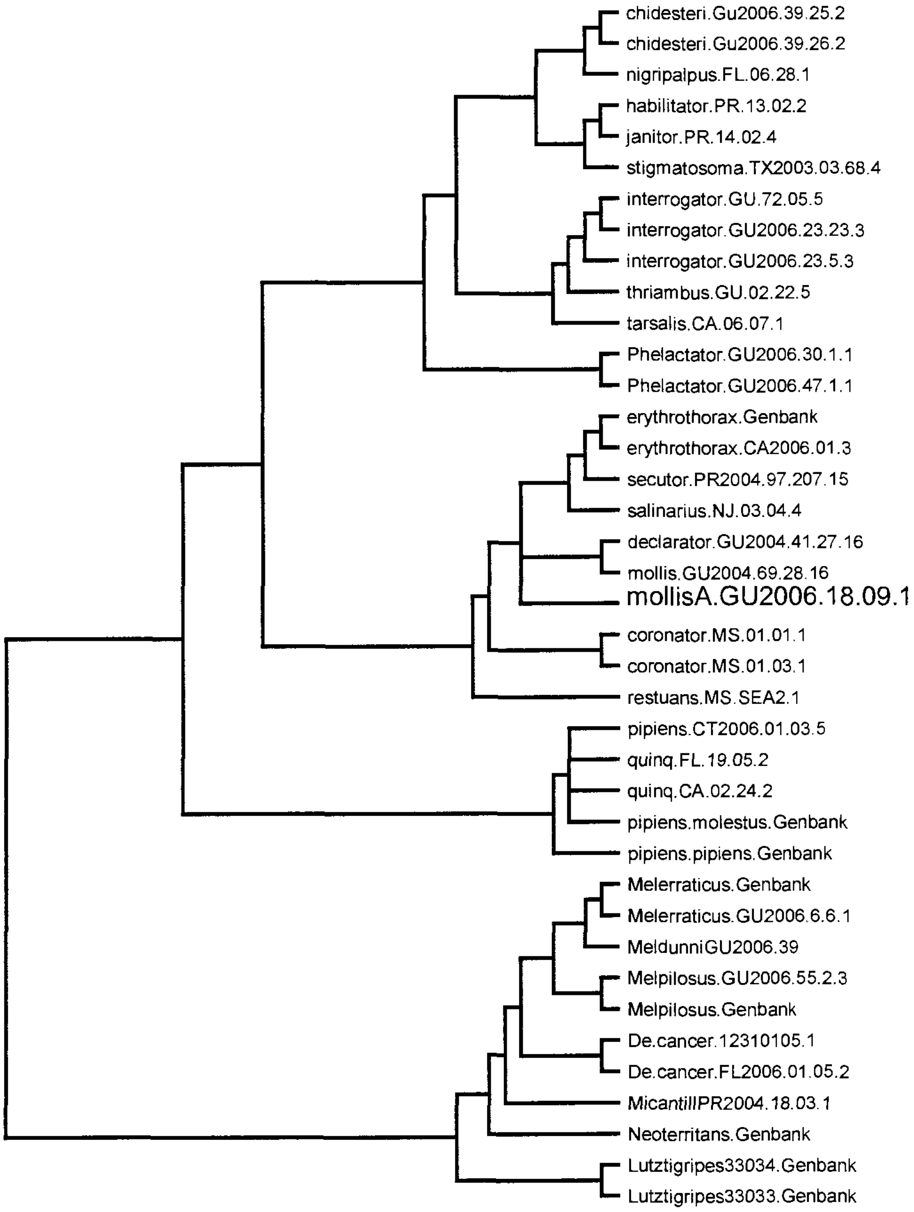

Figure 2.15. Strict consensus of six MP trees found for the *ITS2* data. Length=1657 steps, RC=0.3840.

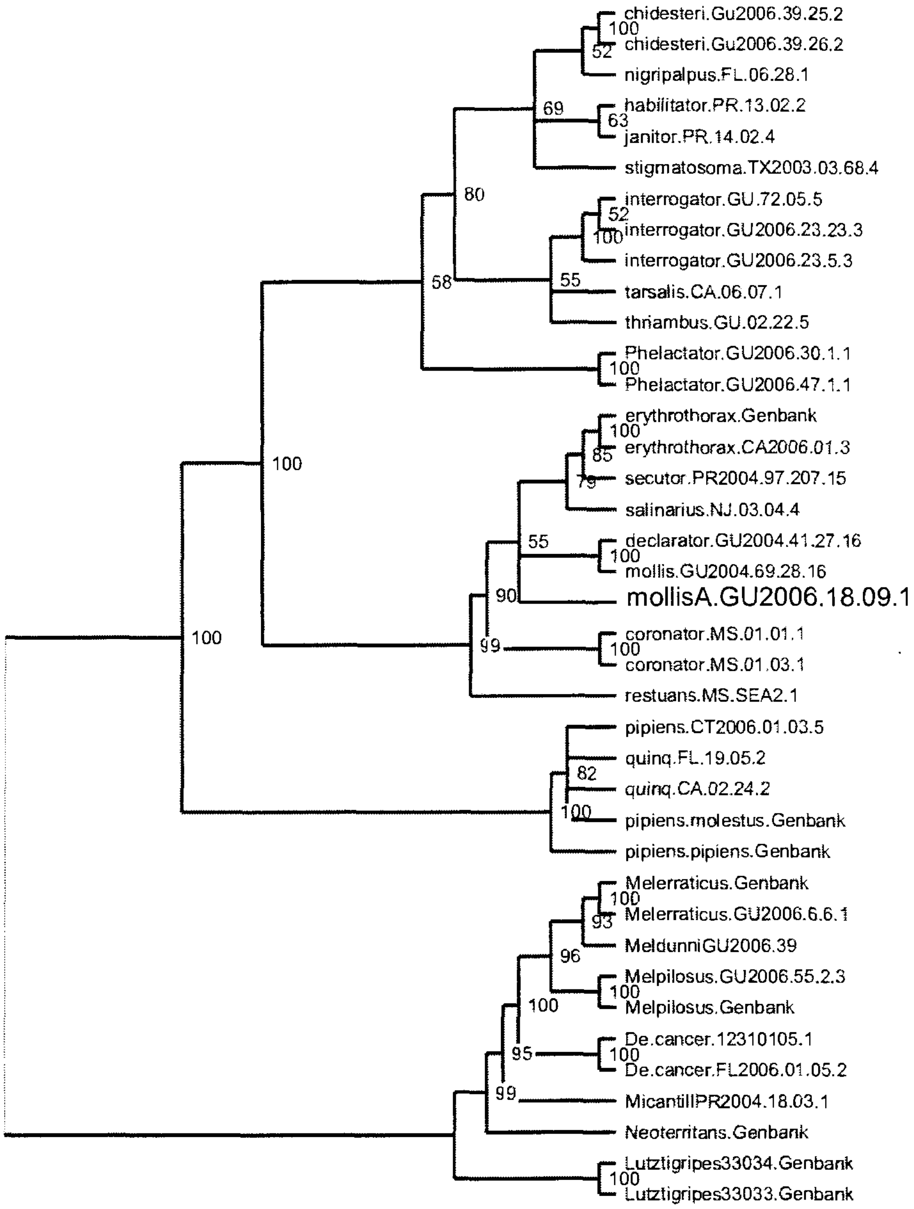

Figure 2.16. 50% majority-rule bootstrap support tree for *ITS2* data. Support values were calculated by running 1000 pseudoreplicates.

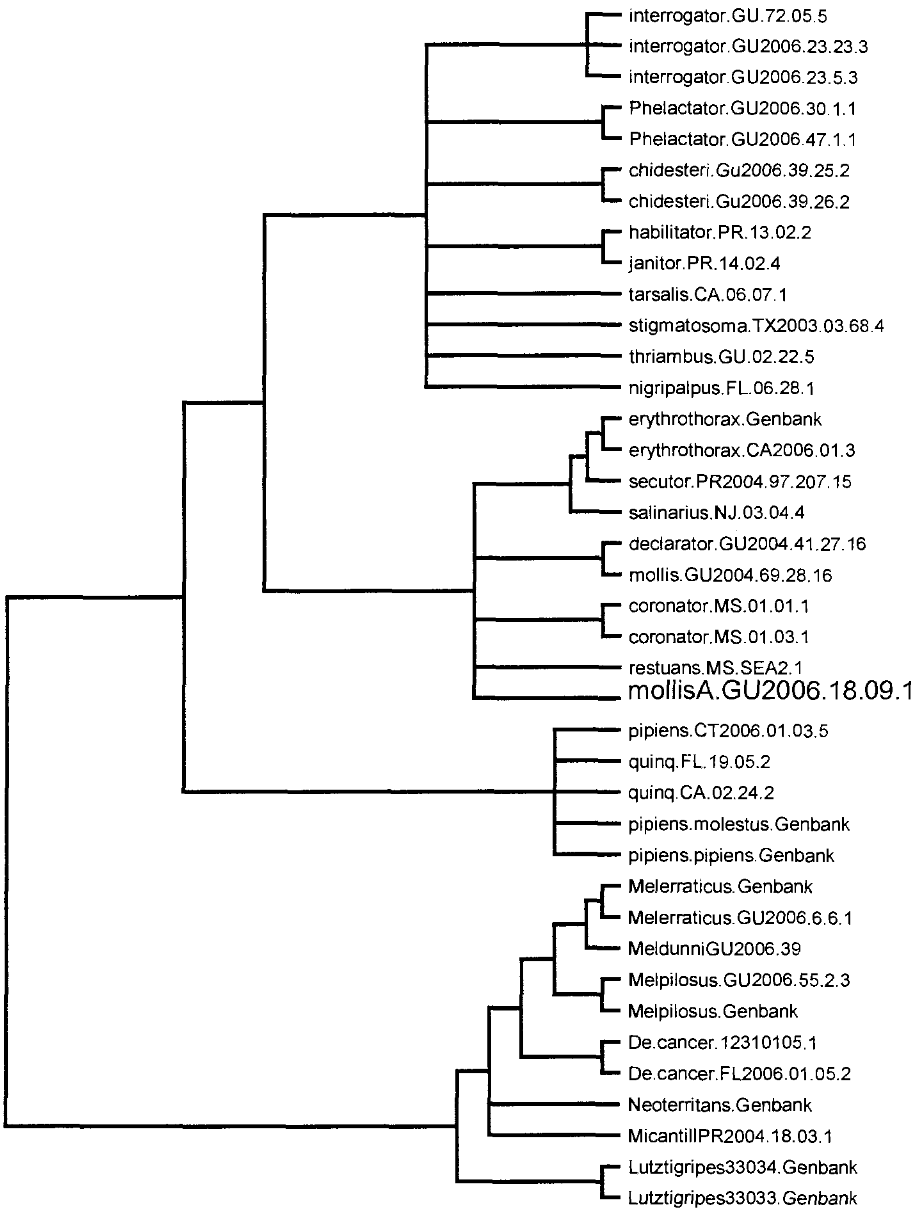

Figure 2.17. Strict consensus tree between Figure 2.15 (*ITS1* data) and Figure 2.16 (*ITS2* data).

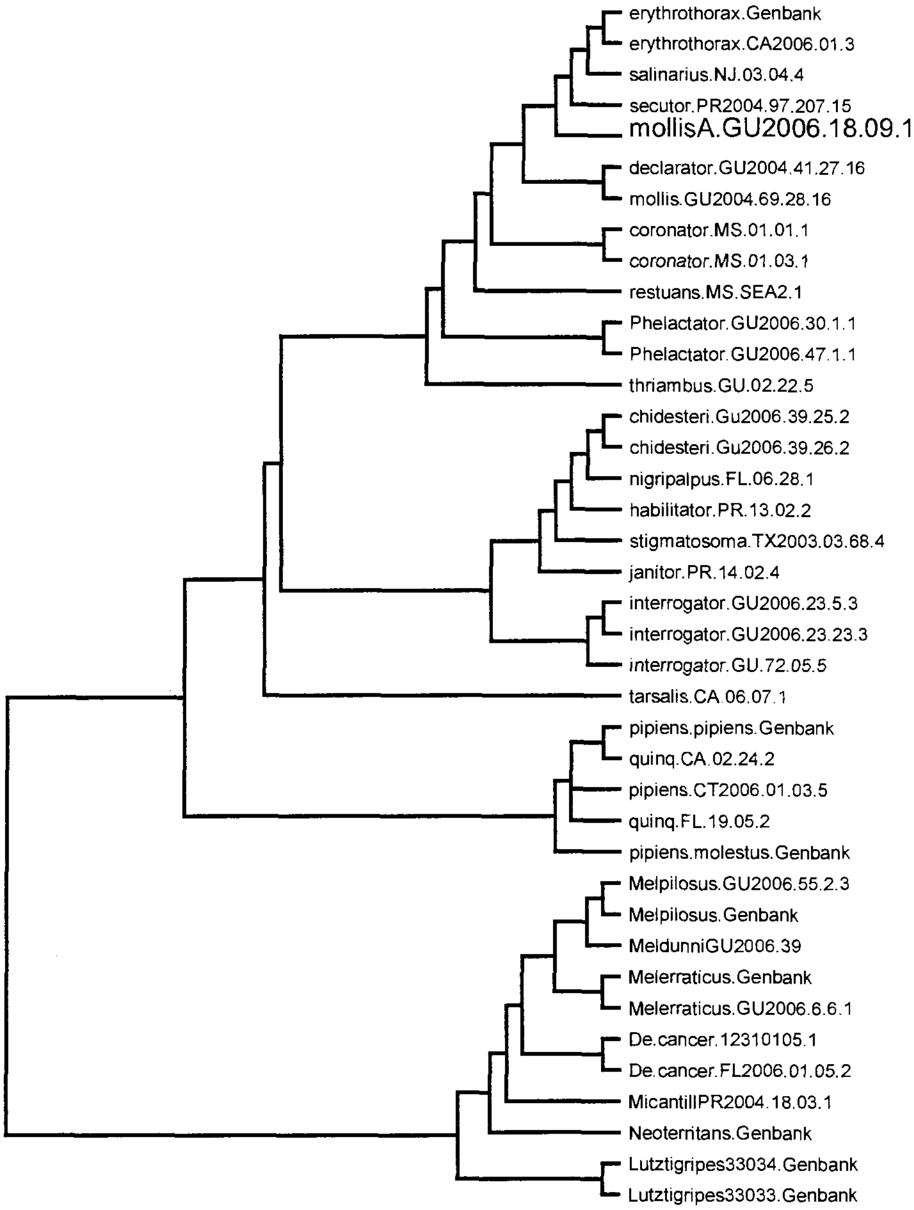

Figure 2.18. Maximum likelihood tree of *ITS2* data, using HKY85 model with gamma distributed rate variation. Negative log likelihood= 4351.11998.

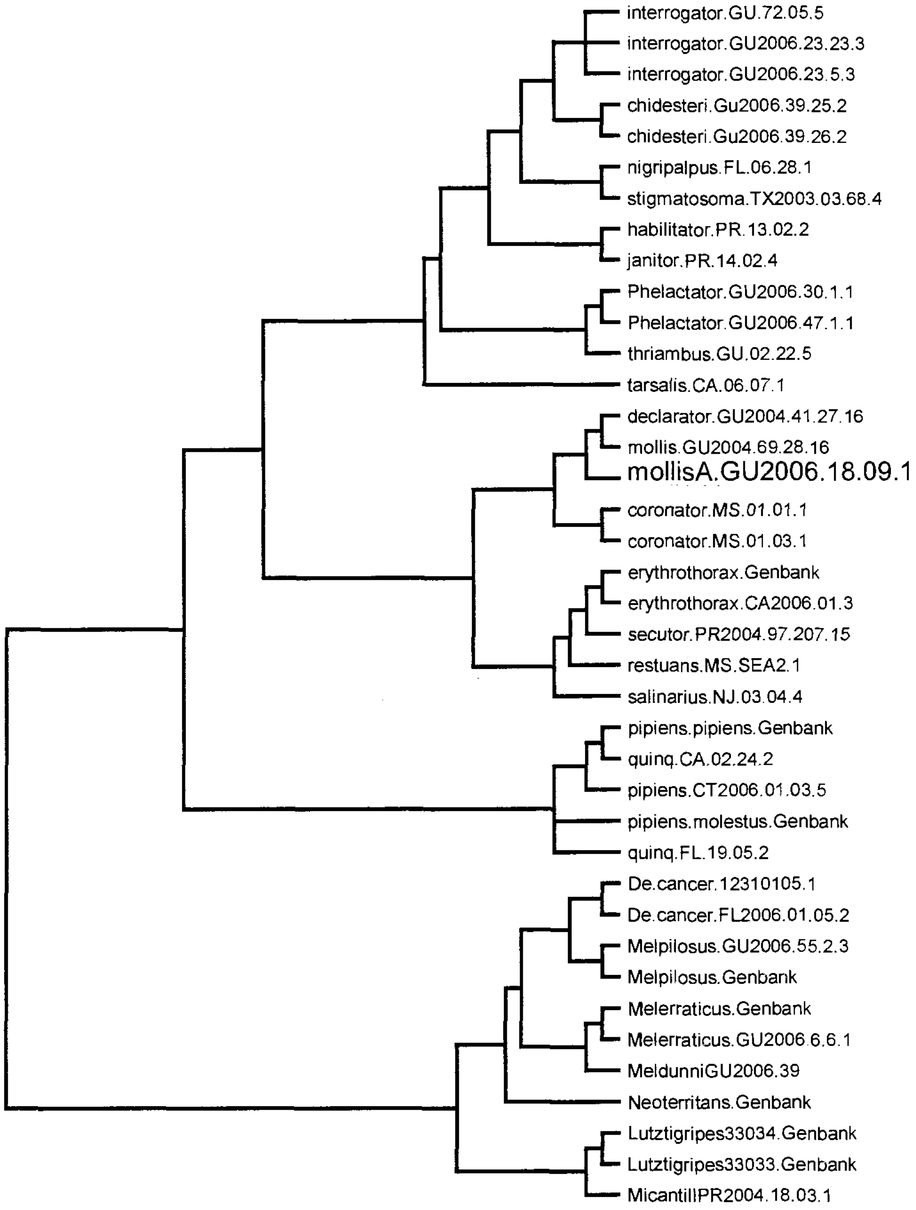

Figure 2.19. Maximum likelihood tree of *ITS1* data, using HKY+I+G model. Negative log likelihood= 10023.73182.

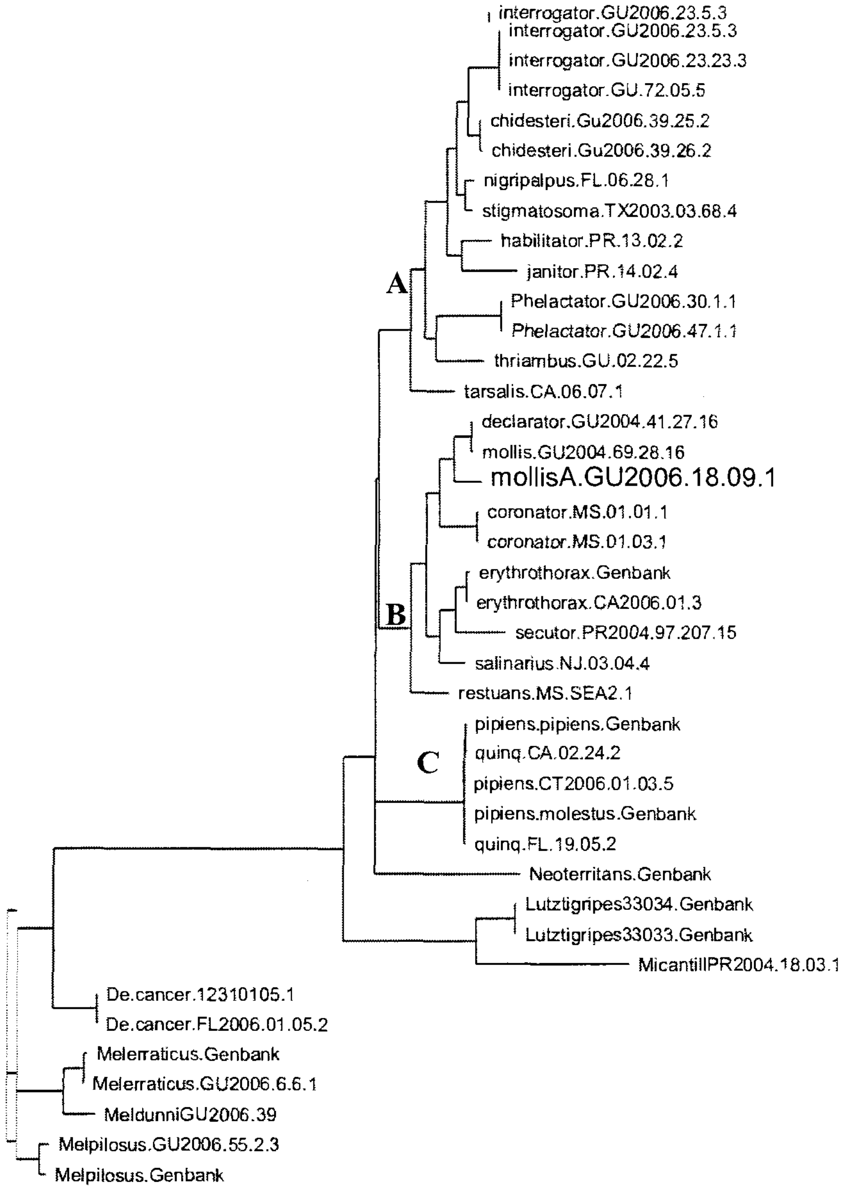

Figure 2.20. Maximum likelihood tree of combined *ITS1* and *ITS2* data, under HKY+I+G model. Negative log likielihood= 14620.68805.

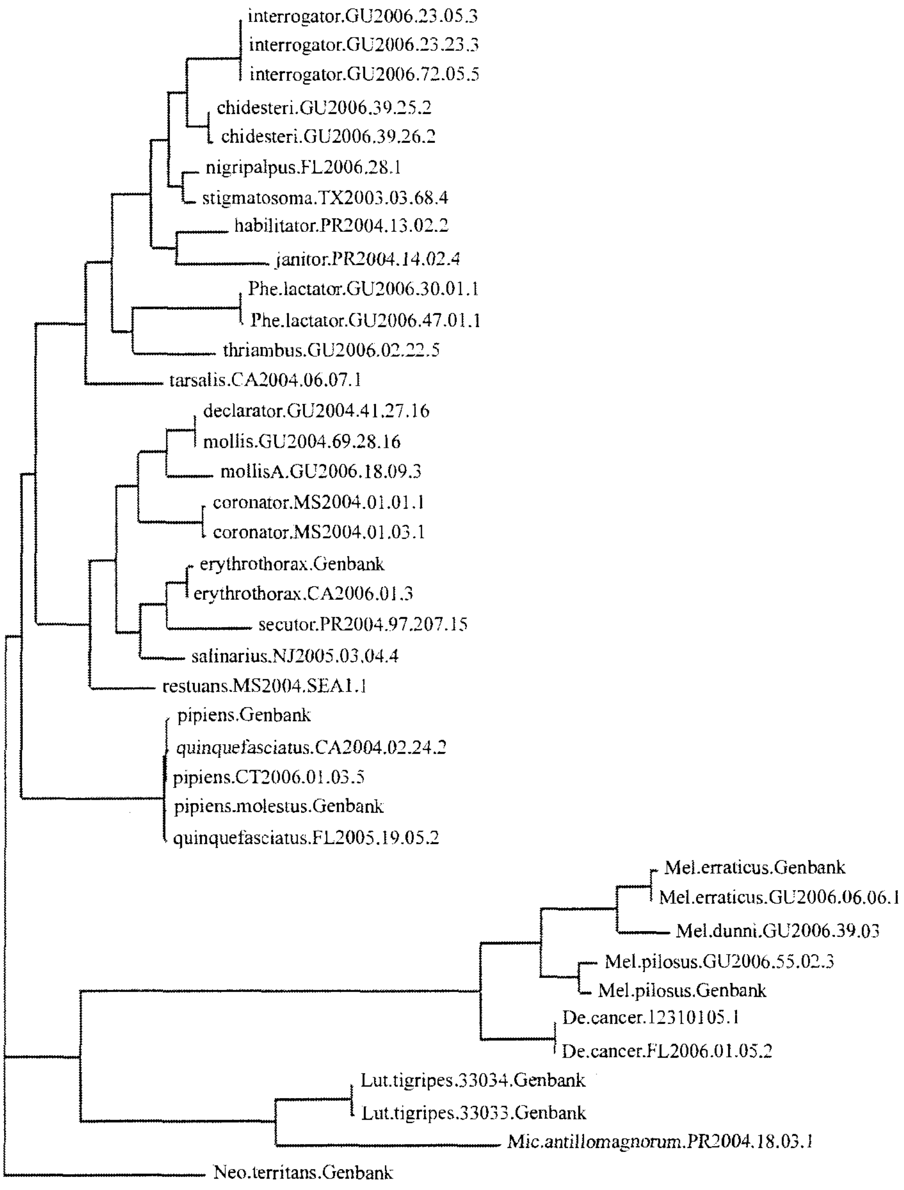

Figure 2.21. Bayesian analysis summary tree of combined *ITS1* and *ITS2* data. Model contained 2 substitution types, a proportion of invariant sites, and gamma distributed rate variation. Node labels indicate posterior probability values.

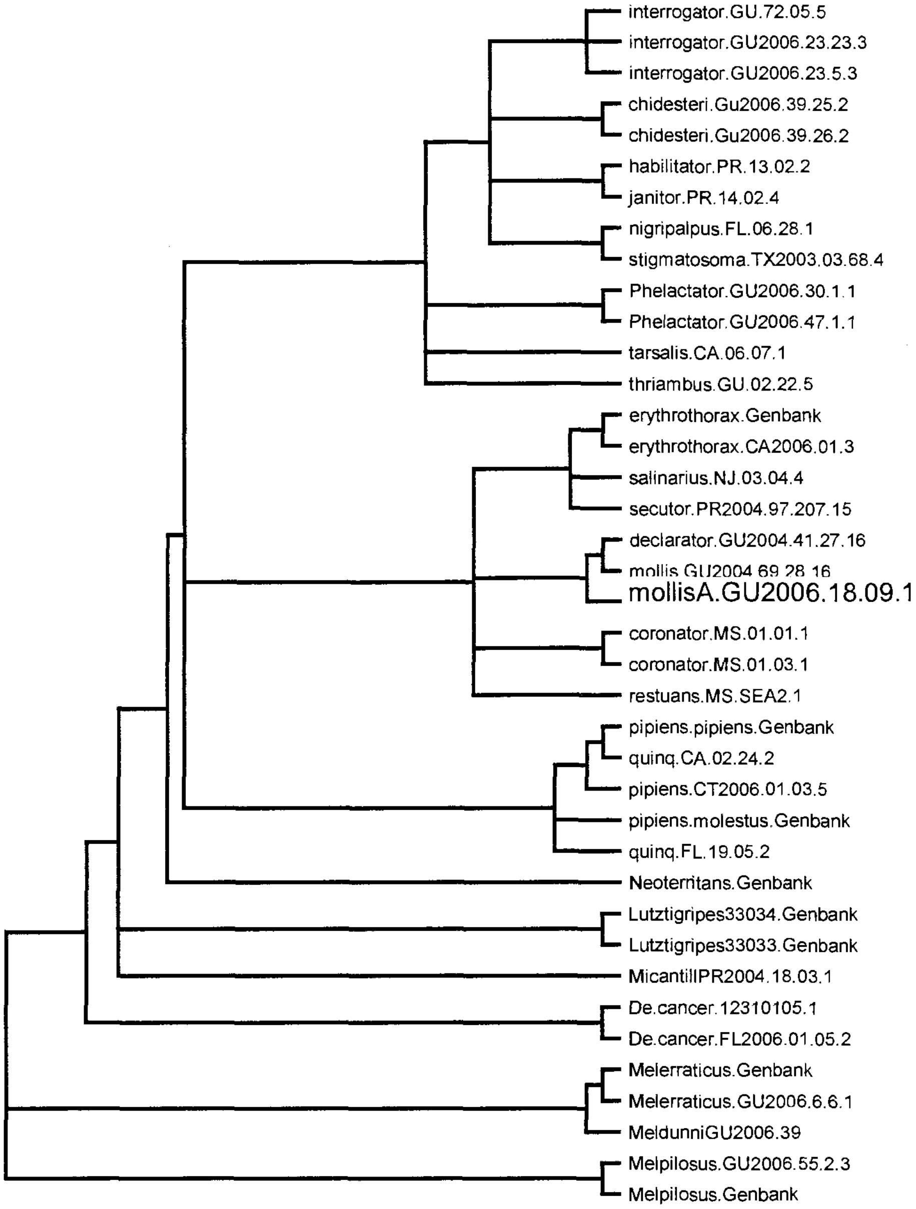

Figure 2.22. Strict consensus among all four methods of phylogenetic analysis studied here, when *ITS1* and *ITS2* are analyzed simultaneously.

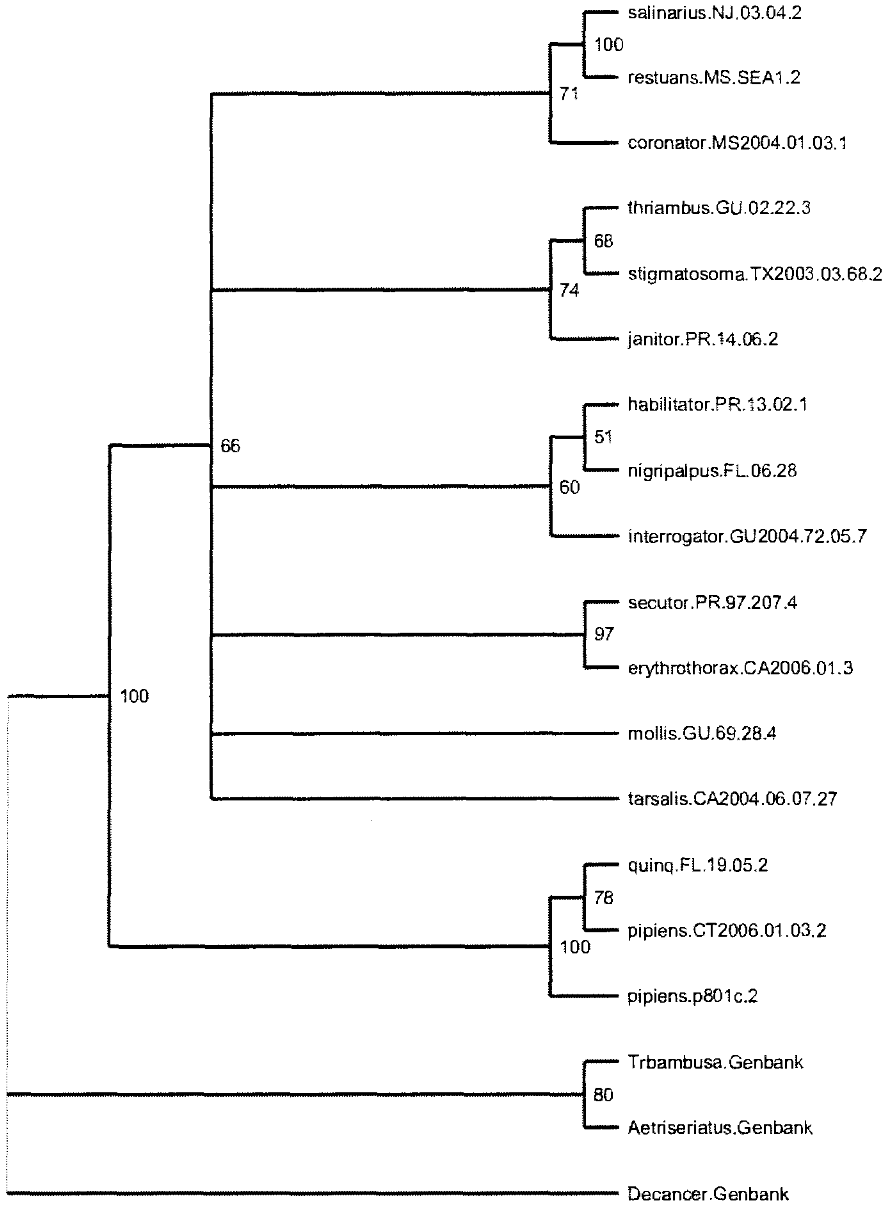

Figure 2.23. 50% majority-rule bootstrap support tree, using 1000 pseudoreplicates, for NJ distance analysis of *white*. Node labels represent bootstrap support values.

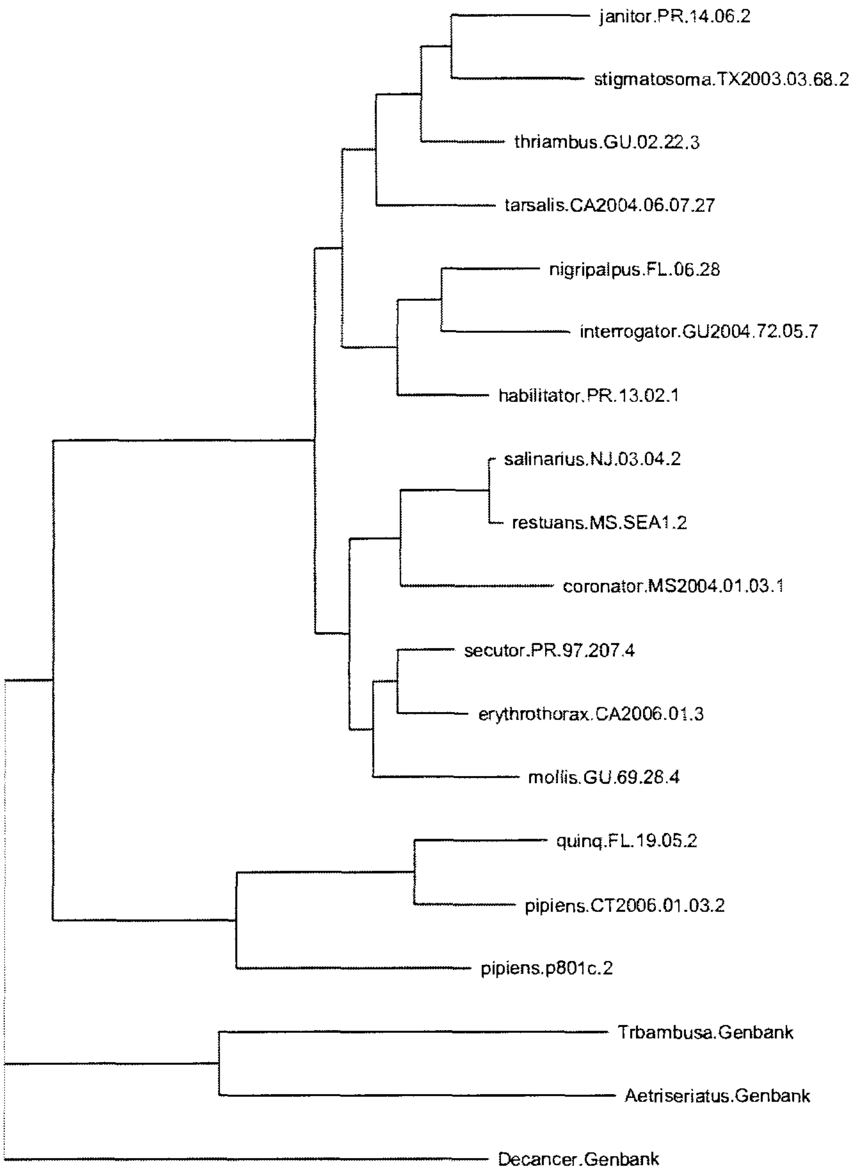

Figure 2.24. One of two MP trees, from entire alignment of *white* data. Length= 1134 steps, CI= 0.6111.

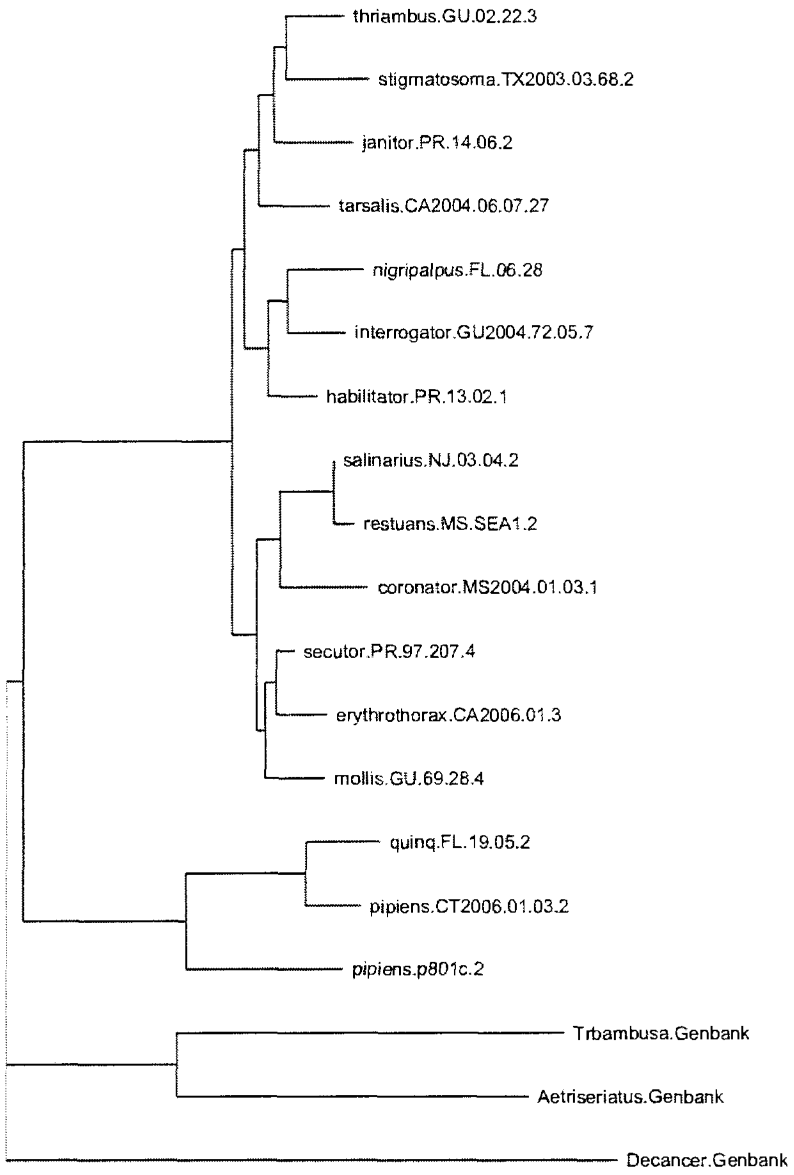

Figure 2.25. Single most parsimonious tree, from entire *white* alignment after reweighting of characters by successive approximations. Length= 488.92937, CI= 0.8523.

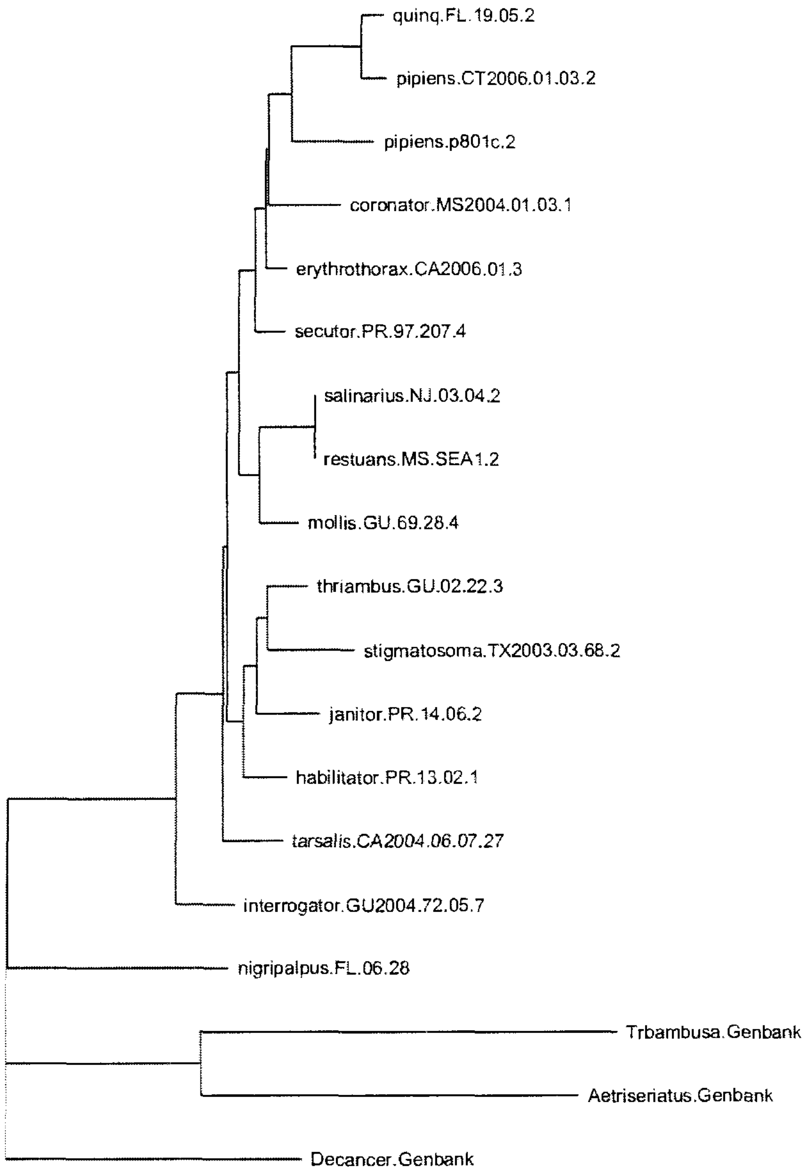

Figure 2.26. Single most parsimonious tree, from coding regions of *white*, after reweighting of characters twice by successive approximations. Length= 281.03830 steps, CI= 0.9086.

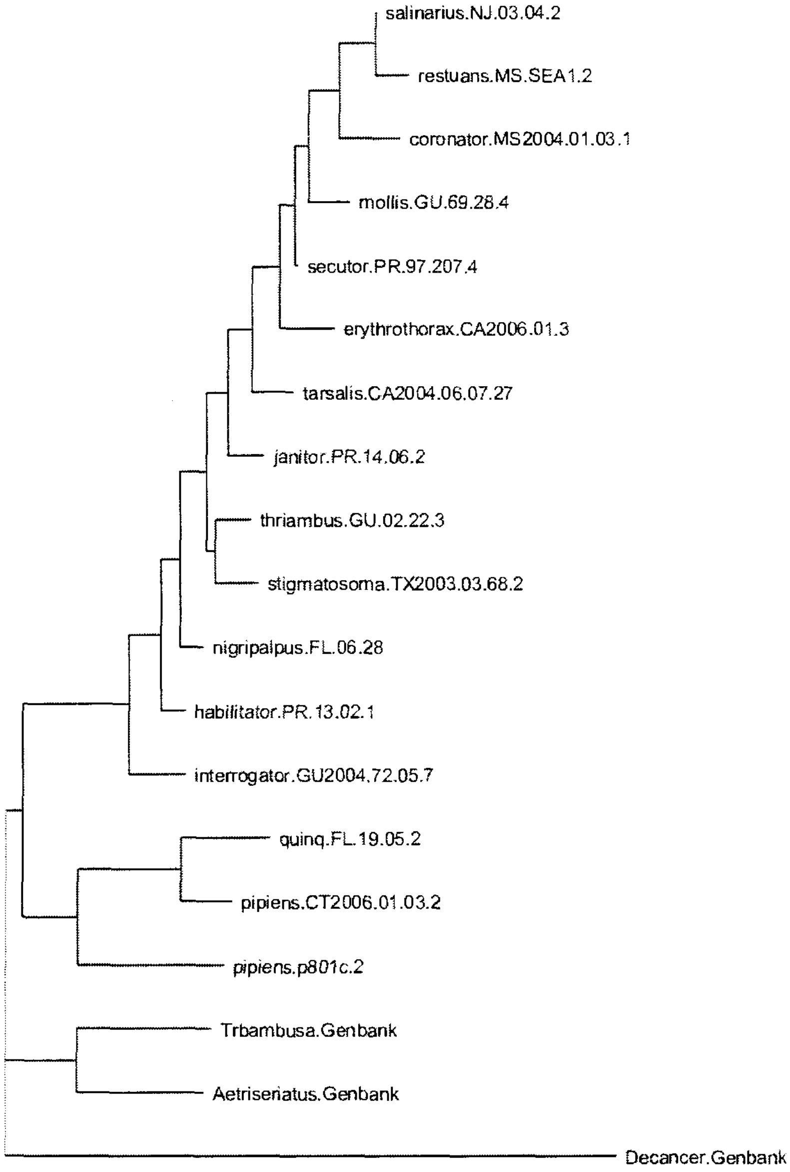

Figure 2.27. Single MP tree, from introns of *white*, after reweighting by successive approximations. Length= 213.70754, CI= 0.7879.

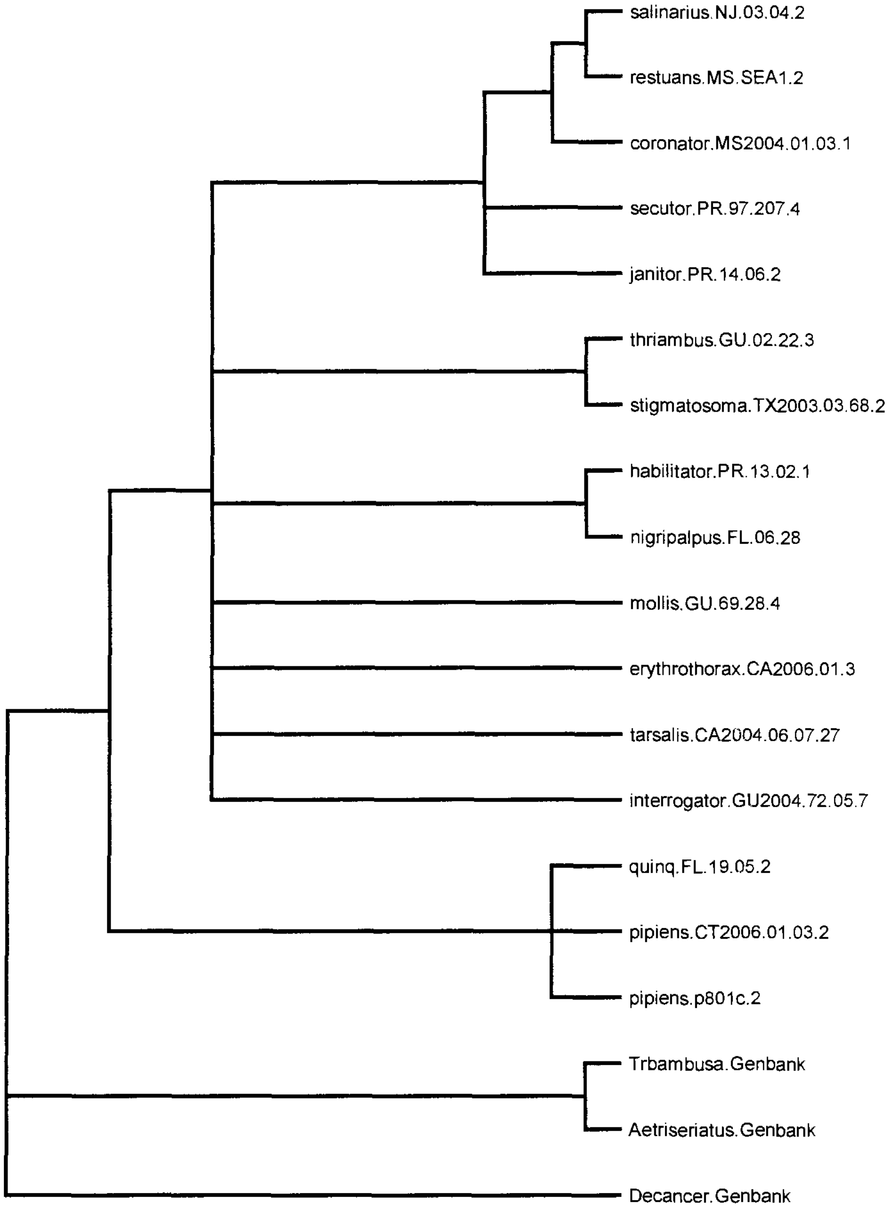

Figure 2.28. Strict consensus of four MP trees of first codon position of *white*, after reweighting characters by successive approximations.

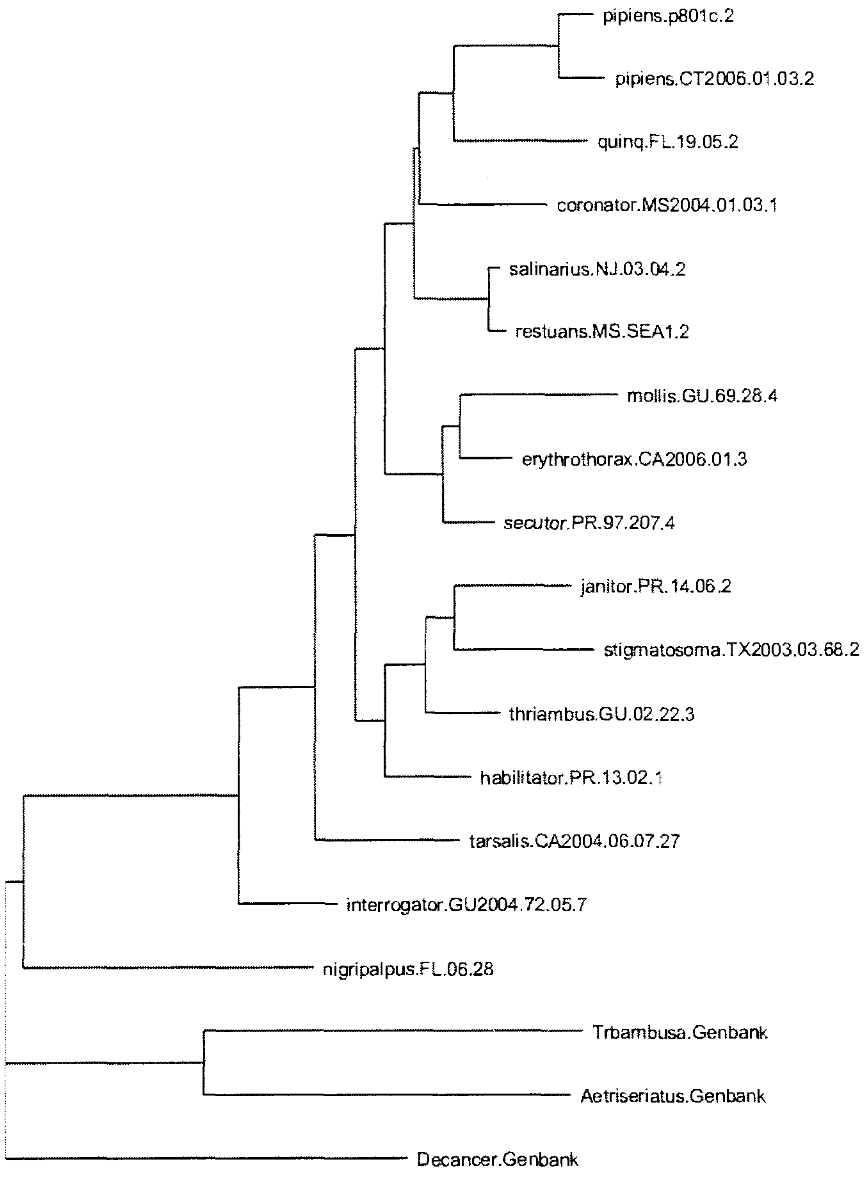

Figure 2.29. One of eight MP trees from third codon position of *white*. Length=549 steps, CI= 0.6357.

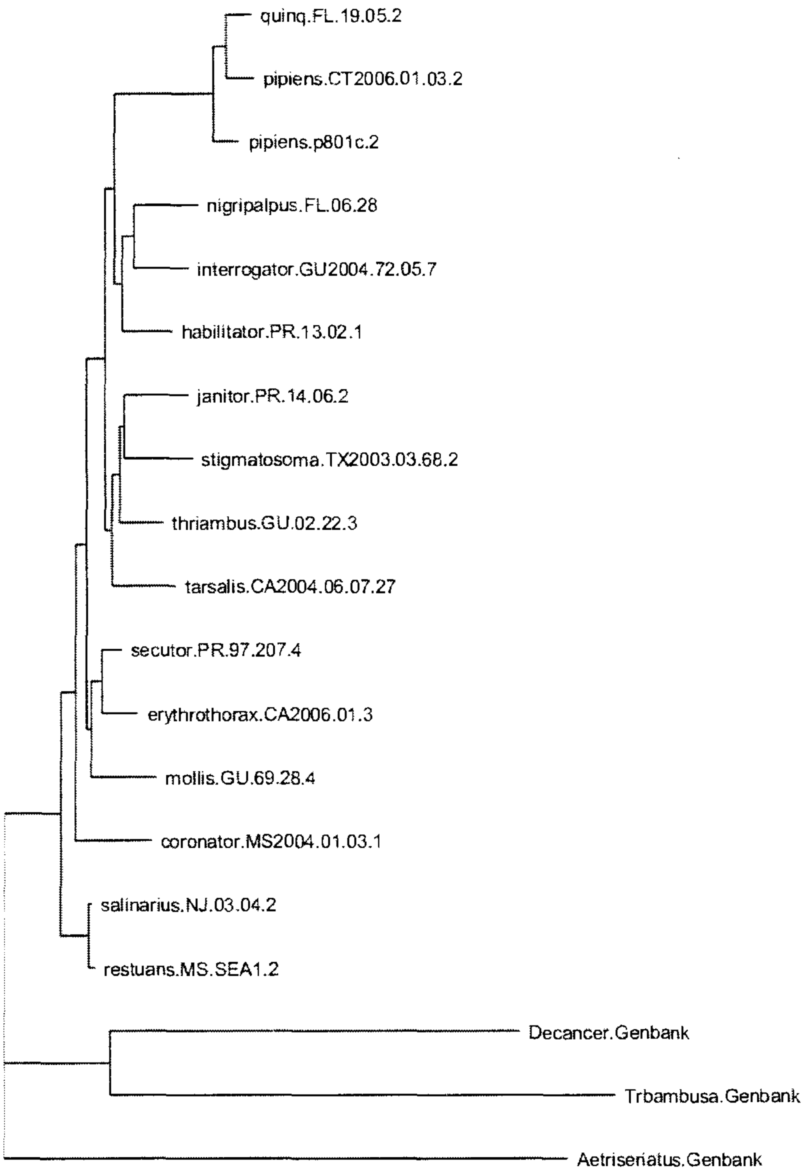

Figure 2.30. Maximum likelihood tree from entire alignment of *white,* using HKY+I+G model. –lnL= 6216.72777.

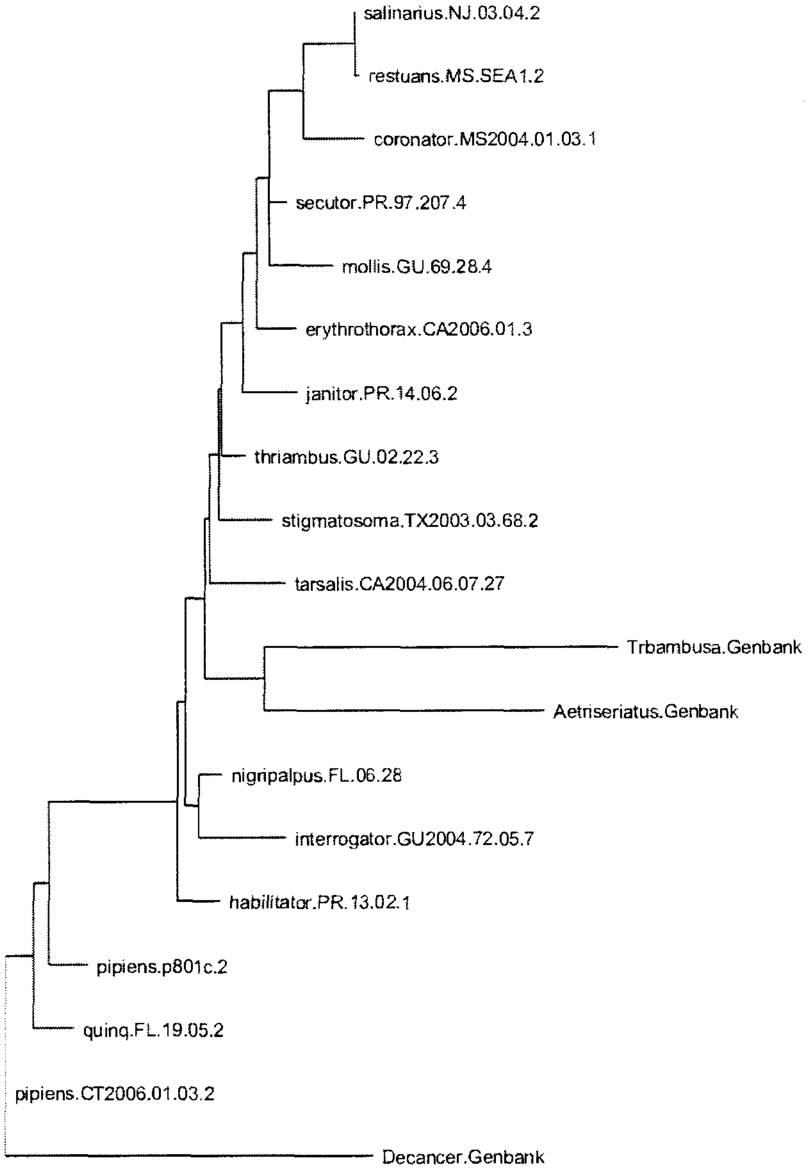

Figure 2.31. Maximum likelihood tree from introns of *white*, using HKY+I+G model. –lnL= 1974.92947.

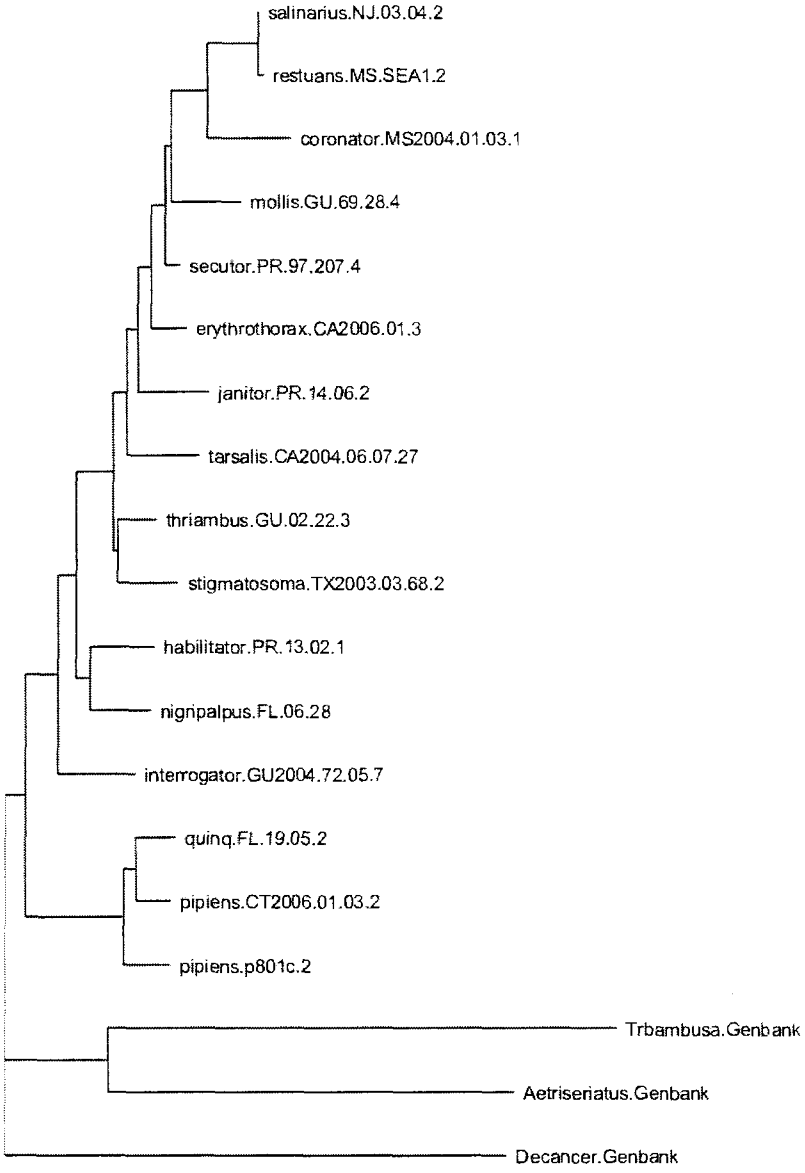

Figure 2.32. Maximum likelihood tree from *white* with third codon position excluded. -lnL= 3578.04000.

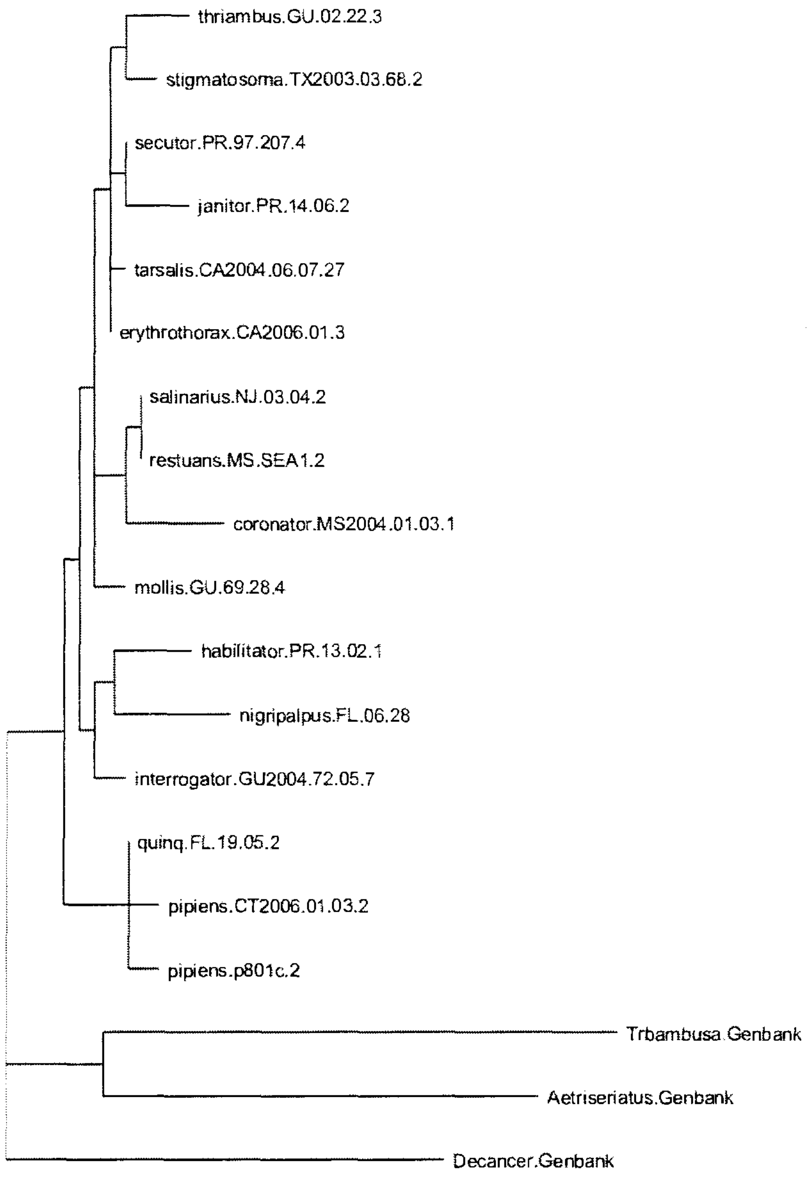

Figure 2.33. Maximum likelihood tree from *white* data, excluding third codon position and introns, and implementing HKY+G model. –lnL= 1455.70818.

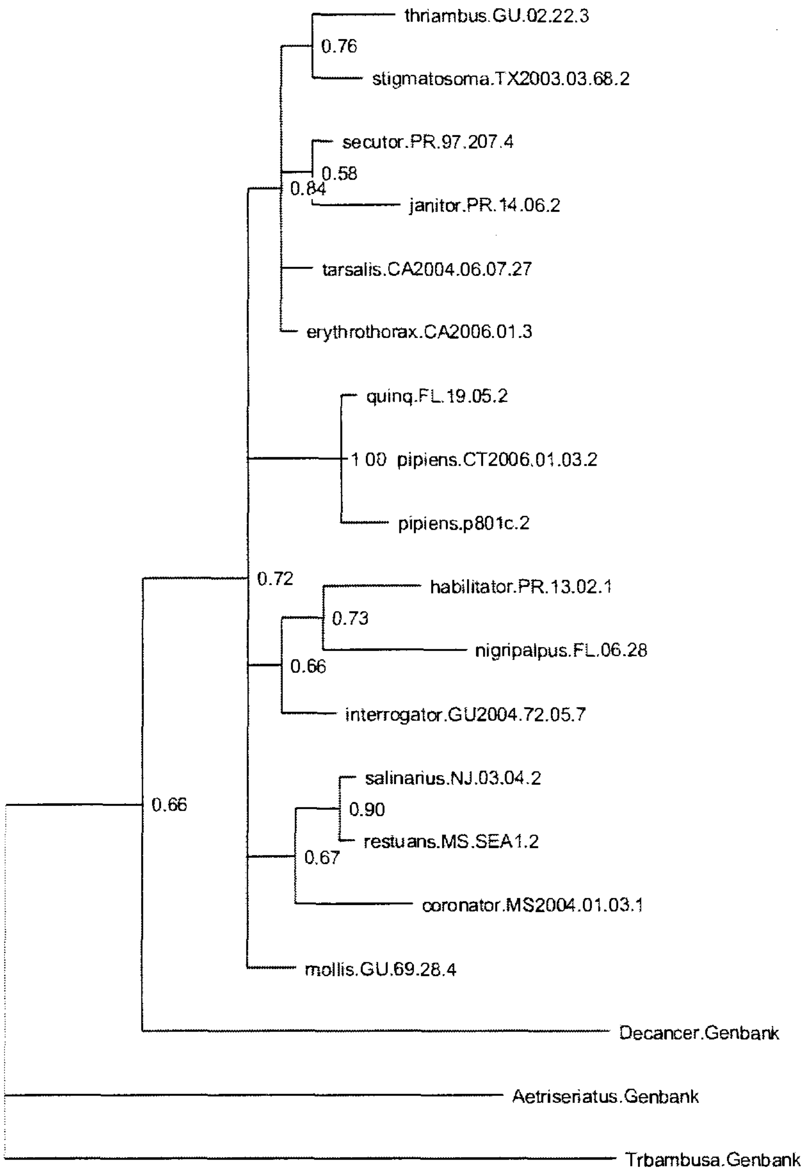

Figure 2.34. Bayesian analysis tree of *white* with third codon position and introns excluded. Node labels represent posterior probability values.

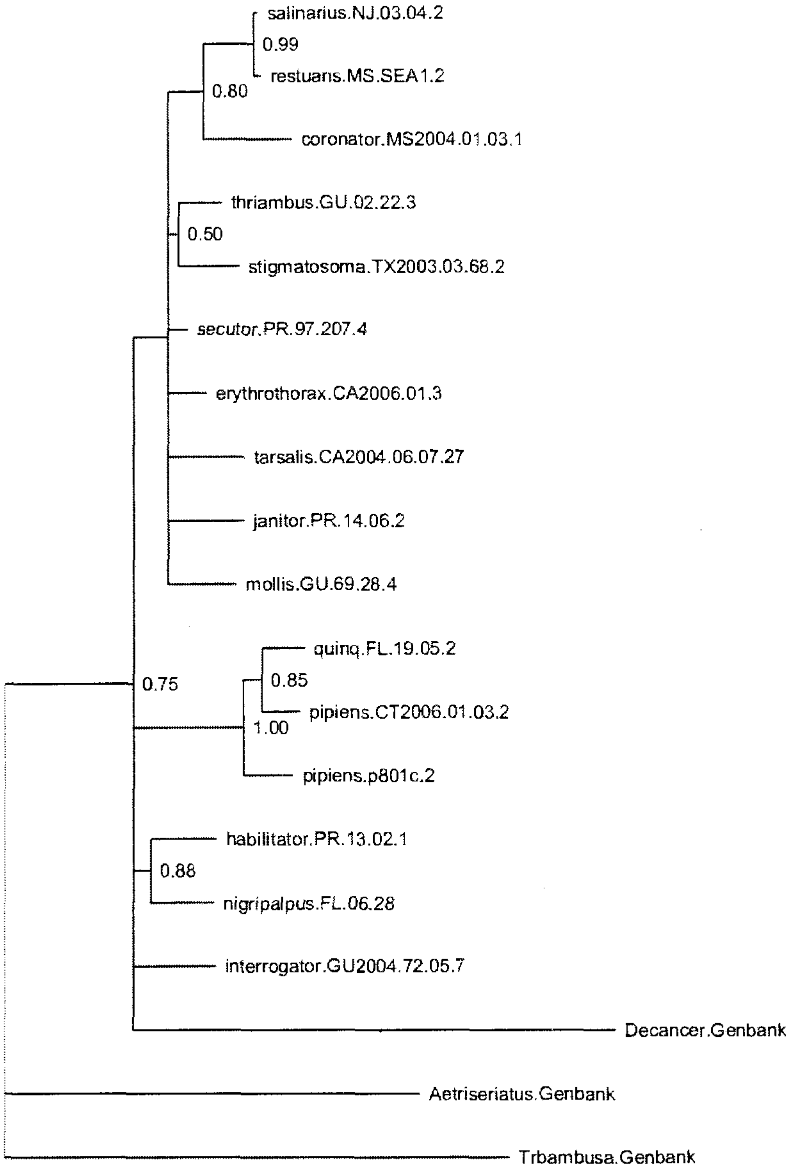

Figure 2.35. Bayesian analysis tree from *white* with third codon position excluded. Node labels represent posterior probability values.

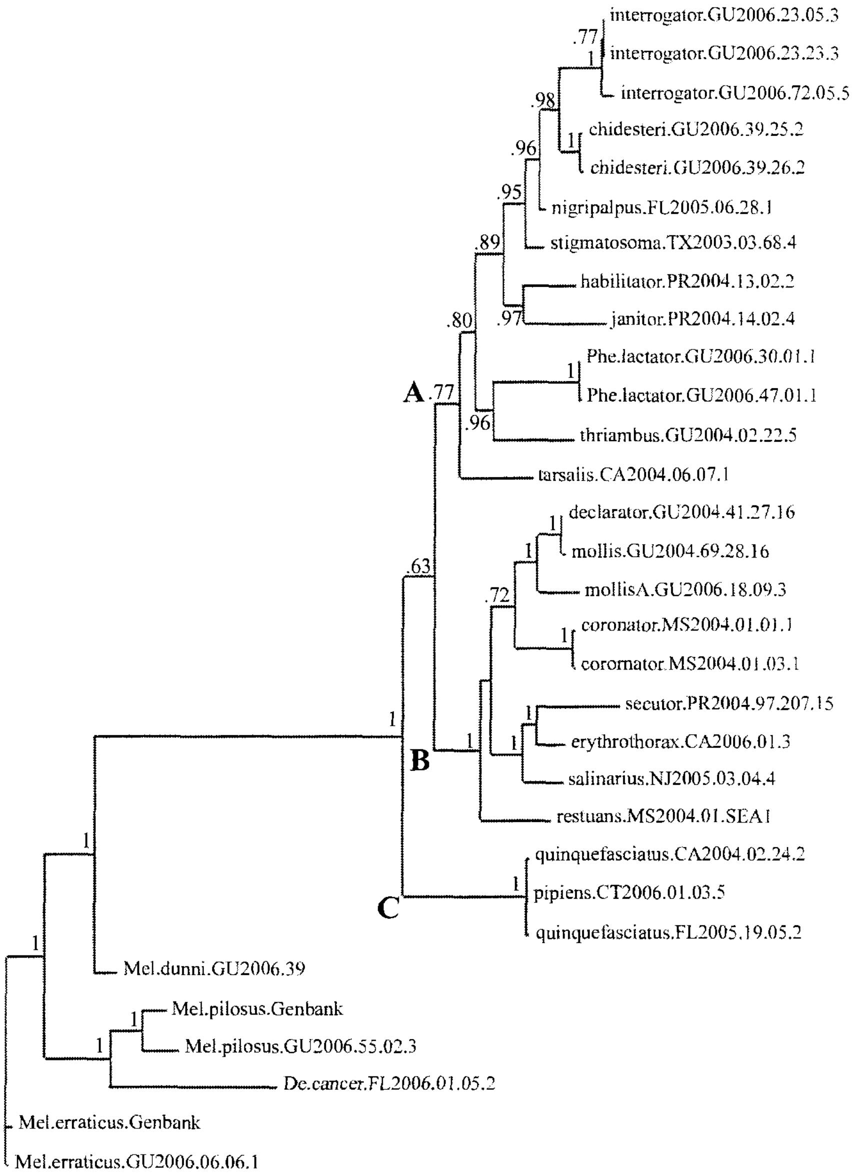

Figure 2.36. Bayesian analysis tree from simultaneous analysis of appended *COII* and *ITS* data sets. Unlinked models, based on the model types suggested by Modeltest, were allowed for *COII*, *ITS1*, and *ITS2*. Node labels represent posterior probability values. Specific lineages have been marked A, B, or C to aid discussion.

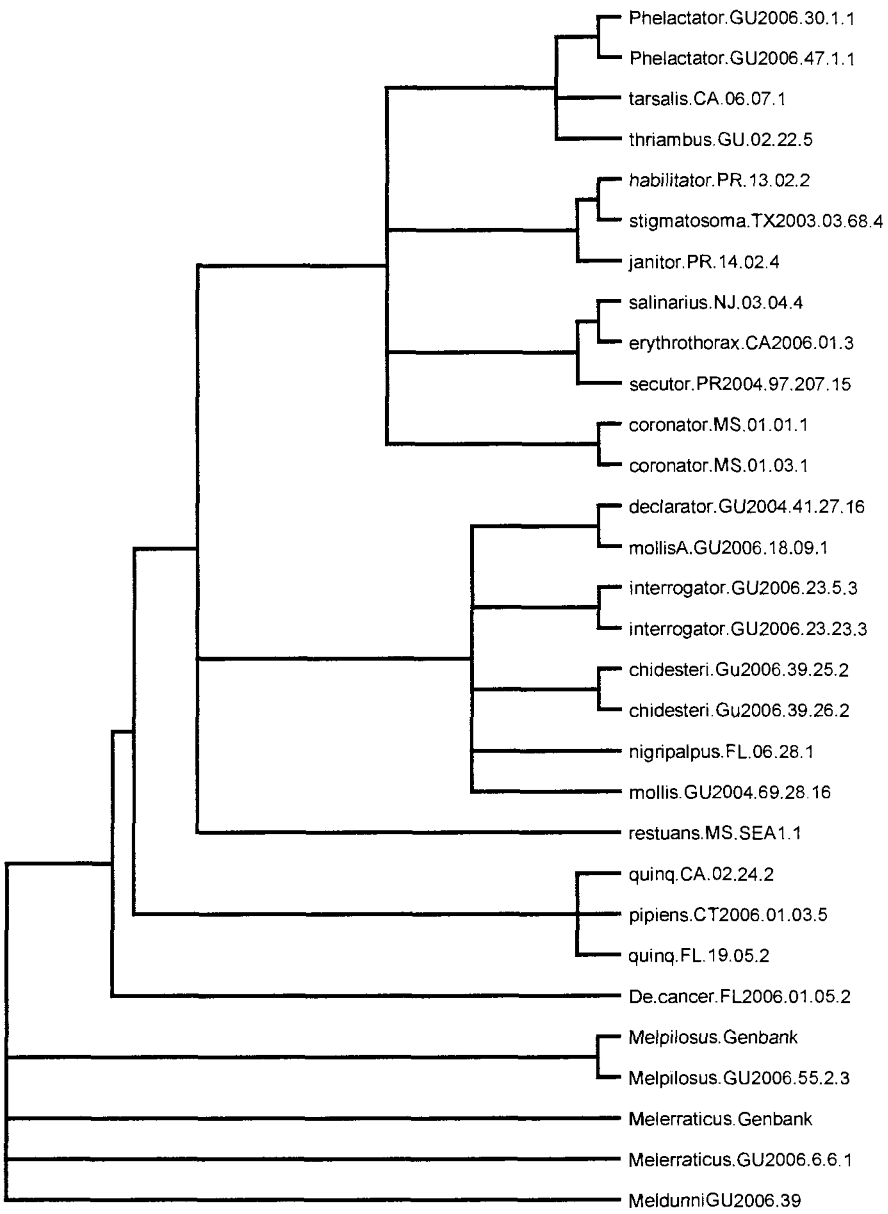

Figure 2.37. Adams consensus tree of the two trees presented in Figures 2.6 (*COII*) and 2.22 (*ITS*).

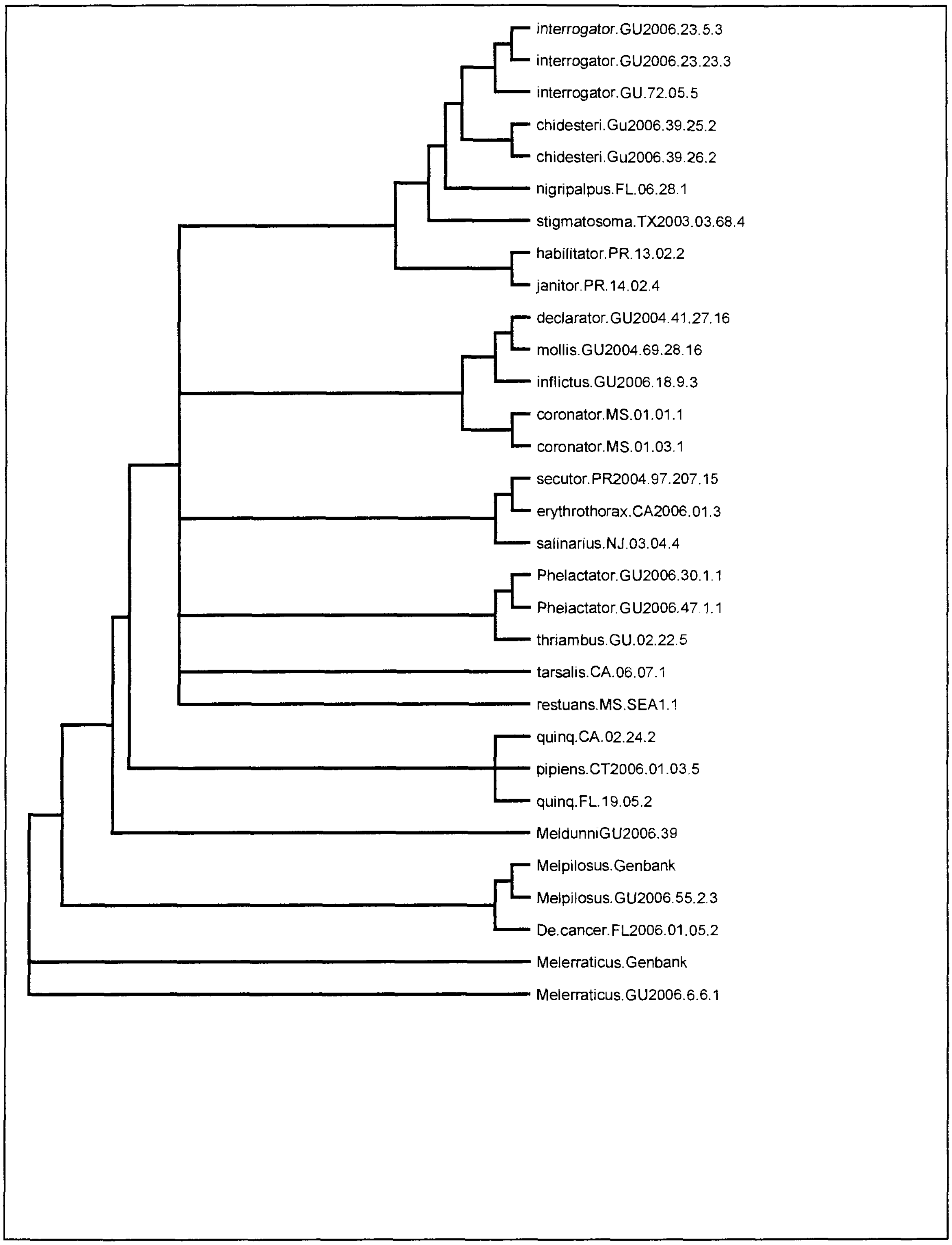

Figure 2.38. Bayesian analysis tree from simultaneous analysis of *COII* and *ITS* data, with poorly supported nodes collapsed.

Figure 2.39. Parsimony-informative sites in total evidence data.

clade A	interrogator.GU2006.23.5.3	C A T G G A T A T A G C G C C G T C T T G T A A C T C T A A
	interrogator.GU2006.23.23.3	. . . .
	interrogator.GU.72.05.5	. . . .
	chidesteri.Gu2006.39.25.2	. . . . . . C . . . C . . T T . . . . C . . . . G .
	chidesteri.Gu2006.39.26.2	. . . . . . C . . . C . . T T . . . . C . . . . G .
	nigripalpus.FL.06.28.1	. . . . . . C . . . C . C . . . . . . C . . . .
	stigmatosoma.TX2003.03.68.4	. . . . A . C . . . C . C . . . . . . C . . . .
	habilitator.PR.13.02.2	. G . . . . . . C . C T A . G . C . . C . . . . G
	janitor.PR.14.02.4	. . . . . . C . C . C . - . . . C . . C . . . . C T G
	Phelactator.GU2006.30.1.1	. . . . C . C C G T . G T C A . C . . G T . . . . G .
	Phelactator.GU2006.47.1.1	. . . . C . C C G T . G T C A . C . . G T . . . . G .
	thriambus.GU.02.22.5	. . . . . . C . C . C . A . A . C . . C . . G . . . .
	tarsalis.CA.06.07.1	T . A . A G C G C T . . C . T . . . . C . . G . T . . . G .
clade B	declarator.GU2004.41.27.16	. T . . . . C A . C A . . . G . C . A . C . . . . G .
	mollis.GU2004.69.28.16	. T . . . . C A . C A . . . G . C . A . C . . . . G .
	mollisA.GU2006.18.09.1	. . . . C A . C A . . . G . C . A . C . . . . G .
	coronator.MS.01.01.1	. . . . C . T C A A C G A C C . A . C . . . . G .
	coronator.MS.01.03.1	. . . . C . T C A A C G G C C . A . C . . . . G .
	secutor.PR2004.97.207.15	T T . . . . . T C T . . T A . A C G G C . A . C T . T . G .
	erythrothorax.CA2006.01.3	. T . . . . C T C T . . T A . A C A . C . A . C T . T . G .
	salinarius.NJ.03.04.4	. T . . . . C T C T . . T A . A C G . C . A . C T . T . G .
	restuans.MS.SEA1.1	. . . . . . G T C T . . T A . A C A . C . A . C . G . . G .
C	quinq.CA.02.24.2	T . C A . C C . . T T . . . . A C A G C . . . . . . T . G .
	pipiens.CT2006.01.03.5	T . C A . C C . . T T . . . . A C A G C . . . . . . T . G .
	quinq.FL.19.05.2	T . C A . C C G . T T . . . . A C A G C . . . . G .
outgroup	MeldunniGU2006.39	T C . C A T G . C T . T . T T . . . C C T C C G T G T C T G
	Melpilosus.Genbank	T C . C A T G . C T . T - T T . C G G C . G C . T G T C T .
	Melpilosus.GU2006.55.2.3	T C . C A T G . C T . T - T T . . G G C . G C . T G T C T .
	De.cancer.FL2006.01.05.2	T T . T . C G . C T A . - T G . A G G C . G C C T G T . T .
	Melerraticus.Genbank	T C . C . . G . C T . T T T T . . A G C T G C G T G T C T G
	Melerraticus.GU2006.6.6.1	T C . C . . G . C T . T T T T . . A G C T G C G T G T C T G

Figure 2.39. Continued.

```
         interrogator.GU2006.23.5.3        C T C T G G C A G C A G T G C A G T G C A T A C T G T T G G
         interrogator.GU2006.23.23.3       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
         interrogator.GU.72.05.5           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
         chidesteri.Gu2006.39.25.2         . . . . . . . . . A . . . . . . . . . . . . . . . . . . . .
         chidesteri.Gu2006.39.26.2         . . . . . . . . . A . . . . . . . . . . . . . . . . . . . .
         nigripalpus.FL.06.28.1            . . . . . . . . . A . . . . . . . . . . . . . . . . . . . .
cladeA   stigmatosoma.TX2003.03.68.4       . . . . . . . . . A . . . . . C . . . . . . . . . . . . . .
         habilitator.PR.13.02.2            T . . . . . . . . A . . . . . C . . . . . . . . . . . . . .
         janitor.PR.14.02.4                T . . . . . . . . A . . . . . C . C . . . . . . . . . . . .
         Phelactator.GU2006.30.1.1         . . . . . . . . . . . . . . . . . . . . . . . . C . C . . .
         Phelactator.GU2006.47.1.1         . . . . . . . . . . . . . . . . . . . . . . . . C . C . . .
         thriambus.GU.02.22.5              . . . . . . . . . . . . . . . C A . . . . . . . C . . . . .
         tarsalis.CA.06.07.1               . . . . . . . . . . . . . . . C . . . . . . . . C . . . . .
       --declarator.GU2004.41.27.16        . . . . . . . . . A . . . . . . . . . . . . . . C . . . . .
         mollis.GU2004.69.28.16            . . . . . . . . . A . . . . . . . . . . . . . . C . . . . .
         mollisA.GU2006.18.09.1            . . . . . . . . . A . . . . . C . . . . . . . . C . . . . .
         coronator.MS.01.01.1              . . . . . . . . . A . . . . T . . . A . . . . . C . . . . A
         coronator.MS.01.03.1              . . . . . . . . . A . . . . T . . . . . . . . . C . . . . A
cladeB   secutor.PR2004.97.207.15          . . . G . . . . . A . . . . . T . C . . . . . . C . . . . .
         erythrothoraxCA2006.01.3          . . . . . . . . . A . . . . . . . . . . . C . . C . . . . .
         salinarius.NJ.03.04.4             . . . . . . . . . . . . . . . . . . . . . C . . C . . . . .
         restuans.MS.SEA1.1                . . . . . . . . . A . . . . . C . . . . . . . . C . . . . .
       --quinq.CA.02.24.2                  . . . . . . . . . A . . . . . C . . . . . C G . A . C . . .
C        pipiens.CT2006.01.03.5            . . . . . . . . . A . . . . . C . . . . . C G . A . C . . .
       __quinq.FL.19.05.2                  . . . . . . . . . A . . . . . C . . . . . C G . A . C . . .
         MeldunniGU2006.39                 T A . . A A T G T A G A C C G . . . A T G . . G A C C A C .
         Melpilosus.Genbank                T A T C A A T G T A G A C C T C A . A T G . . G A A C A C .
         Melpilosus.GU2006.55.2.3          T A T C A A T G T A G A C C T T A . A T G . . G A A C A C .
outgroup De.cancer.FL2006.01.05.2          T . . . A A T G T A G A C C . C A . . T G . . G A . C A . A
         Melerraticus.Genbank              T A . . A A T G T A G A C C . C A . . T G . . G A A C G C .
         Melerraticus.GU2006.6.6.1         T A . . A A T G T A G A C C . C A . . T G . . G A A C G C .
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Figure 2.39. Continued.

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          interrogator.GU2006.23.5.3      T A T G G C A A C A T C A C A A C G C G G G C G C A A C G G
          interrogator.GU2006.23.23.3     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
          interrogator.GU.72.05.5         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
          chidesteri.Gu2006.39.25.2       . . . . . . . . . . . . C T . G . . . . . . . . . . . . . .
          chidesteri.Gu2006.39.26.2       . . . . . . C C . . . . C T . G . . G . . . . . . . . . . .
cladeA    nigripalpus.FL.06.28.1          . . . . . . . . . . . . C T . . . . . . . A . . . . . . . .
          stigmatosoma.TX2003.03.68.4     . . . . . . . . . . . . C T . G . . . . . A . . . . . . . .
          habilitator.PR.13.02.2          . . . . . . . . . . . . C T . . . . . . . . . . . . . . . .
          janitor.PR.14.02.4              . . . . . . . . . . . . G . . . . . . . . . . . . . . . . .
          Phelactator.GU2006.30.1.1       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
          Phelactator.GU2006.47.1.1       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
          thriambus.GU.02.22.5            . . . T . . . . . . . . G . . . . . . . . . . . . . . . . .
          tarsalis.CA.06.07.1             . . . . . . . . . . . . G . . G . . . . . . . . . . . . . .
        — declarator.GU2004.41.27.16      G . . . . . . . . . . . . T . G . . . . . . T . . . . . . .
          mollis.GU2004.69.28.16          G . . . . . . . . . . . . T . G . . . . . . T . . . . . . .
          mollisA.GU2006.18.09.1          G . . . . . . . . . . . T G . G . . . . . . . . A . . . . .
          coronator.MS.01.01.1            G . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
cladeB    coronator.MS.01.03.1            G . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
          secutor.PR2004.97.207.15        G . . . . . . . . . . . T G . . . . . . . . . . . . . . . T
          erythrothorax.CA2006.01.3       G . . . . . . . . . . . T G . . . . . . . . . . . . . G . . .
          salinarius.NJ.03.04.4           G . . . . . . . . . . . T G . . . . . . . . . . . . . G . . .
          restuans.MS.SEA1.1              C . . . . . . . . . . . . . . . . . . . . . . . . A . C . . .
        — quinq.CA.02.24.2                G . C A . . G . . . . . . . . . . . . . . . . . . . . C A . .
C         pipiens.CT2006.01.03.5          G . C A . . G . . . . . . . . . . . . . . . . . . . . C A . .
        _ quinq.FL.19.05.2                G . C A . . G . . . . . . . . . . . . . . . . . . . . C A . .
          MeldunniGU2006.39               . G C . A T G T T G C G T G G . . . . C C A T A . T G T C C
          Melpilosus.Genbank              C C . . A T G T T G C G T G G . . C T C C A T A . T G T C C
outgroup  Melpilosus.GU2006.55.2.3        . C . . A T G T T G C G T G G . . C T C C A T A . T G T C C
          De.cancer.FL2006.01.05.2        C G . . C T G T T G C . C G G . G . . C . C T . T T G T . C
          Melerraticus.Genbank            C G . . A . G T T G C G T G G . T . . C C A T A . T G T C C
          Melerraticus.GU2006.6.6.1       C G . . A . G T T G C G T G G . T . . C C A T A . T G T C C
```

Figure 2.39. Continued.

cladeA	interrogator.GU2006.23.5.3	T A C G G C A C A C C A A C T C G A T A G T A T T T T T T A
	interrogator.GU2006.23.23.3	. . . .
	interrogator.GU.72.05.5	. . . . T . . C . . . .
	chidesteri.Gu2006.39.25.2	. . . . T C . . . .
	chidesteri.Gu2006.39.26.2	. . . . T C . . . .
	nigripalpus.FL.06.28.1	. . . . T . . . .
	stigmatosoma.TX2003.03.68.4	. . . . T . . . . C A . . . . . .
	habilitator.PR.13.02.2	. . . . T . T . . . . . . A . T A . . . . . .
	janitor.PR.14.02.4	. . . . G C . . . . A . T A . . C . . .
	Phelactator.GU2006.30.1.1	. . . . A . . . G T T G . A . . . . . . A . T A . C . . C .
	Phelactator.GU2006.47.1.1	. . . . A . . . G T T G . A . . . . . . A . T A . C . . C .
	thriambus.GU.02.22.5	. G . . A . G . . . . . . - . . T . . . . . C A C C . . . .
	tarsalis.CA.06.07.1	. . . . G . T . . . . T A . . . . . .
cladeB	declarator.GU2004.41.27.16	. . . . A . G . . A . . C . . . .
	mollis.GU2004.69.28.16	. . . . A . G . . A . . C . . . .
	mollisA.GU2006.18.09.1	. . . . A . G . . A . . C . . . .
	coronator.MS.01.01.1	. . . . A . G A C A . C G . . . . A . T . . C . . . .
	coronator.MS.01.03.1	. . . . C . G A C A . C G . . . . A . T . . C . . . .
	secutor.PR2004.97.207.15	C G . . . G T . G T T C C . A . . . . T A . C . . . .
	erythrothorax.CA2006.01.3	. . . . . . G . G A . . C . . . . T A . . . . . T
	salinarius.NJ.03.04.4	. . . . A A G . G A . . C A . . . . T A . . . . . .
	restuans.MS.SEA1.1	. . . . T . G . . A . C G . . . . T A . C . C C .
C	quinq.CA.02.24.2	. . . . A . C . . . G . T . . . . A . T A . . . . . .
	pipiens.CT2006.01.03.5	. . . . A . C . . . G . T . . . . A . T A . . . . . .
	quinq.FL.19.05.2	. . . . A . C . . . G . T . . . . A . T A . . . . . .
outgroup	MeldunniGU2006.39	C . T A A T . G . . G . . T A . . . . . A . T . . C . . . .
	Melpilosus.Genbank	C . T A A A T G . . . . T T A A . T C T . A . . . . C C . T
	Melpilosus.GU2006.55.2.3	C . T A A A T G . . . . C T A A . . C T . A C . . . C . . T
	De.cancer.FL2006.01.05.2	C . T A A . T G . T A . T - A A T T C T A . T . . . . . . T
	Melerraticus.Genbank	C . T A . T . G . . . . . . A A . T C T . . T . C C . . . T
	Melerraticus.GU2006.6.6.1	C . T A . T . G . . . . . . A A . T C T . . T . C C . . . T

Figure 2.39. Continued.

cladeA	interrogator.GU2006.23.5.3	A T A A T G A T G A T T T G T T T T A C T A T A T T A A A C
	interrogator.GU2006.23.23.3	. . . .
	interrogator.GU.72.05.5	G . . . . A . . . . A C . A . . . . . T . . . . T
	chidesteri.Gu2006.39.25.2	. . . . A . . . C . . . .
	chidesteri.Gu2006.39.26.2	. . . . A . . . C . . . .
	nigripalpus.FL.06.28.1	. . . .
	stigmatosoma.TX2003.03.68.4	G . . . . A . . . . . T . . . . . C . T . T
	habilitator.PR.13.02.2	G . . . . C A C . . . . T . T
	janitor.PR.14.02.4	. . . . A . . . . . T . . . . T . T
	Phelactator.GU2006.30.1.1	. . . . C . . . . A . . . . T
	Phelactator.GU2006.47.1.1	. . . . C . . . . A . . . . T
	thriambus.GU.02.22.5	G . . G . A . C . . . . . A . C . . . T . . . . T
	tarsalis.CA.06.07.1	G . . . . T . . . A C . . C . T . . . . T . T
cladeB	declarator.GU2004.41.27.16	. . . . A . . . . . T . . . .
	mollis.GU2004.69.28.16	. . . . T . . . . T
	mollisA.GU2006.18.09.1	. . . . . . T . . . . . . A . . . C . T . . C . . . .
	coronator.MS.01.01.1	G A . . C . . . . A C C . . . T . . . . T
	coronator.MS.01.03.1	G A . . C . . . . A C C . . . T . . . . T
	secutor.PR2004.97.207.15	G . . . C . . . A T . . . A . A . . . T . G . T . . . C C T
	erythrothorax.CA2006.01.3	. . . . C . . C A T . . . A C . . . . T . . . T . . . T C T
	salinarius.NJ.03.04.4	. . . . C . . . A T . . . A C . . . . T . . . T . . . T C T
	restuans.MS.SEA1.1	. . . . . A . . . . G G . A . . . . . T . . . . . C T . G T
C	quinq.CA.02.24.2	G . . . A A T . . . G G . A . C A . . T . . . . C C T T . T
	pipiens.CT2006.01.03.5	G . . . A A T . . . G G . A . C A . . T . . . . C C T T . T
	quinq.FL.19.05.2	G . . . A A T . . . G G . A . C A . . T . . . . C C T T . T
outgroup	MeldunniGU2006.39	G A . . C . . . . A C C . . . T . . . . T
	Melpilosus.Genbank	. . T . . . T . . T G C A A . C . . T T C T . G . C G . T T
	Melpilosus.GU2006.55.2.3	. . T T . . . . A T G C A A . C . . . . . . C T . . . T T T
	De.cancer.FL2006.01.05.2	. . T T . . T . . . A . A A A . . . . T C T . G . . . . . T
	Melerraticus.Genbank	. . T . . . T . A T G C A . C . . C T T . . . . . C T C T T
	Melerraticus.GU2006.6.6.1	. . T . . . T . A T G C A . C . . . T T . . . . . C T C T T

Figure 2.39. Continued.

cladeA	interrogator.GU2006.23.5.3	T C A T A A T T T A T T T G T T T T T A A A T T T A T A A G
	interrogator.GU2006.23.23.3	. . . .
	interrogator.GU.72.05.5	C T . C T . C C . G . . . . C . C . C . . A
	chidesteri.Gu2006.39.25.2	. . . . C . . . . . A . . C . . . .
	chidesteri.Gu2006.39.26.2	. . . . C . . . . . A . . C . . . .
	nigripalpus.FL.06.28.1	. . . . G . . . .
	stigmatosoma.TX2003.03.68.4	. T . . . T . . . . A A . . . T . G C . . T . T . .
	habilitator.PR.13.02.2	A T . . . C . . . . C C . . A . . . . T . . . C . . . T . .
	janitor.PR.14.02.4	. T . . . T . . . . . A . A A A C . . T . . . . T G .
	Phelactator.GU2006.30.1.1	. T . . . T . . C . . . . . . A . . . T . . . . T . .
	Phelactator.GU2006.47.1.1	. T . . . T . . C . . . . . . A . . . T . . . . T . .
	thriambus.GU.02.22.5	. T . . . T . . . . C . . A . . . . . T . . . . C . .
	tarsalis.CA.06.07.1	. T . . . T . . . . A . C . . T . G . . . . C T . .
cladeB	declarator.GU2004.41.27.16	. T . . . . A . . . . C . . . .
	mollis.GU2004.69.28.16	. T . . . . C . . . .
	mollisA.GU2006.18.09.1	. T . . . . A . . . . C . . .
	coronator.MS.01.01.1	. . . . C . . . .
	coronator.MS.01.03.1	. . . . C . . . .
	secutor.PR2004.97.207.15	. T . . . T . . . . . A C A A . . . . T . . . . A . . T . .
	erythrothorax.CA2006.01.3	. T . . . T C C . . . A . A A . . . . T . . . . T . .
	salinarius.NJ.03.04.4	. T . . . T . . . . C A . A A . . . . T . . . . T . .
	restuans.MS.SEA1.1	. T . . . T . C . . . . . A A . . . . T . . . . T . .
C	quinq.CA.02.24.2	. T . C T G . . . . A C . . T . . . .
	pipiens.CT2006.01.03.5	. T . C T G . . . . A C . . T . . . .
	quinq.FL.19.05.2	. T . C T G . . . . A C . . T . . . .
outgroup	MeldunniGU2006.39	. . . . C . . . .
	Melpilosus.Genbank	C T T . . . C C . . . C A . . . . . . C T . . . . T . . T .
	Melpilosus.GU2006.55.2.3	C . T . . C . . . . . . A . . A . C C . T . . C C C . T . .
	De.cancer.FL2006.01.05.2	A T T . . . . . C . C . . A . G . . . T T . . . C T C . T A
	Melerraticus.Genbank	. . . . . . C C . . . A A A A . . . . . T . . . . G C . . .
	Melerraticus.GU2006.6.6.1	. . . . . . C C . . . A A A A . . . . . T . . . . G C . . .

Figure 2.39. Continued.

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         interrogator.GU2006.23.5.3        T A T T C A T A C A T T T A T T A T T T C T T T G A T T T A
         interrogator.GU2006.23.23.3       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
         interrogator.GU.72.05.5           . . . G . . . . T . . . . . . . . . . . . . . A A T . . . .
         chidesteri.Gu2006.39.25.2         . . . . T . . . . . C . C . . . . . . . . . . . . . . . . .
         chidesteri.Gu2006.39.26.2         . . . . T . . . . . C . C . . . . . . . . . . . . . . . . .
         nigripalpus.FL.06.28.1            . . . . T . . . . . . . . . . . . . . . . . . . . . . . . .
cladeA   stigmatosoma.TX2003.03.68.4       C . C A T . . . . . . . . . . . G . A A . . . A . T . A . .
         habilitator.PR.13.02.2            . . . A T . C G T . . . . . . . . . . . . . . A . T A A . .
         janitor.PR.14.02.4                . . . . T C . . T . . . . . . . . . . . . . . A A T C A . .
         Phelactator.GU2006.30.1.1         . . . . T . . T . . . . . . . . . . . . . . C A . T . A . .
         Phelactator.GU2006.47.1.1         . . . . T . . T . . . . . . . . . . . . . . C A . T . A . .
         thriambus.GU.02.22.5              . . . . T . C T T . A . . . . . . . . . . . C A A T . A . .
         tarsalis.CA.06.07.1               . . C A T . . . . . . . . . . . . A . . . . . A . T . A . .
         declarator.GU2004.41.27.16        . . . . . . . . . . . . . . . C . . . . . . . . . . . . . .
         mollis.GU2004.69.28.16            . . . A . . . . . . . . . . . C . . . . . . . . . . . . . .
         mollisA.GU2006.18.09.1            . . . . T . . . . . . . . . . . . . . . . . . . . . . C . .
         coronator.MS.01.01.1              . . . . . . . T T . . . . . . . . . . . . . . A . . . A . .
cladeB   coronator.MS.01.03.1              . . . . . . . T T . . . . . . . . . . . . . . A . . . A . .
         secutor.PR2004.97.207.15          C . . . T T C T T . . . . . . . . . . A . A . A A T . A . .
         erythrothorax.CA2006.01.3         . . . A T . C T . G . . . . . . . . . C T A . A . . . A . G
         salinarius.NJ.03.04.4             . . . A T C C T . . . . . . . . . . . . . A . A . T C A . .
         restuans.MS.SEA1.1                . . . . T . . . . . A . . T . . . . . . . . . A . T . A . G
         quinq.CA.02.24.2                  . T . A . . . T T . A . . . . . . . . . . . C A . T . . A .
C        pipiens.CT2006.01.03.5            . T . A . . . T T . A . . . . . . . . . . . C A . T . . A .
         quinq.FL.19.05.2                  . T . A . . . T T . A . . . . . . . . . . . C A . T . . A .
         MeldunniGU2006.39                 . . . . . . . T T . . . . . . . . . . . . . . A . . . A . .
         Melpilosus.Genbank                . . . . T T C T T . . . C T . . T A A . T A . A A T . . A .
outgroup Melpilosus.GU2006.55.2.3          C . C A T . . T T . . . . . . . T . A A . A . A . T C . A .
         De.cancer.FL2006.01.05.2          C . C A T . . C T G C . C . . . C . . . . C . A A T . C A .
         Melerraticus.Genbank              . . . . T . C . T . . A C . C . T . . C . . . A A T . . A .
         Melerraticus.GU2006.6.6.1         . . . . T . C . T . . A C . C . T . . C . . . A . T . . A .
```

Figure 2.39. Continued.

cladeA	interrogator.GU2006.23.5.3	T A A A T T T T G T T A C T C A T T T A T T A T G T
	interrogator.GU2006.23.23.3	. . . .
	interrogator.GU.72.05.5	. . . . C A . . . . A .
	chidesteri.Gu2006.39.25.2	. . . . A . . . .
	chidesteri.Gu2006.39.26.2	. . . . A . . . .
	nigripalpus.FL.06.28.1	. . . . A . . . .
	stigmatosoma.TX2003.03.68.4	. . . . . C . . A A . C . . . T C
	habilitator.PR.13.02.2	. . . . C C . . A A . . . . T T . . . . . C . . . .
	janitor.PR.14.02.4	. . . . A A . T . . . T . . . . A .
	Phelactator.GU2006.30.1.1	. . . . A . . . . A .
	Phelactator.GU2006.47.1.1	. . . . A . . . . A .
	thriambus.GU.02.22.5	. . . G C . C . A . . T . . . G . A C . . . .
	tarsalis.CA.06.07.1	C . . . . A A . . . . . C . . . G C
cladeB	declarator.GU2004.41.27.16	. . . . A . . . .
	mollis.GU2004.69.28.16	. . . . A . . T . . . .
	mollisA.GU2006.18.09.1	. . . . A . . . .
	coronator.MS.01.01.1	. . . . A . . . . A . . . .
	coronator.MS.01.03.1	. . . . A . . . . A . . . .
	secutor.PR2004.97.207.15	. . . . A . . T . . . . C . . A C
	erythrothorax.CA2006.01.3	. . G . . . . . A . . T . . A . . . . . A C . C . C
	salinarius.NJ.03.04.4	. . . . C . . . A . . T . . A C
	restuans.MS.SEA1.1	C . . . . A A . . . . . T . . . .
C	quinq.CA.02.24.2 □□	. T . . . . C . A . . T . . T . C . . . . A .
	pipiens.CT2006.01.03.5	. T . . . . C . A . . T . . T . C . . . .
	quinq.FL.19.05.2	. T . . . . C . A . . T . . T . C . . . .
outgroup	MeldunniGU2006.39	. . . . A . . G . . . . . A . . . .
	Melpilosus.Genbank	C . T T . . C . A C A . T A T . . A . . A . T . A .
	Melpilosus.GU2006.55.2.3	A . . T . . . . A C A . . A . G C A . . A . T C . .
	De.cancer.FL2006.01.05.2	A T T T . . . C A C A T T A T . . A C G . . T . A C
	Melerraticus.Genbank	C . . . . A C A . T A T . . A A . G . . . . .
	Melerraticus.GU2006.6.6.1	C . . . . A C A . T A T . . A A . G . . . . .

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