AN ABSTRACT OF THE THESIS OF

Weerathep Pongprasert for the degree of Doctor of Philosophy in Entomology presented on February 03, 2000. Title: Phylogenetic Relationships of the Bumblebees (APIDAE: HYMENOPTERA) in the Pacific Northwest of America Inferred from Mitochondrial Cytochrome Oxidase Gene Sequences.

Redacted for Privacy

Abstract approved: _____

Dennis M. Burgett

Systematic studies of bumblebees have been based on morphological characters such as color pattern, wing venation, and male genitalia, and many classification systems have been proposed. However, these classifications are controversial because of the highly polymorphic nature of characters among bumblebee species. The aim of this study is to understand the relationships of bumblebees in the Pacific Northwest (PNW) by phylogenetic analysis inferred from the mitochondrial cytochrome oxidase gene sequences (CO). Because of unique structural alterations in sequence regions that evolve corresponding to species differentiation, the CO genes provide a significant phylogenetic signal for understanding the generic, specific, subspecific and population relationships of bumblebees in the PNW.

In the complete CO gene subunit II (COII) analysis, *Psithyrus* forms a monophyletic sister group with *Bombus* and significant evidence supports the retention of its generic status. The clusters within the genus *Bombus* group were most closely related to the traditional classification system as redefined by Stephen

(1957), Plowright and Stephen (1973), and Thorp *et al.* (1983). The phylogenetic analyses clarified the relationships of bumblebees in the PNW not only at the species level, but also in many subspecies and polymorphic species such as *B. occidentalis*, *B. mixtus*, *B. bifarius nearcticus*, *B. sitkensis*, and *B. californicus*.

Phylogenetic analysis of the partial COII gene and the secondary structure of the $tRNA^{leu}$/COII intergenic region of the subgenus *Bombus* supported the distant relationship of the Nearctic *B. occidentalis* to the Palaearctic *B. terrestris* and there are 4 species of subgenus *Bombus* in the PNW: *B. occidentalis, B. terricola, B. moderatus* and *B. franklini.*

Partial CO gene subunit I and II sequences were used for phylogenetic analysis of *Pyrobombus* species in the PNW. The combined phylogenetic tree explains clearly the relationship of many confusing species and provides not only evidence of adaptive radiation of *B. sitkensis*, *B. mixtus*, and *B. b. nearcticus*, but also the convergence between *B. vosnesenskii* and *B. caliginosus*.

Phylogenetic Relationships of the Bumblebees (APIDAE: HYMENOPTERA) in the Pacific Northwest of America Inferred from Mitochondrial Cytochrome Oxidase Gene Sequences

by

Weerathep Pongprasert

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented February 03, 2000
Commencement June 2000

Doctor of Philosophy thesis of Weerathep Pongprasert presented on February 03, 2000

APPROVED:

Redacted for Privacy

Major Professor, representing Entomology

Redacted for Privacy

Chair of Department of Entomology

Redacted for Privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Redacted for Privacy

Weerathep Pongprasert, Author

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. D. Michael Burgett, for his guidance and support during my Ph.D. program. I would like to thank Dr. Katharine G. Field, who taught me the aspects of molecular techniques and was always generous with her time and comments. I would also like to thank Dr. William P. Stephen, who taught me bumblebee biology and classification, guided me how and where to collect my specimens, helped me to identify the specimens, and provided a great deal of information very useful for this research. Of course, I could not obtain all the species of bumblebees in the Pacific Northwest without his advise, especially the endangered species, *Bombus franklini*. I am also thankful to members of my graduate committee, Dr. John Lattin and Dr. Kenneth B. Johnson, for their advice and assistance during my study.

I am indebted to Dr. Lynn Royce for her encouragement and support, and for her help in preparing these manuscripts. I would like to express my appreciation to Dr. Aaron I. Liston, Dr. Joseph W. Spatafora, Dr. Stephen J. Giovannoni, Dr. Michael Blouin, Dr. Andrew V. Z. Brower and his wife, Dr. Darlene Judd for providing guidance and many comments that were very valuable for me to initiate and proceed with this project. Additionally, I thank Dr. Ena Urbach, Dr. Annette M. Kretzer, Dr. David Gernandt, Dr. Jamie Platt, for teaching me the UNIX operation system and other computer programs, as well as statistical and mathematical models for phylogenetic analyses.

I would also like to thanks to Dr. Juntipa Purintrapiban, Dr. Teerayutt Toojinda, Dr. Wipa Hongtrakul, Dr. Sirimas Katchamart, Williams Robinson, Ramka Leijs, Larry Hodges, Kevin Vergin, Peter D. Hoffman, Lorretta M. Winton, Dr. Sureeporn Katengam, Nanci Adair and all the staff in CGRB for your valuable guidance and supported. I give special thanks to Justen Whittall who not only provided information about phylogeny but also kindly accompanied me in collecting insect specimens. I am grateful to Mathew Lawrence Carlson who provided *B. occidentalis nigroscutatus* from Alaska, Dr. R.W. Thorp who sent me *B. crotchii* and *B. sonorus*, Dr. James Cane who kindly sent me *B. pennsylvanicus* and Professor Burkhart Schricker who provided me *B. terrestris* and *B. lapidarius* from Germany. Thanks to Dr. Vuttipong Areekul and Kemathat Vibhatavanij, engineers who are interested in bees, and kindly provided many bumblebee species from around Pullman, WA and Zion, Utah.

I also thank Raweewan Yuwansiri, Dr. Judith Principal, Jenny M. Gavilanez-Sanchez, Anne Bernhard, and all of my fellow students who helped fulfill my life and experiences at OSU.

Lastly, I express deep appreciation to my parents, my brothers, my sisters and also my grandparents for all of their love, support, and encouragement. I would like them to know how much their support helped me to be here.

CONTRIBUTION OF AUTHORS

This thesis has benefited from advice, suggestions and funding kindly offered by Dr. D. Michael Burgett, my major professor and advisor. Dr. Kate G. Field generously provided all the molecular equipment and expertise need in the lab and also provided detailed critiques of phylogenetic analysis. Dr. William P. Stephen kindly aided in collection, classification, and identification of th bumblebees in the Pacific Northwest, and also provided expertise needed for assistance in the interpretation of the phylogenetic relationships of bumblebees in this analysis.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF FIGURES

LIST OF FIGURES(continued)

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF TABLES

LIST OF TABLES (continued)

DEDICATION

I would like to dedicate this bumblebee work to my beloved father, Vimol Pongprasert, who inspired, motivated, and supported me to reach for and obtain a higher degree of Education.

PHYLOGENETIC RELATIONSHIPS OF THE BUMBLEBEES (APIDAE: HYMENOPTERA) IN THE PACIFIC NORTHWEST OF AMERICA INFERRED FROM MITOCHONDRIAL CYTOCHROME OXIDASE GENE SEQUENCES

CHAPTER 1

INTRODUCTION

Bumblebees are classified in the family Apidae (Bombinae: Bombini), along with honey bees (Apinae: Apini), orchid bees (Bombinae: Euglossini), and stingless bees (Meliponinae) (Kimsey, 1984). They can be distinguished from the other bees in the family Apidae by the following characteristics (Williams, 1991, pp. 10-11):

"Bumblebees have a labrum at least twice as broad as long. The labrum lacks a longitudinal median ridge, although for the females it has a strong transverse basal depression. The clypeus has a transverse subapical depression and the apico-lateral corners are curved back towards the occiput. A malar area (= malar space) separates the compound eye from the base of mandible, often by a distance greater than the breadth of the mandible at its base. The hind wings lack a jugal lobe (= anal lobe). The volsella (=lacinia) of the male genitalia is greatly enlarged and is produced apically beyond the gonostylus (= squama).

Bumblebees are large (body length 7-27 mm) robust insects. Their bodies have a dense covering of variously-colored long plumose hairs, although these are

few or absent on some parts of the ventral surface of the gaster, on parts of the propodeum, on parts of the anterior face of gastral tergum I, and on parts of the head. The sclerites are usually black, or lighter brown on the distal parts of the limbs, but are never marked with bright yellow, red or metallic (= interference) colours. The wings may be transparent (= hyaline) to strongly darkened (= infuscated), but rarely show strongly metallic reflections.

Female bumblebees have 12 antennal 'segments' (= scape, pedicel and 10 flagellomeres) and six visible gastral terga and sterna. Males have 13 antennal 'segments' (= scape, pedicel and 11 flagellomeres) and seven visible gastral terga and sterna."

BIOLOGY OF BUMBLEBEES

Bumblebees are mainly Holoarctic insects comprising about 250 species in the genus *Bombus* Latreille (true bumblebees) and 44 species in the genus *Psithyrus* Lepeletier (cuckoo bumblebees) (Williams, 1985). *Bombus* species are eusocial, with three castes: queen or principal egg-laying females, workers or principal foraging and nesting females, and males. *Psithyrus* species are inquiline social parasites and lack a worker caste; the females usurp bumblebee nests and propagate themselves at the expense of their bumblebee hosts.

The cycle of bumblebee colonies in temperate zone is annual (Michener, 1974). The bumblebee colony is started each year by a fertilized queen who has hibernated over the winter. The queen emerges from her hibernacula in the spring

and at first behaves like many solitary bees, living on nectar and pollen and searching for a suitable nest site, usually abandoned rodent or bird nests. When a suitable site is located, the queen starts construction of the brood nest, deposits eggs, collects nectar and pollen, and takes care of the brood. The development time from egg to adult requires roughly three to five weeks. When the workers appear and take over all the duties of foraging, nest building, and brood care, the queen restricts her activities to egg laying and feeding herself from the stores provided by her worker progeny. All castes receive the same food; no special food, such as the royal jelly in honey bees is required (Alford, 1975). Now they become a true social unit. Additional broods of worker progeny are produced continuously until the number of workers equals or exceeds the number of larvae to be fed. At this point, some unfertilized eggs will be produced and become males whereas fertilized eggs will be new queens. The males leave the nest after emergence and rarely return. New queens take on household duties and foraging activities for a few days before they are ready to mate. Both sexes may mate more than once (Hobbs, 1967). After mating, new queens start increasing their fat bodies for winter survival, and build a small spherical cell in the soil for hibernation (Alford, 1969). The time of entry into hibernation is dependent on the species, nest situation and temperatures (Plath, 1934). At the end of season, the old colony dies out.

Psithyrus females emerge from hibernation in late spring, living as solitary bees by feeding on pollen and nectar until finding a suitable bumblebee host. She invades the bumblebee nest, kills the resident queen, and then lays eggs. The

worker bumblebees continue to forage and take care of the *Psithyrus* brood. The new females of *Psithyrus* mate and survive the winter in the same way as *Bombus* species.

SEASONAL FLIGHT PERIOD

The flight period of bumblebees varies depending on species, sex, and caste. In the early season, flying bumblebees are emerging queens that are foraging or seeking places to establish nests. Foraging bumblebees in the following periods are mostly workers and males. The occurrence of flying queens peaks again in the late season when mated queens seek hibernation sites. In the Pacific Northwest (PNW), according to Stephen (1957), there are two groups of bumblebee, differentiated by the flying season: early spring species and late spring species. Some common species in the Willamette Valley such as *B. flavifrons* Cresson, *B. sitkensis* Nylander, and *B. mixtus* Cresson can be found in the early spring and are gone by early summer. Some species such as *B. occidentalis* Greene and *B. vosnesenskii* Radoszkowski can be found in the early spring also but are abundant until end of summer. Most *Bombus* species in the PNW are active from late April until late summer whereas *Psithyrus* species begin to fly in late May. However, the foundress queens of early spring species such as *B. mixtus* and *B. flavifrons* can also be found later in the year in the high mountains because the spring at the high elevations is delayed.

TAXONOMIC STATUS OF BUMBLEBEE

The taxonomy of bumblebees has been recognized as follows (Borror *et al.*, 1992)

Kingdom Metazoa

Phylum Arthropoda

Class Insecta

Order Hymenoptera

Super-family Apoidea

Family Apidae

Sub-family Bombinae

Genera *Bombus* and *Psithyrus*

Bombinae comprised of the genus *Bombus* and their social parasites *Psithyrus,* consisting of approximately 300 species (Williams, 1985), and are taxonomically well separated from other Apidae (Richards, 1968).

In the early stage of classification around the middle of the 18th century, organisms were classified into categories based on external appearance or morphological characters. Since most bumblebees present colorful patterns on the body, this character was commonly used by many taxonomists such as Radoszkowski (1884), Plath (1922), and Stephen (1957). This character is still a primary taxonomic tool used to classify and identify most species of bumblebee today, since it is very convenient and does not required sophisticated techniques. However, as many more bumblebees were discovered, color patterns alone were

not enough. Therefore, many morphological characters were considered such as male genitalia, wing venation, malar space, antennae, sting sheaths and others; these are found in many classification keys, for instance, Stephen (1957), Thorp *et al.* (1983) and Williams (1985).

CLASSIFICATION BASED ON MOLECULAR CHARACTERS

Most morphological characters are very useful to identify and classify organisms in many taxonomic categories. However, in some situations, they cannot provide adequate information to clarify species or lower categories, especially recently diverged, polymorphic, or convergent species. Bumblebees are included in these difficult situations. During the past 2 decades, taxonomists dealing with bumblebees have tried many methods to clarify the uncertainty of the taxonomic status of some species and to understand more about the speciation of these bees. A new alternative that shows high potential is molecular systematics.

After Mendel's laws were rediscovered in the beginning of the 20th century, they provided the foundation for understanding the molecular basis of life and had a major impact on taxonomic and population studies. Many techniques were developed to clarify taxonomic uncertainty such as chromosome studies, protein analyses, and DNA analyses. Moreover, most of these types of data provide the opportunity to reconstruct phylogenies, allowing a better understanding and clearer explanations of the relationships of organism than traditional classification.

PROTEIN ANALYSES

Many proteins such as isozymes and allozymes can be separated in an electrical field under appropriate conditions and used to identify the differences among organisms. There are more than 50 available enzymes (May, 1992) and these have been applied to study the variation within and among species or populations of insects for almost two decades (Daly, 1991). These isozyme and allozyme analyses have been used to clarify the relationships of bumblebees at many taxonomic levels such as genera, species, and populations. For example, Stephen and Cheldelin (1973) used α-glycerophosphate dehydrogenase (GDH) isozyme patterns to clarify the relationships among 25 bumblebee species. The monophyly of genus *Psithyrus* was nicely confirmed by another study using ten enzymes (Pamilo *et al.*, 1987). Scholl *et al.* (1992) used 21 enzymes to study the subgenus *Bombus* Latreille; they confirmed that *B. franklini* Frison was separated from *B. occidentalis*. Recently, Scholl *et al.* (1995) studied the variation between subspecies of bumblebees (taxa *nevadensis* and *auricomus*) using enzyme electrophoresis and found that these species are genetically different.

CHROMOSOMAL ANALYSES

After discovery of the hypotonic treatment that spreads chromosomes in metaphase, accurate counts of the number and detailed study of morphology of chromosomes were possible (Hoy, 1994). In many cases, chromosomes can be

identified by their relative size, centromere position, and secondary construction and these can be used as taxonomic characters. Unfortunately, there have been few studies of bumblebee chromosomes since Owen (1983) first reported the chromosomal constitution of 17 species of bumblebees and illustrated their karyotypes.

DNA ANALYSES

There are many DNA markers and techniques that allow detection of genetic variations and clarification of problematic classifications, including RFLPs, RAPDs, microsatellites, and DNA sequencing (Hoy, 1994). In bumblebees, Estoup *et al.* (1993) modified RFLP-PCR for characterization of $(GT)_n$ and $(CT)_n$ microsatellites in *Bombus terrestris* (L.) and found that on average, $(GT)_n$ and $(CT)_n$ microsatellites occurred in every 40kb and 500kb. The flanking regions of these microsatellites are similar enough to allow PCR amplification in several other species of *Bombus*. Surprisingly, there are very few studies of DNA sequences of bumblebees. Most bumblebee DNA sequences were limited to use as an outgroup in studies of honey bees, until 1996, when Pederson first used a partial sequence of the mitochondrial cytochrome oxidase I gene (COI) to examine the monophyletic group *Psithyrus*. Recently, the same region of the COI gene and the cytochrome b gene were used by Koulianos (1999) to determine the relationships of bumblebees in subgenus *Pyrobombus* Dalla Torre.

Until now, there has been little information about molecular systematic of bumblebees based on available DNA sequences. Therefore, a series of phylogenetic analyses were conducted using nucleotide-sequencing data from the mitochondrial cytochrome oxidase gene to explore the relationships of bumblebees in the PNW. This was meant not only to clarify the taxonomic status of bumblebee in this region, but also to explain the speciation and evolution of the group.

Following this introduction, Chapter Two of this thesis discusses the use of partial COII gene sequences and the secondary structure of the intergenic region between $tRNA^{leu}$ and the COII gene to study the phylogeny of the subgenus *Bombus* in the PNW and compares the PNW species with some *Bombus* species from Europe. In the third chapter, the phylogeny of the subgenus *Pyrobombus* in the PNW was studied using partial COI and COII gene sequences. The last chapter discusses the phylogenetic analysis of bumblebees in the PNW based on the complete COII gene.

REFERENCES

Alford, D. V. (1969). A study of the hibernation of bumblebees in southern England. *J. Anim. Ecol.* 38: 149-170.

Alford, D. V. (1975). "Bumblebees," Davis-Poynter, London.

Borror, D. J., Triplehorn, C. A., and Johnson, N. F. (1992). "An Introduction to the Study of Insects," Saunders College Publishing, Philadelphia, USA.

Daly, H. V. (1991). Systematics and identification of africanized honey bees. *In* "The African Honey Bee," (M. Spivak, D. J. C. Fletcher, and M. D. Breed, Eds.), pp. 13-44. Westview Press, Boulder.

Estoup, A., Solignac, M., Harry, M., and Cornuet, J. M. (1993). Characterization of $(GT)_n$ and $(CT)_n$ microsatellites in two insect species: *Apis mellifera* and *Bombus terrestris*. *Nucleic Acids Res.* 21: 1427-1431.

Hobbs, G. A. (1967). Ecology of species of *Bombus* (Hymenoptera: Apidae) in southern Alberta. VI. Subgenus *Pyrobombus*. *Can. Entomol.* 99: 1272-1292.

Hoy, M. A. (1994). "Insect Molecular Genetics," Academic Press, USA.

Kimsey, L. S. (1984). The re-evaluation of the phylogenetic relationships in the Apidae (Hymenoptera). *Syst. Entomol.* 9: 435-441.

Koulianos, S. (1999). Phylogenetics of the bumblebee subgenus *Pyrobombus* (Hymenoptera: Apidae) inferred from mitochondrial cytochrome B and cytochrome oxidase I sequences. *Ann. Entomol. Soc. Amer.* 92: 355-358.

May, B. (1992). Starch gel electrophoresis of allozymes. *In* "Molecular Genetic Analysis of Populations: A Practical Approach" (A. R. Hoelzel Ed.) pp. 1-27. IRL Press, Oxford.

Michener, C. D. (1974). "The Social Behavior of the Bees," Harvard Univ. Press, Massachusetts.

Owen, R. E. (1983). Chromosome numbers of 15 North America bumblebee species (Hymenoptera, Apidae, Bombini). *Can. J. Genet. Cytol.* 25: 26-29.

Pamilo, P., Pekkarinen, A., Varvio-Aho, S. L. (1987). Clustering of bumblebee subgenera based on interspecific genetic relationships (Hymenoptera, Apidae: *Bombus* and *Psithyrus*). *Ann. Zool. Fennici.* 24: 19-27.

Pedersen, B. V. (1996). A phylogenetic analysis of cuckoo bumblebees (*Psithyrus*, Lepeletire) and bumblebees (*Bombus*, Latreille) inferred from sequences of the mitochondrial gene cytochrome oxidase I. *Mol. Phylogenet. Evol.* 5: 289-297.

Plath, O. E. (1922). Notes on *Psithyrus*, with records of two new American hosts. *Biol. Bull.* 43: 23-44.

Plath, O. E. (1934). "Bumblebees and Their Ways," The Macmillan Company, New York.

Radoszkowski, O. (1884). Revision des armures copulatrices des mêles du genre *Bombus*. *Bull. Soc. Imp. Nat., Moscou* 59: 51-92.

Richards, O. W. (1968). The subgeneric divisions of the genus *Bombus* Latreille (Hymenoptera: Apidae). *Bull. Br. Mus. (Nat. Hist.) Entomol.* 22: 209-276.

Scholl, A., Thorp, R. W., and Obrecht, E. (1992). The genetic relationship between *Bombus franklini* (Frison) and other taxa of the subgenus *Bombus* s.str. (Hymenoptera: Apidae). *Pan-Pacific Entomol.* 68: 46-51.

Scholl, A., Thorp, R. W., Owen, R. E., and Obrecht, E. (1995). Specific distinctiveness of *Bombus nevadensis* Cresson and *B. auricomus* (Robertson) (Hymenoptera: Apidae) enzyme electrophoretic data. *J. Kans. Entomol. Soc.* 65: 134-140.

Stephen, W. P. (1957). Bumblebees of Western America (Hymenoptera: Apoidae). *Tech. Bull. Oreg. Agric. Exp. Stn.* 40.

Stephen, W. P., and Cheldelin, I. R. (1973). Phenetic grouping in bees of the tribe Bombini based on the enzyme α-Glycerophosphate dehydrogenase. *Biochem. Sys.* 1: 69-76.

Thorp, R. W., Horning, D. S. Jr., and Dunning, L. L. (1983). Bumblebees and cuckoo bumblebees of California. *Bull. Calif. Insect Surv.* 23.

Williams, P. H. (1991). The bumble bees of the Kashmir Himalaya (Hymenoptera: Apidae, Bombini). *Bull. Br. Mus. (Nat. Hist.) Entomol.* 60: 1-204.

Williams, P. H. (1985). A preliminary cladistic investigation of relationships among the bumble bees (Hymenoptera, Apidae). *Syst. Entomol.* 10 : 239-255.

CHAPTER 2

PHYLOGENETIC RELATIONSHIP IN THE SUBGENUS *BOMBUS* (HYMENOPTERA: APIDAE) BASED ON MITOCHODRAL CYTOCHROME OXIDASE SUBUNIT II GENE SEQUENCES

Weerathep Pongprasert, Dennis M. Burgett, Katharine G. Field, and William P. Stephen.

ABSTRACT

The taxonomic status of the bumblebee subgenus *Bombus* (Hymenoptera: Apidae) in the Pacific Northwest of America (PNW) is re-examined in this study. Partial cytochrome oxidase subunit II DNA sequences were used to determine the relationships of the members of this subgenus. Inferred trees reconstructed by neighbor-joining and maximum parsimony methods provided consistency and helped resolve phylogenetic relationships for most of the main species group, although some deep branches were weakly supported by the bootstrap. Results confirmed the traditional classification of the subgenus *Bombus* in the PNW. The subgenus was divided into 4 species: *Bombus occidentalis, B. terricola* Kirby, *B. franklini,* and *B. moderatus* Cresson. All the PNW *Bombus* species in this study, classified as the Nearctic group, were distantly related to *B. lucorum, B. terrestris* and *B. canariensis,* which is known as the Palaearctic group from Europe. In addition, *B. occidentalis* is possibly composed of at least 3 subspecies: *B. o. occidentalis* Greene, *B. o. nigroscutatus* Franklin California type, and *B. o. nigroscutatus* Alaska type. *Bombus o. nigroscutatus* from California is morphologically identical to *B. o. nigroscutatus* from Alaska, but their relationships, based on COII genes in this study, show they are significantly distant from each other.

INTRODUCTION

The subgenus *Bombus* is one of the largest groups of bumblebees, with a long history of debate on its taxonomic status, especially in the PNW region. In this area, four species occur in this subgenus: *Bombus occidentalis, Bombus franklini, Bombus terricola, and Bombus moderatus*. However, a high degree of polymorphism occurs not only among the species in this subgenus, but also within species. This makes it difficult to define the status and relationship of each species. The available classification keys vary depending on the strictness of the color pattern used by taxonomists (Williams, 1998).

The most problematic species is *B. occidentalis*. This species has a very wide geographic distribution, from Alaska to California and Utah. Its range in elevation is from sea level up to 2,700 meters. This species is highly polymorphic throughout its range (Stephen, 1957; Thorp *et al.*, 1983). The polymorphism consists of recombinant formations of the yellow bands on tergum II and III, and pale pubescence on tergum IV (Stephen, 1957). This leads to an unstable taxonomic status of the species *occidentalis,* which disappears in many classification systems, even though it can be distinguished from others simply based on color pattern.

Within this species, two subspecies have been long recognized*: B. o. nigroscutatus* and *B. o. occidentalis. Bombus nigroscutatus* Franklin was reduced to a subspecies by Franklin in 1913. This classification was based on California specimens and individual variants of *B. occidentalis* from the Rocky Mountains

that had similar coloring. Stephen (1957) redefined *B. o. nigroscutatus*, limiting it to the coastal region of California San Francisco Bay area. *Bombus o. nigroscutatus* was given subspecific status rather than specific status because of the hybridization zone that shows gradual intergradation with polymorphic *B. o. occidentalis,* found in Humboldt County and to the north. Without any connection between geographical distributions, the morphology of *B. o. nigroscutatus* found in California is very similar to a form found in northern Canada and Alaska. Since there is no information on this species, we have designated it *B. o. nigroscutatus* Alaska type.

Not only is the subspecific status of this bee unclear, but also its specific status within the subgenus. Milliron (1971) proposed *B. occidentalis* as a subspecies of *B. terricola*. Poole (1996) also placed all *B. occidentalis* as subspecies of *B. terricola*, although the color pattern of *B. terricola*, which is very stable and shows no variation throughout its range, can be used to distinguished it from *B. occidentalis* (Franklin, 1913; Stephen, 1957).

Bombus occidentalis (= *B. terricola*) has been treated as a co-species with *B. franklini* (Milliron, 1971; Poole, 1996) based on morhphological characters. However, *B. franklini* is endemic to a specific area in southern Oregon and has a distinct morphology (Plowright and Stephen, 1980; Stephen, 1957), and also has a remarkably different allozyme pattern from that of *B. occidentalis* (Thorp *et al.*, 1983; Scholl *et al.*, 1990; Scholl *et al.*, 1992). To distinguish *B. franklini* and *B. occidentalis* can often be difficult (Stephen 1957) because *B. occidentalis* shows

gradation in form, resulting in color convergence toward a local Mullerian mimicry group with *B. franklini* (Scholl *et al.*, 1992). *Bombus o. occidentalis* in southern OR shows ferruginous at the abdominal tip and occupies a geographic area overlapping with *B. franklini.* The confusion between these species may not only come from *B. occidentalis*. It may also come from *B. franklini* since Scholl *et al.* (1992) suggested: "One might speculate that *B. franklini* has in fact a more widespread distribution, but become as hidden within the color variation of *B. occidentalis.*"

In addition, the relationship between *B. moderatus,* found in Canada, and *B. lucorum*, a European member of subgenus *Bombus,* is still unclear since there is little significant structural variation between these species. Although allozyme data (Scholl *et al.*, 1990) separates them into two species, *B. moderatus* and *B. lucorum*, many reports list them as synonymous because there is some evidence of distributional variation of *B. lucorum* s. str., which ranges from northern Europe to north of Mongolia (Tkalcu, 1974) and Alaska (Williams, 1991). Therefore, more evidence is required to confirm their status (Williams, 1998).

Because of the controversy surrounding the classification of the species of the subgenus *Bombus*, the aim of this work was to explore and explain their relationship with phylogenetic analysis using the partial mitochondrial cytochrome oxidase subunit II (COII) gene sequences.

The COII gene was chosen to analyze the phylogenetic status of this subgenus because this gene exhibits high interspecific variation that has allowed phylogenetic analysis in many insects such as honey bees (Garnery *et al.*, 1991, and

Estoup *et al.*, 1996), flies (Smith and Bush, 1997), and butterflies (Brower, 1994). The total mtDNA of the closest related genus to bumblebees, *Apis mellifera* (L.), has been completely sequenced (Crozier *et al.*, 1993); this is helpful for the design and confirmation of primers to amplify the specific target sequence by polymerase chain reaction.

MATERIALS AND METHODS

Specimen Collection

The PNW specimens of the subgenus *Bombus* were collected between 1995 and 1998 (Table 2.1). *Bombus franklini, B. moderatus, B. terricola,* two sources of *B. o. nigroscutatus* and three intermediate forms of *B. o. occidentalis* were included in these collections. *Bombus terrestris* was collected from Germany in 1996. In addition, the partial COII gene sequences of *B. lucorum, B. canariensis* and *B. terrestris* based on the study of Estoup *et al.* (1996) were retrieved from the GenBank. Two *Bombus* species from the closely related subgenus *Pyrobombus*, *B. melanopygus* Nylander and *B. vosnesenskii*, and honey bee, *Apis mellifera,* were designated as outgroups. All specimens were stored at –80 C° except *B. terrestris* from Germany, which was preserved in 100% alcohol until use.

Table 2.1 Sources of specimens of subgenus *Bombus* and outgroups (2 species in subgenus *Pyrobombus* and the honey bee)

Name	Code	Location	No. specimens	Remarks
B. occidentalis occidentalis	OCW	Willamette Valley, OR	2	White tip
B. occidentalis occidentalis	OCF	Medford, OR	2	Ferruginous abdominal tip
B. occidentalis occidentalis	OCI	Hell's Canyon, OR	2	Yellow posterior thorax
B. occidentalis nigroscutatus	OCNC	San Francisco Bay, CA	2	
B. occidentalis nigroscutatus	OCNA	Fairbanks, AL, Yukon Territory and North British Columbia	3	
B. franklini	FRK	Medford, OR	1	
B. terricola	TRC	Calgary and Longview, Alberta	2	
B. moderatus	MOD	Kananaskis, Alberta	2	
B. lucorum	LUC	GenBank		Estoup, 1996
B. terrestris type G	TRSG	Germany	1	
B. canariensis	CAN	GenBank		Estoup, 1996
B. terrestris type C	TRSC	GenBank		Estoup, 1996
B. melanopygus	MEL	Coos Bay, OR	1	Outgroup
B. vosnesenskii	VOS	Willamette Valley, OR	1	Outgroup
A. mellifera	APS	Corvallis, OR	1	Outgroup

DNA Extraction

DNA was isolated from the thorax of single individuals. Each thorax was ground in liquid nitrogen and transferred to an microtube with 500 µl STE extraction buffer (0.001 M EDTA, 0.05 M Tris-HCl pH 7.5, 0.1 M NaCl), 75 µl 10% SDS, and 25 µl Proteinase K (10 mg/ml stock solution). The sample was then

incubated at 55 °C with gentle shaking for 2 hrs. The cloudy suspension was extracted twice with equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1) and twice with equal volumes of chloroform: isoamyl alcohol (24:1). Then the clear suspension was precipitated with 1/10 the sample volume of 3 M NaAc and 2.5 times the sample volume of ice cold 100% ethanol (Sambrook *et al.*, 1989 and Hillis *et al.*, 1997). The final DNA was suspended in 50 µl of double distilled water and stored at –20 C°.

Polymerase Chain Reaction

PCR amplifications were performed in 50 µl volumes, containing 10 mM Tris/HCl pH 9, 50 mM KCl, 1.5-2 mM $MgCl_2$, 1.0% Triton® X-100, 200 µM of each of the four deoxyribonucleotide triphosphates, 0.5 µM of each of two different primers, 2.5 U *Taq* polymerase, and 25-50 ng of DNA template. Cycle conditions were as follows: hot starting at 94 °C for 3 min, followed by 30 cycles of denaturation at 92 °C for 45 sec, annealing at 41-44 °C for 30 sec, and extension at 65 °C for 3 min. Additional elongation occurred at 65°C for 3 min after the last cycle. Successful amplification was detected by gel electrophoresis stained with ethidium bromide.

The forward primers were designed from tRNA leucine (Garnery *et al.*, 1991) and a region of COI (Estoup *et al.*, 1996), respectively. The reverse primer

was 317 bp downstream from the start codon of COII (Table 2.2). The amplified regions on the mitochondrial DNA are shown in figure 2.1.

Table 2.2 Oligonucleotide primers for PCR amplification and sequencing used to amplify the partial COII gene of bumblebees in subgenus *Bombus*. SD1=forward primer, ER and GR=reverse primers.

Primer sequence	Code	Remark
5'-GGCAGAATAAGTGCATTG-3'	SD1	Modified from Willis *et al.* (1992), Garnery *et al.* (1991) and Crozier *et al.* (1989)
5'-ATACCACGACGTTATTCAGA-3'	E3	Estoup *et al.* (1996).
5'-AATTCTGGATATTCATAAGATCA-3'	GR	Modified from Crozier *et al.* (1989)

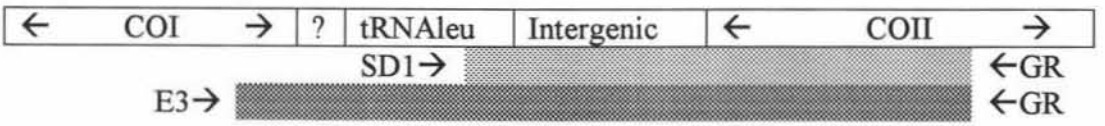


Figure 2.1 The arrangement of COI, COII, 2 tRNAs, and 2 intergenic regions found on the mitochondrial genome of the bee (*Bombus* sp. and *Apis* sp.). The forward and reverse PCR primers and the amplification regions produced from SD1-GR and E3-GR are indicated.

Sequencing

The PCR products were cleaned with QIAquick PCR purification kit (QIAGEN, Vallencia, CA) according to kit instructions. Both strands were sequenced by the cycle termination method using fluorescent-labeled dideoxyribonucleotide triphosphates. The sequence reaction and the processing of the reaction products for electrophoresis were performed following the instructions

for the sequencing kit (ABI PRISM^{tm} Dye Terminator Cycle Sequencing Core Kit, Perkin-Elmer Corp., Norwalk, CT). Electrophoresis and data collection were done on an ABI model 377 DNA Sequencer (Perkin-Elmer Corp.). The raw sequencing data from both directions were compiled using the Staden (version 0.99.2) program (Staden *et al.*, 1997).

Pairwise Sequence Comparison

Sequences composed of the intergenic regions and the partial COII genes were aligned by the Pileup program in the Genetic Computer Group (GCG), Wisconsin package version 9.0 (1996), and re-examined manually. The alignment of the intergenic parts was done with the aid of the RNA secondary structure analysis program, foldRNA, and the squiggle drawing program available in GCG (v. 9). The COII coding region was aligned using translated amino acid sequences based on the invertebrate mitochondrial code (Clary and Wolstenholme, 1985). Pairwise distances were computed using distance matrices based on the partial COII nucleotide sequence. Distances were corrected using Jukes-Cantor (Jukes and Cantor, 1969) and Kimura two-parameter corrections (Kimura, 1980).

Phylogenetic Analysis

The distance tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) with Jukes-Cantor and Kimura two-parameter corrections using the

NEIGHBOR program (v. 3.4). The bootstrap analysis with 100 replicates was done with the BOOTSTRAP program in the PHYLIP (v. 3.5c) computer package (Felsenstein, 1995) available in GCG (v. 9). Parsimony analysis and bootstrap with 500 replicates was performed in PAUP (v. 3.1) (Swofford, 1993) using the heuristic search option with equally-weighted characters, 10 random sequence additions, tree-bisection-reconnection branch swapping, mulpars in effect and maxtrees set at 300. Then the data were analyzed by weighting with the successive approximation method based on the maximum values of the rescaled consistency indices (Farris, 1969; Farris, 1989).

RESULTS

PCR Products

PCR products amplified by SD1-GR primers varied in size from 417 to 488 bp in the subgenus *Bombus* and the two bumblebees in the subgenus *Pyrobombus,* whereas that of honey bee was 550 bp. The primers E3-GR were used only for *B. terrestris,* and the size of PCR product was 697 bp. This difference was caused by variation of the sequences occurring in the intergenic region between $tRNA^{leu}$ and COII region ($tRNA^{leu}$/COII). The size of the partial COII region was constant for all species (Figure 2.2).

```
APS  atttccacat gatttatatt tatatttcaa gaatcaaatt catattatgc tgataattta atttcatttc
MEL  .....a.... ..aac..... a.c..... ..t..... ...t....t. a......... .........
VOS  .....t.... ..aa..... ..c..... ..t..... .t.t....t. ......... .........
TRSC .....t.... ..aac..... .t..... ..t..... .t.t....t. .......c.t .........
TRSG .....t.... ..aac..... .t..... ..t..... .t.t....t. .......c.t .........
CAN  .....t.... ..aac..... .t..... ..t..... .t.t....t. .......c.t .........
LUC  .....t.... ..aa..... .t..c..... ..t..... .t.t...ct. ......... .........
MOD  .....t.... ..aa..... .t..... ..c..... .t.t....t. ...c..... .........
TRC  .....t.... ..aa..... .t..... ..t..... .t.t...ct. ......... .........
FRK  .....t.... ..aa..... ct..... ..t..... .t.t...ct. ......cc.t .........
OCNA .....t.... ..aa..... .t..... ..t..... .t.t...ct. .......c.. .........
OCNC .....t.... ..aa..... .t..... ..t..... .t.t.a.ct. g......c.. .........
OCF  .....t.... ..aa..... .t..... ..t..... .t.t...ct. .......c.. .........
OCW  .....t.... ..aa..... .t..... ..t..... .t.t...ct. .......c.. .........
OCI  .....t.... ..aa..... .t..... ..t..... .t.t...ct. .......c.. .........

APS  ataatatagt tataataatt attattataa tttcaacatt aactgtatat attattttag atttatttat
MEL  .....t..ac a........a ..a..a.... ..attt.... ...aac..t. t......... ....ta.at.
VOS  .....t..ac .........a ..a..a.... ..attttt.. ...aac..t. t.....a... ....ta.at.
TRSC .....t..ac a........a ..a..a.... ..att..t.. ....a...t. t.c...a... ....t.ca..
TRSG .....t..ac a........a ..a..a..t. ..attt.c.. ....a...t. t.c...a... ....t.ca..
CAN  .....t..ac a........a ..a..a.... ..att..t.. ....a...t. t.c...a... ....t.ca..
LUC  ....ct..ac a........a ..a..a.... ..att..t.. ...aa..... t.....a... ....t.cat.
MOD  ....ct..ac .........a ..a..a.... ..att..t.. ...aa..... t.....a.t. ....t.cat.
TRC  .....t..ac .........a ..a..a.... ..att..c.. ...aa..... t.....a... ....t.cat.
FRK  .....c..ac a........a ..a..a.... ..att..t.. ...aa...t. t.....a... .c..t.cat.
OCNA .....c..ac .........a ..a..a.... ..att..c.. ...aac.... t.....a... ....t.cat.
OCNC .....c..ac .........a ..a..g.... ..at...t.. ...aac.... t....aa... ....t.cat.
OCF  .....c..ac .........a ..a..a.... ..att..t.. ...aac.... t.....ac.. ....t.cat.
OCW  .....c..ac .........a ..a..a.... ..att..t.. ...aac.... t.....a... ....t.cat.
OCI  .....c..cc .........a ..a..a.... ..att..t.. ...aac.... t.....a... ....t.cat.

APS  aaacaaattc tcaaatttat ttttattaaa aaatcataat attgaaatta tttgaacaat tattccaatt
MEL  ...t..t..t .t.......a c......... ........ca .......... .......tt. a.c......a
VOS  t..t..t... .t.......a c.c.t..... ........c. .......... .......tc. ..c...t..g
TRSC ...t..t..t .t.......a a.c.t..... ........c. .......... .......tt. a.c...c..a
TRSG ...t..t..t .t.......a a.c.t..... ........c. .......... .......tt. a.c...t..a
CAN  ...t..t..t .t.......a a.c.t..... ........c. .......... .......tt. a.c...c..a
LUC  ...t..t..t .t.......a a.c.t..... ........c. .......... .......tt. a.ca.....a
MOD  ...t.c...t ct....c..a a.c.t..... ...c....c. .......... .c.....tt. a..a.at..a
TRC  ...t..t... .t....c.ta a.c.t..... ...c....c. .......... .......tt. a.ca..t..a
FRK  ...t..t... ctt...c.ta a.c.t..... ........c. .......... .c.....tt. a.ca.....a
OCNA ...t..t... .t....c.ta a.c.t..... ........c. .......... .......tt. a.ca..t..a
OCNC ...t..t... ct....c.ta a.c.t..... ........c. ..c....... .......tt. a.....c..a
OCF  ...t..t... .t....c.ta a.c.t..... ........c. .......... .......tt. a..a..t..a
OCW  ...t..t... .t....c.ta a.c.t..... ........c. .......... .......tt. a..a..t..a
OCI  ...t..t... .t....c.ta a.c.t..... ........c. .......... .......tt. a..a..t..a

APS  attattctat taattatttg ttttccatca ttaaaaa
MEL  ......t..a .......... ...c...... .......
VOS  ......t..a ....a..... .........t .......
TRSC ......t..a .......... ...c.....t .......
TRSG ......t..a .......... ...c.....t .......
CAN  ......t..a .......... ...c.....t .......
LUC  ......t..a .......... ...c.....t .......
MOD  ......t..a .......... ......g..t .......
TRC  ......t..a .......... .........t .......
FRK  ......t..a .......... .........t .......
OCNA ......t..a .......... ...c.....t .......
OCNC ......t..a ....g..... ...c.....t .......
OCF  ......t..a .......... ...c.....t .......
OCW  ......t..a .......... ...c.....t .......
OCI  ......t..a .......... ...c.....t .......
```

Figure 2.2 The nucleotide sequences of a 247-bp segment of the 5' end of the cytochrome oxidase II gene from 14 representatives of the subgenus *Bombus* and 2 species from the subgenus *Pyrobombus* were aligned with the reference sequence of *A. mellifera*. Dots indicate identity of nucleotide base to the sequence from honey bee.

tRNA^{leu}/COII Intergenic Region

The tRNA^{leu}/COII intergenic region has been found in the subgenus *Bombus* as well as in many insects such as the honey bee (Crozier *et al.*, 1989) and flies (Smith and Bush, 1997). This region not only varies in nucleotide length but also nucleotide substitution. However, the variation in the tRNA^{leu}/COII intergenic region occurring in the subgenus *Bombus* is high, making alignment difficult and uncertain. In addition, the sequences of this region for *B. terrestris* type C, *B. canariensis* and *B. lucorum* are not available in the GenBank. Therefore, this region was not included in the phylogenetic analysis. The available sequences of this region from bumblebees in subgenus *Bombus* were used to reconstruct secondary structure using the foldRNA program in GCG. Although this program is designed for reconstructing RNA secondary structure, the algorithm and the free energy calculation enables it to be applied to other DNA sources that can form secondary structures. The tRNA^{leu}/COII intergenic region has the potential to form secondary structures in many insects such as the honey bee and the fly. The scenario of occurrence and formation of this region was proposed by Cornuet *et al.* (1991).

Three forms of secondary structures were found in subgenus *Bombus*: single, and bifurcated stem with or without stock (Figure 2.3). All alliances of subgenus *Bombus* found in the PNW had the bifurcated stem loop structure, whereas the representative from Europe, *B. terrestris,* had the single stem loop as did the two species of *Pyrobombus,* a close relative to subgenus *Bombus,* had the single stem loop.

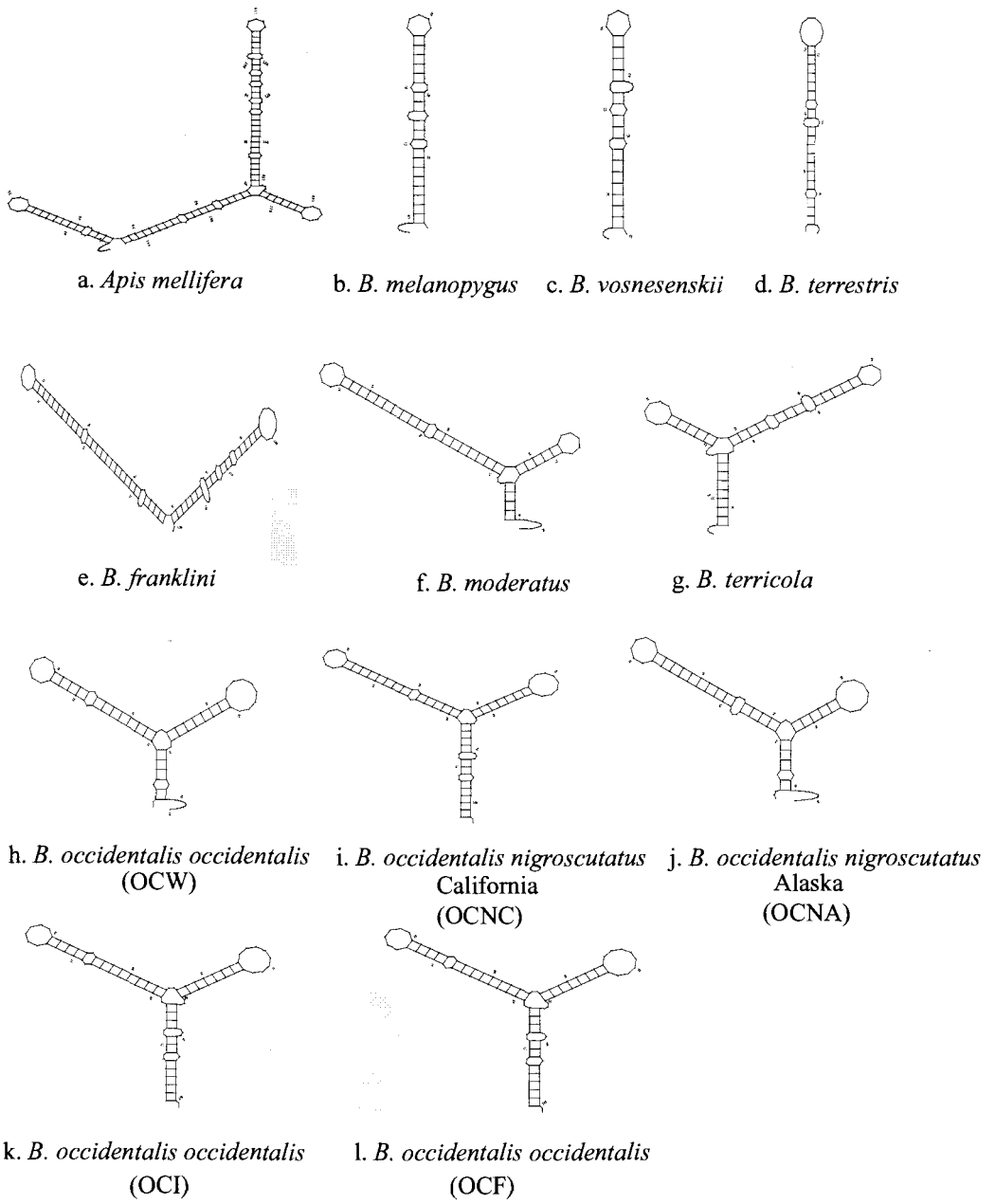


Figure 2.3 The secondary structures of the tRNA leu/COII intergenic regions of the subgenus *Bombus* species in the PNW compared to *B. terrestris* from Europe, 2 species of the subgenus *Pyrobombus*, and *A. mellifera.*

Partial COII Pairwise Analysis

Two hundred forty-seven base pairs of the partial COII region from all 15 species were compared. Eighty-three nucleotide positions (33.6%) were phylogenetically informative with thirty-seven of these being parsimonious informative sites. The mean of base frequencies (A=0.38920, T=0.46856, C=0.11174, and G=0.03050) showed that this partial COII gene is a high A-T rich region (85.8%), which is common in most insects (Crozier *et al.*, 1989; Willis *et al.*, 1992; Schmitz and Moritz, 1996). The nucleotide base substitutions and pairwise distance with the Kimura correction are shown in table 2.3. The pairwise base differences showed an unequal transition/transversion ratio ranging from 0.35 to 7.0; therefore, two types of correction, Jukes-Cantor and Kimura-2 parameter, were applied. However, the two corrections are not greatly different in pairwise distances, and the trees generated from them were similar. Therefore, the distance matrix calculated by Kimura distance method was selected for analysis and presentation.

Neighbor-Joining Analysis

The data set of partial COII gene sequences, 247 base pairs long, was analyzed by neighbor-joining with the Kimura 2-parameter distance correction method. Generally, the neighbor-joining tree demonstrates the relative genetic distances among the analyzed sequences and allows access to the time of

Table 2.3 Estimated sequence divergence (%) with Kimura correction (above) and the numbers of substitutions in the partial COII gene sequences (below) among representative species.

	1	2	3	4	5	6	7	8
APS	-	0.21412	0.2252	0.23041	0.24153	0.22493	0.23041	0.25276
MEL	46	-	0.09024	0.09947	0.09947	0.09485	0.09943	0.16223
VOS	48	21	-	0.09972	0.09972	0.10432	0.09503	0.12837
TRSC	49	23	23	-	0.01638	0.00406	0.05028	0.10464
TRSG	51	23	23	4	-	0.02052	0.06339	0.11389
CAN	48	22	24	1	5	-	0.05461	0.10925
LUC	49	23	22	12	15	13	-	0.07261
MOD	53	36	29	24	26	25	17	-
TRC	48	28	20	17	17	18	10	15
FRK	56	32	28	18	21	19	16	23
OCNA	50	27	21	16	16	17	11	20
OCNC	53	34	28	22	26	23	20	26
OCF	50	29	22	17	19	18	12	19
OCW	49	28	21	16	18	17	11	18
OCI	49	29	22	17	19	18	12	19

Table 2.3 (continued)

	9	10	11	12	13	14	15
APS	0.22502	0.26997	0.23595	0.25277	0.23595	0.23045	0.23054
MEL	0.12291	0.14256	0.11815	0.15221	0.12772	0.12291	0.12769
VOS	0.08586	0.12344	0.09048	0.12365	0.09516	0.09048	0.09503
TRSC	0.07247	0.07720	0.06794	0.09503	0.07247	0.06794	0.07236
TRSG	0.07227	0.09079	0.06779	0.11373	0.08136	0.07680	0.08127
CAN	0.07690	0.08164	0.07236	0.09960	0.07690	0.07236	0.07680
LUC	0.04180	0.06839	0.04617	0.08597	0.05057	0.04617	0.05045
MOD	0.06369	0.09987	0.08673	0.11408	0.08204	0.07739	0.08183
TRC	-	0.05920	0.02066	0.07261	0.03349	0.02917	0.03336
FRK	14	-	0.05476	0.08577	0.05920	0.05476	0.05909
OCNA	5	13	-	0.05028	0.01230	0.00816	0.01226
OCNC	17	20	12	-	0.04594	0.04163	0.04591
OCF	8	14	3	11	-	0.00407	0.00814
OCW	7	13	2	10	1	-	0.00406
OCI	8	14	3	11	2	1	-

divergence of COII genes based on the molecular clock hypothesis (Nei, 1987). The molecular clock of the COII gene is not well established; therefore, no conclusion on the time of divergence of COII among the analyzed sequences can be made. Only branch patterns with the percentage of bootstrap support value were used to demonstrate the phylogenetic relationship of this subgenus in the PNW (Figure 2.4).

All members of subgenus *Bombus* from the PNW, formed a cascade of relationships, separate from the European group; however, the bootstrap support for a separate PNW group was weak (48%). Resolution within the New World group was also weak except for the *B. occidentalis* complex, which formed a group supported by an 83% bootstrap value.

Among the European species, sequences from both *B. terrestris* specimens grouped together with another close relative, *B. canariensis* (100% support); however, the common form of *B. terrestris* (Estoup, 1996) was more closely related to *B. canariensis,* with high support (89%), than to *B. terrestris* collected from Germany. *Bombus lucorum* was placed close to *B. moderatus*, in the PNW group, with 76% support.

Bombus terricola and *B. franklini* did not fall within the *B. occidentalis* group. The *B. occidentalis* alliances formed their own complex with 83% bootstrap support. The common form (OCW) and two intermediate forms (OCI and OCF) of *B. o. occidentalis* clustered together with high bootstrap support (90%).

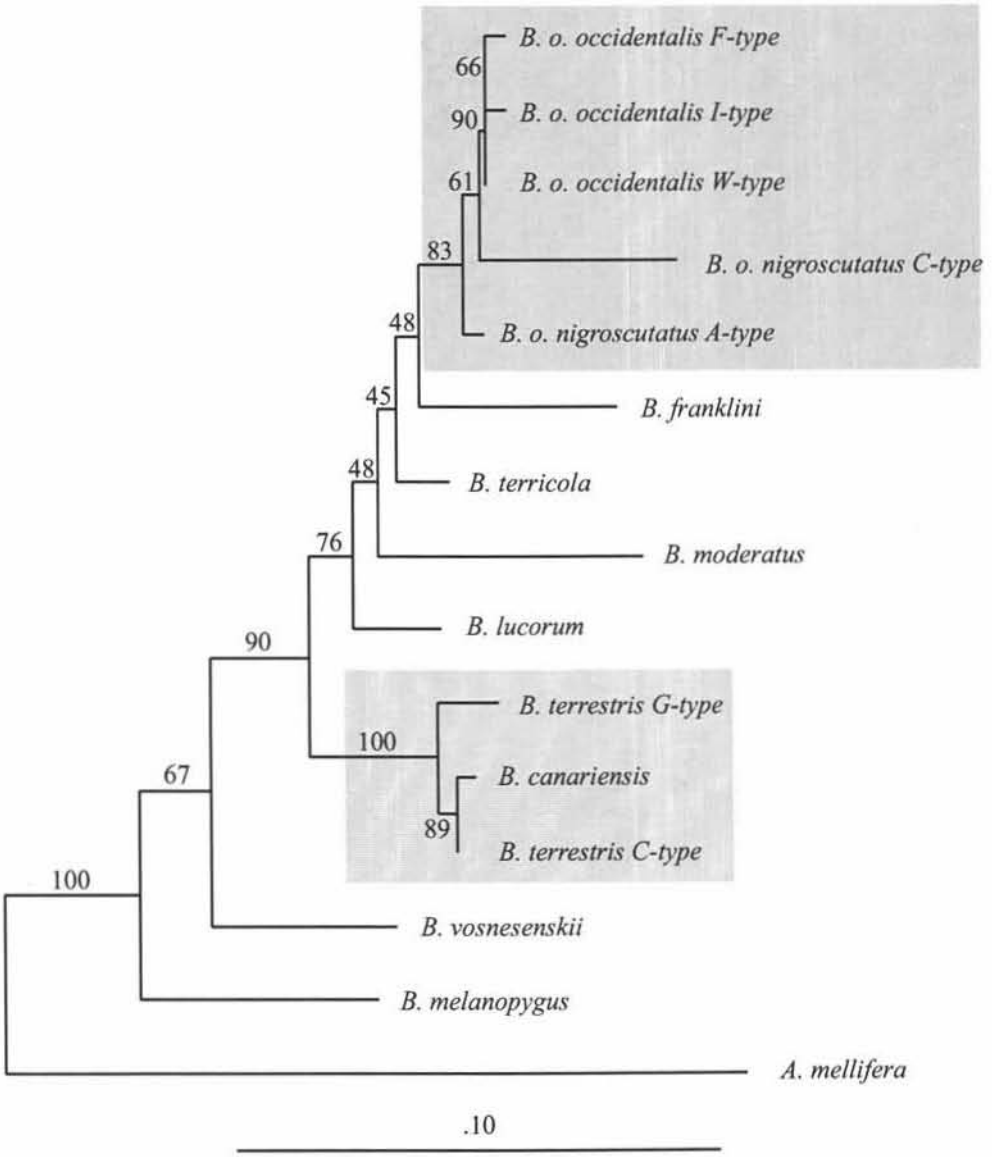


Figure 2.4 Phylogenetic relationship among *Bombus* spp. reconstructed by the neighbor-joining analysis using the Kimura 2-parameter correction.The numbers on the branches represent bootstrap support values.

Two forms of *B. o. nigroscutatus* collected from Alaska (OCNA) and California (OCNC) were not identical. The OCNC had a long branch compared to its alliances. The OCNA formed a short branch connected to the other *occidentalis* with very low bootstrap support.

Maximum Parsimony Analysis

Maximum parsimony analysis of the partial COII data provided five most parsimonious trees of 135 steps (CI = 0.7111, RI = 0.6422, RC = 0.4567 and HI = 0.2889). Differences in the topologies concerned the relationships of *B. moderatus* and *B. lucorum*; however, the trees shared most of the branch patterns of the neighbor-joining tree, although they are strict consensus (Figure 2.5). Moreover, maximum parsimony tree became identical to the neighbor-joining trees, when the maximum parsimony tree was weighted with a rescaled consistency index, which provided only one tree (Figure 2.6). This tree placed all species in the PNW together in the tree, but with only 37% bootstrap support. The *B. terrestris* complex was supported at 95% bootstrap. In the PNW species, all the *B. occidentalis* members formed a monophyletic group with 70% support.

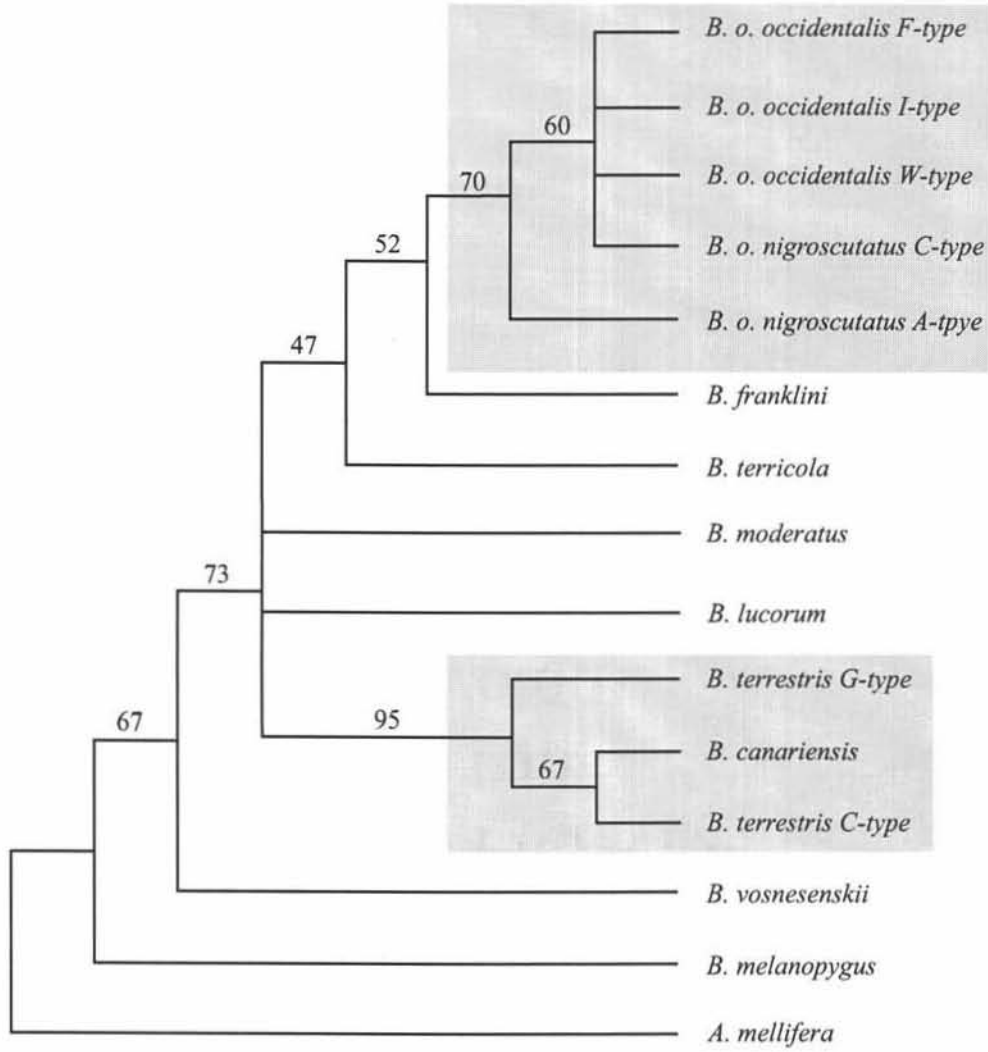


Figure 2.5 The strict consensus tree of the partial COII gene reconstructed from 5 most parsimonious trees resulting from maximum parsimony analysis, with bootstrap support values presented on the branches. Total rearrangements tried 15,356, tree length 135 steps, 5 most parsimonious trees, CI=0.711, RI=0.642, RC=0.457, HI=0.289.

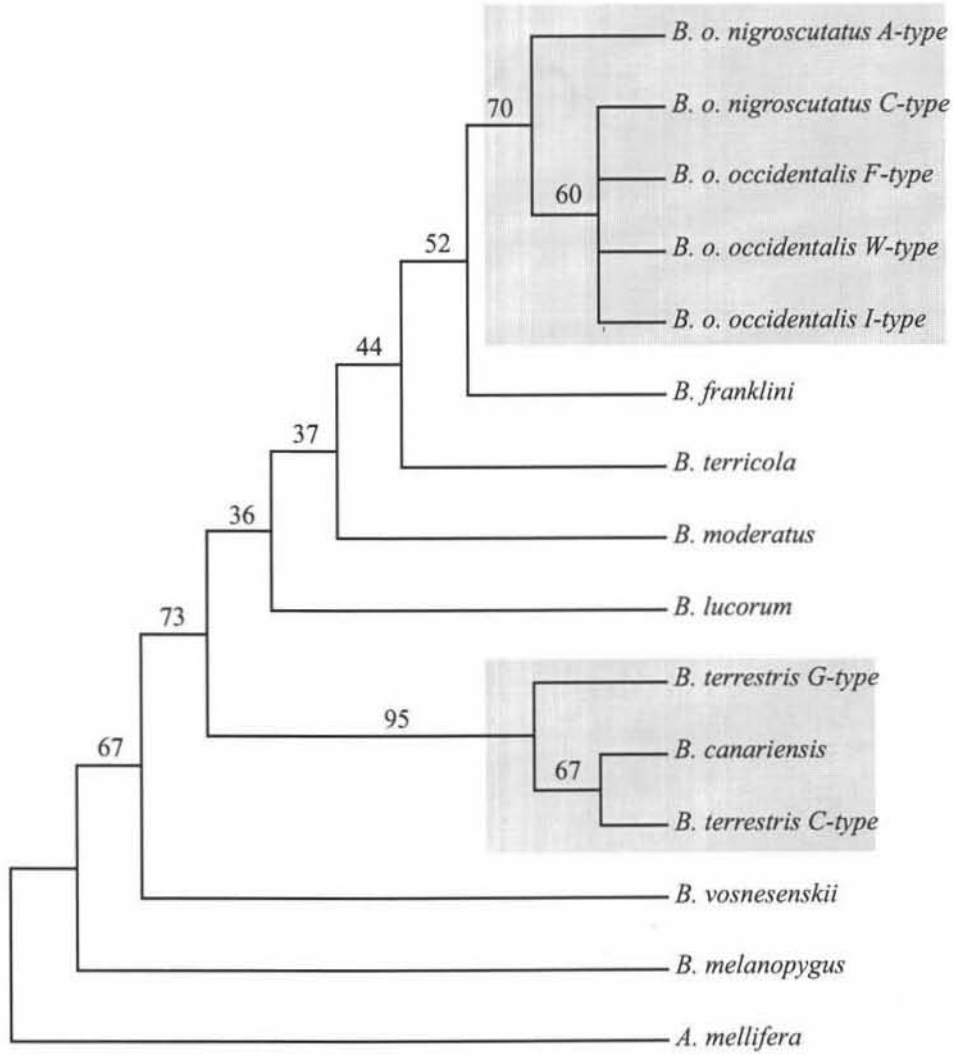


Figure 2.6 Phylogenetic tree reconstructed from the partial COII gene by maximum parsimony analysis and weighted with the RC. The numbers on the branches represent bootstrap value. Total rearangement tries 4,388, tree length 78.22 step, one tree produced, CI=0.926, RI=0.874, RC=0.810, HI=0.074

DISCUSSION

The $tRNA^{leu}$/COII intergenic region was found in all members of subgenus *Bombus*. The formation of this region into secondary structures among the *Bombus* species shows the potential to identify and clarify relationships of this group. Their secondary structures showed a progression from the single stem-loop in *B. terrestris* to the bifurcated stem-loop found in all PNW species. The differences in the secondary structures are based not only on the insertion and deletion of tandem simple sequence repeat units (SSR) such as $(TA)_n$, but also nucleotide base substitution. Since this region is not involved with any transcription process, the variation can happen easily and rapidly. The members of the *B. occidentalis* complex show closely related structures. The secondary structure in OCF and OCI are identical and closely related to those of OCNC whereas OCNA has a secondary structure more closely related to OCW. Moreover, the secondary structures within the *B. occidentalis* complex are not similar to *B. terricola, B. moderatus* and *B. franklini* at all; this implies that they are distantly related. The transformations of the structures among the closely related alliance in subgenus *Bombus* correspond to the phylogenetic relationship based on partial COII region and can be used to support the divergence evidence on the tree.

North America species vs. European species

The branch order of the trees reconstructed from partial COII gene of the subgenus *Bombus* by both, neighbor-joining and maximum parsimony, and the secondary structures of the intergenic region, reveal the distant relationship between the Nearctic and Palaearctic group. Although the topologies are not similar to the tree reconstructed from numeric cluster analysis based on the wing venation (Plowright and Stephen, 1973) or those from enzymatic studies (Scholl *et al.*, 1992), their phylogenetic trees share some branch patterns and show potential to divide these species into two groups as well. However, the position of *B. lucorum* in the tree is anomalous; it groups with the Nearctic species with relatively high bootstrap support (Figure 2.4). This suggests the need for more taxa and longer sequences to clarify this relationship.

The Status of *B. lucorum* and *B. moderatus*

With a high bootstrap value at the branch between *B. lucorum* and the *B. terrestris* complex, it appears that *B. lucorum* is more closely related to *B. moderatus* than to *B. terrestris*. This result agrees with Sladen (1919) who first proposed to place *B. moderatus* closer to *B. lucorum* rather than *B. terrestris*. However, the relationship of these two species is not close enough to draw the conclusion that they are synonymous species as suggested by Milliron (1971). Since these two species have 17 nucleotide bases differences in the COII gene

sequence, making their relative distance around 0.07, this suggests that they should be classified as different species. This conclusion is strongly supported by the discontinuity of species distribution (Tkalcu, 1974) and also the allozyme studies (Scholl *et al.* 1990).

The Status of *B. terricola* with *B. terrestris* and *B. occidentalis*

Bombus terricola has been considered not only to be closely related to *B. terrestris* (Sladen, 1919) but also to be co-specific with *B. occidentalis* (Milliron, 1971; Poole, 1996) based on morphological evidence. The phylogenetic trees of this study reconstructed by both neighbor-joining and maximum parsimony confirm that *B. terricola* is neither closely related to *B. terrestris* nor *B. occidentalis* and not in agreement with those suggestions. *Bombus occidentalis* and *B. terrestris* both form strongly supported clades, (83% and 100% in neighbor-joining tree and 70% and 95% in maximum parsimony tree, respectively) and *B. terricola* is outside both these clades. This suggests that all of these bumblebees are separate species. This result is in agreement with the allozyme studies (Scholl, 1990), and morphological studies (Franklin, 1913; Stephen, 1957), although *B. terricola* in this study does not form a sister group with *B. occidentalis* exactly as it did in the allozyme study of Scholl (1990).

The Status of *B. franklini*

The data from partial cytochrome oxidase II gene sequences confirms that *B. franklini* is distinct from *B. occidentalis* with high bootstrap support. This species has different comb architecture from *B. occidentalis* and multivariate analysis of wing venation places this species well away from subgenus *Bombus*. Moreover, when combining this COII data with other evidence such as male genitalia (Plowright and Stephen, 1980), an extremely limited geographical distribution (Stephen, 1957; Thorp, 1970) and no evident interbreeding with *B. occidentalis* in several sympatric locations (Thorp, 1983), this species appears unique.

However, there is one form of *B. occidentalis* (OCF) which is similar to *B. franklini* and occupies the same habitat. This confusion may lead to misidentification of OCF as *B. franklini* as mention by Stephen (1957). The phylogenetic tree in this study demonstrated clearly that OCF belongs to *B. occidentalis* and could not be the polymorphic form of *B. franklini*, which mimics *B. occidentalis* and becomes hidden within the variation of *B. occidentalis*.

The Status within *B. occidentalis*

Based on both neigbhor-joining and parsimony analyses, partial COII sequences demonstrated that *B. occidentalis* is monophyletic, (83% and 70% support value, respectively). These data strongly support that *B. occidentalis* is

separated from *B. terricola* and *B. franklini.* The branching within the group was very compact, making it difficult to establish the polarity of branching of the group members. Even though OCNA tends to separate from the others and appears as the primitive sister group in both trees, its branch support is too weak to draw this conclusion. The relative distances among the members in this group are unbalanced, since the branch of OCNC is very long compared to other members. This long branch attraction may affect the analysis by producing an artifact in the branch order of the tree. This problem may be solved by extending the sequence longer for analysis in order to gain more informative sites (see chapter 4). OCNA looks similar to OCNC but they occupy distant, non-overlapping geographic areas, Alaska and California respectively (Figure 2.7). The area between them is filled with many forms of *B. o. occidentalis*. This suggests that the ancestor of OCNA and OCNC occupied the area from Alaska to California and was separated by a geographical barrier such as climatic change during the glacial period. Two main lineages evolved: one survived in the north whereas another survived in the south. All other forms of *B. occidentalis* may be the products of species adaptation since many islands in the PNW could have been formed during glaciation. When the glaciers retreated all these isolated populations became the polymorphic forms of this species, which occupy the area between Alaska and California. The evidence of the branch support values along with their geographical distribution and gradual intergrading polymorphism (Stephen, 1957) suggests that *B. o. occidentalis* and *B. o. nigroscutatus* (OCNC) should retain their relationships as subspecies.

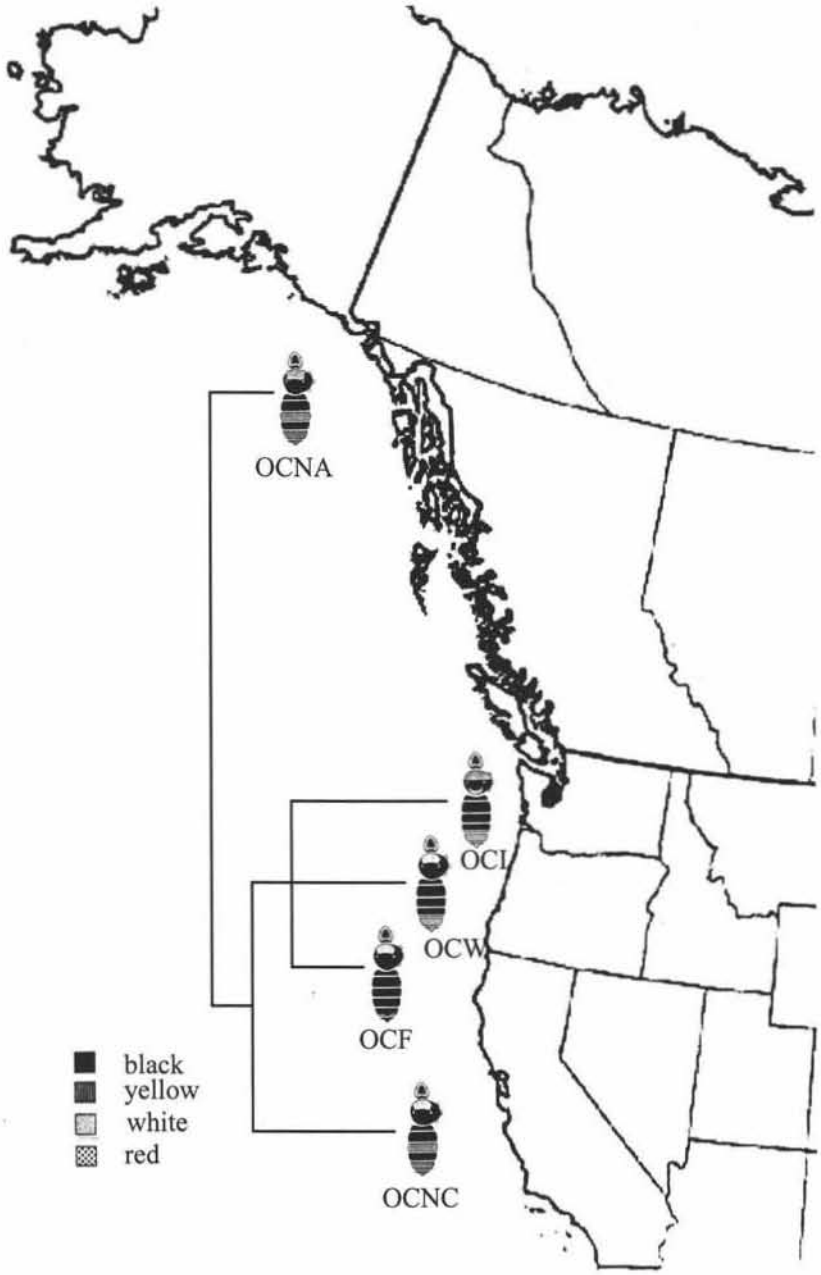


Figure 2.7 The phylogeographic distribution of *Bombus occidentalis* and its relatives in the PNW. *Bombus o. nigroscutatus* Alaska type (OCNA) is morphologically identical to *Bombus o. nigroscutatus* California type (OCNC) but disjunct. The area between is occupied by *B. o. occidentalis* and its polymorphs, *Bombus o. occidentalis* W-type (OCW), *Bombus o. occidentalis* I-type (OCI), and *Bombus o. occidentalis* F-type, (OCF)

OCNA could be considered as another subspecies of *B. occidentalis*. The OCNA has similar morphology to OCNC but shows differences in COII sequences analysis. However, because of the appearance of the long branch of OCNC on the tree, which may perturb the branching order on the tree, it is therefore appropriate to conclude the relationships among *B. occidentalis* when more information is available.

CONCLUSIONS

This study demonstrates the utility of nucleotide sequence data from the partial cytochrome oxidase subunit II for reconstructing a phylogeny of the subgenus *Bombus* in the PNW. Although some of the branch support values presented in the phylogenetic trees reconstructed from the different analytical methods are weak, their topologies were still consistent. In addition, the intergenic region may be useful to confirm the reliability of the tree via its secondary structure, although the sequence was excluded from the phylogenetic analysis because it could not be aligned. The results of this study clarified many of the relationships among the species of the subgenus *Bombus*. However, the weak support in some branches suggests that relationships within this group should be confirmed by using another gene sequence or the complete COII gene sequence, and by adding more taxa to the analysis.

ACKNOWLEDGEMENTS

I would like to thank Dr. B. Schricker who kindly sent *B. terrestris* from Germany and also M. L. Carlson who kindly collected *B. o. nigroscutatus* from Alaska and inspired me to go to northern British Columbia and the Yukon Territory.

REFERENCES

Brower, A.V.Z. (1994). Phylogeny of *Heliconius* butterflies inferred from mitochondrial DNA sequences (Lepidoptera: Nymphalidae). *Mol. Phylogenet. Evol.* 3: 159-174.

Clary, D. O., and Wolstenholme, D. R. (1985). The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. *J. Mol. Evol.* 22: 252-271.

Cornuet, J. M., Garnery, L., and Solignac, M. (1991). Putative origin and function of the intergenic region between COI and COII of *Apis mellifera* L. mitochondrial DNA. *Genetics* 1128: 393-403.

Crozier, R. H., and Crozier, Y. C. (1993). The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organization. *Genetics* 133: 97-117.

Crozier, R. H., Crozier, Y. C., and Mackinlay, A. G. (1989). The CO-I and CO-II region of honeybee mitochondrial DNA: evidence for variation in insect mitochondrial evolutionary rates. *Mol. Biol. Evol.* 6: 399-411.

Estoup, A., Solignac, M., Cornuet, J. M., Goudet, J., and Scholl, A. (1996). Genetic differentiation of continental and island populations of *Bombus terrestris* (Hymenoptera: Apidae) in Europe. *Molec. Ecol.* 5: 19-31.

Farris, J. S. (1969). A successive approximations approach to character weighting. *Syst. Zool.* 18: 374-385.

Farris, J. S. (1989). The retention index and the rescaled consistency index. *Cladistics* 5: 417-419.

Felsenstein, J. (1995). "PHYLIP computer package program (version 3.5c)," University of Washington, Seattle.

Franklin, H. J. (1913). The Bombidae of the New World. *Trans. Am. Entomol. Soc.* 38: 177-486.

Garnery, L., Vautrin, D., Cornuet, J. M., and Solignac, M. (1991). Phylogenetic relationships in the genus *Apis* inferred from mitochondrial DNA sequence data. *Apidologie* 22: 87-92.

Hillis, D. M., Mable, B. K., Larson, A., Davis, S. K., and Zimmer, E. A. (1996). Nucleic Acids IV: Sequencing and Cloning. *In* "Molecular Systematics"(D. M. Hillis, C. Moritz, and B. K. Mable, Eds.), pp. 321-381. Sinauer Associates Inc., Sunderland, Massachusetts.

Jukes, T. H., and Cantor, C. R. (1969). Evolution of Protein Molecules. *In* "Mammalian Protein Metabolism" (H. N. Munro, Ed.), pp. 21-123. Academic Press, New York.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16: 111-120.

Lui, H., and Beckenbach, A. T. (1992). Evolution of the mitochondrial cytochrome oxidase II gene among 10 orders of insects. *Mol. Phylogenet. Evol.* 1: 41-52.

Milliron, H. E. (1971). A monograph of the Western Hemisphere bumblebees (Hymenoptera: Apidae; Bombini). I. The genera *Bombus* and *Megabombus* subgenus *Bombias*. *Mem. Entomol. Soc. Can.* 82: 1-80.

Nei, M. (1987). "Molecular Evolutionary Genetics," Columbia Univ. Press, New York.

Plowright, R. C., and Stephen, W. P. (1973). A numerical taxonomic analysis of the evolutionary relationships of *Bombus* and *Psithyrus* (Apidae: Hymenoptera). *Can. Entomol.* 105: 733-743.

Plowright, R. C., and Stephen, W. P. (1980). The taxonomic status of *Bombus franklini* (Hymenoptera: Apidae). *Can. Entomol.* 112: 475-479.

Poole, R. W. (1996). "Nomina Insecta Nearctica [sic], a checklist of the insects of North America. Volume 2: Hymenoptera, Mecoptera, Megaloptera, Neuroptera, Rhaphidioptera, Tricoptera," 793pp. Rockville, Maryland.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstruction phylogenetic trees. *Mol. Biol. Evol.* 4: 406-425.

Sambrook, J., Fritsch, E., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press, New York.

Schmitz, J. and Moritz, R. F. A. (1996). Molecular phylogeny of Vespidae (Hymenoptera) and the evolution of sociality in wasps. *Mol. Phylogenet. Evol.* 9: 183-191.

Scholl, A., Obrecht, E., and Owen, R. E. (1990). The genetic relationship between *Bombus moderatus* Cresson and the *Bombus lucorum* auct species complex (Hymenoptera: Apidae). *Can. J. Zool.* 68: 2264-2268.

Scholl, A., Thorp, R. W., and Obrecht, E. (1992). The genetic relationship between *Bombus franklini* (Frison) and other taxa of the subgenus *Bombus* s. str. (Hymenoptera: Apidae). *Pan-Pacific Entomol.* 68: 46-51.

Sladen, F. W. L. (1919). Notes on the Canadian representatives of British species of bees. *Can. Entomol.* 51: 124-130.

Smith, J. J., and Bush, G. L. (1997). Phylogeny of the genus *Rhagoletis* (Diptera: Tephritidae) inferred from DNA sequences of mitochondrial cytochrome oxidase II. *Mol. Phylogenet. Evol.* 7: 33-43.

Staden, R., Bonfield, J., and Kathryn, B. (1997). "Staden computer package (version 0.99.2)," Medical Research Council, Laboratory of Molecular Biology, Cambridge, UK.

Stephen, W. P. (1957). Bumblebees of Western America (Hymenoptera: Apoidae). *Tech. Bull. Oreg. Agric. Exp. Stn.* 40.

Swofford, D. L. (1993). "PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1," Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

Thorp, R. W. (1970). The type locality of *Bombus franklini* and notes on putative Arizona records of other Bombini. *Pan-Pacific Entomol.* 46: 177-180.

Thorp, R. W., Horning, D. S. Jr., and Dunning, L. L. (1983). Bumblebees and cuckoo Bumblebees of California. *Bull. Calif. Insect Surv.* 23.

Tkalcu, B. (1974). Ergebnisse der 1. Und 2. mongolisch-tschechoslowakischen entomologisch-botanischen Expedition in der Mongolei. Nr. 29. Hymenoptera, Apidae, Bombinae. *Sb. Faun. Praci Entomol. Odd. Eleni.* 15: 25-58.

Williams, P. H. (1991). The bumble bees of the Kashmir Himalaya (Hymenoptera: Apidae, Bombini). *Bull. Br. Mus. (Nat. Hist.) Entomol.* 60:1-204.

Williams, P. H. (1998). An annotated checklist of bumblebees with an analysis of patterns of description (Hymenoptera: Apidae, Bombini). *Bull. Br. Mus. (Nat. Hist.) Entomol.* 67: 79-152.

Willis, L. G., Winston, M. L. and Honda, B. M. (1992). Phylogenetic relationships in the honeybee (Genus *Apis*) as determined by the sequence of the cytochrome oxidase II region of mitochondrial DNA. *Mol. Phylogenet. Evol.* 1: 169-178.

Wisconsin Package version 9.0. (1996). "Genetics Computer Group (GCG)," Madison, WI.

CHAPTER 3

PHYLOGENETIC ANALYSIS OF THE BUMBLEBEE SUBGENUS *PYROBOMBUS* (HYMENOPTERA: APIDAE) BASED ON MITOCHODRIAL CYTOCHROME OXIDASE SUBUNIT I AND II GENE SEQUENCES

Weerathep Pongprasert, D. Michael Burgett, Katharine G. Field,
and William P. Stephen.

ABSTRACT

The subgenus *Pyrobombus* is the largest group of bumblebees in the world, having a wide distribution in both the New and Old Worlds. More than half of the identified species in North America occur in the Pacific Northwest of America (PNW). Identification of species of this subgenus is difficult because of the very high level of variation in the group, thought to be caused by adaptive radiation into the highly varied habitats in the PNW. A phylogenetic analysis of 14 species of this subgenus in the PNW, inferred from molecular evidence of mitochondrial cytochrome oxidase subunit I and II gene sequences (COI and COII), was carried out not only to clarify species status but also to understand their evolutionary relationships.

In addition, this analysis allowed comparison of the utility of these two genes with morphology and geographic distribution evidence in understanding the relationships of the members of this subgenus in the PNW, and finally, determination of the potential of these two genes for future phylogenetic study.

Both neighbor-joining and maximum parsimony analyses resulted in consistent but different phylogenetic trees when applied to each gene. The incongruence of those trees was detected by the partition homogeneity test (p-value = 0.002). The conflict between COI and COII gene trees mainly occurred at the placement of *Bombus bifarius* and *Bombus centralis* Cresson. When these two species were removed, the new data sets provided congruent results in all combined data sets and their partitions. A possible placement of the conflicting taxa in the

congruent tree was based on other evidence such as morphology and other molecular data.

The final phylogenetic trees provided clear relationships among all taxa and explained the radiation of many closely related and polymorphic species.

INTRODUCTION

As polymerase chain reaction (PCR) and DNA sequencing has become more practical and many more genes are used, the study of molecular systematics has increased dramatically. Mitochondrial DNA (mtDNA) has become a popular choice for a wide range of taxonomic, population, and evolutionary studies in animals because many aspects of its structure and evolution have made it a valuable evolutionary tool. For example, it occurs in multiple copies per cell, it is independent from the nuclear DNA, rapidly evolving, maternally inherited and lacks recombination (Moritz *et al.*, 1987; Harrison, 1989; Simon, 1991). There are many genes in mtDNA that show potential use in molecular systematics from higher taxonomic (Ballard *et al.*, 1992) to intraspecific categories (e.g. Avise *et al.*, 1987). While no molecular marker is universally superior to all others under all circumstances, some genes are clearly a better choice for study than others (Brower and DeSalle, 1994). Normally, the choice frequently has been based on the availability of universal primers and the prior knowledge of the level of variability appropriate for a particular study.

Protein-coding regions in mtDNA provide an additional advantage by being constrained to a reading frame that can make homology assessment easier with help of amino acid sequences. Furthermore, since each amino acid is coded by three nucleotide bases, protein coding regions provide the opportunity to separately analyze the different positions in the codons (Bull *et al.*, 1993). For these reasons, there are many molecular studies on animals and insects using mtDNA protein coding sequences (e.g. Liu and Beckenbach, 1992; Szymura *et al*, 1996; Vogler and Pearson, 1996; Randi, 1996)

In this study, we have used two mtDNA-coding regions, the COI and COII genes, for a phylogenetic analysis of species in the bumblebee subgenus *Pyrobombus* in the PNW. This subgenus is the largest group of bumblebees, composed of 43 species worldwide, with most of them found in the Nearctic region of North America (Williams, 1998). The PNW species makes up more than half of the described species of subgenus *Pyrobombus* in North America (Stephen, 1957). The number of species varies from reviser to reviser and range from 12-15 (Williams, 1998; Poole, 1996; Richards, 1968).

Most species are highly polymorphic, the systematics of these species becomes more difficult because closely related species have partial or completely overlapping distributions (Stephen, 1957).

Various morphological characters, especially male genitalia, were introduced as key characters which were often sufficient to separate many species in the bees (Michener, 1944). However, in the subgenus *Pyrobombus*, structural

variability can occur not only between populations, but also within a single population. It is sometimes impossible to distinguish species and often the last resort, male genitalia, had failed (Stephen, 1957).

Bombus melanopygus is a remarkable example. This species exhibits variation of the red/black pile on abdominal terga 2 and 3, that gradually changes from red to black according to the geographic location, from Canada to California, respectively. Even though this character is controlled by a single diallele locus (Owen and Plowright, 1980), it appears to be relatively homogeneous at the level of allozyme analysis within this species, possibly because of active gene flow among the various populations (Owen *et al*, 1992). There are many allozyme studies on the subgenus *Pyrobombus* (Snyder, 1974; Pamilo *et al.*, 1978; Pamilo *et al.*, 1984; Owen, 1985; Owen *et al.*, 1992) but only a few include PNW species. Moreover, most results show little allozyme variation within the subgenus *Pyrobombus* and none mention a phylogenetic analysis among species.

Numerical taxonomic analysis based on wing venation was the first significant evidence to confirm that all members of the subgenus *Pyrobombus* found in the PNW form their own group that clusters with groups from the other regions. Surprisingly, this subgenus forms a sister group with the subgenus *Bombus* (Plowright and Stephen, 1973).

Recent cladistic analyses based on morphological characters of bumblebees were done by Williams in 1985 and 1994 but no species of subgenus *Pyrobombus* from the PNW were included. The first molecular phylogenetic analysis of

bumblebees using partial COI gene sequences was limited to the species from Europe (Pedersen, 1996). Recently, four species of bumblebees in subgenus *Pyrobombus* from the PNW were included in an analysis of phylogenetic relationships with European species based on COI and cytochrome *b* (Koulianos, 1999). The pattern of relationships among these four species shared some similarities to the cluster analysis of Plowright and Stephen (1973). No one has done a molecular phylogenetic analysis using all the PNW species of the subgenus *Pyrobombus*.

We examined the possibility that DNA sequences of partial mitochondrial COI and COII (with and without the intergenic regions) would explain the relationships of bumblebees in the subgenus *Pyrobombus* in the PNW. In addition, a combined analysis was performed with an incongruent length different analysis (ILD), or partition homogeneity test, to determine the heterogeneity of all data sets and consider the possibility of combining data sets in order to gain more phylogenetic signal and higher resolution.

The 3'end of the COI gene region, around 600 bp in length, was selected based on the evolutionary pattern and conserved primers for phylogenetic studies (Lunt *et al.*, 1996). Since this region appears to be the most amino acid variable site of COI, the level of variation in its nucleotides should be higher when the degenerate genetic code is taken into account. Therefore, this region is potentially suitable for phylogenetic analysis. Furthermore, the appropriate primers designed from the conserved region, U7 and U10, suggested by Lunt and his colleagues, can

be modified for bumblebees by using the reference sequence from the honey bee, the most closely related genus (Crozier and Crozier, 1993). This region has also been successful in describing the phylogenetic relationship of Coleoptera (Howland and Hewitt, 1995).

The 5' end of the COII gene region was compared with the partial COI gene sequence, based on the success of previous studies in many insects such as butterflies (Brower, 1994), tiger beetles (Vogler and Pearson, 1996), and honey bees (Willis, 1992; Garnery 1991). The potential of this gene for phylogenetic analysis is due to the nucleotide variation not only in the COII gene itself but also in the adjacent $tRNA^{leu}$/COII intergenic region, since the amplification of the COII gene by PCR requires a forward primer starting from $tRNA^{leu}$ and a reverse primer from inside COII gene. These extraordinary characteristics make this region very useful for phylogenetic study at the interspecific level (Figure 3.1).

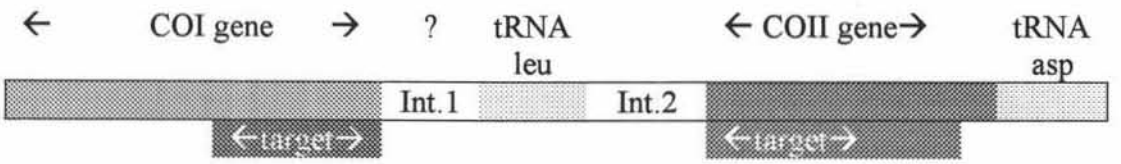


Figure 3.1 The arrangement of COI, COII, 2tRNAs, and 2 intergenic regions found on the mitochondrial genome of bees (*Bombus* sp. and *Apis* sp.). The target areas are the sites used for this phylogenetic analysis.

MATERIALS AND METHODS

Specimens collection

Specimens of the subgenus *Pyrobombus* in the PNW (36 specimens of 14 species) were collected in the spring and summer during 1995 to 1998, covering British Columbia, Alberta, Washington, Idaho, and Oregon (Table 3.1). *Bombus bifarius bifarius* Cresson, *B. flavifrons dimidiatus* Ashmead, *B. edwardsii* Cresson, *and B. vandykii* Frison were not included in this study because they were rare during the four years of collection. *Bombus impatiens* Cresson was obtained from Alabama to compare with species in the PNW. The honey bee, *A. mellifera* was designated as outgroup. Most of the specimens were stored in the freezer at –80 C° except *B. impatiens* (from Alabama) and all of the specimens from Canada which were instead preserved in 100% alcohol until DNA extraction.

DNA Extraction

DNA was isolated from the thorax of single individuals. The thorax of the bee was ground in liquid nitrogen and transferred to an microtube with 500 µl STE extraction buffer (0.001 M EDTA, 0.05 M Tris-HCl pH 7.5, 0.1 M NaCl), 75 µl 10% SDS, and 25 µl Proteinase K (10 mg/ml stock solution). The sample was then incubated at 55 C° with gentle shaking for 2 hrs. The suspension was extracted twice with equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1) and

Table 3.1 Sources and number of bumblebee specimens of subgenus *Pyrobombus* and the outgroup used in this analysis.

Name	Code	# specimen	Location	Remark
Apis mellifera	APS	1	Corvallis, OR	ID to reference from Crozier and Crozier (1993)
B. bifarius nearcticus type M	BNEM	1	Mt. Hood, OR	
B. bifarius nearcticus type H	BNEH	1	Eagle Cap, OR	
B. huntii	HUN	3	Klamath Falls, OR, Spokane, WA, Fernie, British Columbia	ID
B. ternarius	TRN	2	Kananaskis, Alberta and Golden, British Columbia	ID
B. vosnesenskii	VOS	3	Corvallis, Crater Lake and Newport, OR	ID
B. centralis	CEN	1	Hell's Canyon, OR	
B. sitkensis (with no band)	SITN	1	Corvallis, OR	
B. sitkensis (having bands)	SITB	3	Corvallis, Iron Mt., Mt. Hood, OR	ID
B. flavifrons flavifrons	FFL	2	Hell's Canyon, OR and Kananaskis, Alberta	ID
B. caliginosus	CLG	2	Fort Stephen and Cannon Beach, OR	ID
B. vagans vagans	VAG	3	Hope and Kamloops, British Columbia	ID
B. impatiens	IMP	1	Alabama	
B. melanopygus	MEL	3	Coos Bay, Corvallis and Mt. Hood, OR	ID
B. sylvicola	SYL	3	Kamloops, Golden, and Fernie, British Columbia	ID
B. frigidus	FIG	1	Banff, Alberta	
B. mixtus type E	MXE	3	Crater Lake, Iron Mt., OR	ID
B. mixtus type C	MXC	3	Corvallis, OR, Golden, British Columbia, Glacier National Park, Alberta	ID

ID = all specimens provide identical sequence of both COI and COII genes.

twice with an equal volumes of chloroform: isoamyl alcohol (24:1). Then the clear suspension was precipitated with 1/10 the sample volume of 3 M NaAc and 2.5 times the sample volume of ice cold 100% ethanol (Sambrook *et al.*, 1989; Hillis *et*

al., 1996). The final DNA was suspended in 50 µl of double distilled water and stored at –20 C°.

Polymerase Chain Reaction (PCR)

PCR amplifications of the partial COII region were performed in 50 µl volumes containing: 10 mM Tris/HCl, pH 8.3; 50 mM KCl; 7mM $MgCl_2$; 0.1 mg/ml gelatin; 200 µM of each of the four deoxyribonucleotide triphosphates; 0.5 µM of each of two different primers; 2.5 units of *Taq* polymerase; and 50 ng/µl of DNA template. This reaction was modified from Kocher *et al.* (1989) and Garnery *et al.* (1991). Forward primers [5'-GGCAGAATAAGTGCATTG-3' and 5'-GAATAAGTGCATTGAACTTA – 3'] are at tRNA leucine modified from Willis *et al.* (1992) and Garnery *et al.* (1991) whereas reversed primers [5'-AATTCTGG ATATTCATAAGATCA-3' and 5' – CATAAGATCAATATCATTGATG – 3'] are inside the COII gene at the position 303 and 315 bp downstream from the start codon. Thermocycle conditions were as follows: hot starting at 94 °C for 3 min, followed by 30 cycles of denaturation at 92 °C for 45 sec, annealing at 41-44 °C for 30 sec, and extension at 65 °C for 3min. The reaction was alllowed to extend for an additional 3 min at 65°C and then held at 4 °C.

For the partial COI region, PCR reactions and thermocycle conditions were performed following the suggestions of Lunt *et al.* (1996) as well as forward and reverse primers (U7 and U10). However, in some species, multiple PCR products

were produced, caused by some non-specific nucleotide base on those primers. Therefore, primers were modified based on the sequence of *A. mellifera* (Crozier *et al.*, 1989; Crozier *et al.*, 1993), which improved the success of amplification of the partial COI region (only one product was amplified). The modified primers are [5' – TACAGTCGGATTAGATGTTGATAC – 3'] and [5' – TTAAATTCAATGCAC TAATCTGCC – 3'].

All PCR products were examined by gel electrophoresis, stained with ethidium bromide. The band results on the gel showed that both COI and COII PCR products of the *Pyrobombus* species vary in sizes.

Sequencing

The PCR products were cleaned with the QIAquick PCR purification kit (QIAGEN, Vallencia, CA) according to the kit instructions. Both strands were sequenced by the cyclic termination method using fluorescence labeled dideoxyribonucleotide triphosphates. The sequence reaction and the processing of the reaction products for electrophoresis were performed following the instructions for the sequencing kit (ABI PRISM^{tm} Dye Terminator Cycle Sequencing Core Kit, Perkin-Elmer Corporation, Rockville, MD). Electrophoresis and data collections were done on an ABI model 377 DNA Sequencer (Perkin-Elmer Corporation). The raw sequencing data from both directions were compiled by the Staden program (version 0.99.2) (Staden *et al.*, 1997)

Pairwise Sequence Comparison

The consensus sequences resulting from the Staden program were compiled and aligned with the pileup command in the Genetics Computer Group program (GCG), Wisconsin package version 9.0 (1996), and re-examined manually. In the COII region, the alignment of the intergenic part was done with the aid of two RNA secondary structure analysis programs, foldRNA and squiggle drawing program, available in GCG (v. 9). The coding regions of COI and COII were aligned using translated amino acid sequences based on the invertebrate mitochondrial code (Clary and Wolstenholme, 1985). The COI sequences were aligned well with aid from the amino acid sequences; however, the COI/tRNA^{leu} intergenic region was unalignable because the length and nucleotide bases of this region were so variable. Pairwise sequence comparisons were performed using distance matrices with total character change and mean of distance. The three classes of the sites of the codon and amino acid sequences were analyzed by a similarity plot in GCG (v.9) in order to determine the variability of the base codon position among COI, COII genes and amino acid residues occurring within this subgenus.

Phylogenetic Analysis

The phylogenetic analyses were performed separately using partial COI gene (COI), partial COII gene (COII), and partial COII gene with intergenic region

(COIIint) and then combined analysis of COI&COII and COI&COIIint. Distance trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) with the Kimura two parameter correction (Kimura, 1980) using the NEIGHBOR program (version 3.4). The bootstrap analysis, with 100 replicates, was done by the BOOTSTRAP function in PHYLIP computer package (Felsenstein, 1995) available in GCG (v. 9). Parsimony trees were constructed and bootstrapped with 500 replicates using PAUP (version 3.1) (Swofford, 1993) with heuristic searches with equal weighted characters, 10 random sequence additions, tree-bisection-reconnection branch swapping, mulpars in effect and maxtrees set at 1,000. Gaps were coded as a fifth character state. Then the data were analyzed by weighting with the successive approximation method based on the maximum values of their rescaled consistency indices (RC) (Farris, 1969; Farris, 1989). The incongruent length different analyses (ILD) between the two sets of combined data, COI&COII, and COI&COIIint, were carried out in PAUP* (Swofford, 1999) in order to compare the trees reconstructed from different gene regions (Farris, 1994).

RESULTS

The Sequences from PCR

The COI sequences from all taxa of bumblebees had intergenic regions between the end of COI and tRNA leucine (Figure 3.1). The length varied from 9 bp in *B. v. vagans* F. Smith up to 95 bp in *B. mixtus,* while the outgroup, *A.*

mellifera had none. This high variation, basically caused by the SSR, $(TA)_n$ and $(TAA)_n$, made this region unalignable; it could not be used in the phylogenetic analysis. The partial COI region data set of *Pyrobombus* species, composed of 664 bp, was aligned with the sequence of *A. mellifera*, 670 bp long. When all sequences were aligned, COI sequences from all bumblebee species had 2 amino acids less than *A. mellifera,* at base positions 644 and 665, close to the 5'end stop codon terminus. Therefore, six gaps were added to make even lengths of all sequences for alignment (Figure 3.2). The data set provided 183 variable character sites (27.3%) with 78 parsimony informative sites (11.6%) (Table 3.2).

The PCR products of COII revealed that all *Pyrobombus* species apparently have an intergenic region between $tRNA^{leu}$ and the COII gene (Figure 3.1). This intergenic region is found in many other insects such as honey bees (Crozier *et al.*, 1989; Cornuet *et al.*, 1991), and flies (Smith and Bush, 1997). This region is very long in *A. mellifera* (193 bp) and forms multiple stem-loops, whereas in *Pyrobombus* species, it varied from 59 bp to 84 bp; however, only one stem-loop was found. The size of this region in *A. mellifera* differs from *Pyrobombus* species because of the insertion of an 128 bp sequence next to $tRNA^{leu}$. When this inserted region is removed, all taxa could be aligned allowing the analysis of COII with the intergenic region.

```
Apis  tcgagcatat tttacttcag caacaataat cattgctgta ccaacaggaa ttaaagtttt tagatgatta
 MXE  ......t... .....a.... .......... t........t ..t....... .......a.. ..........
 MXC  ......t... .....a.... .......... t......... ..t....... .......a.. ..........
 VSU  ......t... ..c..a.... ....t..... t........t ..t....... .......a.. ..........
 TRN  ......t... .....a.... .......... t........t ..t....... .......a.. ..........
 HUN  ......t... .....a.... .......... t........t ..t....... .......a.. ..........
BNEM  ......t... .....a.... .t........ t........t ..t....... .......a.. ..........
BNEH  ......t... .....a.... .t........ t........t ..t....... .......a.. ..........
 SYL  ......t... .....a.... .t........ t.....a... ..t..t.... .......a.. ......g...
 MEL  ......t... .......... ....t..... t.....a... ..t..t.... .......a.. ..........
 CEN  ......t... .......... ....t..... t.....a... ..t..t.... .......a.. ..........
 IMP  ......t... .......... .t........ t.....a..t ..t...tt.. .......a.. ..........
 VGN  ......t... .....a.... .t........ t.c......t ..t....... .......a.. ...t......
 CLG  ......t... .....a.... .t........ t......... ..t....... .......a.. ..........
 FIG  ......t... .....a.... ....t..... t......... ..t....... .......a.. ..........
 FFL  a.....t... .....a.... .t........ t......... ..t....... .......a.. ..........
SITN  a.....t... .....a.... .t........ t......... ..t....... .......a.. ..........
SITB  a..t..t... .....a.... .t........ t......... ..t....... .......a.. ..........

Apis  gcaacttatc atggttcaaa attaaaatta aatatttcaa ttttatgatc actaggtttt attatactat
 MXE  .......... .......... .a....t..t .....a.... ..a.t..... .a.t...... ...t..a...
 MXC  ..t..a.... .......... .ac...t..t .....ca... ..a.t..... .a.t..a... ...t..a...
 VSU  .......... .......... .a....t..t ......a... c.a.t..... ta.t...... ...t..a...
 TRN  .......... .c........ .a....t..t ......a... .ca.t..... .a.t...... ...t..a...
 HUN  .......... .c........ .a....t..t ......a... .ca.t..... .a.t...... ...t..a...
BNEM  .......... .......t.. ......t..t ......a... ..a.t..... .a.t...... ...t..a...
BNEH  .......... .......t.. ......t..t ......a... ..a.t..... .a.t...... ...t..a...
 SYL  .......... .......t.. .a....t..t ......a... ..a.t..... .a.t...... ...t..a...
 MEL  .......... .......... .a....t..t ......a... ..a.t..... .a.t...... ...t..a...
 CEN  .......... .......... .a....t..t ......a... ..a.t..... .a.t...... ...t..a...
 IMP  .....a.... .......... .a....t... ......a... ..a.t..... .a.t...... ...t..a...
 VGN  .......... .......t.. .a....t..t ......a... ..a.t..... .a.t...... ...t..a...
 CLG  .......... .......t.. .a....t... ......a... ..a.t..... .a.t...... ...t..a...
 FIG  .......... .......t.. .a....t..t ......a... ..a.t..... .a.t...... ...t..a...
 FFL  .......... .......t.. .a....t..t ......a... ..a.t..... .a.t...... ...t..a...
SITN  .......... .......t.. .a....t..t ......a... ..a.t..... .a.t...... ...t..a...
SITB  .......... .......t.. .a....t..t ......a... ..a.t..... .a.t...... ...t..a...

Apis  ttactattgg tggattaaca ggaattatat tatcaaattc ttctattgat attattcttc atgatacata
 MXE  ....a..... a......... ...g.a.... .......... a..a...... ......t.a. .......t..
 MXC  .......... a......... ...g.a.... ....t..... a..a...... ......t.a. ..........
 VSU  .c..a..... .........g ...g.a.... ....t..... a..a...... ......t.a. .......c..
 TRN  ....a..... a......... ..tg.a.... ....t..... a..a...... ......t.a. .......t..
 HUN  ....a..... a......... ..tg.a.... ....t..... a..a...... ......t.a. .......t..
BNEM  ....a..... a......... ...g.a.... .......... a..a...... ......t.a. .......t..
BNEH  ....a..... a......... ...g.a.... .......... a..a...... ......t.a. .......t..
 SYL  ....a..... a......... ...g.a.... ....t..... a..a...... ......t.a. ....c..t..
 MEL  ....a..... a......... ...g.a...c ....t..... a..a...... ......t.a. .......t..
 CEN  ....a..... a......... ...g.a...c ....t..... a..a...... ......t.a. .......t..
 IMP  ....a..... a......... ...g.a.... .......... a..a...... ......t.a. .......t..
 VGN  ....a..... a......... ...g.a.... .......... a..a...... ......t.a. .......t..
 CLG  ....a..... a......... ..gg.a.... .......... a..a...... ......t.a. .......t..
 FIG  ....a..... a......... ...g.a.... .......... a..a...... ......t.a. .......t..
 FFL  ....a..... a......... ...g.a.... .......... a..a...... ......t.a. .......t..
SITN  ....a..... a......... ...g.a.... .......... a..a...... ......t.a. .......t..
SITB  ....a..... a......... ...g.a.... .......... a..a...... ......t.a. .......t..
```

Figure 3.2 Partial COI gene sequences aligned by Pileup program in GCG 9. Dots represent bases identical to the *A. mellifera* sequence; hyphens represent gaps. All bumblebees in subgenus *Pyrobombus* are 6 nucleotide bases less than *A. mellifera.*

```
Apis  ttacgttgtt ggacattttc attatgttct ttcaataggt gcagtatttg caattatttc aagatttatt
 MXE  ...t..a..a .......... .......... a........a ..g..t.... .c......a. t.........
 MXC  ...t..a..a .......... .......at. a........a .....t.... .t......a. t.....a...
 VSU  ...t..a.... ........c. .......at. a........a .....t.... .t......a. t.........
 TRN  ...t..a.... .......... ....c..a.. a........a .....t.... .t......a. ..........
 HUN  ...t..a.... .......... ....c..a.. a........a .....t.... .t......a. ..........
BNEM  ...t..a.... .......... ........t. a........a .....t.... .t......a. t.........
BNEH  ...t..a.... .......... ........t. a........a .....t.... .t......a. t.........
 SYL  c.....a.... .......... .......at. a..t.....a .......... .t......a. t.........
 MEL  ...t..a..a ........c. .......at. a..t.....a .......... ........a. t.........
 CEN  ...t..a..a ........c. .......at. a..t.....a .......... ........a. t.........
 IMP  ...t..a..a ..t........ .......at. a..t.....a .....t.... .t......a. t........c
 VGN  ...t..a.... ..t....... ........t. a........a .....t.... .t......a. t.........
 CLG  ...t..a.... .......... ........t. a..t.....a .....t.... .t......a. t.........
 FIG  ...t..a.... .......... ........t. a..t.....a .....t.... .t......a. t.........
 FFL  ...t..a.... .......... ........t. a..t.....a .....t.... .t......a. t.........
SITN  ...t..a.... .......... ........t. a..t.....a .....t.... .t......a. t.........
SITB  ...t..a.... .......... ........t. a..t.....a .....t.... .t......a. t.........

Apis  cattgatatc cattaattac tggattatta ttaaatatta aatgattaaa aattcaattt attataatat
 MXE  ........c. ..a....... a..t...a.. ......caa. .......... .......... ..........
 MXC  .......... .......... a..t...a.. ......caa. .......... .......... ..........
 VSU  ..c....... ..a....... a..t...a.. c.....caa. .......... .......... ..........
 TRN  .......... ..a....c.. a..t...a.. ......caa. .......... .......... ..........
 HUN  .......... ..a....c.. a..t...a.. ......caa. .......... .......... ..........
BNEM  .......... ..a....... a..t...a.. .....ccaa. .......... .......... ..........
BNEH  .......... ..a....... a..t...a.. .....ccaa. .......... .......... ..........
 SYL  .......... .......... a..t...a.. ......caa. .......... .......... ..c.......
 MEL  .......... .t........ a..t...a.. ......caa. .......... .......... ..........
 CEN  .......... .t........ a..t...a.. ......caa. .......... .......... ..........
 IMP  .......... ..a....... a..tc..a.. ......caa. .......... .......... ..........
 VGN  .......... .......... a..t...a.. ......caa. .......... .......... ..........
 CLG  .......... ....g..... a..t...a.. ......caa. .......... .......... ..........
 FIG  .......... .......... a..t...a.. ......caa. .......... .......... ..........
 FFL  .......... .......... a..t...a.. ......caa. .......... .......... ..........
SITN  ........c. .....g.... a..t...a.. ......caa. .......... .......... ..........
SITB  ........c. .....g.... a..t...a.. ......caa. .......... .......... ..........

Apis  ttattggagt aaatctaact ttctttcctc aacatttttt aggactaata tctataccac gacgttattc
 MXE  .......t.. ....a..... ..t..c.... .......... ...ttc.... ........t. ....a.....
 MXC  .......t.. ....a....a ..t..... .......... ....t..... ..a.....t. ....a.....
 VSU  .......t.. ....a..... ..t..c..a. .......... ...tt..... ........t. ....a..c..
 TRN  .......t.. ...ca....c ..t..c.... .......... ...tt..... ........t. ....a.....
 HUN  .......t.. ...ca....c ..t..c.... .......... ...tt..... ........t. ....a.....
BNEM  .......t.. ....t....a ..t..... .......... ....t..... ........t. ....a.....
BNEH  .......t.. ....t....a ..t..... .......... ....t..... ........t. ....a.....
 SYL  .......t.. ....a....a ..t..... ....c..... ....t..... ..a.....t. ....a..c..
 MEL  .......t.. t...a....a ..t..c.... .......... ....t..... ........t. ....a.....
 CEN  .......t.. t...a....a ..t..c.... .......... ....t..... ........t. ....a.....
 IMP  .......t.. ....a..... ..t..c.... ....c..... ....t..... ........t. .t..a.....
 VGN  .......t.. ....t....a ..t..... .......... ....t..... ........t. ....a.....
 CLG  .......t.. ....t....a ..t..... .......... ....t..... ........t. ....a.....
 FIG  .......t.. ....t....a ..t..... .......... ....t..... ........t. ....a.....
 FFL  .......t.. ....t....a ..t..... .......... ....t..... ........t. ....a.....
SITN  .......t.. ....t....a ..t..... .......... ....t..... ........t. ....a.....
SITB  .......t.. ....t....a ..t..... .......... ....t..... ........t. ....a.....
```

Figure 3.2 (continued)

```
Apis  agactatcca gattcttatt actgttgaaa ttcaatttca tctataggat caataatttc attaaataga
 MXE  ...t.....t .......... .t........ ..t....... ..a..t...g .......... .a........
 MXC  t..t.....t .......... .t........ ..t.g..... ..a..t...g .t........ .a........
 VSU  ...t.....t .......... .t........ ..t......t ..a..t...g .......... .a........
 TRN  ...t.....t .......... .t........ ..t......t ..a..t...g .......... .a........
 HUN  ...t.....t .......... .t........ ..t......t ..a..t...g .......... .a........
BNEM  ...t...... .......... .t........ ..t......t ..a..t..tg .t........ .a........
BNEH  ...t...... .......... .t........ ..t......t ..a..t..tg .t........ .a........
 SYL  ...t.....t .......... .t........ ..t....... ..g..t...g .t........ .a........
 MEL  ...t.....t .......... .t........ ..t....... ..a..c...g .t........ .a....c...
 CEN  ...t.....t .......... .t........ ..t....... ..a..c...g .t........ .a....c...
 IMP  ...t.....t .......... .t........ ..t....... ..a..c...g .......... .a........
 VGN  ...t...... .......... .t........ ..t......t ..a..t...g .t........ .a........
 CLG  ...t...... .......... .t........ ..t......t ..a..t...g .t........ .a........
 FIG  ...t...... .......... .t........ ..t......t ..a..t...g .t........ .a........
 FFL  ...t...... .......... .t........ ..t......t ..a..t...g .t........ .a........
SITN  ...t...... .......... .t........ ..t......t ..a..t...g .t........ .a........
SITB  ...t...... .......... .t........ ..t......t ..a..t...g .t........ .a........

Apis  ataatttttt taatttttat tattttagaa agattaattt ctaaacgaat attattattt aaattcaacc
 MXE  ...t.a.... .c........ ....g.t... .......... ........t. .g........ .....tc.t.
 MXC  ...t.a.... .......... ......t... ...a...... .........a tg..a.t... .....tc...
 VSU  ...t.a.... .t........ ......t... .......... ........t. .g.ta..... .....tc...
 TRN  ...t.a.... .t........ ......t... .......... ........t. .g..a..... .....tc.t.
 HUN  ...t.a.... .t........ ......t... .......... .a......t. .g..a..... .....tc.t.
BNEM  ...t.a.... .t........ ......t... .......... .......... tg.t...... .....tc.t.
BNEH  ...t.a.... .t........ ......t... .......... .......... tg.t...... .....tc.t.
 SYL  ...t.a.... .c........ ......t... .......... ........t. .g..a..... .....tc.t.
 MEL  ...t.a.... .c........ ......t... .......... .a........ tg..a..... .....tc.t.
 CEN  ...t.a.... .c........ ......t... .......... .......... tg..a..... .....tc.t.
 IMP  ...t.a..c. .t........ ......t... .......... .......... .g.ta..... .....tc.t.
 VGN  ...t.a.... .t........ ......t... .......... .......... tg.t...... .....tc.t.
 CLG  ...t.a.... .t........ ......t... .......... .......... ta.t...... .....tc.t.
 FIG  ...t.a.... .t........ ......t... .......... .......... tg.t...... .....tc.t.
 FFL  ...t.a.... .t........ ......t... .......... .......... tg.t...... .....tc.t.
SITN  ...t.a.... .t........ ......t... .......... .......... tg.t...... .....tc.t.
SITB  ...t.a.... .t........ ......t... .......... .......... tg.t...... .....tc.t.

Apis  aatcatcact tgaatgatta aatttttac cacctctaga tcattcacat ttagaaattc cattattaat
 MXE  ....t..t.. .......... ...aa..at. .t..at.... a......tta a.t....... .....a.t..
 MXC  ....t..t.. .......... ...aa..at. .t..at.... ......ttta a.t....... .t...a.t..
 VSU  ....t..t.. .......... ...aa..at. .t..at.... .......tta a.t....... .c...a.tt.
 TRN  ....t..tt. a......... ...aa..at. .t..at.... .......tta a.g....... .....a.t..
 HUN  ....t..tt. a......... ...aa..at. .t..at.... .......tta a.t....... .....a.t..
BNEM  ....t..t.. .......... ...aa..at. .t..aa.t.. ......ttta a.t....... .....a.t..
BNEH  ....t..t.. .......... ...aa..at. .t..aa.t.. ......ttta a.t....... .....a.t..
 SYL  ....t..t.. .......... ...aa..at. .....t.t.. ...c...tta a.t.....a. .....a.tt.
 MEL  .......t.. .......... ...aa..at. .....t..a. .......tta a.t....... .....a.t..
 CEN  .......t.. .......... ...aa..at. .....t..a. .......tta a.t....... .....a.t..
 IMP  ....t..t.. .......... ...aac.at. .t...t.... ...c...tta a.t....... .t...a.t..
 VGN  ....t..t.. .......... ...aa..at. .t..aa.... .......tta a.t....... .....a.t..
 CLG  ....t..t.. .......... ...aa..at. .t..at.t.. .......tta a.t....... .....a.t..
 FIG  ....t..t.. ....g..... ...aa..at. .t..aa.t.. .......tta a.t....... .t...a.t..
 FFL  ....t..t.. .......... ...aa..at. .t..aa.t.. .......tta a.t....... .t...a.t..
SITN  ....t..t.. .......... ...aa..at. .t..aa.t.. .......tta a.t....... ....ta.t..
SITB  ....t..t.. .......... ...aa..at. .t..aa.t.. .......tta a.t....... .....a.t..
```

Figure 3.2 (continued)

```
Apis   taaaaattta aatttaaaat caattttaat taaattttaa
 MXE   a..t...aa. .tc---...a at.....t.. ...t---...
 MXC   a..t...aa. .tc---...a acc....t.. ...t---...
 VSU   a......aa. .tc---...a at.....t.a ...t---...
 TRN   a......aa. .tc---...a at.....t.a ...t---...
 HUN   a......aa. .tc---...a at.....t.a ...t---...
BNEM   ...t...aa. .t.---...a at.....t.a ....---...
BNEH   ...t...aa. .t.---...a at.....t.a ....---...
 SYL   a..t...aa. .t.---...a at.....c.a ....---...
 MEL   a..t...aa. .t.---...a ac.....t.a ....---...
 CEN   a..t...aa. .t.---...a ac.....t.a ....---...
 IMP   a.cc...aa. .t.---...a ac.....t.a ....---...
 VGN   a..t...aa. .t.---...a at.....t.a ....---...
 CLG   ...t...aa. .t.---...a at.....t.a ....---...
 FIG   a..t...aag ...---...a at.....t.a ....---...
 FFL   at.t...aag .t.---...a at.....t.a ...t---...
SITN   a..t...aag .t.---...a at.....t.a ....---...
SITB   a..t...aag .t.---...a at.....t.a ....---...
```

Figure 3.2 (continued)

Table 3.2 Summary of tree statistics of the partial COI, and COII gene sequences and their combined data in maximum parsimony analysis

	Total char.	**Var. site**	**Infor. site**	**Steps**	**CI**	**RI**	**RC**	**HI**
COI	670	183 27%	78 11.6%	273	0.725	0.681	0.494	0.275
COII	320	100 31%	41 12.8%	157	0.720	0.674	0.485	0.280
COII ext.	433	144 33%	60 13.9%	231	0.723	0.685	0.495	0.277
Combined COI&COII	990	283 28.5%	119 12%	453	0.688	0.619	0.426	0.312
Combined COI&COII(int)	1103	327 29.6 %	138 12.5%8	533	0.685	0.616	0.422	0.315

The COII data set was 320 bp for all taxa and could be perfectly aligned (Figure 3.3). The COII data set was composed of 100 variable character sites (31.3%) with 41 parsimony informative sites (12.8%). When the intergenic region

was included and aligned, the new data set was 433 bp with 144 variable sites (33.2%) and 60 parsimony informative sites (13.9%).

```
Apis  gaattttaaa ttcaatctta aagat--tta atctttttat taaaattaat aataaaataa aacaaaatat
 MXE  ......a... ......aa.. .....at..t .......-.. ...t.--..a t..tt..... ..tt...a..
 MXC  ......a... ......aa.. .....at..t .......-.. ...t.--..a t..tt..... ..t....a..
 VSU  ......a... ......ta.. ..a...t... ..t....-.. ...t.--... t..tt..... ..tt.c--..
 TRN  ......a... ......ta.. ......t..t .......-.. ...t.--... t..tt..... ..t..t--..
 HUN  ......a... ......ta.. ......t..t .......-.. ...t.--... t..tt..... ..t..c--..
BNEM  ......a... ......aa.. ......t..t .......-.. ...t.--..c t..tt....t ..tt.t.-..
BNEH  ......a... ......aa.. ......t..t .......-.. ...t.--..c t..tt....t ..tt.t.-..
 SYL  ......a... ......aa.. ......t..t .......-.. ...t.--tt. t..tt..... ...t.c.-..
 MEL  ......a... ......ta.. ......t..t t......-.. ...t.--... ...tt..... ..tt.c.-..
 IMP  ......a... .....caa.. ......t..t .......-.. ...t.--... t..tt..... ..at.t.-..
 VGN  ...c..a... .....ata.. .......-.t .......-.. ...t.--... t..tt..... ...t-t.-..
 CLG  ......a... .....ata.. .......-.t .......-.. ...t.--... t..tt..... ...t-t.-t.
 CEN  ......a... .....ata.. .......-.t .......-.. ...t.--... t..tt..... ...t-t.-t.
 FIG  ......a... .....ata.. .......-.t .......-.. ...t.--... -..tt..... ...t-t.-..
 FFL  ......a... .....ata.. .......-.t .......-.. ...t.--... -..tt..... ...t-t.-..
SITB  ......a... .....ata.. .......-.t .......-.. ...t.--..c -..tt..... ...t-t.-..
SITN  ......a... .....ata.. .......-.t .......-.. ...t.--..c -..tt..... ...t-t.-..

Apis  aatcagaata tatttattaa aatttaa--- ---tttatta aa-atttcca catgatttat atttatattt
 MXE  tt.-t....t ...aat.c.. .taaa.ctaa ata..... ..a.....t. .....aa... .....c....
 MXC  t---t....t ...aat.c.. .taaa..taa ata..... ..a.....t. .....aa... .....c....
 VSU  ..--t....t .c.aat.c.. .taaatttta a..-..... ..a.....t. .....aa... .....c....
 TRN  ..--t....t .caaat.c.t .taaattttt a..-..... ..a.....t. .....aa... .....c....
 HUN  ..--t....t .caaat.c.t .taaattttt a..-..... ..a.....t. .....aa... .....c....
BNEM  ..--t....t ..gaat.c.. .taaattttt a..-..... ..a.....t. .....aa... .a.g.c....
BNEH  ..--t....t ..gaat.c.. .taaattttt a..-..... ..a.....t. .....aa... .a.g.c....
 SYL  tt--t....t .t.aat.c.. .taaatt... ata..... ..a.....t. .....aac.. ...a.c....
 MEL  tt.tt....t .c.aat.c.. ttaaatt..a ata..... ..a.....a. .....aac.. ...a.c....
 IMP  tt--t....t .t.aat.c.. .taaatttta a..-..... ..a.....t. .....aa... ...a.c....
 VGN  gt.tt....t ..caat.c.. .taaa.ttta at.-..... ..a.....t. .....aa... ...a.c....
 CLG  gt.tt..g.t .t.aat.c.. .taaa.t... ...-..... ..a.....t. .....aa... .a.a.c....
 CEN  gt.tt....t ...aat.c.. .taaa.ttta at.-..... ..a.....t. .....aa... ...a.c....
 FIG  gt.tt....t ...aat.c.. .taaa.tt.a a..-..... ..a.....t. .....aa... ...a.c....
 FFL  gt.tt....t ...aat.c.. .taaa.tt.a a..-..... ..a.....t. .....aa... ...a.c....
SITN  gt.tt....t ...aat.c.. .taaa.tt.a a..-..... ..a.....t. .....aa... ...a.c....
SITB  gt.tt....t ...aat.c.. .taaa.tt.a a..-..... ..a.....t. .....aa... ...a.c....

Apis  caagaatcaa attcatatta tgctgataat ttaatttcat ttcataatat agttataata attattatta
 MXE  .....t.... ....t.t... .t.a..... c.t..... ....c...t. .ac..... ..a..a..a.
 MXC  .....t.... ....t.t... .t.a.....c c.t..... ........t. .ac..... ..a..a..a.
 VSU  .....t.... ....t.t... .t..... ..... .....t. .ac..... ..a..a..a.
 TRN  .....t.... ....t.tc.. .t..... .....c. .....ct. .ac..... ..a..a..a.
 HUN  .....t.... ....t.tc.. .t..... ..... .....ct. .ac..... ..a..a..a.
BNEM  .....t.... ....g.t... .t.a..... ..... .....t. .ac..... ..a..a....
BNEH  .....t.... .c..c.t... .t.a..... ..... .....t. .aca..... ..a..a..a.
 SYL  .....t.... ....t.t... .t.a..... ..... .....t. .ac..... ..a..a..a.
 MEL  .....t.... .....t.... .t.a..... ..... .....t. .aca..... ..a..a..a.
 IMP  .....t..t. ....t.t.c. .t.a..... ..... .....t. .aca..... ..a..a..a.
 VGN  .....t.... ....t.t... .t.a..... c.t..... .....ct. .ac..... ..a..a..a.
 CLG  .....t.... ....t.t... .t.g..... ..... .....t. .ac..... ..a..a..a.
 CEN  .....t.... ....t.t... .t.a..... ..... .....t. .ac..... ..a..a..a.
 FIG  .....t.... ....t.tc.. .t.a..... ..... .c....t. .ac..... ..a..a..a.
 FFL  .....t.... ....t.tc.. .t.a..... ..... .c....t. .ac..... ..a..a..a.
SITN  .....t.... ....t.tc.. .t.a..... ..... .c....t. .ac..... ..a..a..a.
SITB  .....t.... ....t.tc.. .t.a..... ..... .c....t. .ac..... ..a..a..a.
```

Figure 3.3 Partial COII sequences with the intergenic regions aligned by Pileup in GCG9. Dots represent bases identical to the *A. mellifera* sequence; hyphens represent gaps. The COII region starts at position 114.

```
Apis  taatttcaac attaactgta tatattattt tagatttatt tataaacaaa ttctcaaatt tatttttatt
 MXE  .....attt. c.....aac. .t.t.....a .......ta. a.....t..t ..t.t..... ..ac......
 MXC  .....attt. t.....aac. .t.t.....a .......ta. a.....t..t ..t.t..... ..ac......
 VSU  .....atttt t.....aac. .t.t.....a .......ta. at.t..t..t ....t..... ..ac.c.t..
 TRN  .....attt. t.....aac. .t.t.....a .......ca. .tat..t..t .a..t..... ..ac.c.t..
 HUN  .....attt. t.....aac. .t.t.....a .......ca. .tat..t..t .a..t..... ..ac.c.t..
BNEM  .t...attt. t.....aac. .t.t.....a .......ta. atat..t..t ....t..... ..ac.c....
BNEH  .....attt. t.....aact .t.t.....a .......ta. .tat..t..t ..t.t..... ..ac.c....
 SYL  .....attt. ......aac. .t.t..... .......ca. at....t..t ..t.t..... ..ac......
 MEL  .....attt. ......aac. .t.t..... .......tac at....t..t ..t.t..... ..ac......
 IMP  .t...attt. t.....aa.. .t.t.....a .......ta. .tgt..t..t .at.t..... ..ac.c..c.
 VGN  .....attt. t.....aa.. .t.t..... .......ta. a.....t..t .at.t..... ..ac......
 CLG  .....attt. t.....aa.. .t.t..... .......ta. a.....t..t .at.t..... ..ac......
 CEN  .....attt. t......a.. .t.t..... g......ca. a.....t..t .at.t..... ..ac......
 FIG  .....attt. t.....aa.. .t.t..... .......ca. a.....t..t .at.t..... ..ac......
 FFL  .....attt. t.....aa.. .t.t..... .......ca. a.....t..t .at.t..... ..ac......
SITN  .....attt. t.....aa.. .t.t..... .......ca. a.....t..t .at.t..... ..ac......
SITB  .....attt. t.....aa.. .t.t..... .......ca. a.....t..t .at.t..... ..ac......

Apis  aaaaaatcat aatattgaaa ttatttgaac aattattcca attattattc tattaattat ttgttttcca
 MXE  ......c... .c........ .c........ tt.a.c...t ..a......t ..a....... ..........
 MXC  ......c... .c........ .c.....g.. tc.a.c...t ..a......t ..a....... ...g......
 VSU  .......... .c........ .......... tc...c...t ..a......t ..a....... ..........
 TRN  .......... .ca....... ......c... tt.a.....t ..a......t ..a....... c.........
 HUN  .......... .ca....... .......... tt.a.....t ..a......t ..a....... c.........
BNEM  .......... .ca....... .......... tt.a.c...t ..a......t ..a....... ..........
BNEH  .........c .c........ ....c..... tc.a.c...t ..a......t ..a....c.. .c....c...
 SYL  .......... .ca....... .......... tt.a.c.... ..a....... ..a....... c..c......
 MEL  .......... .ca....... .......... tt.a.c.... ..a......t ..a....... ......c...
 IMP  .......... .ca....... .......g.. ct.a.c...t ..a......t ..a....... ..........
 VGN  .......... .c........ .......... tt.a...... ..a......t ..a....... ......c...
 CLG  .......... .c........ .......... .t.a...... ..a......t ..a....... ......c...
 CEN  .......... .c........ .......... tt.a.c.... ..a......t ..a....... ..........
 FIG  .......... .cc....... .......... tt.a.c.... ..a......t ..a....... ..........
 FFL  .......... .cc....... .......... tt.a.c.... ..a......t ..a....... ..........
SITN  .......... .ca....... .......... tt.a.c.... ..a......t ..a....... ..........
SITB  .......... .ca....... .......... tt.a.c.... ..a......t ..a....... ..........

Apis  tcattaaaaa ttttatattt aattgatgaa attgtaaatc ctttttttc aattaaatca attggtcatc
 MXE  ..t....... ..c.....a c......... ...a..... ...a..... t.....g.t ..........
 MXC  ..t....... .........a t......... ...a..... ...a..... t.....g.t ..........
 VSU  ..t....... .........a t......... ...a..... ...a..... t.....g.t ..........
 TRN  ..t....... .........a t..c...... ...a..... ...a..... t.....g.t .....c....
 HUN  ..t....... .........a t......... ...a..... ...a..... t.....g.t .....c....
BNEM  ..t....... .........a t......... ...a..... ...a..... t.a....g.t .....c....
BNEH  ..t....... .........a t......... ...a.c.... ...a..... t.....g.t .....c....
 SYL  ..t....... .........a t......... ...a..... ...a..... t.....g.t ..........
 MEL  .......... .........a t......... ...a..... ...a..... t.....g.t ..........
 IMP  ..t....... .........a t......... ...a..... ...a..... t.....g.t ..........
 VGN  ..t....... .........a t......... ...a..... ...a..... .....g.t ..........
 CLG  ..t....... .........a t......... ...a..... ...a..... .....g.t ..........
 CEN  ..t....... .........a t......... ...a..... .a.a..... .....g.t ..........
 FIG  ..t....... .........a t......... ...a..... ..aa..... t.....g.t ..........
 FFL  ..t....... .........a t......... ...a..... ...a..... .....g.t ..........
SITN  ..t....... .........a t......... ...a..... ...a..... .....g.t ..........
SITB  ..t....... .........a t......... ...a..... ...a..... .....g.t ..........
```

Figure 3.3 (continued)

```
Apis  aatgatattg atcatatgaa tatccagaat ttaataatat tgaatttgat tcatatatac taaattataa
 MXE  .......... ...t...... .......... ........ta .......... .........t ........g.
 MXC  .......... .......... .......... ........ta .......... .........a ........g.
 VSU  .......... ...t...... ..c....... .......cta .......... ..t......t ........g.
 TRN  .......... ...t...... ..c....... ........ta .......... ..t......t ........g.
 HUN  .......... ...t...... ..c....... ........ta .......... ..t......t ........g.
BNEM  .....at... ...t...... ..c....... ........ta .......... .........t ........g.
BNEH  .......... ...t...... ..c....... ........ta .......... .........t ........g.
 SYL  .......... ...t...... .......... ....c...ta .......... ..t......t ........g.
 MEL  .......... ...t...... .......... ........ta .......... ..t......t ......t.g.
 IMP  .......... ...t...... .......... ........ta .......... ..t......a ........g.
 VGN  .......... .......... .......... .c......ta .......... ..t......t ........g.
 CLG  .......... ...t...... .......... ........ta .......... ..t.....gt ........g.
 CEN  .......... ...t...... .......... ........ta .......... ..t......t ........g.
 FIG  .......... ...t...... .......... ........ta .......... .........t ........g.
 FFL  .......... ...t...... .......... ..t.....ta .......... ..t......t ........g.
SITN  .......... ...t...... .......... ........ta .......... ..t......t ........g.
SITB  .......... ...t...... .......... ........ta .......... ..t......t ........g.

Apis  taatttaaac caatttcgtt tactagaaac tgataatcga atagtaattc caataaaaat cccactacgt
 MXE  atcaa.t..t ........ac .tt....... ......c... t..a.t.... ..t.t..... tt..a....a
 MXC  atcaa.t..t ........ac .tt....... .......... t..a.t.... ..t.t..... tt..a....a
 VSU  a.caa....t ........a. ..t....... .......... t..a.t..c. ..t.t..... tt.ta....a
 TRN  a.caa.t..t ........a. ..t....... .......... t..a.t.... ..t.t..... tt.ta....a
 HUN  a.caa.t..t ........a. ..t....... .......... t..a.t.... ..t.t..... tt.ta....a
BNEM  gtcaa....t ........a. ..t....... .......... t.ga.t.... ..t.t..... .t..a....a
BNEH  gtcaa....t ........a. ..t....... .......... t.ga.t.... ..t.t..... .t..a....a
 SYL  atcaa.t..t ........a. .......... .........t c.ta.t.... ..t.t..... ta.ta....a
 MEL  att.a.t..t ........a. ..t....... .........t ...a.t.... ..t.t..... tt.ta....a
 IMP  atcaa.t..t ........ac .tt....... .......... ...a.t..c. ..t.t..... ta.ta....a
 VGN  atcaa.t... ........ac .tt....... .......... ..ta.t.... ..t.t..... ta.ta....a
 CLG  atcaa.t..t ........ac .tt....... .......... ..ta.t.... ..t.t...g. ta.ta....a
 CEN  attaa.t..t ........ac .tt....... .......... ..ta.t.... .tt.t..... ta.ta....a
 FIG  atc.a.t..t ........ac .tt....... .......... t..a.t..c. ..t.t..... tt..a....a
 FFL  atcaa.t..t ........ac .tt....... .......... ..ta.t.... ..t.t..... ta.ta....a
SITN  atcaa.t..t ........ac .tt....... .......... ..ta.t.... ..t.t..... ta.ta....a
SITB  atcaa.t..t ........ac .tt....... .......... ..ta.t.... ..t.t..... ta.ta....a

Apis  ttaattacaa catcaacaga tgtaattcat tcatgaacag ttccatcctt aggtattaaa gttgatgcag
 MXE  a.....gt.t .t........ .......... ........ta .......t.. ...a...... ........ta
 MXC  a......ttt ......g... .......... .........a .......a.. .......... ..a..c..ta
 VSU  a....cgttt .....tt... .......... ........ta .......a.. ...a...... ..a.......
 TRN  a......ttt .....tt... .......... ........ta .......a.. ...a...... ..a.....t.
 HUN  a......ttt .....tt... ...t...... ........ta .......a.. ...a...... ..a.....t.
BNEM  a......ttt .t...tt... .......... ..c......a .......a.. ...a...... ..a.....c.
BNEH  a......ttt .t...tt... .......... ..c......a .......a.. ...a...... ..a.....c.
 SYL  a......ttt ......t... .......... ........ca .......a.. ...a...... ..a.....ta
 MEL  a......ttt ......t... .......... ........ta .......a.. ...a...... ..a......a
 IMP  a......ttt .....tt... .......... ........ta .......a.. ...a...... ..a......a
 VGN  a......ttt ....t.t... .........c ..t.....ta .......a.. .......... ..a......a
 CLG  a......ttt ....t.t... .......... ..t.....ta ....t..t.. ...a...... ..a......a
 CEN  a....c.ttt ....t.t... .......... ........ta .......a.. ...a...... ..a......a
 FIG  a.....gt.t tt........ .......... ........ta .......t.. ...a...... ........ta
 FFL  a......tt. ....t.t... ......c... ..t.....ta .......... ...a...... ..a......a
SITN  a......ttt ....t.t... ......c... ..t.....ta .......... ...a...... ..a......a
SITB  a......tt. ....t.t... ......c... ..t.....ta .......... ...a...... ..a......a
```

Figure 3.3 (continued)

```
Apis  ttccaggacg aattaatcaa ttaaatttaa ttagaaaacg tccaggaatt ttttttggtc aatgttcaga
 MXE  ....t..... .......... .........t .ct.t.tt.. ...t...... .a........ .......t..
 MXC  ....t..... .......... .........t .ct.c.tt.. a..t...... .a......a. .......t..
 VSU  ....t..t.. .g.a...... .........t ..t.t.tt.. .......... .a........ .......t..
 TRN  ....t..t.. .......... .........t ...ct.tt.. .......... .a......a. .......t..
 HUN  ....t..t.. .......... .........t ...ct.tt.. .......... .a......a. .......t..
BNEM  ....c..... ...c..c... c.........t .ct.t.tc.. a......... .a......g. .......t..
BNEH  ....c..... ...c..c... c.........t .ct.t.tc.. a......... .a......g. .......t..
 SYL  ....t..t.. .......... .........t ..t.t.tt.. a..t...... .a......a. ....c..c..
 MEL  ....t..... ......c... c.........t ..t.t.tt.. a..t...... .a........ .......t..
 IMP  ....t..c.. .......... .........t .ct.t.tt.. a..t...... .a........ ....c..t..
 VGN  ....t..t.. .......... .........t ..t.t.tt.. a..t...... .a......a. .......t..
 CLG  ....t..t.. .......... .........t ..t.t.tt.. a......... .a......a. .......t..
 CEN  ....c..t.. .......... .........t ..t.t.tt.. a......... .a......a. .......t..
 FIG  ....t..... .......... .........t .ct.t.tt.. a..t...... .a........ .......t..
 FFL  ....t..t.. ...c...... .........t ..t.t.tt.. a......... .a......a. .......t..
SITN  ....t..t.. .......... .........t ..t.t.tc.. a......... .a......a. .......t..
SITB  ....t..t.. ...c...... .........t ..t.t.tt.. a......... .a......a. .......t..

Apis  aatttgtggt ataaatcata gatttatacc aattataatt gaatcaactt catttcaata ttttttaaat
 MXE  .........g .......... ....c..... t.......t.a ...ag..... .t.a.g...t a..c.c....
 MXC  .........g .......... ....c..... t.......t.a ...ag...a. .t.a.a.t.t a.........
 VSU  .......... ........c. .......... t.......t.a ...ag..... .t.a.g...c a...a.....
 TRN  ......c..a .......... ....c..... t.......t.a ...ag..... .t.a.g...t a...a.....
 HUN  ......c..a .......... ....c..... t.......t.a ...ag..... .t.a.g...t a...a.....
BNEM  .........g ........c. .......... t.......t.a ...ag..... .t.a.g...t a.........
BNEH  .........g ........c. .......... t.......t.a ...ag..... .t.a.g...t a.........
 SYL  ...c.....a ........c. .......... ........t.a ...agt.... .t.a.g...c a.........
 MEL  ...c.....a .......c.. .......t.. ........t.a ...ag..... .caaag...t a.........
 IMP  .........a .......... .......... t.......t.a ...ag..... .t.a.g...t a.........
 VGN  .........a .......... .......... t.......t.a ...agt.... .t.a.g...t a.........
 CLG  .......... .......... .......... t.......t.a ...agt.... .t.a.g...t ..........
 CEN  .......... .......... .......... t.......t.a ...agt.... .t.a.g...t a.........
 FIG  .........g .......... .t........ ........t.a ...ag...a. .tga.g...t a..c.c....
 FFL  .......... .......... .......... t.......t.a ...agt.... .t.a.g...t a.........
SITN  .......... .......... .......... t.......t.a ...agt.... .t.a.g...t a.........
SITB  ......c... .......... .......... t.......t.a ...agt.... .t.a.g...t ..........

Apis  tgagta---a ataaacaaat c------taa aaaattagtt aa
 MXE  ...a.taaa. .a...---.. a...ata.g. .......... ..
 MXC  ...a.taaa. .a.t.---.. a...tat.g. .......... ..
 VSU  ...a.taaa. .....---.. t.......g. .......... ..
 TRN  ...a.taaa. .....---.. t.......g. .......... ..
 HUN  ...a.taaa. .....---.. t.......g. .......... ..
BNEM  ..ga.taaa. .....---.. a...att.g. .......... ..
BNEH  ..ga.taaa. .....---.. a...att.g. .......... ..
 SYL  ...a.taaa. .a...---.. t.......g. .......... c.
 MEL  ...a.taaa. .a...---.. t.......g. .......... ..
 IMP  ...a.taaa. .a...---.. attaata.g. .......... ..
 VGN  ...a.taaa. .a...---.. t.......g. .......... ..
 CLG  ...a.taaa. .a...---.a t.......g. .......... ..
 CEN  ...a.taaa. .a...---.. t.......g. .......... ..
 FIG  ...a.taaa. .....---.. t...ata.g. ...t...... ..
 FFL  ...a.taaa. .a..t---.. t.......g. .......... ..
SITN  ...a.taaa. .a...---.. t.......g. .......... ..
SITB  ...a.taaa. .a...---.. t.......g. .......... ..
```

Figure 3.3 (continued)

Pairwise Comparison

A similarity plot of nucleotide base positions within the codons of COI showed that nucleotide bases at the second codon position are the most conserved (98% similarity) followed by the first codon position (95.8%) while third codon position, shared only 88% similarity (Figure 3.4a, b, and c). The relative similarities of nucleotide base positions of COII were similar to that of COI: 96.5%, 97.6% and 88.8% for the first, second and third codon, respectively (Figure 3.5a, b, and c). Most of the base substitutions in both COI and COII were silent substitution, occurring mainly at the 3rd codon position (80%). Since amino acids can be coded by degenerate codons, this high substitution of nucleotides at the third codon position has little effect on amino acid sequence. However, amino acid sequences in COI were more conserved than those in COII, with percent of similarity at 96% and 92%, respectively (Figure 3.6a and b).

The base frequencies of A, C, G, and T in COI were 36%, 11%, 10% and 43%, while in COII, they were 38%, 10%, 5% and 47%. Both COI and COII had high frequencies of AT, 79% and 85% respectively, which is common in many insect mitochondrial genomes (Lui and Beckenbach, 1992).

In pairwise distance comparisons of COI among several *Pyrobombus* species (Table 3.3), the total nucleotide base differences varied from 0-47 bp. There was no difference between *B. b. nearcticus* Handlirsch collected from different sites but the highest differences of 47 bp, occurred between *B. impatiens* and *B. mixtus* type C.

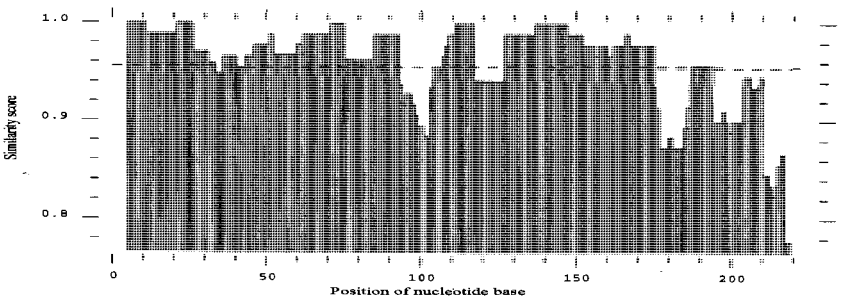


Figure 3.4a Similarity plot of nucleotide bases at the first codon position of the partial cytochrome oxidase I. Average similarity is 95.8% (dashed line).

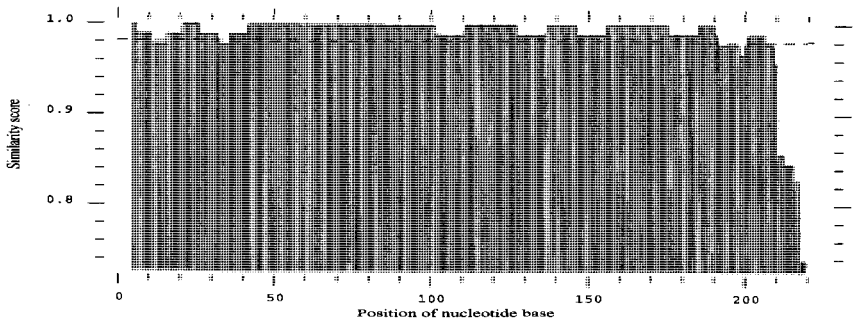


Figure 3.4b Similarity plot of nucleotide bases at the second codon position of the partial cytochrome oxidase I. Average similarity is 98.0% (dashed line).

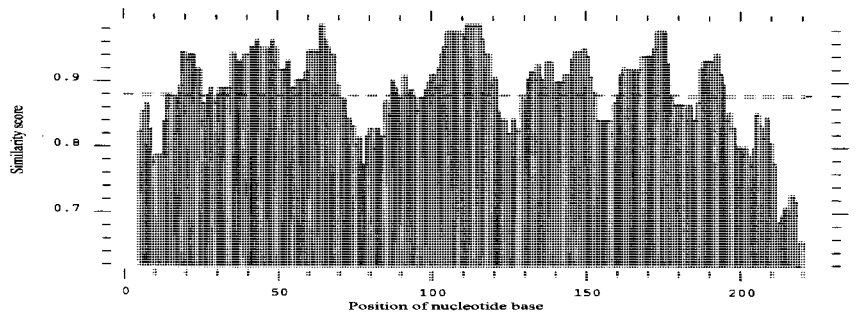


Figure 3.4c Similarity plot of nucleotide bases at the third codon position of the partial cytochrome oxidase I. Average similarity is 88% (dashed line)

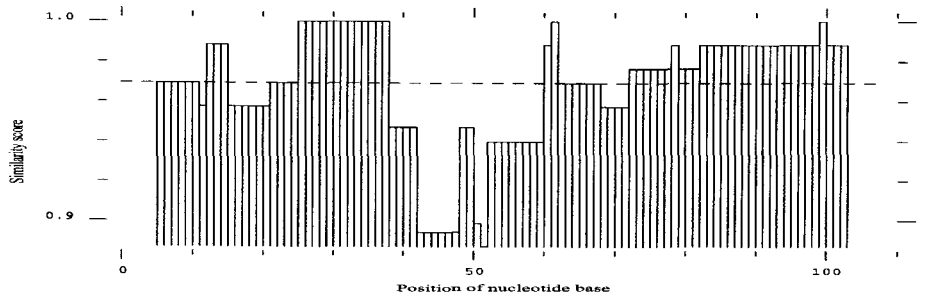


Figure 3.5a Similarity plot of nucleotide bases at the first codon position of the partial cytochrome oxidase II. Average similarity is 97% (dashed line).

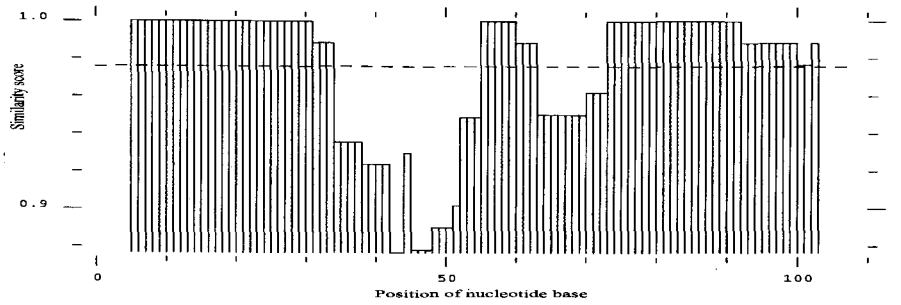


Figure 3.5b Similarity plot of nucleotide bases at the second codon position of the partial cytochrome oxidase II. Average similarity is 97.6% (dashed line).

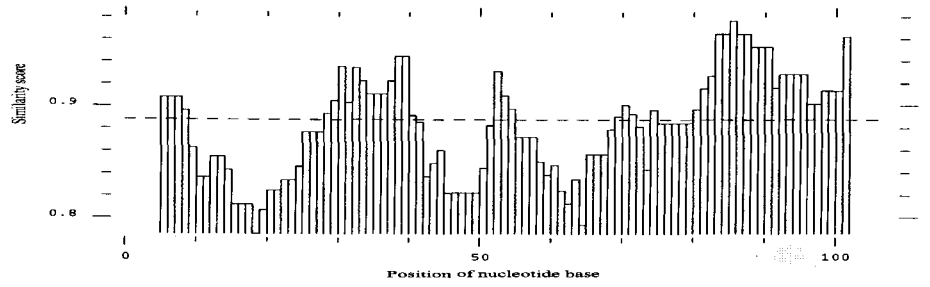


Figure 3.5c Similarity plot of nucleotide bases at the third codon position of the partial cytochrome oxidase II. Average similarity is 88.8% (dashed line).

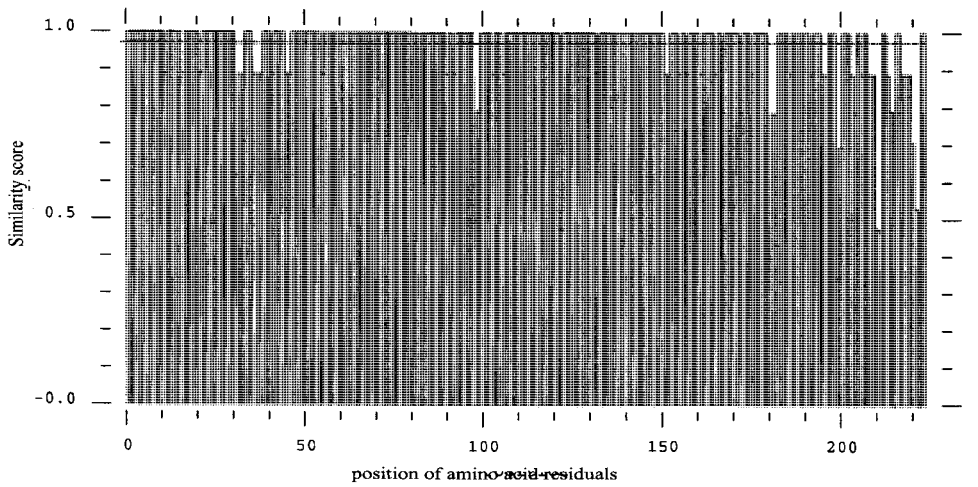


Figure 3.6a The similarity plot of amino acid residues of the partial cytochrome oxidase I, the average of similarity is 96% (hyphen line)

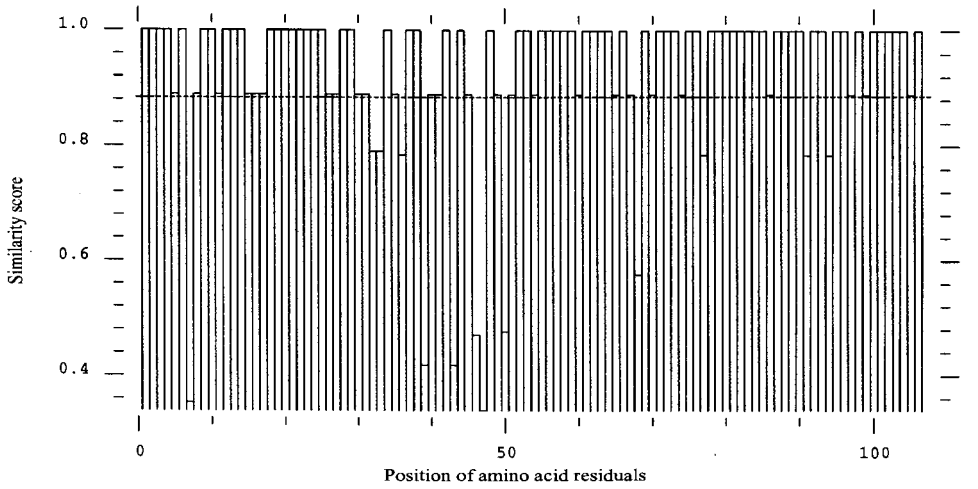


Figure 3.6b The similarity plot of amino acid residues of the partial cytochrome oxidase II, the average of similarity is 92% (hyphen line)

Table 3.3 Pairwise distances between taxa analyzed from thecytochrome oxidase I gene sequences. Total character differences are below the diagonal and mean character differences are above the diagonal.

	1	2	3	4	5	6	7	8	9
Apis		0.14759	0.15964	0.16416	0.15663	0.15813	0.14608	0.14608	0.15813
MXE	98		0.05723	0.04819	0.03765	0.03765	0.04669	0.04669	0.06325
MXC	106	38		0.06777	0.06476	0.06476	0.06024	0.06024	0.06928
VSU	109	32	45		0.04367	0.04367	0.05873	0.05873	0.06928
TRN	104	25	43	29		0.00301	0.05271	0.05271	0.06627
HUN	105	25	43	29	2		0.05271	0.05271	0.06627
BNEM	97	31	40	39	35	35		0.00000	0.05572
BNEH	97	31	40	39	35	35	0		0.05572
SYL	105	42	46	46	44	44	37	37	
MEL	100	38	41	45	43	41	40	40	33
CEN	99	37	40	44	42	42	39	39	32
IMP	107	38	47	44	42	42	39	39	42
VGN	97	29	38	37	33	33	10	10	35
CLG	95	33	40	41	36	36	12	12	33
FIG	97	33	38	38	37	37	14	14	35
FFL	100	32	37	39	36	36	13	13	34
SITN	100	32	41	42	38	38	13	13	34
SITB	100	32	41	42	38	38	13	13	34

Table 3.3 (continued)

	10	11	12	13	14	15	16	17	18
Apis	0.15060	0.14910	0.16114	0.14608	0.14307	0.14608	0.15060	0.15060	0.15060
MXe	0.05723	0.05572	0.05723	0.04367	0.04970	0.04970	0.04819	0.04819	0.04819
MXc	0.06175	0.06024	0.07078	0.05723	0.06024	0.05723	0.05572	0.06175	0.06175
VSU	0.06777	0.06627	0.06627	0.05572	0.06175	0.05723	0.05873	0.06325	0.06325
TRN	0.06476	0.06325	0.06325	0.04970	0.05422	0.05572	0.05422	0.05723	0.05723
HUN	0.06175	0.06325	0.06325	0.04970	0.05422	0.05572	0.05422	0.05723	0.05723
BNEM	0.06024	0.05873	0.05873	0.01506	0.01807	0.02108	0.01958	0.01958	0.01958
BNEH	0.06024	0.05873	0.05873	0.01506	0.01807	0.02108	0.01958	0.01958	0.01958
SYL	0.04970	0.04819	0.06325	0.05271	0.04970	0.05271	0.05120	0.05120	0.05120
MEL		0.00151	0.05572	0.05422	0.05422	0.05120	0.05572	0.05572	0.05572
CEN	1		0.05422	0.05271	0.05271	0.04970	0.05422	0.05422	0.05422
IMP	37	36		0.05271	0.05572	0.05873	0.05723	0.06024	0.06024
VGN	36	35	35		0.01807	0.01807	0.01657	0.01657	0.01657
CLG	36	35	37	12		0.01807	0.01657	0.01657	0.01657
FIG	34	33	39	12	12		0.01054	0.01355	0.01355
FFL	37	36	38	11	11	7		0.00904	0.00904
SITN	37	36	40	11	11	9	6		0.00301
SITB	37	36	40	11	11	9	6	2	

The mean character differences ranged from 0.00 to 0.06928. The two forms of *B. mixtus* (MXC and MXE) had a 38 bp differences with a 0.05723 mean character difference, whereas two forms of *B. sitkensis* (SITN and SITB) had only a 2 bp difference, with a 0.00310 mean character difference. In contrast, some species are more similar: *B. melanopygus* and *B. centralis*, and *B. ternarius* Say and *B. huntii* Greene, had only 1 and 2 dissimilar base pairs with very low mean character differences.

COI gene sequences revealed differences at the intraspecific level better than the interspecific level in *Pyrobombus* species. This result suggests that the rate of nucleotide substitution in the COI gene is not correlated to morphological changes in these *Pyrobombus* species. The substitution of nucleotides in DNA may not be related to the overall speciation or change in morphology (Nei, 1987). The transition and transversion ratios among most taxa were lower than 1 (0.00 to 1.64). AT-transversions were most frequent; in CT-transitions was high but there were no occurrences of CG.

In the COII region (Table 3.4), the total nucleotide differences within subgenus *Pyrobombus* varied from zero between different forms of *B. sitkensis* (SITN and SITB) to 28 bp in many of the species comparisons. The average nucleotide differences ranged from 0 to 0.08750. The pairwise distances in COII were not in agreement with the COI data in some comparisons, especially among *B. melanopygus*, *B. centralis* and *B. f. flavifrons*. The *B. centralis* sequence differed

Table 3.4 Pairwise distances between taxa analyzed from the cytochrome oxidase II gene. Total character differences are below the diagonal and the mean character differences are above the diagonal.

	1	2	3	4	5	6	7	8	9
Apis		0.18125	0.18438	0.16875	0.19375	0.18438	0.19375	0.20937	0.16875
MXE	58		0.02500	0.05313	0.08434	0.07500	0.07187	0.08438	0.05625
MXC	59	8		0.04688	0.08434	0.07500	0.07187	0.07812	0.05625
VSU	54	17	15		0.05000	0.04062	0.05000	0.06250	0.05625
TRN	62	27	27	16		0.00938	0.06250	0.08125	0.06875
HUN	59	24	24	13	3		0.05313	0.07187	0.05937
BNEM	62	23	23	16	20	17		0.05937	0.06562
BNEH	67	27	25	20	26	23	19		0.08438
SYL	54	18	18	18	22	19	21	27	
MEL	54	20	21	20	28	25	22	24	10
CEN	51	18	18	19	25	22	24	28	13
IMP	63	24	22	19	22	19	19	23	21
VGN	53	15	15	20	24	21	25	27	16
CLG	50	19	19	19	25	22	23	25	16
FIG	55	18	18	19	22	19	23	28	12
FFL	53	18	18	19	22	19	23	28	12
SITN	53	18	18	19	21	18	22	28	11
SITB	53	18	18	19	21	18	22	28	11

Table 3.4 (continued)

	10	11	12	13	14	15	16	17	18
Apis	0.16875	0.15937	0.19688	0.16563	0.15625	0.17188	0.16563	0.16563	0.16563
MXE	0.06250	0.05625	0.07500	0.04688	0.05937	0.05625	0.05625	0.05625	0.05625
MXC	0.06562	0.05625	0.06875	0.04688	0.05937	0.05625	0.05625	0.05625	0.05625
VSU	0.06250	0.05937	0.05937	0.06250	0.05937	0.05937	0.05937	0.05937	0.05937
TRN	0.08750	0.07812	0.06875	0.07500	0.07812	0.06875	0.06875	0.06562	0.06562
HUN	0.07812	0.06875	0.05937	0.06562	0.06875	0.05937	0.05937	0.05625	0.05625
BNEM	0.06875	0.07500	0.05937	0.07812	0.07187	0.07187	0.07187	0.06875	0.06875
BNEH	0.07500	0.08750	0.07187	0.08438	0.07812	0.08750	0.08750	0.08750	0.08750
SYL	0.03125	0.04062	0.06562	0.05000	0.05000	0.03750	0.03750	0.03438	0.03438
MEL		0.05313	0.06562	0.05000	0.05000	0.05000	0.05000	0.04688	0.04688
CEN	17		0.06250	0.02812	0.02812	0.02500	0.01875	0.01875	0.01875
IMP	21	20		0.06562	0.06250	0.05937	0.05937	0.05625	0.05625
VGN	16	9	21		0.01875	0.03438	0.02812	0.02812	0.02812
CLG	16	9	20	6		003438	0.02812	0.02812	0.02812
FIG	16	8	19	11	11		0.00625	0.00938	0.00938
FFL	16	6	19	9	9	2		0.00313	0.00313
SITN	15	6	18	9	9	3	1		0.00000
SITB	15	6	18	9	9	3	1	0	

from the sequence of *B. f. flavifrons* and *B. melanopygus* by 6 and 17 bp, respectively, with mean differences of 0.01875 and 0.05313, respectively. In contrast, the COI sequences of *B. centralis* differed from the sequence of *B. melanopygus* and *B. f. flavifrons* by 1 and 36 bp, respectively, with mean differences of 0.00151 and 0.05572, respectively. This suggests that these two genes have a difference history of nucleotide substitutions in these three species, possibly caused by multiple hits, which cannot be detected by pairwise analysis. The transition and transversion ratios for COII from all taxa are higher than in COI. However, the AT-transversions and CT-transitions occur in the same manner as in COI. CG-transversions are also found in some positions in COII but never in COI.

Phylogenetic Analysis COI Tree

A phylogenetic tree was reconstructed from the partial COI gene sequences using neighbor-joining with the Kimura 2-parameter correction. This tree separated *Pyrobombus* species into 3 main clades with bootstrap support values of 35%, 37%, and 100% (Figure 3.7). The first clade was composed of both types of *B. mixtus*, *B. vosnesenskii*, *B. ternarius* and *B. huntii*. The second clade comprised *B. sylvicola* Kirby, *B. melanopygus*, *B. centralis* and *B. impatiens*. The third clade was composed of *B. b. nearcticus*, *B. v. vagans*, *B. caliginosus* Frison, *B. frigidus* F. Smith, *B. f. flavifrons* and *B. sitkensis*. The first clade was weakly supported by the bootstrap value when both types of *B. mixtus* were included.

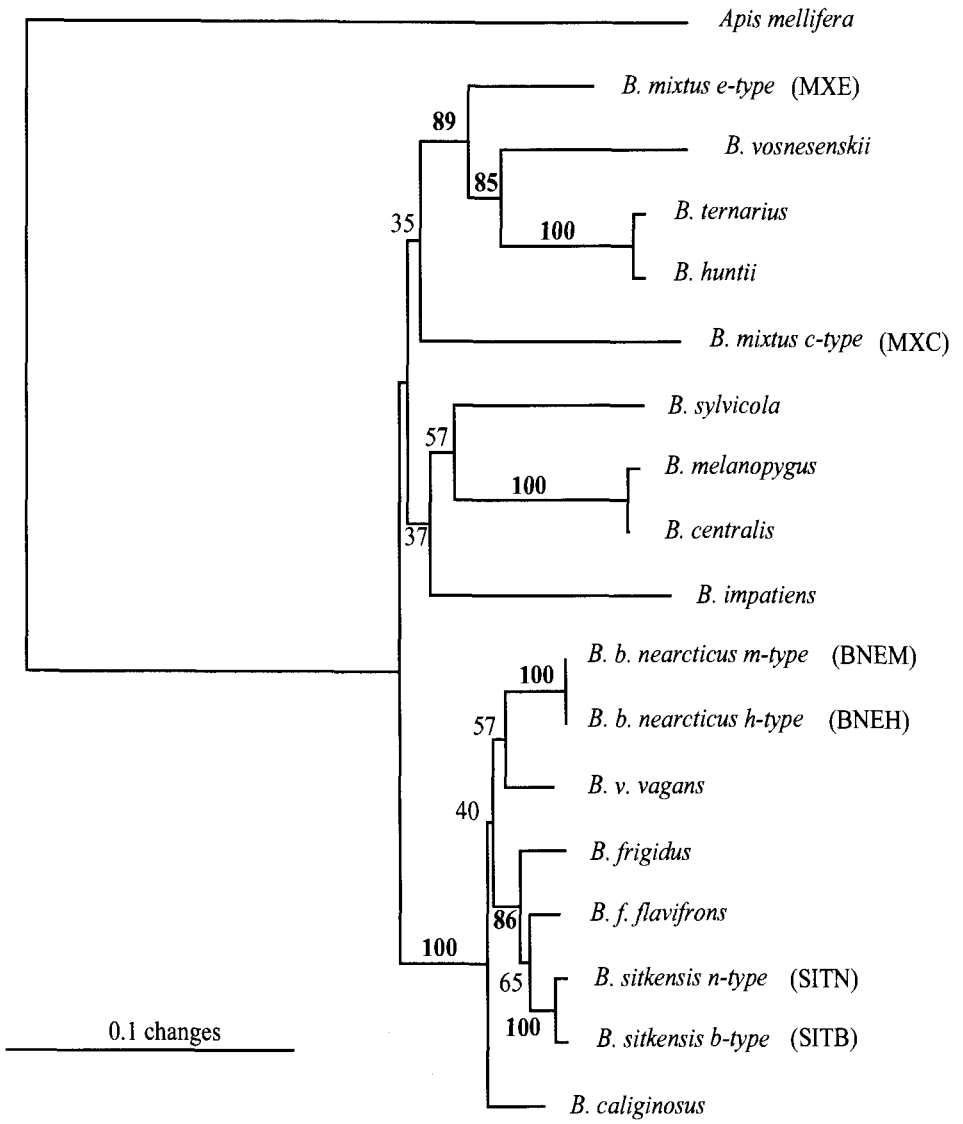


Figure 3.7 Phylogenetic tree reconstructed from the partial COI gene sequences by the neighbor-joining method using the Kimura 2-parameter correction. The numbers represent bootstrap support values.

However, when *B. mixtus* (c-type) was not included in this clade, all branches were highly supported by the bootstrap. The second clade also had weak support. *Bombus impatiens,* a distant species representative from the East Coast, appears to be the cause. However, this case is different from the first one because there is no help from an intraspecific relationship as found in *B. mixtus* to consider its position. In this case, *B. impatiens* may represent an independent lineage.

Maximum parsimony analysis with 96,243 total rearrangements found the best trees at a tree length of 273 steps. At this tree length, there were 12 most parsimonious trees with consistency index (CI) = 0.725, retention index (RI) = 0.681, rescaled consistency index (RC) = 0.494, and homoplasy index (HI) = 0.275. The differences among those parsimonious trees occurred at the low support branches of *B. v. vagans, B. caliginosus, B. sylvicola, B. f. flavifrons, B. frigidus,* and *B. sitkensis.* However, after one round of successive approximation weighted with the RC value, a single tree was produced which was identical to one of the 12 most parsimonious trees. The resulting tree showed a different branch order from the neighbor-joining tree, especially in *B. mixtus* (c-type) (Figure 3. 8). This taxon was placed outside of the whole ingroup; however, the branch support for this conflicting taxon was very weak. If this branch was collapsed, *B. mixtus* (c-type) could cluster with *B. mixtus* (e-type), resulting in a tree similar to the neighbor-joining tree.

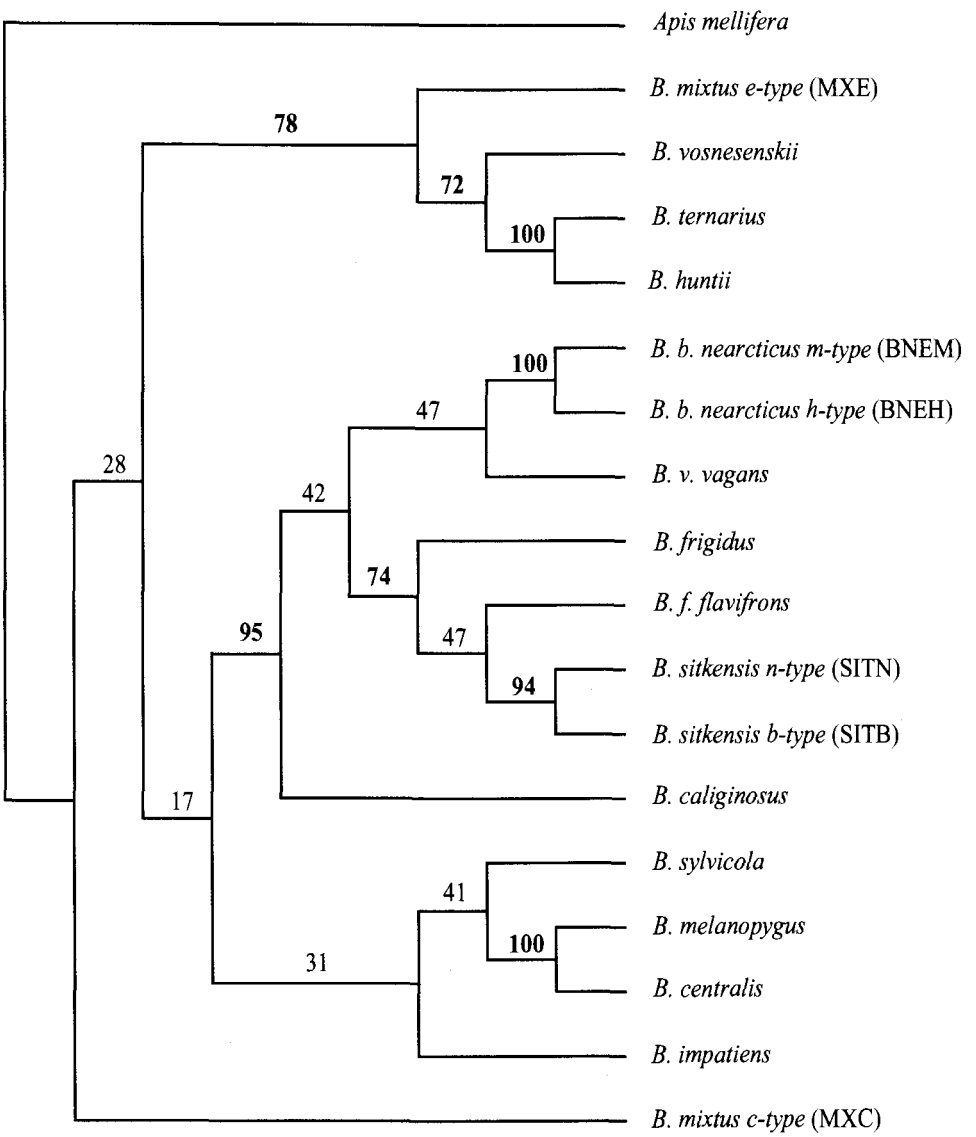


Figure 3.8 Phylogenetic tree reconstructed from the partial COI gene sequences by the maximum parsimony method (total number of arrangements tried, 96,243, tree length 273 steps, 12 most parsimonious trees, CI=0.725, RI=0.681, RC=0.494, HI=0.275). The single tree shown here was produced from one round of successive approximation with the RC value. The numbers are bootstrap values.

Phylogenetic Analysis COII tree

Phylogenetic trees produced from the partial COII gene by both methods were different from those of the partial COI gene. This gene contains variable and parsimonious informative sites that provided slightly higher resolution for the branch patterns. The neighbor-joining method with Kimura 2-parameter correction produced at least 4 main groups of *Pyrobombus* species (Figure 3. 9). Both types of *B. mixtus* formed their own clade with 98% bootstrap support value. The adjacent clade with 78% support value was composed of *B. vosnesenskii, B. ternarius, B. huntii, B. b. nearcticus* and *B. impatiens*. Within this clade, *B. ternarius* and *B huntii* were placed as closely related species with 100% support. Both *B. b. nearcticus* types clustered together with high support and the distances between them were more obvious than in the COI data. Clustering of *B. impatiens* with this group rather than with *B. sylvicola* and *B. melanopygus* was strongly supported. The third clade comprised *B. sylvicola* and *B. melanopygus*, with 71% bootstrap support. The final clade was composed of 6 species. *Bombus caliginosus* is clustered with *B. v. vagans* and forms a sister group with another group that includes *B. centralis, B. frigidus, B. f. flavifrons,* and *B. sitkensis*. In this analysis, *B. centralis* was closely related to *B. f. flavifrons* rather than *B. melanopygus* as found in the COI data. *Bombus frigidus* is clustered with *B. f. flavifrons* and formed a sister group with both forms of *B. sitkensis*.

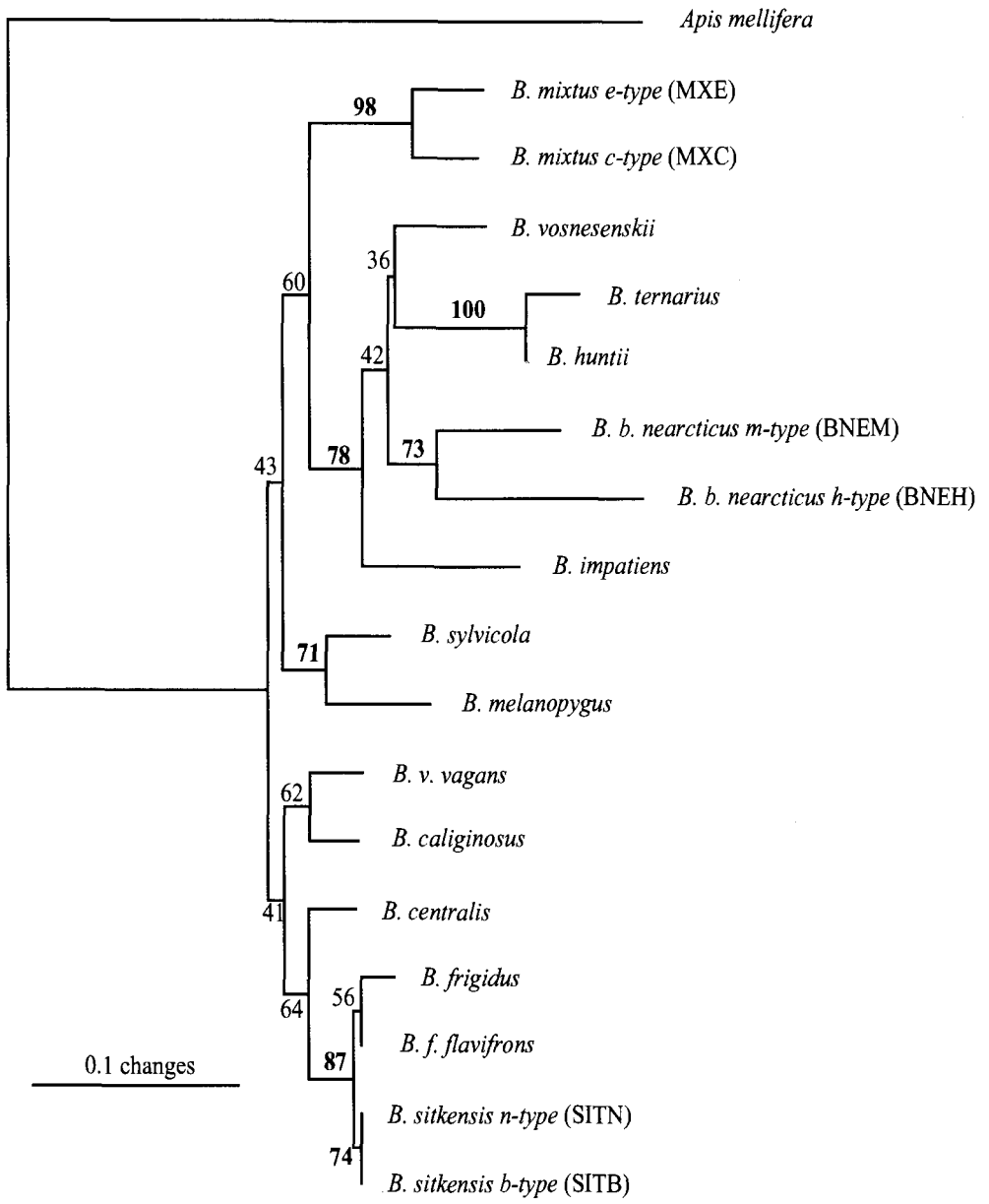


Figure 3.9 Phylogenetic tree reconstructed from the partial COII gene sequences by the neighbor-joining method using the Kimura 2-parameter correction. The numbers are bootstrap values.

The maximum parsimony method, with 132,329 total arrangements, yielded the best tree length of 157 steps, which produced 48 most parsimonious trees with CI= 0.720, RI = 0.674, RC = 0.485, and HI = 0.280. To increase the resolution, one round of the successive weighting method was applied. This produced 3 trees, different only in the branching orders of *B. f. flavifrons, B. sitkensis,* and *B. frigidus*. The consensus tree exhibited the same branch pattern of the main clades as in the neighbor joining tree (Figure 3.10). The bootstrap support of the first, second, third, and fourth main clades were 97%, 71%, 73%, and 38%, respectively.

Phylogenetic Analysis COII-Extend Tree

Unlike the COI gene, PCR products from the partial COII gene provide the adjacent intergenic region. This region is composed of many variable sites, allowing an increase in the resolution of the tree, and clarifying the relationships at the intraspecific level. After this region was added to the partial COII region, phylogenetic trees were reconstructed using neighbor joining with Kimura 2-parameter correction. The resulting tree was similar to those of the partial COII (Figure 3.11), whereas the best tree from maximum parsimony analysis was slightly different in branch order but the clustering of the main clades remained the same. This extended intergenic region increases the robustness of bootstrap support on many branches. The number of most parsimonious trees resulting from the maximum parsimony analysis under condition of equal weights dropped from 48 trees to ten with tree length = 231 steps, CI = 0.723, RI = 0.685, RC = 0.495, HI =

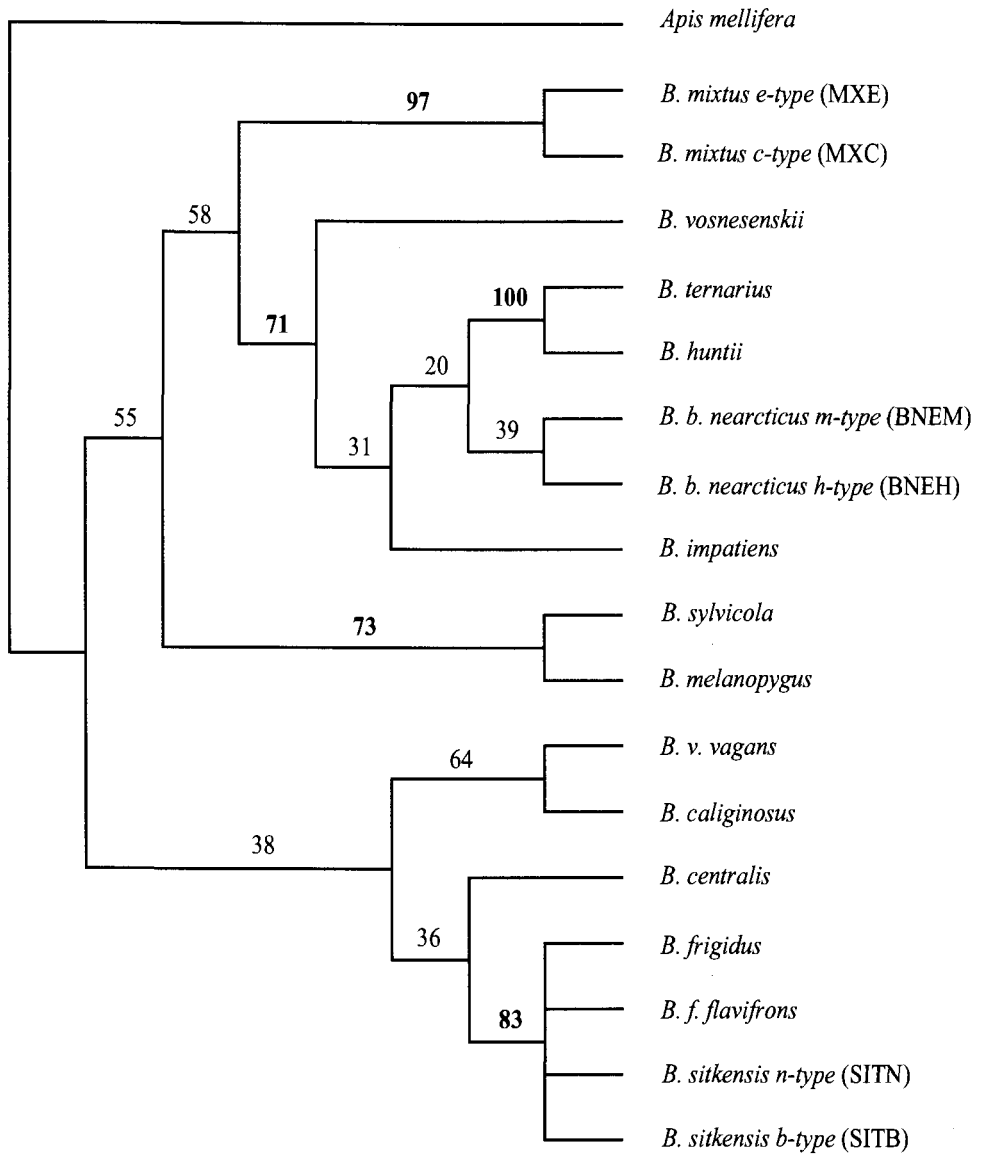


Figure 3.10 Phylogenetic tree reconstructed from the partial COII gene sequences by the maximum parsimony method (total number of arrangements tried, 132,329, tree length 157 steps, 48 most parsimonious trees, CI=0.720, RI=0.647, RC=0.485, HI=0.280). The single tree shown here was a consensus tree from 3 trees (differing in the clade of *B. frigidus, B. f. flavifrons,* and *B. sitkensis*) produced by one round of successive approximation with the RC value. The numbers on the branches are bootstrap values.

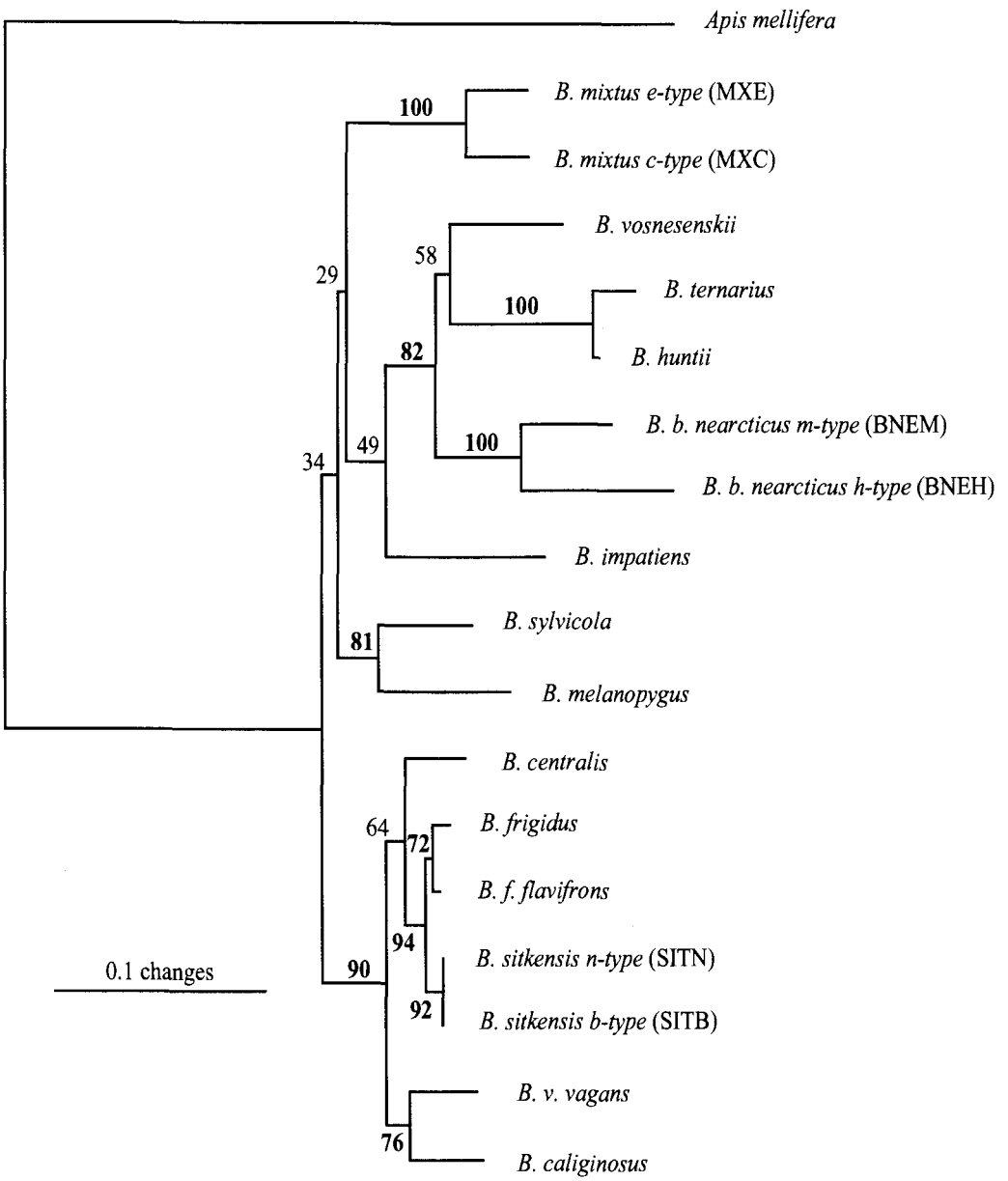


Figure 3.11 Phylogenetic tree reconstructed from the partial COIIint gene sequences by the neighbor-joining method using the Kimura 2-parameter correction.The numbers on the branches are bootstrap values.

0.277. In addition, when a posteriori weight through one round of successive approximation with the RC value was applied to this data set, the maximum parsimony analysis resulted in 2 most parsimonious trees different only at the branching orders between *B. frigidus* and *B. f. flavifrons*. The consensus tree from these 2 most parsimonious trees had a branch order identical to the neighbor-joining trees produced by COII and COIIint as well as the parsimony tree of COII. (Figure 3.12).

Combined Analysis and Partition-Homogeneity Test between Partial COI and Partial COII gene

Combining data sets is one alternative to make more characters available for phylogenetic analysis. This increase in information enhances the phylogenetic signal and reduces the sampling error in analysis, resulting in a more accurate estimate of phylogeny of the group (Barrett *et al.*, 1991; de Queiroz, 1993; Huelsenbeck and Hillis, 1993). Moreover, the combined analysis takes advantage of both genes to produce a higher resolution tree and increase the branch support values (Doyle *et al.*, 1994; Schmitz and Moritz, 1998). In some cases, it may uncover unique patterns that cannot be detected in the original data (Olmstead and Sweere, 1994).

In this study we performed 2 sets of combined analysis: partial COI and COII gene (COI/COII), and partial COI and COII gene with intergenic region (COI/COIIint). The sequence matrices contained 990 and 1,103 positions,

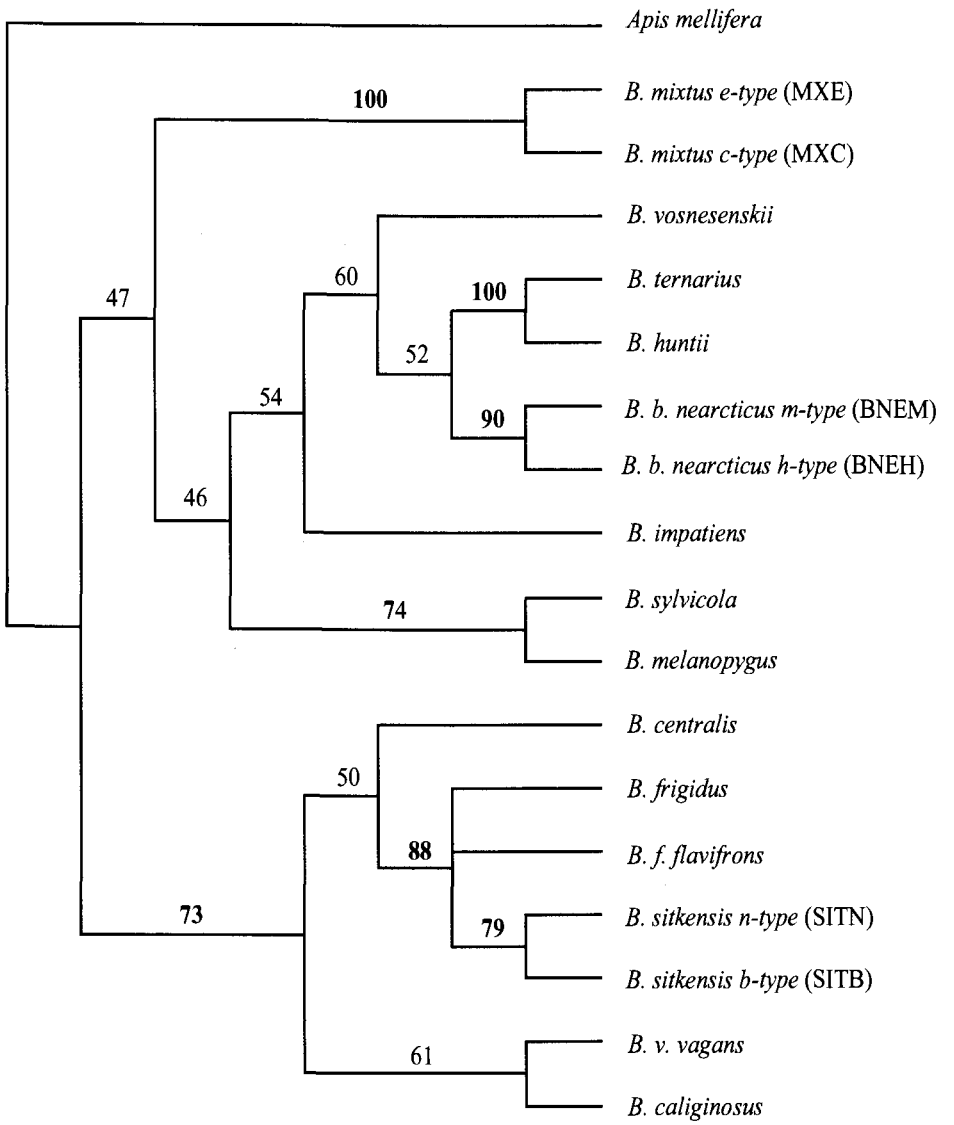


Figure 3.12 Phylogenetic tree reconstructed from the partial COIIint gene sequences by the maximum parsimony method (total number of arrangements tried, 39,882, tree length 231 steps, 10 most parsimonious trees, CI=0.723, RI=0.685, RC=0.495, HI=0.277). The single tree shown here was a consensus tree from 2 trees (differing in the clade of *B. frigidus* and *B. f. flavifrons*) produced by one round of successive approximation with the RC value. The numbers on the branches are bootstrap values.

the variable sites increased to 283 and 327 sites, and parsimony informative sites increased to 119 and 138 sites respectively. The combined trees reconstructed from both data sets were totally different from the trees from the original data sets. However, branch resolution and branch support was improved.

Neighbor-joining analysis of the combined data sets showed clear branching orders for closely related taxa such as *B. b. nearcticus*, *B. mixtus*, and *B. centralis*, although the two data sets gave conflicting placement of *B. impatiens* (Figure 3.13 and Figure 3.14).

Parsimony analysis of COI/COII under equal weight condition, after 40,269 rearrangements, produced best trees of 452 steps, 22 steps longer than the sum of the original data sets. This resulted in 4 most parsimonious trees with CI = 0.688, RI = 0.619, RC = 0.426, and HI = 0.312. These trees differed only in the branch arrangement among *B. v. vagans*, *B. caliginosus*, *B. frigidus*, and *B. f. flavifrons*. After one round of successive approximation with the RC, these four trees converged to a single most parsimonious tree that differed from the above neighbor-joining trees only by the low branch support for *B. impatiens* (Figure 3.15)

Parsimony analysis of COI/COIIint, after 49,648 arrangements were tried, resulted in 13 most parsimonious trees with CI = 0.685, RI = 0.616, RC = 0.422, HI = 0.315. The best trees had tree length of 533 steps, 29 steps longer than the sum of their partitions. After one round of successive approximation with the RC weight,

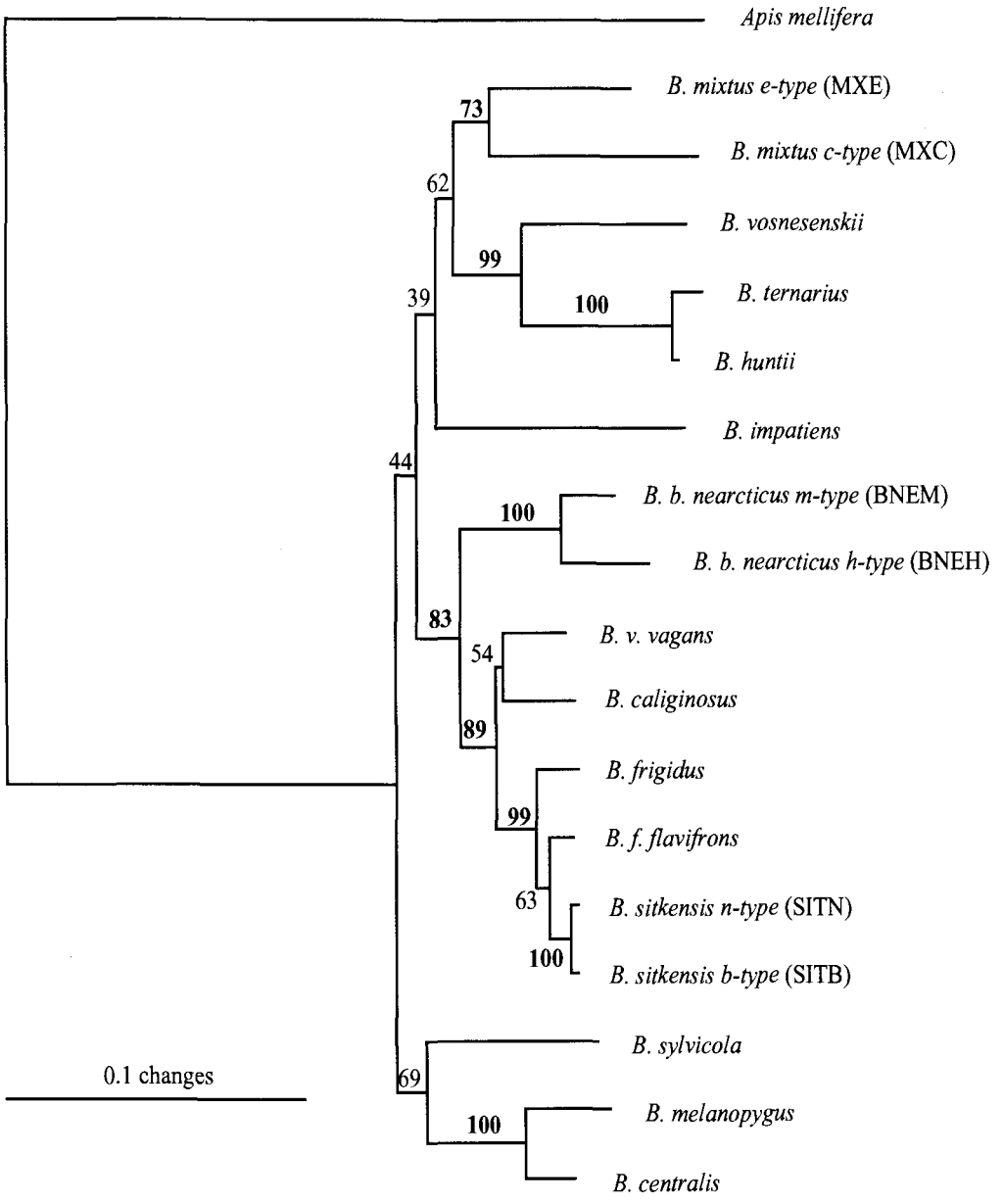


Figure 3.13 Phylogenetic tree reconstructed from the combined COI and COII sequence data sets by the neighbor-joining method using the Kimura 2-parameter correction. The numbers on the branches are bootstrap values.

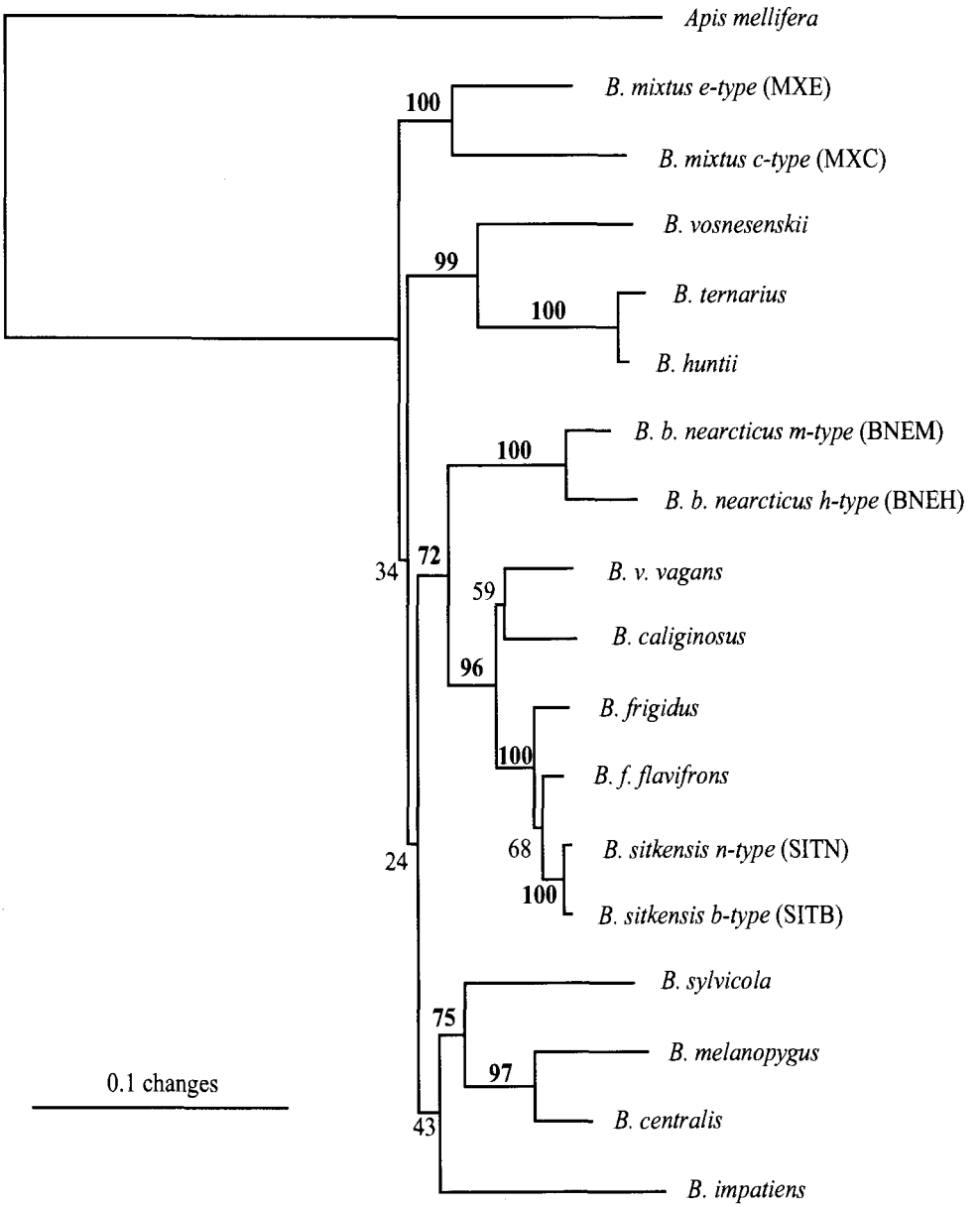


Figure 3.14 Phylogenetic tree reconstructed from the combined COI and COIIint sequence data sets by the neighbor-joining using the Kimura 2-parameter correction. The numbers on the branches are bootstrap values.

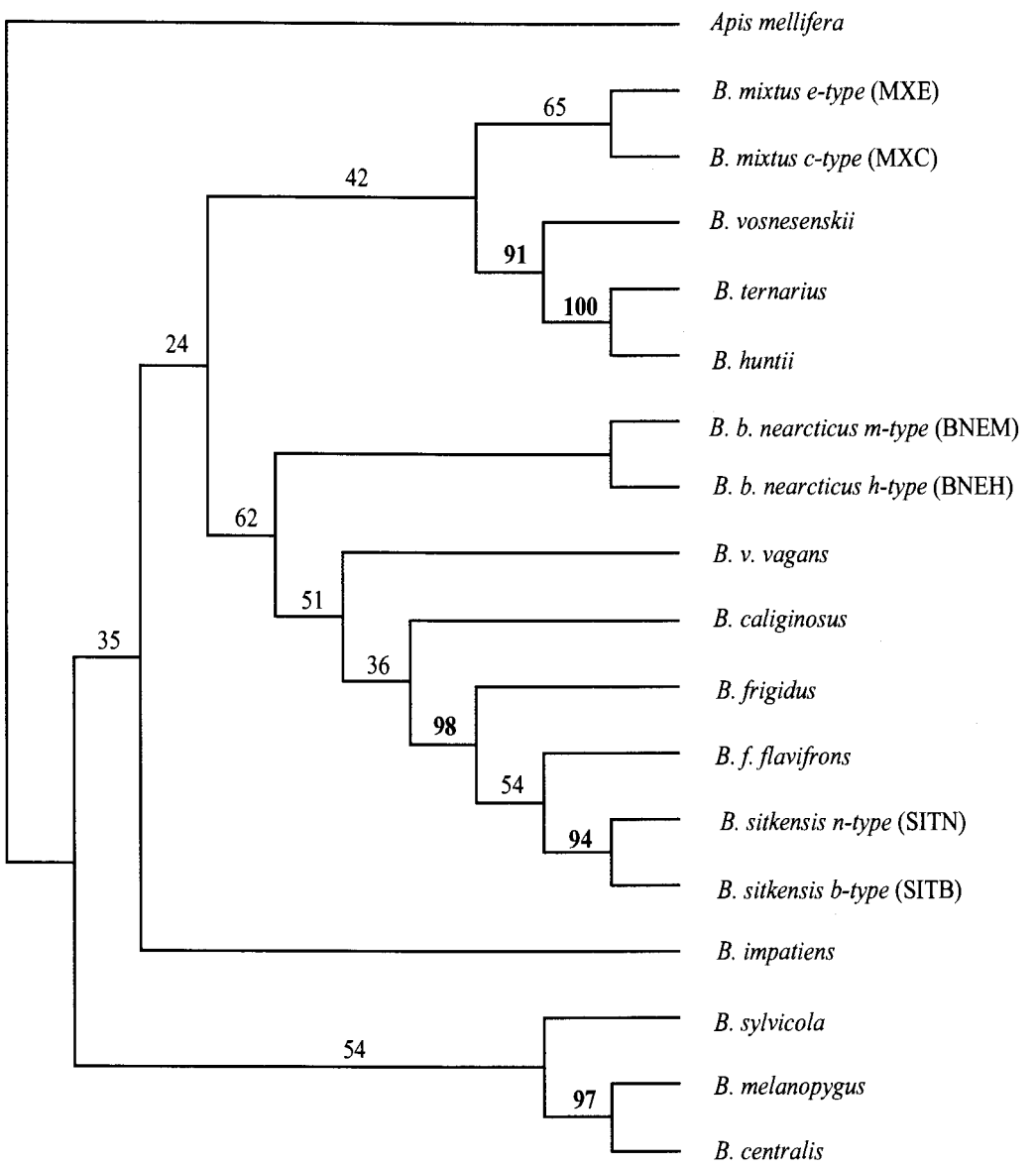


Figure 3.15 Phylogenetic tree reconstructed from the combined COI and COII sequence data sets by the maximum parsimony method (total number of arrangements tried, 40,269, tree length 452 steps, 4 most parsimonious trees, CI=0.688, RI=0.619, RC=0.246, HI=0.312). The single tree shown here was produced by one round of successive approximation with the RC value. The numbers are bootstrap values.

only one unique tree was produced which was almost identical to the neighbor joining trees (Figure 3.16).

However, after the congruence of these COI/COII and COI/COIIint combined trees was examined by the partition homogeneity test, the null hypothesis of congruence between data partitions was rejected ($p = 0.002$) for both combined sets. This means that the partitions of the data sets were heterogeneous and should be considered as different classes of evidence, combining them may provide misleading phylogenetic estimation (Bull *et al.*, 1993). Therefore, in a statistical sense, it may not be appropriate to combine these two data sets and perform phylogenetic relationship analysis. These combined trees may not be acceptable as the best representative of the true tree (Huelsenbeck *et al.*, 1996).

DISCUSSION

Combined Analysis

COI and COII are parts of mitochondrial DNA and share many closely related properties and functions such as location on the mt-genome, environmental conditions, constrained function, occurrence, and evolution. These genes are adjacent on the mt-genome and when they are translated into proteins, the proteins must interact with many others to form the functional core of the cytochrome oxidase complex. In order to keep the final complex functional, every step of change in these COI and COII genes is constrained. Therefore, COI and COII

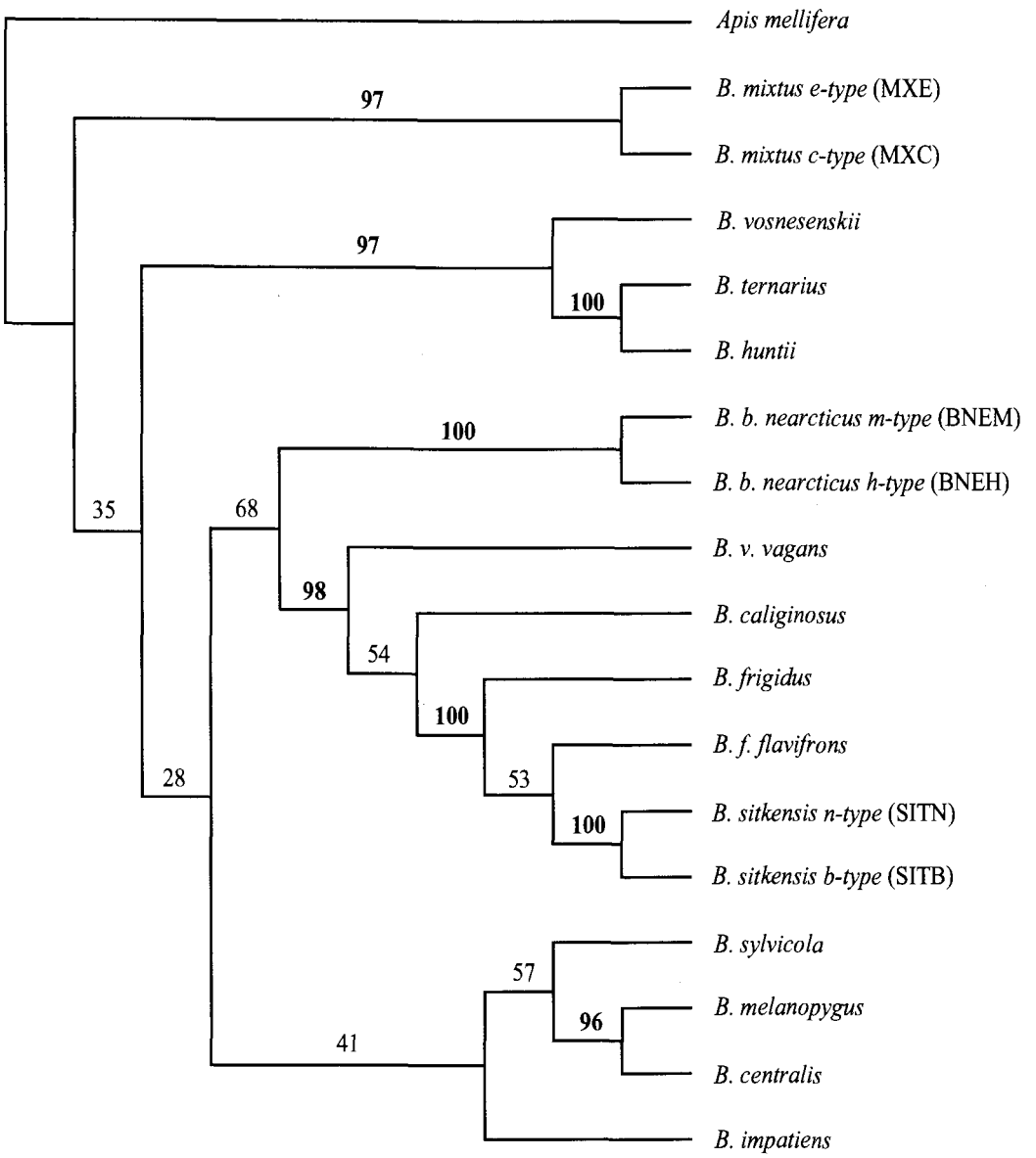


Figure 3.16 Phylogenetic tree reconstructed from the combined COI and COIIint sequence data sets by the maximum parsimony method (total number of arrangements tried, 49,648, tree length 533 steps, 13 most parsimonious trees, CI=0.685, RI=0.616, RC=0.422, HI=0.315). The single tree shown here was produced by one round of successive approximation with the RC value. The numbers on the branches are bootstrap values.

should act as a single linked genetic unit with the same phylogenetic history. However, each gene is composed of many parts. Each part has a unique characteristic in order to form a specific structure and function after being translated into protein (Moritz *et al.*, 1987; Harrison, 1989; Simon, 1991; Wolstenholme, 1992; Lunt *et al.*, 1996). The partial genes used in this study were selected from the most variable part of each gene, 3' end in COI and 5' end in COII. They therefore do not necessarily have the same history.

We found that the partial COI tends to be more conserved than the partial COII, since it contains a lower percentage of variable sites at amino acid sequences. The trees produced from both genes are significantly different and each has high branch support for the conflicting taxa. These conflicts may be strong enough to cause problems in the combined analysis. As mentioned by Bull *et al.* (1993) in his simulation study, combined analysis of genes having different evolutionary rates is less likely to provide a true tree.

The partition homogeneity analysis found significant differences in the partitioned data sets. The conflict mainly occurred in branch order of *B. b. nearcticus* and *B. centralis*, which were very highly supported by bootstrap values. This incongruency possibly occurred from lineage sorting or different branching history. Moreover, sampling error and stochastic error may be involved too (de Queiroz *et al.*, 1995) because at least two taxa, *B. mixtus* (c-type) and *B. impatiens*, on the trees from each gene recovered by the different methods, were slightly

different. However, the two latter factors had very little influence because the branch support values of those species were very low.

After *B. centralis* and *B. b. nearcticus* were excised from the combined data set, the null hypothesis of their partition homogeneity was accepted with p-values = 0.27 and 0.155 for COI/COII, and COI/COIIint combination sets, respectively. Certainly, these new combined data sets were more appropriate and gave a better estimated of phylogenetic analysis than the original one because the new combined data sets were the sum of the data with the same history of branching order (Rodrigo *et al.*, 1993). The increased number of characters in these combined sets raises the number of variables in the data set. In a statistical sense, it therefore reduces sampling error and stochastic error, resulting in a more reliable tree from phylogenetic analysis (de Queiroz, 1993; Huelsenbeck and Hillis, 1993). Phylogenetic trees reconstructed from both new combined data sets, and also their partitions, are all likely to have the same branch pattern and form the main clade in the same pattern with all phylogenetic analysis methods (Figure 3.17a-f and Figure 3.18a-d).

The combined phylogenetic trees support the idea that there are two forms of *B. mixtus* and *B. sitkensis* in the PNW. *Bombus ternarius* and *B. huntii* are recently diverged and their closest relative is *B. vosnesenskii*. *Bombus sylvicola* shares a recent ancestor with *B. melanopygus*. *Bombus v. vagans* is surprisingly closely related *to B. caliginosus*. *Bombus frigidus* is closely related to *B. f. flavifrons* and *B. sitkensis*. Since *B. impatiens* has a distribution limited to the East

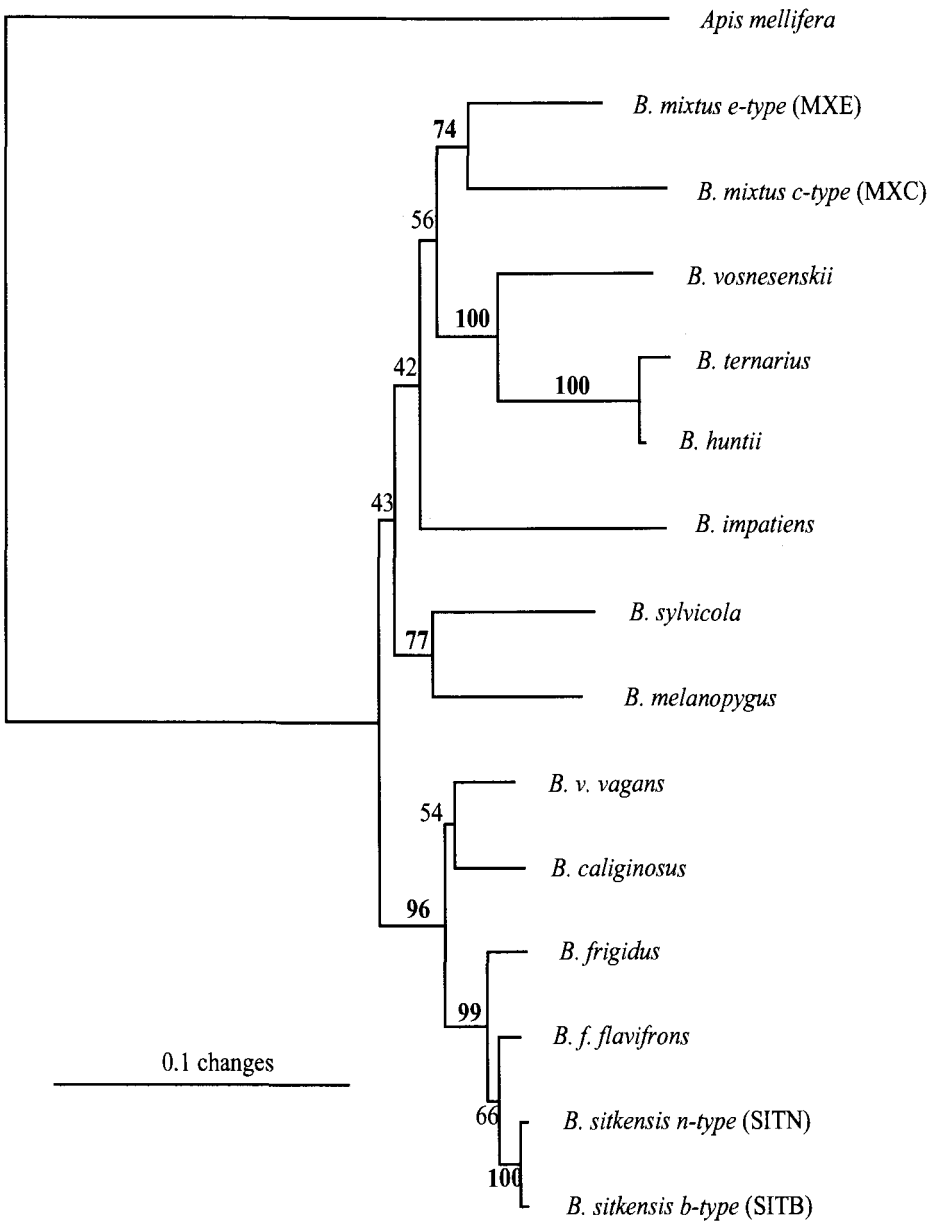


Figure 3.17a Phylogenetic tree reconstructed from the combined COI and COII sequence data sets (with deletion of 3 conflicting taxa, *B. centralis* and 2 of *B. b. nearcticus*) by the neighbor-joining method using the Kimura 2-parameter correction. The numbers are bootstrap support values.

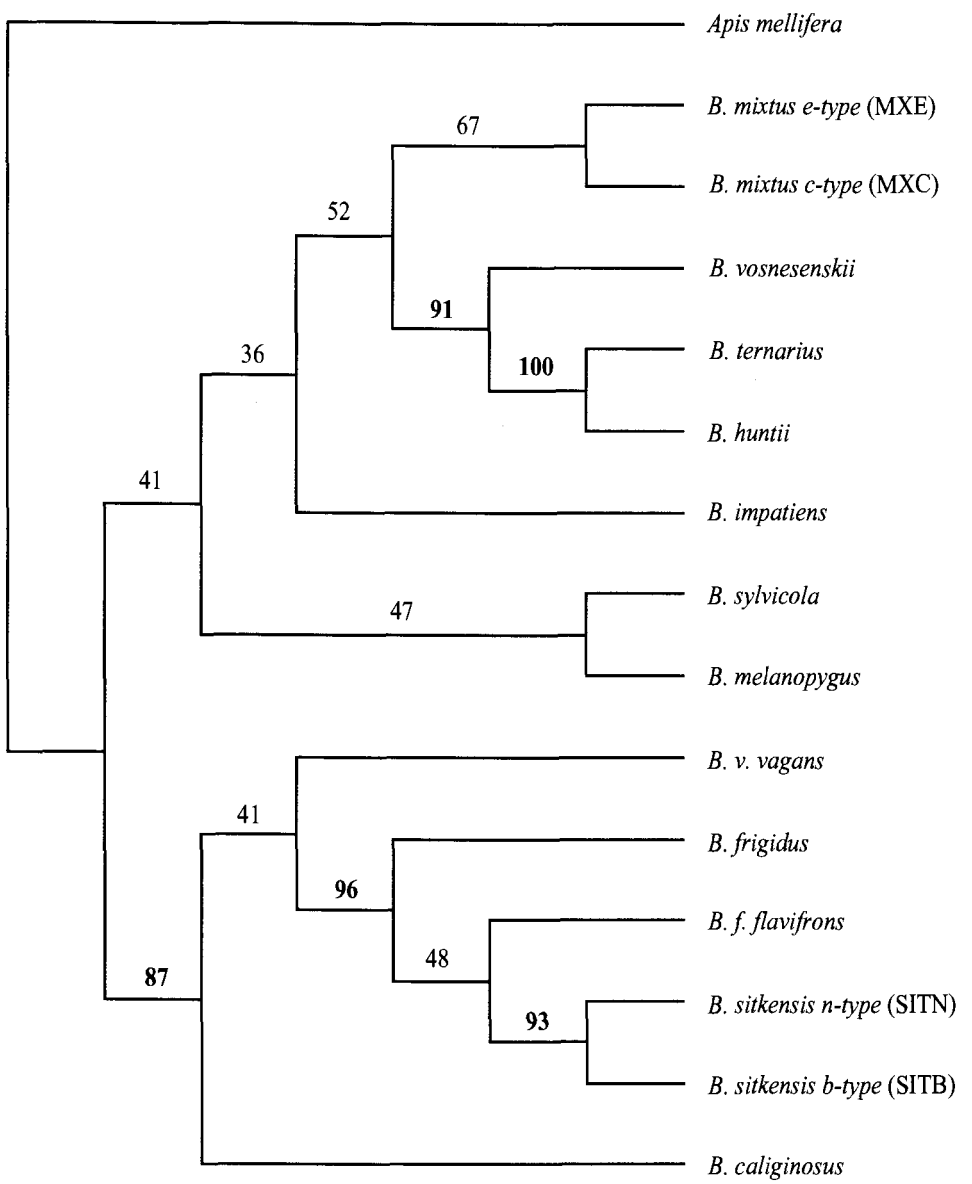


Figure 3.17b Phylogenetic tree reconstructed from the combined COI and COII sequence data sets (with deletion of 3 conflicting taxa) by the maximum parsimony method (total number of arrangements tried, 18,485, tree length 404 steps, 6 most parsimonious trees, CI=0.728, RI=0.615, RC=0.448, HI=0.272). The single tree shown here was produced by one round of successive approximation with the RC value.
The numbers on the branches are bootstrap values.

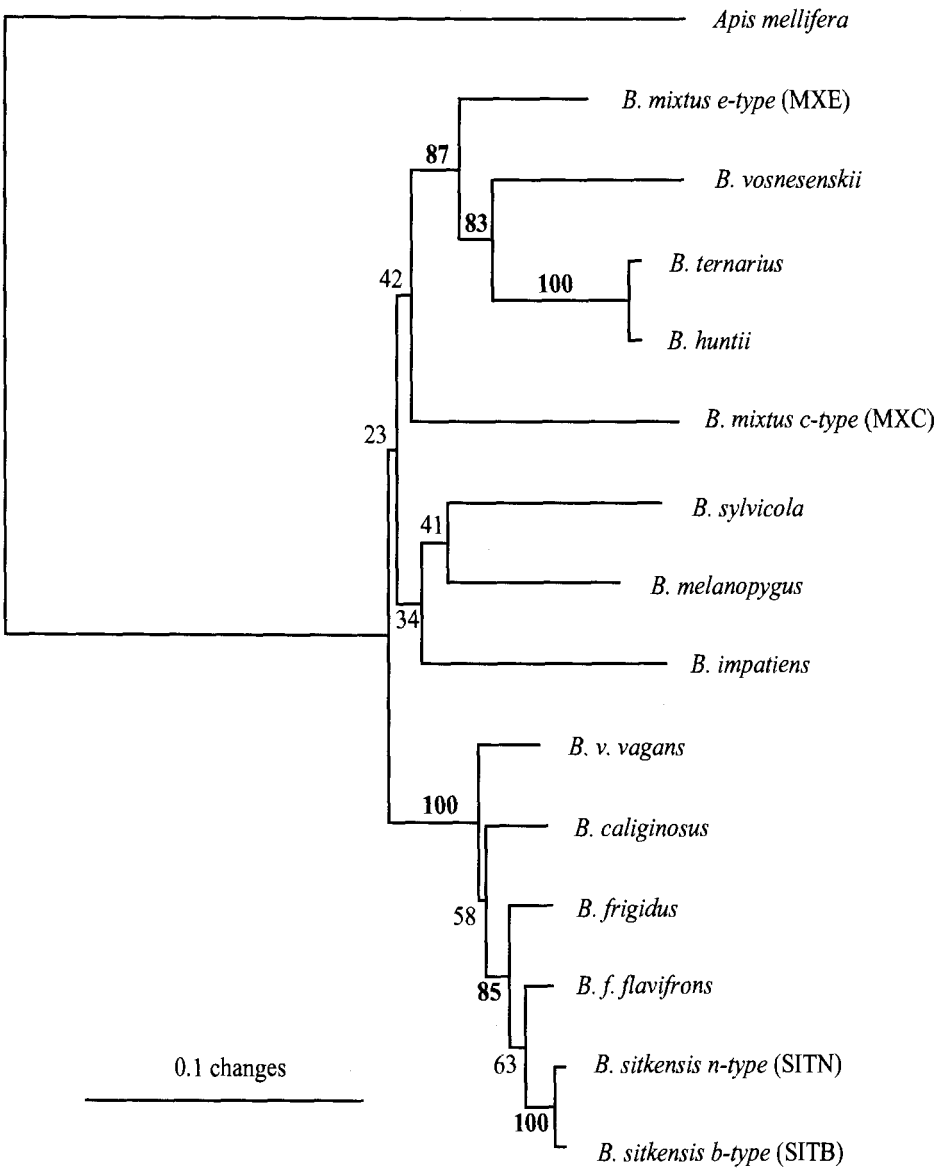


Figure 3.17c Phylogenetic tree reconstructed from the COI gene sequence (with deletion of 3 conflicting taxa) by the neighbor-joining method using the Kimura 2-parameter correction. The numbers on the branches are bootstrap values.

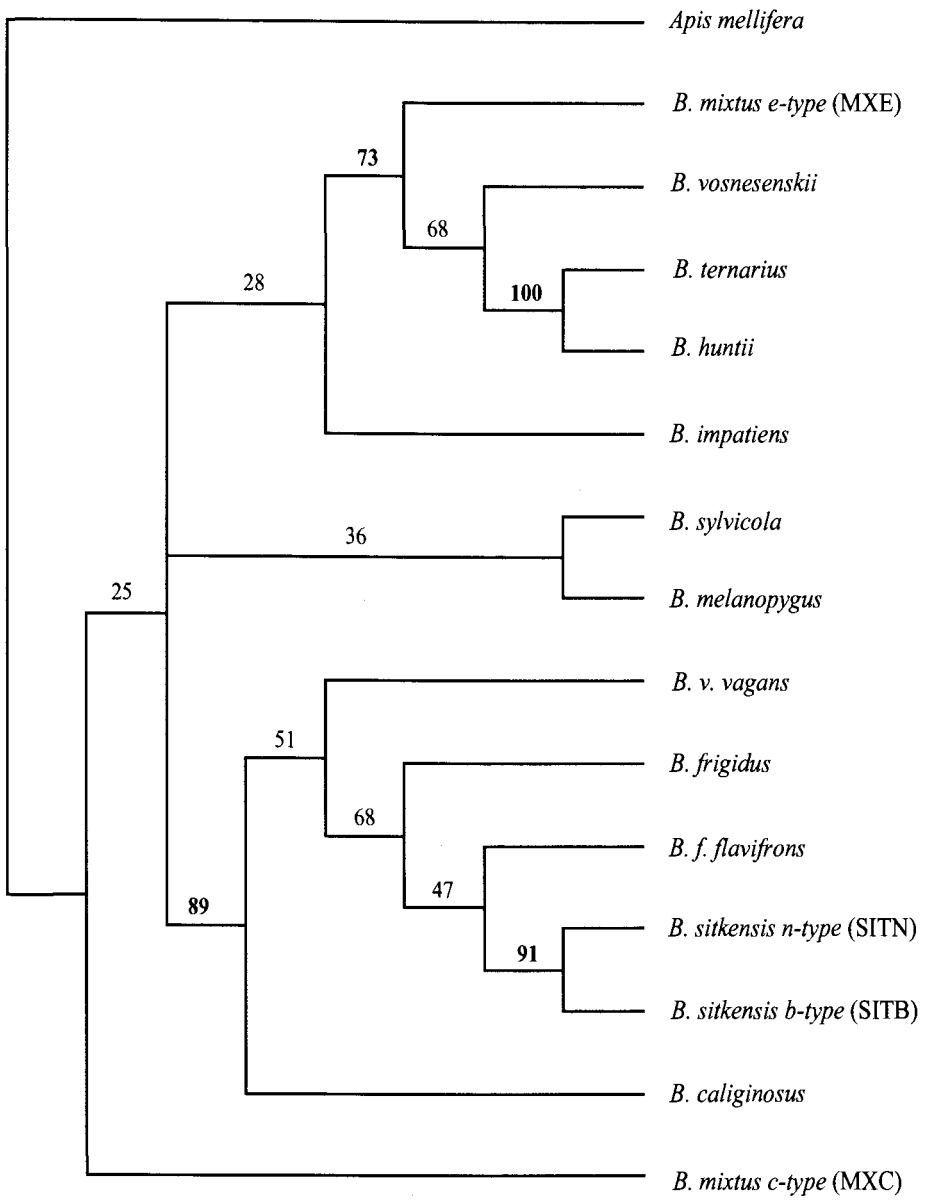


Figure 3.17d Phylogenetic tree reconstructed from the partial COI gene sequence (with deletion of 3 conflicting taxa) by the maximum parsimony method. The single tree shown here was produced by one round of successive approximation with the RC value. The numbers on the branches are bootstrap values.

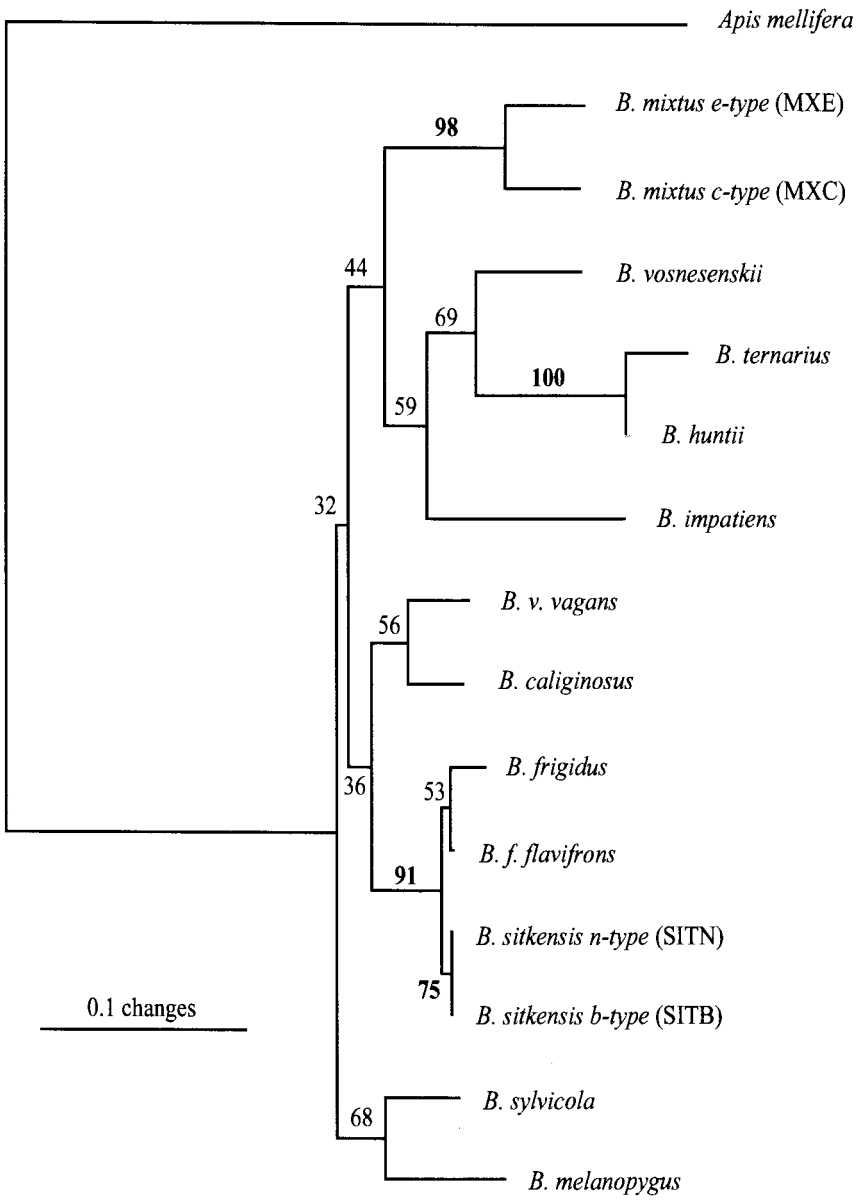


Figure 3.17e Phylogenetic tree reconstructed from the COII gene sequences (with deletion of 3 conflicting taxa) by the neighbor-joining method using the Kimura 2-parameter correction. The numbers are bootstrap values.

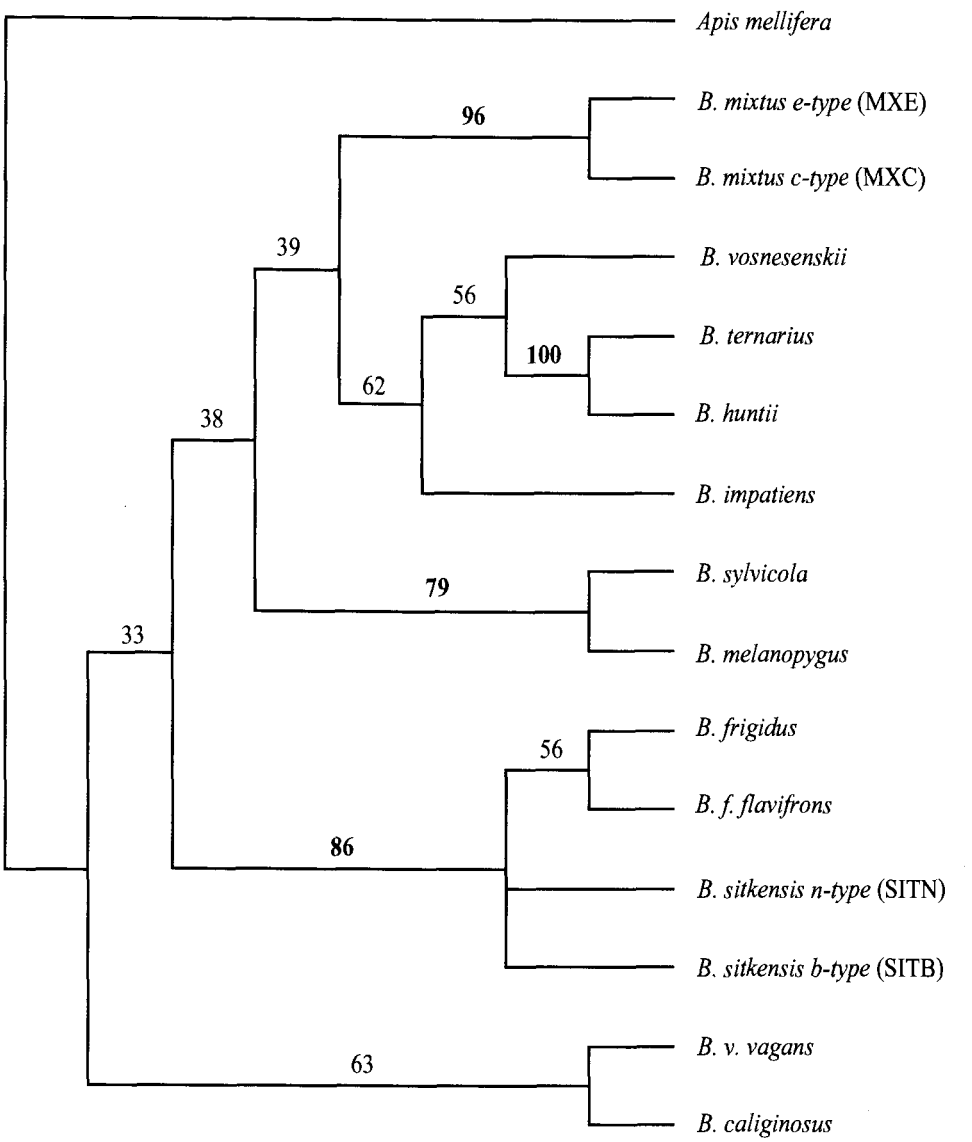


Figure 3.17f Phylogenetic tree reconstructed from the COII gene sequences (with deletion of 3 conflicting taxa) by the maximum parsimony method. The single tree shown here was produced by one round of successive approximation with the RC value. The numbers are bootstrap values.

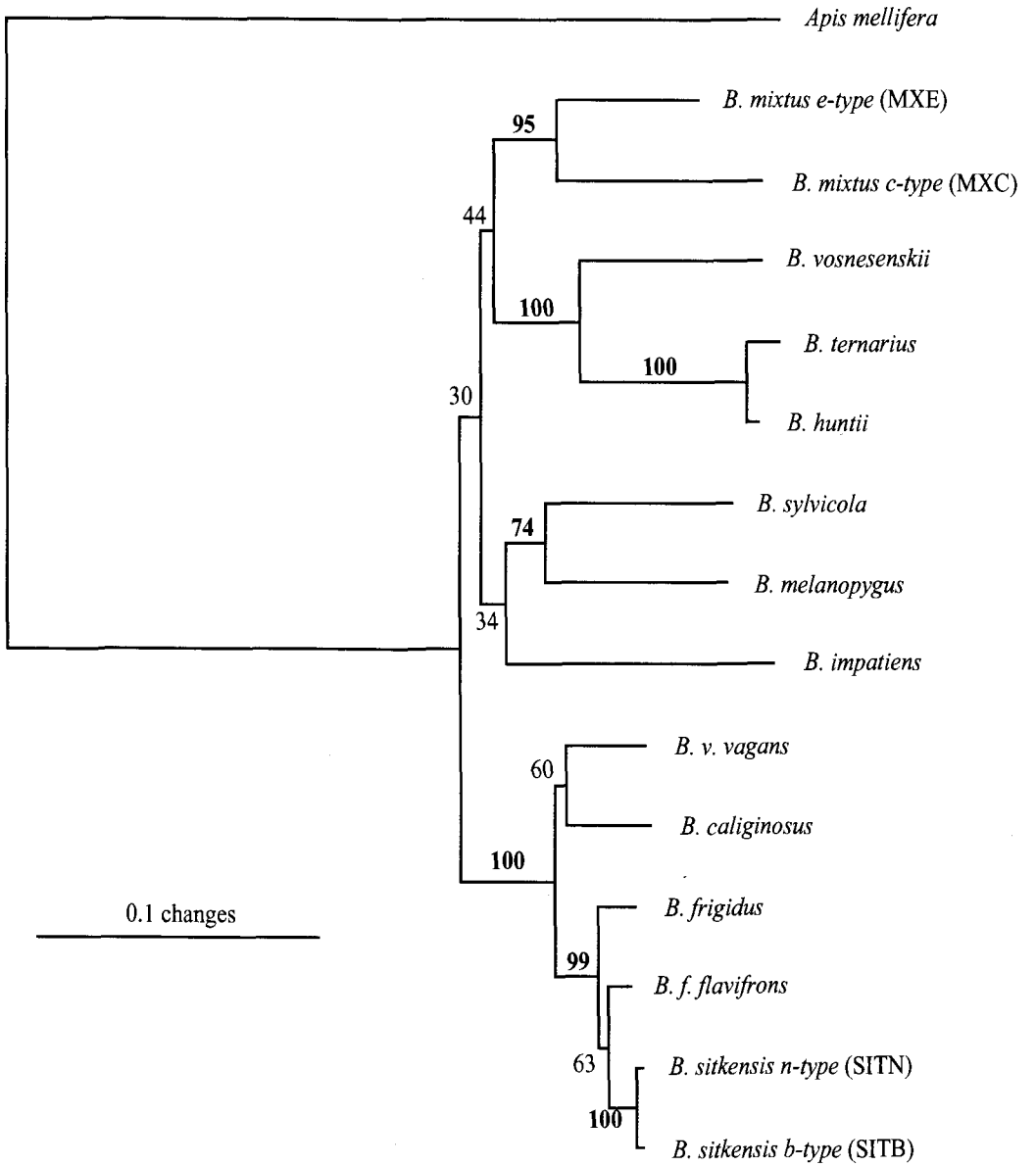


Figure 3.18a Phylogenetic tree reconstructed from the combined COI and COIIint sequence data sets (with deletion of 3 conflicting taxa) by the neighbor-joining using the Kimura 2-parameter correction. The numbers are bootstrap values.

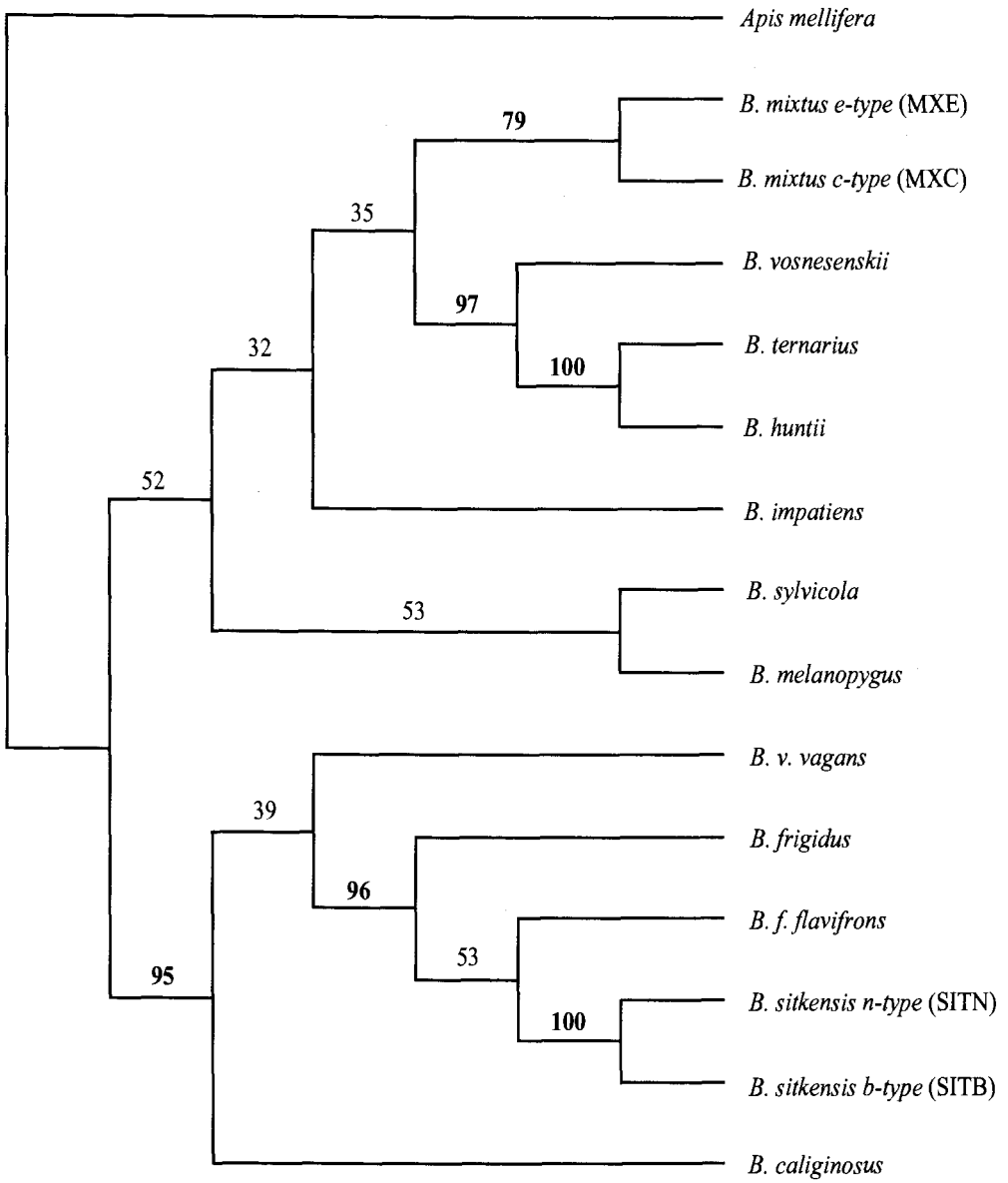


Figure 3.18b Phylogenetic tree reconstructed from the combined COI and COIIint sequence data sets (with deletion of 3 conflicting taxa) by the maximum parsimony method. The single tree shown here produced by one round of successive approximation with the RC value. The numbers are bootstrap values.

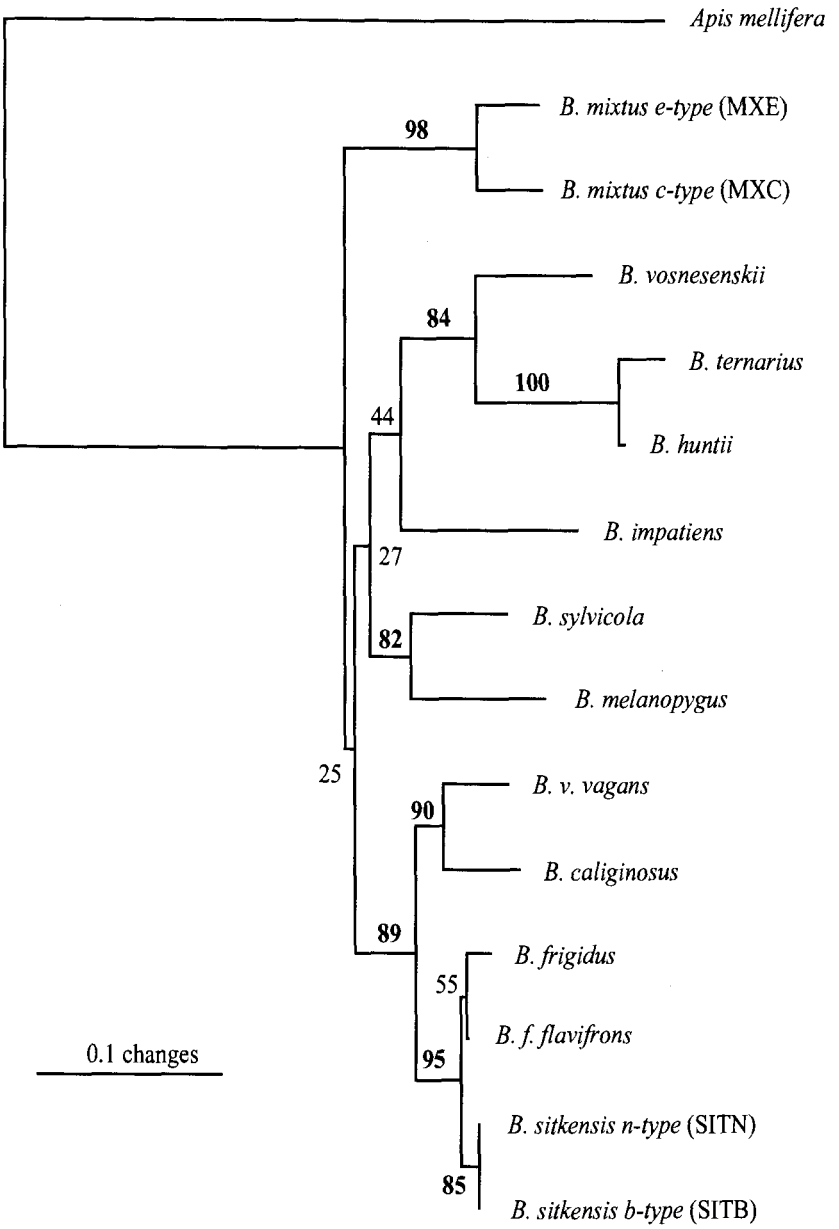


Figure 3.18c Phylogenetic tree reconstructed from the COIIint sequence (with deletion of 3 conflicting taxa) by the neighbor-joining method using the Kimura 2-parameter correction. The numbers are bootstrap values.

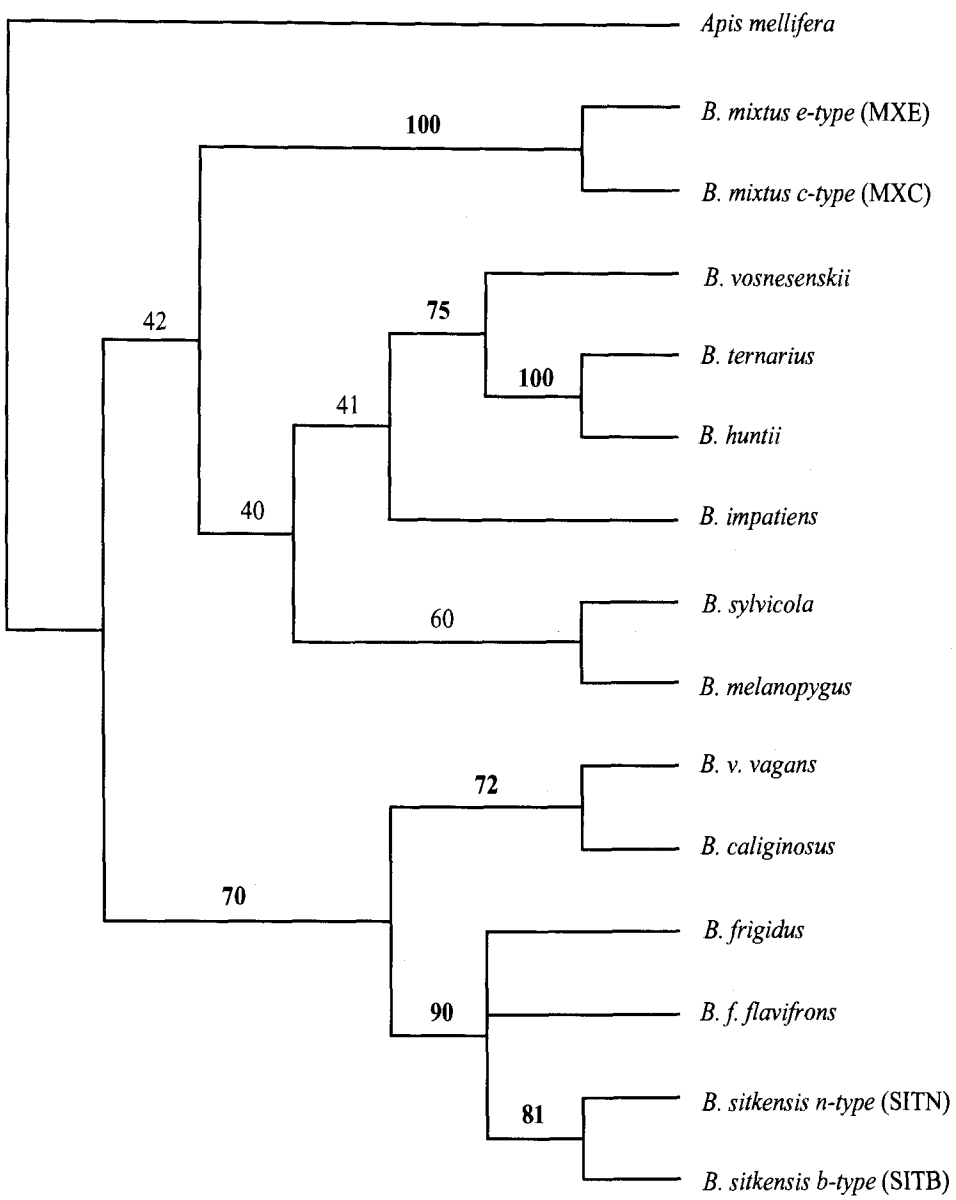


Figure 3.18d Phylogenetic tree reconstructed from the COIIint sequence (with deletion of 3 conflicting taxa) by the maximum parsimony method. The single tree shown here was produced by one round of successive approximation with the RC value. The numbers are bootstrap values.

Coast, its remote relationship with *Pyrobombus* species in the PNW results in unstable branching of this species such that it may cluster with either the *B. melanopygus* group or the *B. vosnesenskii* group. However, when considered from the COII and COIIint trees and branching order from combined trees that the conflicting taxa were excised, *B. impatiens* is highly supported to place with the *B. vosnesenskii* group rather than with the *B. melanopygus* group.

Of course, the phylogenetic information on those excised taxa is lost by this combined analysis. However, their placement in the congruent tree can be inferred from other taxonomic information such as morphological or other molecular data.

Bombus centralis has been considered more closely related to *B. f. flavifrons* based on morphological data (Stephen, 1957) and numeric analysis of wing venation (Plowright and Stephen, 1973). The morphology of *B. centralis* is most similar to *B. f. flavifrons* and can be differentiated only by the yellow color over the anterior face of the mesoscutum and the short malar space. The male genitalia of this species demonstrates a close relationship to *B. f. flavifrons* rather than *B. melanopygus* (Figure 3.19). This evidence all supports the phylogenetic trees inferred from COII and COIIint, rather than the tree from COI data that grouped *B. centralis* with *B. melanopygus*.

Another conflicting species, *B. b. nearcticus*, was placed with the *B. f. flavifrons* group by COI data whereas COII placed this species with the *B. huntii* group. There is also other evidence supporting the COII data but no support for the COI placement of *B. b. nearcticus*. The structure of male genitalia (Stephen, 1957)

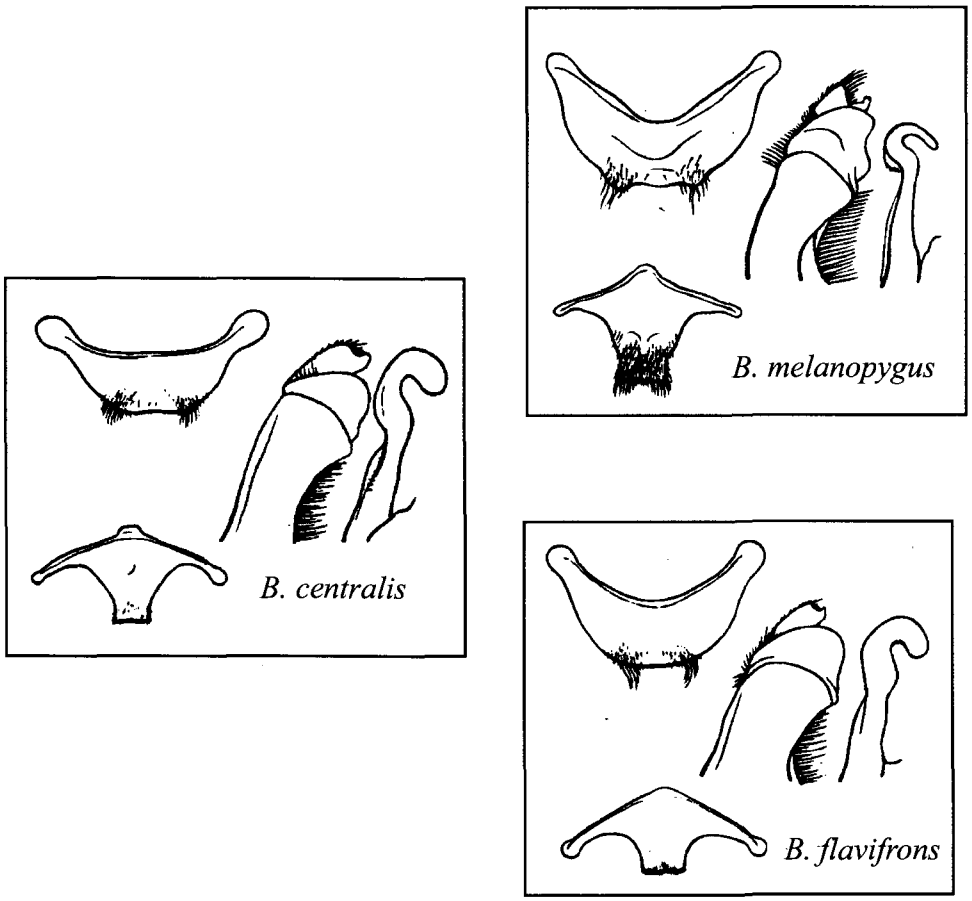


Figure 3.19 The structure of male genitalia of *Bombus centralis* is more closely related to that of *Bombus flavifrons* than to *Bombus melanopygus* (Stephen, 1957).

indicates that this species is more closely related to the *B. huntii* group (Figure 3.20). Numeric analysis on wing venation also supports this placement (Plowright and Stephen, 1973). Moreover, the tree reconstructed from cytochrome b and also another region of COI by Koulianos (1999) confirms that this species is close to *B. huntii* and *B. ternarius* rather than *B. frigidus* and *B. flavifrons.*

After placing the conflicting taxa back into the most congruent tree based on the above discussion, the resulting tree is more similar to the tree reconstructed from COII and COIIint than that from COI. This suggests that the phylogeny of subgenus *Pyrobombus* in the PNW inferred from COII and COIIint is consistent with many more data types and more acceptable, than the tree inferred from COI.

Evolution of subgenus *Pyrobombus* in the Pacific Northwest

The phylogenetic tree resulting from this study provides congruent evidence along with morphological and molecular studies that enable the development of a hypothetical scenario for the evolution of many species of *Pyrobombus* in the PNW. Many species or taxa in this analysis appear to have recently diverged. Their divergence can be explained with geographic evidence in terms of allopatric, parapatric and sympatric relationships. The members of this subgenus are clustered together and form their own cluster as a monophyletic group with an outgroup. This study shows no root relationship as ancestor lineage link between species from the East Coast, represented by *B. impatiens*, and species of the PNW. Based on our molecular data and phylogenetic trees in this study, *Pyrobombus* species in the

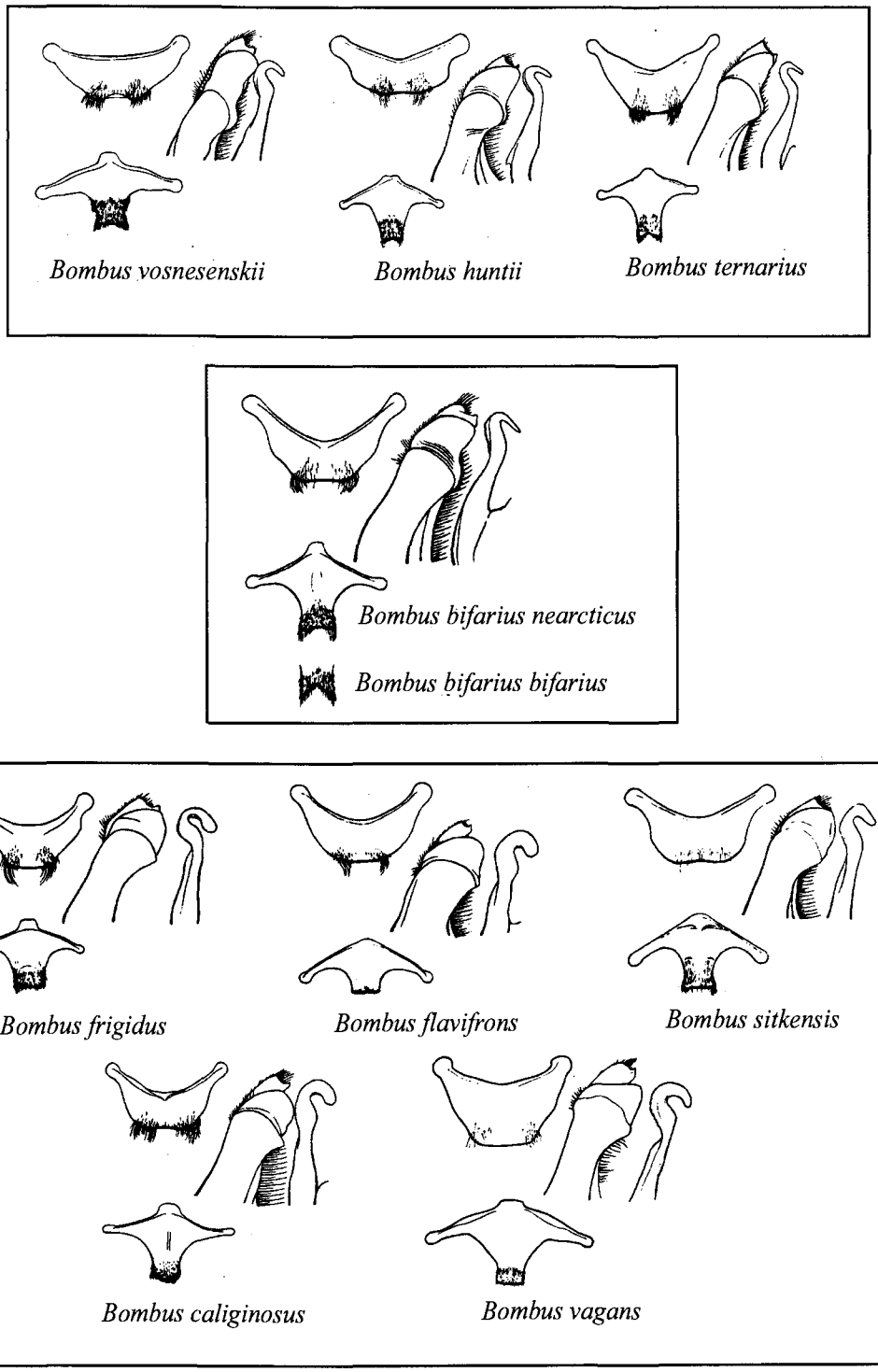


Figure 3.20 The structure of male genitalia of *B. bifarius* is closer to that of the *B. huntii* group than the *B. flavifrons* group (Stephen, 1957).

PNW tend to form 4 subclades clearly presenting the evidence of recent divergence and providing clear inter and intraspecific relationships.

The number of base pair differences, mean differences in pairwise analysis, and phylogenetic tree show clearly that many species in the PNW are currently in the process of radiation. For example, *B. mixtus, B. b. nearcticus* and *B. sitkensis* are found as polymorphic species occupying various habitats, and this variation in each species can be detected by analysis based on the COI and COII genes. *Bombus mixtus* (e-type), found only at high elevations, such as Three Sisters and Crater Lake, has a yellow band at the 1st abdominal tergum with small yellow curved band at the 2nd abdominal tergum, and the thoracic intercalar band is sharp and clear. The c-type, found along the valley at lower elevations, has yellow bands on the 1st and 2nd abdominal terga, and the thoracic intercalar band is fuzzy. The pairwise distance analysis of these two types suggests that they are widely separated from each other. The separation occurs since c-type is active during late spring until early summer whereas e-type shows up in late summer through mid fall because the spring season at the high elevations is later than in the valley. They are almost completely separated as allopatric species with very little chance of gene flow.

Bombus bifarius nearcticus, like *B. mixtus* e-type, is limited to higher elevations. This isolation could have occurred recently by glacial movement, because glacial movement occurred not only from the south northward but also from low to high elevation (Ahnert, 1998). *Bombus b. nearcticus* is limited to the higher elevations on mountains such as Mt. Hood, OR and Eagle Cap, OR.

Hybridization between these populations was possibly infrequent. This isolation allowed each population to evolve differently, resulting in allopatric speciation. Although the morphology of individual *B. b. nearcticus* from Mt. Hood and Eagle Cap was identical, differences were detected in the COII gene. This divergence may have occurred recently because in some areas such as in northern Canada, variation in this species can be found, resulting in many intermediate forms between the two subspecies, *B. b. bifarius* and *B. b. nearcticus* (Stephen, 1957).

Bombus sitkensis provides another controversy. Polymorphism is seen in the variation of the yellow bands on the abdominal terga 3rd to 6th. This variation makes species identification difficult based on only color pattern because it can be confused with *B. f. dimidiatus* (Stephen, 1957). In this analysis, four specimens that showed variation of these yellow bands were used.

A Few differences were detected by COI gene analysis but not by the COII gene. When considering their habitat and the flying season, all of the polymorphic types show up in the same time from late spring to late summer and occupy the same habitat. Therefore, this species would be an example of gradual radiation and evolution as long as hybridization exists in their entire area of distribution.

Bombus ternarius and *B. huntii* are very closely related species based on morphological characters. *B. huntii* can be separated from *B. ternarius* only by the yellow pile on face and vertex, completely yellow scutellum and slightly longer malar space. However, these two species are markedly allopatric because *B. huntii* occupies the southern part of the Pacific Northwest, USA (WA, OR, ID, and CA),

whereas *B. ternarius* is mainly found in the northern part, Canada (Stephen, 1957). In addition, unlike *B. mixtus* or *B. sitkensis*, their genital structures are different, which increases the probability of reproductive isolation. Their placement on the in COI and COII phylogenetic trees are very close and different only 1 and 3 nucleotide bases, respectively. These two species likely to be very recently diverged species and the speciation process occurred via geographic isolation.

Our results from COI and COII confirmed that *B. caliginosus* and *B. vosnesenskii* are convergent species. As suggested by Stephen (1957) and Thorp *et al.* (1983), these two species are not closely related species, although their banding patterns look identical. In fact *B. caliginosus* is more closely related to the *B. f. flavifrons* group rather than *B. vosnesenskii*, especially when considering male genitalia and the divergent branch pattern from the phylogenetic trees in this study. *Bombus caliginosus* is limited to the PNW coast whereas *B. vosnesenskii* is abundant from the valley to coast, covering the entire distribution area of *B. caliginosus*. Their similarity of banding patterns may be the result of convergence or mimicry.

CONCLUSIONS

The current study provides clear evidence of the relationships of bumblebees in the subgenus *Pyrobombus* in the PNW inferred from phylogenetic analyses of two mitochondrial DNA genes. The analyses compared sequences from two close mt-genes, COI and COII that have the same environmental conditions,

occurrence, and evolutionary background. However, the phylogenetic trees resulting from the COI gene sequence were not congruent to those from the COII gene. Although COII was reanalyzed by adding the intergenic region, the differences still occurred, but the recovered trees were improved with higher resolution and stronger branch support values. Even though different analytical methods were applied, the results between the two genes remained different. When these two genes' data sets were combined and tested by partition homogeneity analysis, the result confirmed that these two genes have different phylogenetic histories, demonstrated by three conflicting taxa. When the three conflicting taxa were removed and the new combined data sets were re-analyzed, all reconstructed trees were congruent. Of course, the phylogenetic information of those three species was lost. We solved this problem by adding those conflicting species into the congruent tree considering their position based on other taxonomic information such as morphological evidence.

The final phylogenetic trees explain not only the relationship of many confusing species, but also provide evidence of ada[tive radiation in many species in comparison to their ecology and geographical distribution. This analysis also can detect differences between individuals of many species; for example, in *B. sitkensis*, *B. mixtus*, and *B. b. nearcticus*. However, in many polymorphic species such as *B. melanopygus*, the phylogenetic signal from these two genes in this analysis does not separate the polymorphs. It may be useful to study genes that are

more variable than COI and COII genes, allozymes, or finer molecular techniques such as microsatellites to explain their variation.

The COII gene will be used for studying the phylogenetic relationships of genus *Bombus* and *Psithyrus* in the PNW in the future. In this study, the COII gene would be advantageous over the COI gene. For example, the results from the COII gene were more congruent with other taxonomic data, and also provided an option to increase the phylogenetic signal for analysis by extending sequences into the intergenic region between $tRNA^{leu}$ and COII gene.

ACKNOWLEDGEMENTS

I would like to thank Dr. James Cane who kindly sent specimen of *B. impatiens* from Alabama. Special thanks you to Dr. Lynn Royce for her help and support, and who kindly collected *B. sitkensis and B. mixtus.*

REFERENCES

Ahnert, F. (1998). "Introduction to Geomorphology," Arnold, London.

Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, J. E., Reeb, C. A., and Saunders, N. C. (1987). Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annu. Rev. Ecol. Syst.* 18: 489-522.

Ballard, J. W., Olsen, G. J., Falth, D. P., Odgers, W. A., Rowell, D. M., and Atkinson, P. W. (1992). Evidence from 12S ribosomal RNA sequences that Onychophorans are modified arthropods. *Science* 258: 1345-1348.

Barrett, M., Donoghue, M. J., and Sober, E. (1991). Against consensus. *Syst. Zool.* 40: 486-493.

Brower, A. V. Z., and DeSalle, R. (1994). Practical and theoretical considerations for choice of a DNA sequence region in insect molecular systematics, with a short review of published studies using nuclear gene regions. *Ann. Entomol. Soc. Amer.* 87: 702-716.

Brower, A.V.Z. (1994). Phylogeny of *Heliconius* butterflies inferred from mitochondrial DNA sequences (Lepidoptera: Nymphalidae). *Mol. Phylogenet. Evol.* 3: 159-174.

Bull, J. J., Huelsenbeck, J. P., Cunningham, C. W., Swofford, D. L., and Waddell, P. J. (1993). Partitioning and combining data in phylogenetic analysis. *Syst. Biol.* 42: 384-397.

Clary, D. O., and Wolstenholme, D. R. (1985). The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. *J. Mol. Evol.* 22: 252-271.

Cornuet, J. M., Garnery, L., and Solignac, M. (1991). Putative origin and function of the intergenic region between COI and COII of *Apis mellifera* L. mitochondrial DNA. *Genetics* 1128: 393-403.

Crozier, R. H., and Crozier, Y. C. (1993). The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organization. *Genetics* 133: 97-117.

Crozier, R. H., Crozier, Y. C., and Mackinlay, A. G. (1989). The CO-I and CO-II region of honeybee mitochondrial DNA: evidence for variation in insect mitochondrial evolutionary rates. *Mol. Biol. Evol.* 6: 399-411.

de Queiroz, A. (1993). For consensus (sometimes). *Syst. Biol.* 42: 368-372.

de Queiroz, A., Donoghue, M., and Kim, J. (1995). Separate versus combined analysis of phylogenetic evidence. *Annu. Rev. Ecol. Syst.* 26: 657-681.

Doyle, J. A., Donoghue, M. J., Zimmer, E. A. (1994). Integration of morphological and rRNA data on the origin of angiosperms. *Ann. Mo. Bot. Gard.* 81: 419-450.

Farris, J. S. (1969). A successive approximations approach to character weighting. *Syst. Zool.* 18: 374-385.

Farris, J. S. (1989). The retention index and the rescaled consistency index. *Cladistics* 5: 417-419.

Farris, J. S., Källersjö, M., Kluge, A. G., and Bult, C. (1994). Testing significance of incongruence. *Cladistics* 10: 315-319.

Felsenstein, J. (1995). "PHYLIP computer package program (version 3.5c)," University of Washington, Seattle.

Garnery, L., Vautrin, D., Cornuet, J. M., and Solignac, M. (1991). Phylogenetic relationships in the genus *Apis* inferred from mitochondrial DNA sequence data. *Apidologie* 22: 87-92.

Harrison, R. G. (1989). Animal mtDNA as a genetic marker in population and evolutionary biology. *Trends Ecol. Evol.* 4: 6-11.

Hillis, D. M., Mable, B. K., Larson, A., Davis, S. K., and Zimmer, E. A. 1996. Nucleic acids IV: sequencing and cloning. *In:* "Molecular Systematics" (D. M. Hillis, C. Moritz, and B. K. Mable, Eds.), pp. 321-381, Sinauer Associates, Inc., Sunderland, Massachusetts.

Howland, D. E., and Hewitt, G. M. (1995). Phylogeny of Coleoptera based on mitochondrial cytochrome oxidase I sequence data. *Insect Mol. Biol.* 4: 203-215.

Huelsenbeck, J. P., and Hillis, D. M. (1993). Success of phylogenetic methods in the four-taxa case. *Syst. Biol.* 42: 267-264.

Huelsenbeck, J. P., Bull, J. J., and C. W. Cunningham. (1996). Combining data in phylogenetic analysis. *Trends Ecol. Evol.* 11: 152-157.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16: 111-120.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X. and Wilson, A. C. (1989). Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. USA* 86: 6196-6200.

Koulianos, S. (1999). Phylogenetics of the bumblebee subgenus *Pyrobombus* (Hymenoptera: Apidae) inferred from mitochondrial cytochrome B and cytochrome oxidase I sequences. *Ann. Entomol. Soc. Amer.* 92: 355-358.

Lui, H., and Beckenbach, A. T. (1992). Evolution of the mitochondrial cytochrome oxidase II gene among 10 orders of insects. *Mol. Phylogenet. Evol.* 1: 41-52.

Lunt, D. H., Zhang, D.-X., Szymura, J. M., and Hewitt, G. M. (1996). The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. *Insect Mol. Biol.* 5: 153-165.

Michener, C. D. (1944). Comparative external morphology, phylogeny, and a classification of the bees (Hymenoptera). *Bull. Amer. Mus. Nat. Hist.* 82: 157-326.

Moritz, C., Dowling, T.E., and Brown, W. M. (1987). Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Annu. Rev. Ecol. Syst.* 18: 269-292.

Nei, M. (1987). "Molecular Evolutionary Genetics," Columbia Univ. Press, New York.

Olmstead, R. G., and Sweere, J. A. (1994). Combining data in phylogenetic systematics: an empirical approach using three molecular data sets in the Solanaceae. *Syst. Biol.* 43: 467-481.

Owen, R. E. (1985). Difficulties with the interpretation of patterns of genetic variation in the eusocial Hymenoptera. *Evolution* 39: 201.

Owen, R. E., and Plowright, R. C. (1980). Abdominal pile color dimorphism in the bumble bee, *Bombus melanopygus*. *J. Hered.* 71: 241-247.

Owen, R. E., Mydynski, L. J., Packer, L., and McCorquodale, D. B. (1992). Allozyme variation in bumblebees (Hymenoptera: Apidae). *Biochem. Genet.* 30: 443-453.

Pamilo, P., Varvio-Aho, S. L., and Pekkarinen, A. (1978). Low enzyme variability in Hymenoptera as a consequence of haplodiploidy. *Hereditas* 88: 93.

Pamilo, P., Varvio-Aho, S. L., and Pekkarinen, A. (1984). Genetic variation in bumblebees (*Bombus, Psithyrus*) and putative sibling species of *Bombus lucorum*. *Hereditas* 101: 245.

Pedersen, B. V. (1996). A phylogenetic analysis of cuckoo bumblebees (*Psithyrus*, Lepeletire) and bumblebees (*Bombus*, Latreille) inferred from sequences of the mitochondrial gene cytochrome oxidase I. *Mol. Phylogenet. Evol.* 5: 289-297.

Plowright, R. C. and Stephen, W. P. (1973). A numerical taxonomic analysis of the evolutionary relationships of *Bombus* and *Psithyrus* (Apidae: Hymenoptera). *Can. Entomol.* 105: 733-743.

Poole, R. W. (1996). "Nomina Insecta Nearctica [sic], a checklist of the insects of North America. Volume 2: Hymenoptera, Mecoptera, Megaloptera, Neuroptera, Rhaphidioptera, Tricoptera," Rockville, Maryland.

Randi, E. (1996). A mitochondrial cytochrome B phylogeny of the *Alectoris* partridges. *Mol. Phylogenet. Evol.* 6: 214-227.

Richards, O. W. (1968). The subgeneric divisions of the genus *Bombus* Latreille (Hymenoptera: Apidae). *Bull. Br. Mus. (Nat. Hist.) Entomol.* 22: 209-276.

Rodrigo, A. G., Kelly-Borges, M., Bergquist, P. R., and Bergquist, P. L. (1993). A randomisation test of the null hypothesis that two cladograms are simple estimates of a parametric phylogenetic tree. *N. Z. J. Bot.* 31: 257-268.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstruction phylogenetic trees. *Mol. Biol. Evol.* 4: 406-425.

Sambrook, J., Fritsch, E. and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press, New York.

Simon, C. (1991). Molecular systematics at the species boundary: exploiting conserved and variable regions of the mitochondrial genome of animals via direct sequencing from amplified DNA. *In* "Molecular Techniques in Taxonomy" (G. M. Hewitt, A. W. B. Johnston, and J. P. W. Young, Eds.), pp.33-71, Springer Verlag, Berlin.

Smith, J. J. and Bush, G. L. (1997). Phylogeny of the genus *Rhagoletis* (Diptera: Tephritidae) inferred from DNA sequences of mitochondrial cytochrome oxidase II. *Mol. Phylogenet. Evol.* 7: 33-43.

Smithz, J., and Moritz, R. F. A. (1996). Molecular phylogeny of Vespidae (Hymenoptera) and the evolution of sociality in wasps. *Mol. Phylogenet. Evol.* 9: 183-191.

Snyder, T. P. (1974). Lack of allozyme variability in three bee species. *Evolution* 28: 687.

Staden, R., Bonfield, J., and Kathryn, B. (1997). "Staden computer package (version 0.99.2)," Medical Research Council, Laboratory of Molecular Biology, Cambridge, UK.

Stephen, W. P. (1957). Bumblebees of Western America, *Tech. Bull. Oreg. Agric. Exp. Stn.* 40.

Swofford, D. L. (1993). "PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1," Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

Swofford, D. L. (1999). "PAUP*4: Phylogenetic Analysis Using Parsimony, Version 4d65," Sinauer Associated, Inc. Publishers, Massachusetts.

Szymura, J. M., Lunt, D. H., and Hewitt, G. M. (1996). The sequence and structure of the meadow grasshopper (*Chorthippus parallelus*) mitochondrial srRNA, ND2, COI, COII, ATPase8 and 9 tRNA genes. *Insect Mol. Biol.* 5: 127-139.

Thorp, R. W., Horning, D. S. Jr., and Dunning, L. L. (1983). Bumblebees and cuckoo bumblebees of California. *Bull. Calif. Insect Surv.* 23.

Vogler, A. P., and Pearson, D. L. (1996). A molecular phylogeny of the tiger beetles (Cicindelidae): congruence of mitochondrial and nuclear rDNA data sets. *Mol. Phylogenet. Evol.*6: 321-338.

Williams, P. H. (1985). A preliminary cladistic investigation of relationships among the bumble bees (Hymenoptera, Apidae). *Syst. Entomol.* 10 : 239-255.

Williams, P. H. (1994). Phylogenetic relationships among bumble bees (*Bombus* Latr.): a reappraisal of morphological evidence. *Syst. Entomol.* 19: 327-344.

Williams, P. H. (1998). An annotated checklist of bumblebees with an analysis of patterns of description (Hymenoptera: Apidae, Bombini). *Bull. Br. Mus. (Nat. Hist.) Entomol.* 67: 79-152.

Willis, L. G., Winston, M. L., and Honda, B. M. (1992). Phylogenetic relationships in the honeybee (Genus *Apis*) as determined by the sequence of the cytochrome oxidase II region of mitochondrial DNA. *Mol. Phylogenet. Evol.* 1: 169-178.

Wisconsin Package version 9.0. (1996). "Genetics Computer Group (GCG)," Madison, WI.

Wolstenholme, D. R. (1992). Animal mitochondrial DNA: structure and evolution. *Int. Rev. Cytol.* 141: 173-216.

CHAPTER 4

PHYLOGENETIC RELATIONSHIPS OF THE BUMBLEBEES (APIDAE: HYMENOPTERA) IN THE PACIFIC NORTHWEST OF AMERICA BASED ON MITOCHONDRIAL CYTOCHROME OXIDASE II GENE SEQUENCES

Weerathep Pongprasert, Dennis M. Burgett, Katharine G. Field
and William P. Stephen.

ABSTRACT

Systematic studies of bumblebees have been based on morphological characters such as color pattern, wing venation, and male genitalia, and many classification systems have been proposed. However, these classifications are controversial because of the highly polymorphic nature of color pattern, size, and seasonality among bumblebee species. The aim of this study is to understand the relationships of bumblebees in the Pacific Northwest (PNW) by phylogenetic analysis inferred from nucleotide-sequencing data of the mitochondrial cytochrome oxidase II gene (COII). Because of unique structural alterations in sequence regions that evolve corresponding to species differentiation, the COII gene provides a significant phylogenetic signal for understanding the relationships of bumblebees in the PNW, at the generic, specific, subspecific and population levels.

Psithyrus forms a monophyletic sister group with *Bombus* and significant evidence supports the retention of its generic status. Classification of the genus *Bombus* was most closely related to the traditional system as redefined by Stephen (1957), Plowright and Stephen (1973), and Thorp *et al.* (1983). The phylogenetic analyses clarified the relationships of bumblebees in the PNW not only at the species level, but also in many subspecies and polymorphic species such as *B. occidentalis*, *B. mixtus*, *B. bifarius nearcticus*, *B. sitkensis*, and *B. californicus* F. Smith.

INTRODUCTION

All bees diverged from a wasp ancestor approximately 100 million years ago (Michener and Grimaldi, 1988), and were associated with the dominance of angiosperms during the Cretaceous. The development of pollen-collecting structures such as plumose hair, broadened hind legs for pollen collection, mouthpart modification and also behavior, support placing bees in one superfamily, Apoidea (Order: Hymenoptera) (Winston, 1987), with 10 or 11 families (Michener, 1974). Bumblebees are placed in the family Apidae (Bombinae: Bombini), along with honey bees (Apinae: Apini), orchid bees (Bombinae: Euglossini), and stingless bees (Meliponinae) (Kimsey, 1984).

More than 300 species of bumblebees worldwide have been identified based on morphological characters. In Western America, bumblebees were arranged by Stephen (1957) into 3 sections, 7 subgenera, and 33 species. Conventional taxonomy classified bumblebees into 1 tribe (Bombini), 2 genera (*Bombus* and *Psithyrus*), and 35 subgenera (Richards, 1968). Milliron (1971, 1973a, 1973b) proposed dividing bumblebees into 2 tribes (Bombini and Psithyrini). Bombini were classified into 3 genera and 5 subgenera. However, in a new approach by Williams (1994), bumblebees were classified only in one tribe, (Bombini), one genus (*Bombus*), and 38 subgenera (Table 4.1).

Systematic studies of bumblebees have been based on morphological characters including color patterns (Stephen, 1957; Thorp *et al.,* 1983), that are expressed by either polygenic or single-gene control (Owen and Plowright, 1980;

Table 4.1 Three major revisions & classifications of the genera *Bombus* and *Psithyrus*

Method	Tribe	Genus	#Subgenus	#Group
Richards (1968)	Bombini	*Bombus*	35	-
		Psithyrus	-	
Milliron (1971-1973)	Bombini	*Bombus*	1	1
		Megabombus	2	6
		Pyrobombus	2	16
		Psithyrus	-	
Williams (1994)	Bombini	*Bombus*	38*	-

*Including *Psithyrus*

Owen and Plowright, 1988). Morphology frequently allows instantant identification of bumblebee species and does not require as much experience or sophisticated equipment. However, the high variation in color pattern among bumblebee species (Owen and Plowright, 1988), size (Sutcliffe and Plowright, 1988), and seasonality (Stephen, 1957) have made their classification difficult. The polymorphic color patterns of bumblebees in the PNW is confusing: not only do some species resemble each other, but also recently diverged species have intermediate forms. For example, *B. flavifrons*, *B. sitkensis*, *B. californicus*, and *B. caliginosus* require the expertise of a professional entomologist working on bumblebee taxonomy in order to differentiate species (Heinrich, 1994). Many more morphological characters such as wing venation, male genitalia, and male antennae have been applied to classify bumblebees (Stephen, 1957; Plowright and Stephen, 1973), and new classification systems were proposed (Milliron, 1971; Milliron, 1973a; Milliron, 1973b; Williams, 1985). But the status of most taxonomic categories from the generic to subspecific levels is still unclear and classification varies from author

to author. For example, the monophyletic or polyphyletic status of *Psithyrus* is unclear and the specific status of many pairs such *B. fervidus* (F.) and *B. californicus*, *B. pennsylvanicus* De Geer and *B. sonorus* Say, *B. terricola* and *B. occidentalis* remains unresolved (Williams, 1998).

Phylogenetic analysis provides an alternative method to clarify relationships among species and higher taxa of bumblebees; however, most available phylogenetic trees are based on morphological and behavioral characters (e.g. Hobbs, 1964; Milliron, 1971; Williams, 1994) and some subgeneric systems do not fit well in the estimated phylogeny (Williams, 1991). Recently, cladistic relationships of bumblebees based on morphological characters were reconstructed, resulting in the reduction of the genus *Psithyrus* to subgeneric rank within the genus *Bombus* (Williams, 1994). Phylogenetic relationships of bumblebees in the PNW have been derived from isozyme data (Stephen and Cheldelin, 1973) and by numerical taxonomic analysis based on wing venation (Plowright and Stephen, 1973).

More recently, molecular techniques have been developed with high potential to clarify this confusion. Many techniques in molecular biology, such as PCR, PCR-RFLP, PCR-RAPD, AFLP and DNA sequencing can be applied to either genomic DNA or mitochondrial DNA. These techniques provide essential information for study at the genetic level to explain genetic diversity, population structure, species identification, subspecies identification, biotype identification,

and phylogenetic reconstruction of many organisms (Hoy, 1994) including bumblebees.

The aim of this study is to explore the relationships of bumblebees in the PNW via phylogenetic analysis using nucleotide-sequencing data from the mitochondrial cytochrome oxidase II gene.

The mitochondrial DNA gene was chosen because it is small, measuring approximately 16kb in length, maternally inherited, without recombination or heterogeneity. It generally codes for 13 proteins, 22 tRNAs, 2 ribosomal subunits, and a control region. Introns are generally absent and the gene order is often conserved between taxa (Brown, 1985). These characteristics have led mitochondrial DNA to be used as a tool for answering important systematic questions about interspecific variation and phylogenetic relationships in a wide range of animal groups.

The mitochondrial cytochrome oxidase II gene (COII) was used based on the success of previous studies in many insects such as butterflies (Brower, 1994), tiger beetles (Vogler and Pearson, 1996), and honey bees (Willis *et al.*, 1992; Garnery *et al.*, 1991). Moreover, in the two previous chapters (2 and 3), only a part of the COII gene was used, but it provided significant information to explain the relationship of two bumblebee subgenera, *Bombus* and *Pyrobombus*. In this study, we extended the sequence length to cover the entire COII gene in order to get more information and better branch support values.

Phylogenetic trees reconstructed from the COII gene by using the neighbor-joining method with Kimura 2-parameter and Log-Det transformation and the maximum parsimony method explained nicely the relationship between genera (*Bombus* and *Psithyrus*), subgenera and species of bumblebee in the PNW. Moreover, the results provide guidance for future studies at the population level of many species such as *B. mixtus*, *B. bifarius nearcticus*, and *B. occidentalis*.

MATERIALS AND METHODS

Bumblebee Specimen Collection.

Adult bumblebees of 30 species in the genera *Bombus* and *Psithyrus* were collected from California, Oregon, Washington, Idaho, British Columbia, Alberta, Yukon, and Alaska during 1995-1999. *Bombus impatiens* was obtained from Alabama from Dr. James Cane. *Bombus terrestris* and *B. lapidarius* (L.) were sent from Germany by Professor Burkhart Schricker. *Bombus sonorus* and *Bombus crotchii* Cresson were sent from California by Professor R. W. Thorp. The honey bee, *Apis mellifera*, and the carpenter bee, *Xylocopa* (Nyctomelitta) *tranquebarica* (F.), collected from Chiengmai, Thailand were desinated as outgroups. The specimens were kept either at -80 °C or in 100% alcohol to preserve genetic material until use (Table 4.2). Two specimens of *Apis mellifera* were used as positive control to compared the COII sequence result from the PCR with sequence retrieved from the GenBank, since the PCR amplifications for this region required

very high concentration of magnesium. Magnesium was required to increase the affinity between the *Taq*-DNA polymerase and the DNA template; our concern was that the high amounts used could cause errors in the amplification reaction.

Table 4.2 Subgenus name and species name of bumblebees and outgroup, location, and number of specimens used in this study.

Subgenus*	Species	location	Code	No. specimens
Bombias	*B. nevadensis*	Hell's Canyon, OR and Fernie, British Columbia	NEV	2
Fervidobombus	*B. fervidus*	Hell's Canyon and Redmond, OR	FER	2
	B. californicus "intermediate"	Hell's Canyon, OR	CALI	2
	B. californicus consanguineus	Fernie, British Columbia	CALO	1
	B. californicus californicus	Willamette Valley, OR	CALA	1
	B. sonorus	California	SON	2
	B. pennsylvanicus	Alabama	PEN	2
Psithyrus	*P. suckleyi* Greene	Golden, British Columbia	SUC	1
	P. insularis	Coos Bay, and Newport, OR	INS	2
Subterraneobombus	*B. appositus*	Madras and Albany, OR	APP	2
Pyrobombus	*B. bifarius nearcticus type M*	Mt. Hood, OR	BNEM	4
	B. bifarius nearcticus type H	Hell's Canyon and Eagle Cap, OR	BNEH	4
	B. melanopygus	Coos Bay, Willamette Valley, and Mt Hood, OR	MEL	3
	B. mixtus type C	Willamette Valley, OR, Golden, British Columbia, and Glacier National Park, Alberta	MXC	4
	B. mixtus type E	Three Sisters, Crater Lake, Mt. Iron, OR	MXE	3
	B. caliginosus	Fort Stephen, and Cannon Beach, OR	CLG	2
	B. sitkensis type B	Willamette Valley, and Mt. Hood, OR	SITB	2
	B. sitkensis type N	Willamette Valley, and Mt. Hood, OR	SITN	2
	B. centralis	Hell's Canyon, OR	CEN	1
	B. sylvicola	Kamloops, Golden, and Fernie, British Columbia	SYL	3
	B. ternarius	Kananaskis, Alberta and Golden, British Columbia	TRN	2

Table 4.2 (continued)

Subgenus*	Species	location	Code	No. specimens
Pyrobombus (continued)	*B. vagans vagans*	Hope and Kamloops, British Columbia	VGN	2
	B. vosnesenskii	Corvallis, Crater Lake and Newport, OR	VOS	3
	B. flavifrons flavifrons	Hell's Canyon, OR and Kananaskis, Alberta	FFL	2
	B. impatiens	Alabama	IMP	1
	B. huntii	Klamath Fall, OR, Spokane, WA, Fernie, British Columbia	HUN	3
Bombus	*B. terricola*	Calgary and Longview, Alberta	TRC	2
	B. franklini	Medford, OR	FRK	1
	B. moderatus	Kananaskis, Alberta	MOD	2
	B. occidentalis nigroscutatus type A	Fairbanks, AK, Yukon Territory and North British Columbia	OCNA	3
	B. occidentalis nigroscutatus type C	San Francisco Bay, CA	OCNC	2
	B. occidentalis occidentalis type W	Willamette Valley, and Newport, OR	OCW	2
	B. occidentalis occidentalis type F	Medford, OR	OCF	2
	B. occidentalis occidentalis type I	Hwy 26, Portland, and Hell's Canyon, OR	OCI	2
	B. terrestris	Germany	TRS	1
Crotchiibombus	*B. crotchii*	Madera, CA	CRO	2
Separatobombus	*B. griseocollis*	Corvallis, and Hell's Canyon, OR	GRI	2
	B. morrisoni type O	Bend, OR	MOR O	2
	B. morrisoni type U	Zion, Utah	MOR U	2
Melanobombus	*B. lapidarius*	Germany	LAP	1
Out group	*A. mellifera*	Willamette Valley, and GenBank	APS	3
	X. tranquebarica	Chiengmai, Thailand	CAR	1

* Subgenus classification as proposed by Stephen (1957) and Richards (1968)

Bumblebees DNA Extraction

DNA was isolated from the thorax of single individuals. The bee thorax was ground in liquid nitrogen and transferred to a microtube with 500 µl STE extraction

buffer (0.001 M EDTA, 0.05 M Tris-HCl pH 7.5, 0.1 M NaCl), 75 µl 10% SDS, and 25 µl Proteinase K (10 mg/ml stock solution). The sample was then incubated at 55 C° with gentle shaking for 2 hrs. The suspension was extracted twice with equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1) and twice with equal volumes of chloroform: isoamyl alcohol (24:1). Then the supernatant was precipitated with 1/10 the sample volume of 3 M NaAc and 2.5 times the sample volume of ice cold 100% ethanol. The suspension was pelleted at 12,000 rpm for 15 min, washed twice with 70% ethanol, and dried under vacuum. (Sambrook *et al.*, 1989; Hillis *et al.*, 1996). The final DNA was suspended in 50 µl of double distilled water and stored at –20 C°.

Quantification of Bumblebees DNA.

DNA quality was determined by gel electrophoresis and DNA concentration was measured by absorbance at 260 nm and 280 nm. The purity of each sample was estimated by the ratio of a 260/280.

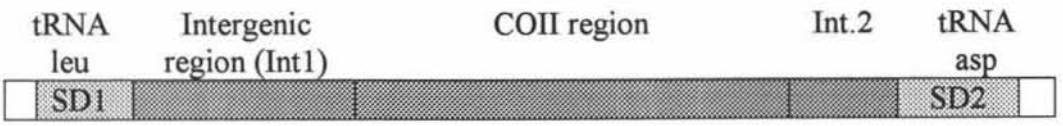
Polymerase Chain Reaction (PCR).

PCR amplifications were performed in 50 µl volume, containing 10 mM Tris/HCl pH 9, 50 mM KCl, 7 mM $MgCl_2$, 1.0% Triton® X-100, 200 µM of each of the four deoxyribonucleotide triphosphates, 0.5 µM of each of two different primers, 2.5 units of *Taq* polymerase, and 25-50 ng of DNA template. Cycle

conditions were as follows: beginning at 94 °C for 3 min, followed by 30 cycles of denaturation at 92 °C for 45 sec, annealing at 41-44 °C for 30 sec, and extension at 65 °C for 3 min. Additional elongation occurred at 65°C for 3 min after the last cycle. Successful amplification was detected by agarose gel electrophoresis stained with ethidium bromide.

Since primers for mitochondrial DNA of bumblebees were not available, the initial PCR primers were designed from *A. mellifera* mitochondrial DNA (Crozier *et al.*, 1989). The forward primer was located at tRNA leucine: SD1, 5'-GGC AGA ATA AGT GCA TTG-3' (Garnery *et al.*, 1991). The reverse primer was located at tRNA aspartic acid: SD2, 5'-GGC CGT TTG ACA AAC TAA TGT TAT-3' (modified from Willis *et al.*, 1992). These primers amplified the target region (Figure 4.1) from all species in this study. The forward primer used to confirm 3' end of sequence were GF1 (5'-CAT CAA TGA TAT TGA TCT TAT-3') and GF2 (5'-TTG ATC TTA TGA ATA TCC AG-3') modified from the reverse primer designated by Garnery *et al.* (1991). The reversed primer used to confirm 5'end of sequence was the ER (3'-GTT CAT GAA TGA ATT ACA TC-5') modified from the primer H1 (Estoup *et al.*, 1996).

Figure 4.1 The target region between $tRNA^{leu}$ and $tRNA^{asp}$ (dark gray) amplified by SD1 and SD2 (light gray) which covers the intergenic region and the entire mitochondrial COII gene.



Sequencing

The PCR products were cleaned with QIAquick PCR purification kit (QIAGEN®, Valencia, CA) according to kit instructions. For each species, both strands were sequenced by the cycle termination method using fluorescent-labeled dideoxyribonucleotide triphosphates. The sequence reaction and the processing of the reaction products for electrophoresis were performed following the instructions for the sequencing kit (ABI PRISM™ Dye Terminator Cycle Sequencing Core Kit, Perkin-Elmer, Norwalk, Conn.). Electrophoresis and data collection were done on an ABI model 377 DNA Sequencer (Perkin-Elmer). The raw sequencing data from both directions were edited using the Staden program (version 0.99.2) (Staden, 1997).

Pairwise sequence Comparison

Sequences composed of intergenic regions and COII genes were compiled, aligned with a pileup program in GCG (v. 10), Wisconsin package program (1998), and re-examined manually. The alignment of the intergenic parts was done with aid of the RNA secondary structure analysis program, foldRNA, and the squiggle drawing program available in GCG (v. 9). The COII coding region was aligned using translated amino acid sequence based on the invertebrate mitochondrial code (Clary and Wolstenholme, 1985). The codon frequencies were analyzed by the CODON FREQUENCY (v. 1) in GCG (v. 10).

Similarity analysis was performed using the multiple comparison option in GCG (v. 10) in order to determine the variation among overall nucleotide bases in the COII gene sequence, as well as among codon positions and amino acid sequences.

Pairwise distances were computed using distance matrices based only on COII nucleotide sequence. Distances were corrected using the Kimura two-parameter method (Kimura, 1980) and Log-Det transformation (Lockhart *et al.*, 1994). Base frequencies and base differences for pairs of sequence (transition and transversion rate) were determined by PAUP*4 (Swofford, 1999).

Phylogenetic Analysis

Distance trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) with Kimura two-parameter corrections and Log-Det tranformation using the neighbor-joining option in PAUP*4 (Swofford, 1999) as well as the bootstrap analysis with 100 replicates. Parsimony trees were constructed and bootstraps analysis with 500 replicates were performed in PAUP*4, using the heuristic search option with equally-weighted characters, 10 random sequence additions, tree-bisection-reconnection branch swapping, mulpars in effect and maxtrees set at 1,000. Then the data were analyzed by weighting with the successive approximation method based on the maximum values of the rescaled consistency indices (Farris, 1969; Farris, 1989).

RESULTS

PCR Product and Sequence Data

Primers SD1 and SD2 recovered the DNA target from all species very well. However, in some species, sequences were not clear at both end sites. They therefore were confirmed by using GF1, GF2, and ER, three internal primers designed specifically from the bumblebee cytochrome oxidase II gene sequences. Two sequences amplified from all *A. mellifera* were identical to the sequences in GenBank (Crozier *et al.*, 1989) confirming the accuracy of the PCR reaction and indicating that the increase of magnesium in the reaction did not induce product error.

The length of PCR product from all species varied from 809 to 1,479 bp (Figure 4.2). This variation occurred mainly at the intergenic region between $tRNA^{leu}$ and the start codon of COII. All bumblebees of the PNW have this intergenic region except species of subgenus *Fervidobombus* Skorikov and *Bombias* Robertson, where $tRNA^{leu}$ was adjacent to the start codon of COII. The variation in this intergenic region mainly occurred from the insertion and deletion of microsatellites $(TA)_n$ and $(TAA)_n$ that make this region unalignable. This region therefore was omitted from the phylogenetic analysis in this study, because of uncertainty of positional homology.

Figure 4.2 The size of cytochrome oxidase II gene PCR products amplified from 40 taxa of bumblebees and 4 outgroups, 3 honeybees and 1 carpenter bee, (a=outgroup, b=*Psithyrus*, c=*Melanobombus*, d=*Separatobombus*, e=*Crotchiibombus*, f=*Fervidobombus*, g=*Subterraneobombus*, h=*Bombias*, i=*Bombus*, j=*Pyrobombus*)

The size of the COII gene among bumblebee species also varied after alignment, which was improved by incorporating amino acid sequences information (Figure 4.3). Most variation occurred at the end of the COII gene, because some amino acid residues were deleted or inserted at this site, causing shifting of the stop codon position in most bumblebee species compared to the outgroup. Deletions occurring inside the COII gene were found in *B. nevadensis* Cresson, which lost one amino acid in the middle of the sequence. All deletions and insertions were adjusted by addition of gaps to make all sequences even in length. The connection between the 3' end of COII and $tRNA^{asp}$ also varied. In *A. mellifera*, the 3' end of the COII gene is directly connected to $tRNA^{asp}$ (Crozier *et al.*, 1989) but the 3' end of the COII gene in all bumblebees in this study overlapped with $tRNA^{asp}$. The final length of the aligned sequences was 702 bp, consisting of 370 variable sites (52.70%) and 275 parsimony informative sites (39.17%). When outgroups were excluded, the percentage of those variable sites was little decreased (Table 4.3). The variable sites occurred mostly at the third codon position, an average of 3.51 and 2.03 times more than the second and first codon position respectively. There were 5.03 and 2.39 times more parsimony informative sites at the third codon position than the second and first codon positions respectively. The overall similarity of COII sequences in this study was 86% (Figure 4.4). However, the second codon was very highly conserved, with 92% similarity, followed by the first codon (88%). The third codon was more variable, having only 78% similarity among sequences (Figure 4.5.1-4.5.3).

```
CAR   attgcaactg gagaaatatt ttttttttcaa aattcaaact ctccttattc tgataactta atttcatttc ataatttagt aataataatt atatctataa tcataattat
APS1  ...t.c..at ..ttt..... .a.a...... g.a.....t. .ata....g. ......t... .......... .....a.... t......... ..tat..... .ttc..cat.
APS2  ...t.c..at ..ttt..... .a.a...... g.a.....t. .ata....g. ......t... .......... .....a.... t......... ..tat..... .ttc..cat.
APSR  ...t.c..at ..ttt..... .a.a...... g.a.....t. .ata....g. ......t... .......... .....a.... t......... ..tat..... .ttc..cat.
SUC   ...t....at ..a.t..... ......c... g.......t. .atac..... a......... .......... ........ac t........a ..tat..... .t..t.cac.
INS   ..at....at ..a.t...c. ..ca..c..g g.....g.t. .attc..... a.....t... .......... ........ac t........a ..tata..t. .t..t.ca..
NEV   ...t.t..at ..a.t....a .a.a...... g....t..t. ..ttc..... a.....tc.. .......... ........ac .........a ....t..... .t....c.t.
PEN   .....t..at ..a.t..... .a.a...... g.......ta .att..... ......t..g .......... ........ac t........a ...gta.... .t..t.c...
SON   .....t..at ..a.t..... .a.a...... g.......t. .att..... ......t... .......... ........ac t........a ...gta.... .t..t.c...
FER   ...t.t..at ..a.t...g. .a.a...... g.......t. .att..... a.....t... .......... ........ac t........a ...gt..... .t..t.c.t.
CALI  ...t.t..at ..a.t...a. .a.a...... g.......t. .att..... a.....t... .......... ........ac t........a ...at...g. .t..t.c.t.
CALO  ...t.t..at ..a.t...a. .a.a...... g.......t. .att..... a.....t... .......... ........ac t........a ...at...g. .t..t.c.t.
CALA  ...t.t..at ..a.t...a. .a.a...... g.......t. .att..... a.....t... .......... ........ac t........a ...at...g. .t..t.c.t.
APP   ...t.t..at ..a.t..... aa.a...... g.......t. ..tt..... a.....t... .......... ........ac .........a ...ata.... .t..ttcat.
LAP   ...t.t..at ..a.t...a. .a.a...... g.......t. ..ttc..... a.....tc.t .......... ........ac .........a ...ata.... .t..t.c.c.
CRO   ...t.t..at ..a.t...a. aa.a..c... g....t..t. .attc..... a.....tc.t ........c. ........ac t........a ...gta.... ....t.c.t.
GRI   ...t.c..at ..a.t...a. .a.a...... g....t..t. .att..... a.....tc.t .......... ........ac t........a ...ata.... .t..t.cct.
MORO  ...t.t..at .ga.t..... aa.a...... g......... .att..... ......tc.t ........c. .c......ac t........a ...ata.... .t..t.c.t.
MORU  ...t.t..at .ga.t..... aa.a...... g......c.. .att..... ......tc.t ........c. .c......ac t........a ...ata.... .t..t.c.t.
IMP   ...t.t..at ..a.t..... aaca...... g....t..t. ..tt.c.... a.....t... .......... ........ac .........a ...ata..t. .t..ttc.t.
BNEM  ...t.t..at ..a.t...a. gaca...... g.......t. .gtt..... a.....t... .......... ........ac t........a ...at...t. .t..ttc.t.
BNEH  ...t.t..at ..a.t...a. gaca...... g......... .ctt..... a.....t... .......... ........ac .........a ...ata.... .t..ttc.t.
VOS   ...t.t..at ..a.t..... .aca...... g.......t. ..tt..... ......t... .......... ........ac .........a ...ata.... .t..tt..t.
TRN   ...t.t..at ..a.t..... .aca...... g.......t. ..ttc..... ......t... .....c.... ....c...ac t........a ...ata.... .t..ttc.t.
HUN   ...t.t..at ..a.t..... .aca...... g.......t. ..ttc..... ......t... .......... ....c...ac t........a ...ata.... .t..ttc.t.
SYL   ...t.t..at ..a.c..... aaca...... g.......t. ..tt..... a.....t... .......... ........ac t........a ...ata.... .t..ttcat.
MEL   ...t....at ..a.c..... aaca...... g.......t. .att..... a.....t... .......... ........ac .........a ...ata.... .t..ttcat.
VGN   ...t.t..at ..a.t..... aaca...... g.......t. ..tt..... a.....tc.t .......... ....c...ac t........a ...ata.... .t..ttc.t.
CLG   ...t.t..at ..a.t...a. aaca...... g.......t. ..tt..... g.....t... .......... ........ac t........a ...ata.... .t..ttc.t.
CEN   ...t.t..at ..a.t..... aaca...... g.......t. ..tt..... a.....t... .......... ........ac t........a ...ata.... .t..ttc.t.
FFL   ...t.t..at ..a.t..... aaca...... g.......t. ..ttc..... a.....t... ........c. ........ac t........a ...ata.... .t..ttc.t.
SITN  ...t.t..at ..a.t..... aaca...... g.......t. ..ttc..... a.....t... ........c. ........ac t........a ...ata.... .t..ttc.t.
SITB  ...t.t..at ..a.t..... aaca...... g.......t. ..ttc..... a.....t... ........c. ........ac t........a ...ata.... .t..ttc.t.
MXE   ...t.t..at ..a.t..... .aca...... g.......t. ..tt..... a.....tc.t .......... .c......ac t........a ...ata.... .t..ttcct.
MXC   ...t.t..at ..a.t..... .aca...... g.......t. ..tt..... a......c.t .......... ........ac t........a ...ata.... .t..ttc.t.
TRS   ...t.t..at ..a.c..... ...a...... g.......t. ..tt..... ......tc.t .......... ........ac .........a ...ata..t. .t..ttcct.
FRK   ...t.t..at ..a.t..... c..a...... g.......t. ..tt...c.. .......c.t .......... .....c..ac .........a ...ata.... .t..t.c.t.
MOD   ...t.t..at ..a.t..... ...a...... g.c.....t. ..tt..... ...c..t... .......... ....c...ac t........a ...ata.... .t..t.c.t.
TRC   ...t.t..at ..a.t..... ...a...... g.......t. ..tt...c.. ......t... .......... ........ac t........a ...ata.... .t..t.cct.
OCNC  ...t.t..at ..a.t..... ...a...... g.......t. ..tt.a.c.. g.....tc.. .......... .....c..ac t........a ...atg.... .t....c.t.
OCNA  ...t.t..at ..a.t..... ...a...... g.......t. ..tt...c.. ......tc.. .......... .....c..ac t........a ...ata.... .t..t.cct.
OCF   ...t.t..at ..a.t..... ...a...... g.......t. ..tt...c.. ......tc.. .......... .....c..ac t........a ...ata.... .t..t.c.t.
OCN   ...t.t..at ..a.t..... ...a...... g.......t. ..tt...c.. ......tc.. .......... .....c..cc t........a ...ata.... .t..t.c.t.
OCW   ...t.t..at ..a.t..... ...a...... g.......t. ..tt...c.. ......tc.. .......... .....c..ac t........a ...ata.... .t..t.c.t.
```

Figure 4.3 The nucleotide sequences of the COII gene from all bumblebees and outgroups, 3 honeybees and one carpenter bee. Dots refer the identity of nucleotide base to the carpenter bee sequence.

```
 CAR  aattatttta attttattca atttatttac aaatttatac acaaatcgat ttttattaaa aaatcacgct attgaaatca tttgaacaat tattccaata cttattctaa
APS1  ..c.g.a.at ...a.t..ag .........t ...caa..t. t.....tt.. .......... ......taa. .........t. .......... .........t a........t
APS2  ..c.g.a.at ...a.t..ag .........t ...caa..t. t.....tt.. .......... ......taa. .........t. .......... .........t a........t
APSR  ..c.g.a.at ...a.t..ag .........t ...caa..t. t.....tt.. .......... ......taa. .........t. .......... .........t a........t
 SUC  t.catca..t t..a.t..ag .c.c.a..ca c..caa..t. tt....tt.a a.......... ......ta.a .........t. .......t.. ...c..tgc. ..a..ct...
 INS  ..catca..t t..a.cc.ag .......att t...aa..t. tt....tt.a .....c.... ......tata .........t. .......t.. ag.a...tc. a.a..c....
 NEV  ..ca.....t t..a.t..tg ....ta.a.a t...aa..tt tt.....t.a a.......... ......ttta .........t. .......tt. a.......... a.....t...
 PEN  ..ca.ga.at t..a....gg ..a.t...tg t...ca..tt tt....tt.a .......... ......ta.. .........t. .......tt. ag........ a.....t...
 SON  ..ca.ga.at t..a....ag ..a.t...tg t...ca..tt tt....tt.a .......... ......ta.. .........t. .......tt. ag........ a.....t...
 FER  ..ca..a..t t..a.t..ag ....ta..tg t...aa...t tt....tt.g .......... ......ta.. .........tg .......tt. a.....t... a.....t...
CALI  ..ca..a..t t..a.t..ag ....ta..tg t...aa...t tt....tt.g .......... ......ta.. .........tg .......tt. a.......... a.....t...
CALO  ..ca..a..t t..a.t..ag ....ta..tg t...aa...t tt....tt.g .......... ......ta.. .........tg .......tt. a.......... a.....t...
CALA  ..ca..a..t t..a.t..ag ....ta..tg t...aa...t tt....tt.g .......... ......ta.. .........tg .......tt. a.......... a.....t...
 APP  ..ca..a..t t..a.t..ag ....t.ca.t ....aa..tt tt....tt.. ca........ ......ta.. .........t. .........t. a.....t... a.....t...
 LAP  ..catca..t t..a....ag ....ta.a.t ....aat.tt tt...ctt.a ca........ ......ta.. ..c....... .......tt. a..c..t... a.....t...
 CRO  ..catca..t t..a.c..tg ....c.a..t c...aat.tt tt.....t.a c......... ......ta.. .........t. .......tt. a..a..t... a....ct...
 GRI  ..catca..t t..a.t..ag ....t.c.tt t...aa...t ct....tt.. ca........ ......ta.c .........tg .......tc. a..a....c. a.....t...
MORO  ..catca..t t.ca.ta.ag ....t.c.tt t...aat..t tt....tt.a .ac..... ......ta.. ..c.....t. .........t. a.......... t.c...t..t
MORU  ..catca..t t.ca.ta.ag ....t.c.tt t...aat..t tt....tt.a .ac..... ......ta.. ..c.....t. .........t. a.......... t.c...t..t
 IMP  ..ca..a..t t..a.ta.ag ....ta..tg t...aat..t tt....tt.a c.c..c.... ......ta.a .........t. ....g..ct. a.c...t... a.....t...
BNEM  ..ca.ca..t t..a.ta.ag ....ta.ata t...aat.t. tt....tt.a c.c..... ......ta.a .........t. .......tt. a.c...t... a.....t...
BNEH  ..ca.c...t t..a.ta.ag ....ta..ta t...aat.tt tt....tt.a c.c..... ......a.. .........t. .c.....tc. a.c...t... a.....t...
 VOS  ..ca.ca..t t..a.ta.ag ....ta.att t...aat.t. tt....tt.a c.c.t.... ......ta.. .........t. .......tc. ..c...t... a.....t...
 TRN  ..ca.ca..t t..a.ta.ag ....ca..ta t...aat... tt....tt.a c.c.t.... ......ta.a .........t. ...c...tt. a.....t... a.....t...
 HUN  ..ca.ca..t t..a.ta.ag ....ca..ta t...aat... tt....tt.a c.c.t.... ......ta.a .........t. .......tt. a.....t... a.....t...
 SYL  ..ca.ca..t t..a.t..ag ....ca.att ....aat.tt tt....tt.a c......... ......ta.a .........t. .......tt. a.c..... a.........
 MEL  ..ca.ca..t t..a.t..ag ....tacatt ....aat.tt tt....tt.a c......... ......ta.a .........t. .......tt. a.c..... a.....t...
 VGN  ..ca..a..t t..a.t..ag ....ta.a.t ....aat..t tt....tt.a c......... ......ta.. .........t. .......tt. a.......... a.....t...
 CLG  ..ca..a..t t..a.t..ag ....ta.a.t ....aat..t tt....tt.a c......... ......ta.. .........t. .........t. a.......... a.....t...
 CEN  ..c...a..t t..a.t.gag ....ca.a.t ....aat..t tt....tt.a c......... ......ta.. .........t. .......tt. a.c..... a.....t...
 FFL  ..ca..a..t t..a.t..ag ....ca.a.t ....aat..t tt....tt.a c......... ......ta.c .........t. .......tt. a.c..... a.....t...
SITN  ..ca..a..t t..a.t..ag ....ca.a.t ....aat..t tt....tt.a c......... ......ta.a .........t. .......tt. a.c..... a.....t...
SITB  ..ca..a..t t..a.t..ag ....ca.a.t ....aat..t tt....tt.a c......... ......ta.a .........t. .......tt. a.c..... a.....t...
 MXE  ..ca.ca..t t..a.ta.ag ....ta.a.t ....aat.tt tt....tt.a c......... ...c..ta.. .......... .......tt. a.c...t... a.....t...
 MXC  ..ca.ca..t t..a.ta.ag ....ta.a.t ....aat.tt tt....tt.a c......... ...c..ta.. .......... ....g..tc. a.c...t... a.....t...
 TRS  ..c...a..t t.ca.ta.ag ....t.ca.t ....aat.tt tt....tt.a a.c.t..... ......ta.. .........t. .......tt. a.c...t... a.....t...
 FRK  ..ca..a..t t..a.ta.ag .c..t.catt ....aat.t. ctt....tta a.c.t..... ......ta.. .........t. .c.....tt. a.ca..... a.....t...
 MOD  ..ca..a.at t..a.ta.tg ....t.catt ....ac..tt ct.....t.a a.c.t..... ...c..ta.. .........t. .c.....tt. a..a.at... a.....t...
 TRC  ..ca..a.at t..a.ta.ag ....t.catt ....aat.t. tt.....tta a.c.t..... ...c..ta.. .........t. .......tt. a.ca..t... a.....t...
OCNC  ..ca.ca.at t..a..a.ag ....t.catt ....aat.t. ct.....tta a.c.t..... ......ta.. ..c.....t. .......tt. a.....c... a.....t...
OCNA  ..ca.ca.at t..a.ta.ag ....t.catt ....aat.t. tt.....tta a.c.t..... ......ta.. .........t. .......tt. a.ca..t... a.....t...
 OCF  ..ca.ca.at t..a.tacag ....t.catt ....aat.t. tt.....tta a.c.t..... ......ta.. .........t. .......tt. a..a..t... a.....t...
 OCN  ..ca.ca.at t..a.ta.ag ....t.catt ....aat.t. tt.....tta a.c.t..... ......ta.. .........t. .......tt. a..a..t... a.....t...
 OCW  ..ca.ca.at t..a.ta.ag ....t.catt ....aat.t. tt.....tta a.c.t..... ......ta.. .........t. .......tt. a..a..t... a.....t...
```

Figure 4.3 (continued)

```
 CAR  tatttatttg ttttccttca ttaaaaattt tatattatat tgatgaaatt ttaaatccaa ttttttcaat taaatctatt ggacaccaat gatattgatc atatgaatat
APS1  ..a..... ......a... .......... ......ta.. .......... g.......tt .......... ......a... ..t..t.... .......... ..........
APS2  ..a..... ......a... .......... ......ta.. .......... g.......tt .......... ......a... ..t..t.... .......... ..........
APSR  ..a..... ......a... .......... ......ta.. .......... g.......tt .......... ......a... ..t..t.... .......... ..........
 SUC  .ta..... ......a... .......... .......... .......... .........t ..a.aa.... ....a.a..a ..c..t.... .......... t.........
 INS  .c..... ......a... .......... .......c.. c......... c.........t ..a.aa.t.. ....a.a..a ..c..... ...t...... t.........
 NEV  .ta.c..c.. .........t .......... ....c..... .......... ........tt a......... ....g.a..a .....t.... .......... t.........
 PEN  ..a..... .........t .......... .......... .......... ........tt a......... ....g..... .....t.... .......... t.........
 SON  ..a..... .........t .......... .......... .......... ........tt a......... ....g..... ..g..t.... .......... t.........
 FER  ..a..... .........t .......... .......... .......... a.......tt a......... ....g..... ..t..t.... .......... t.........
CALI  ..a..... .........t .......... .......... .......... a.......tt a......... ....g..... ..t..t.... .......... t.........
CALO  ..a..... .........t .......... .......... .......... a.......tt a......... ....g..... ..t..t.... .......... t........c
CALA  ..a..... .........t .......... .......... .......... a.......tt a......... ....g..... ..t..t.... .......... t.........
 APP  ..a..... .........t .......... .......... .......... a.......tt a......t.. ....g..... ..t..t.... .......... .........c
 LAP  ..a..... c.....a... .........c .t........ .......... a........t a......... ....g..... .....t.... ....c..... ..........
 CRO  ..a..... c..c..a..t c.........c .......... .......... a.......tt a......t.. ....g.a... ..t..t.... ....c..... t.........
 GRI  ..a.c..c.. c.....c... c......... .......... ...c...... a........t a......... ....g..... ..t..t.... ....c..... t.........
MORO  ..a..... ...c..a..c .......... .......c.. .......... .ct......t a......... ....g..... .....t.... .......... t........c
MORU  ..a..... g..c..a..c .......... .......c.. .......... .ct......t a......... ....g..... .....t.... .......... t........c
 IMP  ..a..... ......a..t .......... .......... .......... a.......tt a......t.. ....g..... ..t..t.... .......... t.........
BNEM  ..a..... ......a..t .......... .......... .......... a.......tt a......t.a ....g..... ..c..t.... ..at...... t........c
BNEH  ..a.c...c. ...c..a..t .......... .......... .......... a.c.....tt a......t.. ....g..... ..c..t.... .......... t........c
 VOS  ..a..... ......a..t .......... .......... .......... a.......tt a......t.. ....g..... ..t..t.... .......... t........c
 TRN  ..a....c.. ......a..t .......... .......... c......... a.......tt a......t.. ....g..... ..c..t.... .......... t........c
 HUN  ..a....c.. ......a..t .......... .......... .......... a.......tt a......t.. ....g..... ..c..t.... .......... t........c
 SYL  ..a....c.. c.....a..t .......... .......... .......... a.......tt a......t.. ....g..... ..t..t.... .......... t.........
 MEL  ..a..... ...c..a... .......... .......... .......... a.......tt a......t.. ....g..... ..t..t.... .......... t.........
 VGN  ..a..... ...c..a..t .......... .......... .......... a.......tt a......... ....g..... ..t..t.... .......... ..........
 CLG  ..a..... ...c..a..t .......... .......... .......... a.......tt a......... ....g..... ..t..t.... .......... t.........
 CEN  ..a..... ......a..t .......... .......... .......... a........t a......... ....g..... ..t..t.... .......... t.........
 FFL  ..a..... ......a..t .......... .......... .......... a.......tt a......... ....g..... ..t..t.... .......... t.........
SITN  ..a..... ......a..t .......... .......... .......... a.......tt a......... ....g..... ..t..t.... .......... t.........
SITB  ..a..... ......a..t .......... .......... .......... a.......tt a......... ....g..... ..t..t.... .......... t.........
 MXE  ..a..... ......a..t .........c .......c.. .......... a.......tt a......t.. ....g..... ..t..t.... .......... t.........
 MXC  ..a..... g.....a..t .......... .......... .......... a.......tt a......t.. ....g..... ..t..t.... .......... ..........
 TRS  ..a..... ...c..a..t .......... .......... .......... a.......tt a......... ...g...... ..t..t.... .......... t.........
 FRK  ..a..... ......a..t .........c .......... .......... a.......tt a....c.... ......a... ..t..t.... .......... t.........
 MOD  ..a..... ......g..t .......... .......... .......... a.......tt a......... ......a... ..t..t.... .......... t.........
 TRC  ..a..... ......a..t .......... .......... .......... a.......tt a......... ......a... ..t..t.... .......... t.........
OCNC  ..a.g.... ...c..a..t .......... .......... .......... a.......tt a......... ......a... ..c..t.... .g........ t.........
OCNA  ..a..... ...c..a..t .......... .......... .......... a.......tt a......... ......a... ..c..t.... .......... t.........
 OCF  ..a..... ...c..a..t .......... .......... .......... a.......tt a......... ......a... ..c..t.... .......... t.........
 OCN  ..a..... ...c..a..t .......... .......... .......... a.......tt a......... ......a... ..t..t.... .g........ t.........
 OCW  ..a..... ...c..a..t .......... .......... .......... a.......tt a......... ......a... ..t..t.... .g........ t.........
```

Figure 4.3 (continued)

```
 CAR  tcagattttt tagaagttga atttgactca tatataatta aaaataatct tattaatctt tttcgactat tagatgttga taatcgaata gttattccta taaaaactgc
APS1  c....a...a ata.ta.... ......t... ......c.a. .tt.....aa .t.a..c.aa .....tt..c ....aac... .......... ..a.....a. ......tcc.
APS2  c....a...a ata.ta.... ......t... ......c.a. .tt.....aa .t.a..c.aa .....tt..c ....aac... .......... ..a.....a. ......tcc.
APSR  c....a...a ata.ta.... ......t... ......c.a. .tt.....aa .t.a..c.aa .....tt..c ....aac... .......... ..a.....a. ......tcc.
 SUC  c....a.... ata.ta.c.. ......a..t ......t.a. .ct.c...ta ..aa....aa ..c...t... ....aaca.. .......t.. a.......at .t....t.ta
 INS  c....a.... .ta.ta.... ......a... ......c.a. .tt....atc a.aa....aa ..c..... ....aaca.. ......tt.. a..g.a..at .t..t.t.ta
 NEV  c.t..a...a atatt---.. ......t... ...t....a. .tt..g.aa. a.a.....aa .....tt... ....aac... .......t.. a........t .t....t.t.
 PEN  c....a...a ata.tta... ......t..t ......t.a. .tt..c.ata ..aag...aa ......t... ....aac... .......t.. a.......at .t...gt.t.
 SON  c....a...a ata.tta... ......t..t ......t.a. gtt..c.ata ..aag...aa ......t... ....aac... .......t.. a.......at .t...gt.t.
 FER  c....a...a ata.tta... ......t..t ......t.a. .tt..g.aaa a.aag...aa ......t... ....aac... c......t.. a.......at .t....t.t.
CALI  c....a...a ata.tta... ......t..t ......t.a. .tt..ggaaa a.aag...aa ......t... ....aac... .......t.. a.......at .t..ggt.t.
CALO  c....a...a ata.tta... ......t..t ......t.a. .tt..ggaaa a.aag...aa ......t... ....aac... .......t.. a.......at .t..ggt.t.
CALA  c....a...a ata.tta... ......t..t ......t.a. .tt..ggaaa a.aag...aa ......t... ....aac... .......t.. a.......at .t..ggt.t.
 APP  c....a...a ata.tt.... ......t... ......t.a. .tt..g.at. a.aag...aa .....t..t. ....aaca.. .......t.. a.......at .t....t.t.
 LAP  c....a...a ata.ttac.. ......t... ......t.a. .tt.c...tc a.......aa .....t..c. ....aac... .......c.. a.......at .t....t.t.
 CRO  c....a...a ata.tta... ......t... ......t.a. .tt....atc at.a....aa ........tc ....aac... .......t.. a.......at .c....t.t.
 GRI  c.t..a...a ata.ctac.. .......... ......t.a. .tt..g.atc at.a....aa .....t..tc ....aac... c......t.. a.......at .t....tca.
MORO  c....a...a ata.tt.... ...c...... ......t.a. .tt.....tc at.a....aa .....g...c ....aac... .......c.. a.c.....at .t....t.t.
MORU  c....a...a ata.tt.... .c.c...... ......t.a. .tt.....tc at.a....aa .....g...c ....aac... .......c.. a.c.....at .t....t.t.
 IMP  c....a...a ata.tta... ......t..t ........a. .tt..g.atc a.......aa ........t. ....aac... .......... a....c..at .t....t.a.
BNEM  c....a...a ata.tta... ......t... ......t.a. .tt..g.gtc a..a....aa ......t... ....aac... .......t.g a.......at .t....tct.
BNEH  c....a...a ata.tta... ......t... ......t.a. .tt..g.gtc a..a....aa ......t... ....aac... .......t.g a.......at .t....tct.
 VOS  c....a...a ata.cta... ......t..t ......t.a. .tt..g.aac a..a....aa ......t... ....aac... .......t.. a....c..at .t....t.t.
 TRN  c....a...a ata.tta... ......t..t ......t.a. .tt..g.aac a.......aa ......t... ....aac... .......t.. a.......at .t....t.t.
 HUN  c....a...a ata.tta... ......t..t ......t.a. .tt..g.aac a.......aa ......t... ....aac... .......t.. a.......at .t....t.t.
 SYL  c....a...a aca.tta... ......t..t ......t.a. .tt..g.atc a.......aa ......t..c ....aac... ......tc.t a.......at .t....t.a.
 MEL  c....a...a ata.tta... ......t..t ......t.a. .ttt.g.at. ........aa ......t... ....aac... ......t... a.......at .t....t.t.
 VGN  c....a..ca ata.tta... ......t..t ......t.a. .tt..g.atc a.....c.aa ........t. ....aac... .........t a.......at .t....t.a.
 CLG  c....a...a ata.tta... ......t..t .....gt.a. .tt..g.atc a.......aa ........t. ....aac... .........t a.......at .t...gt.a.
 CEN  c....a...a ata.tta... ......t..t ......t.a. .tt..g.at. a.......aa ........t. ....aac... .........t a........t .t....t.a.
 FFL  c....a.... ata.tta... ......t..t ......t.a. .tt..g.atc a.......aa ........t. ....aac... .........t a.......at .t....t.a.
SITN  c....a...a ata.tta... ......t..t ......t.a. .tt..g.atc a.......aa ........t. ....aac... .........t a.......at .t....t.a.
SITB  c....a...a ata.tta... ......t..t ......t.a. .tt..g.atc a.......aa ........t. ....aac... .........t a.......at .t....t.a.
 MXE  c....a...a ata.tta... ......t... ......t.a. .tt..g.atc a.......aa ........t. ....aac... ...c...t.. a.......at .t....t.t.
 MXC  c....a...a ata.tta... ......t... ........a. .tt..g.atc a.......aa ........t. ....aac... .......t.. a.......at .t....t.t.
 TRS  c....a...a ata.cta... ......t... ......t.a. .ttg.g.atc a..a....aa ........t. ....aac... .......t.. a.......at .t....t.t.
 FRK  c....a...a ata.tta... ......t... ......t.a. .tt.....tc ...a....aa ..c.....c. ....aac... .......t.. a.......at .t....t.t.
 MOD  c....a...a ata.tta... ......t... ......t.a. .tt..g.atc ........aa ........t. ....aac... .......t.. a.......at .t....t.t.
 TRC  c....a...a ata.tta... ......t... ......t.a. .tt..g.atc ........aa ..c.....t. ....gac... c......t.. a.......at .t....t.t.
OCNC  c....a...a ata.tta... ......t..t ......t.a. .tt..g.atc a.......aa ........t. ....aac... .......t.. a.......at .t....t.t.
OCNA  c....a...a ata.tta... ......t... ......t.a. .tt..g.atc ........aa ........t. ....aac... .......t.. a....c..at .t....t.t.
 OCF  c....a...a ata.tta... ......t... ......t.a. .tt..g.atc ........aa ........t. ....aac... .......t.. a....c..at .t....t.t.
 OCN  c....a...a ata.tta... ......t... ......t.a. .tt..g.atc ........aa ........t. ....aac... .......t.. a....c..at .t....t.t.
 OCW  c....a...a ata.tta... ......t... ......t.a. .tt..g.atc ........aa ........t. ....aac... ...c...t.. a....c..at .t....t.t.
```

Figure 4.3 (continued)

```
 CAR  aatccgatta attactactt ctgcagatgt tattcattca tgagttgttc cttcaatagg agtaaaaatt gatgctgttc caggacgaat taatcaattt aatttattta
APS1  .c.a..t... .....a..a. .aa..... a......... ...aca.... .a..ct.... ta.t...g.. .....a.... .......... .........a ......a...
APS2  .c.a..t... .....a..a. .aa..... a......... ...aca.... .a..ct.... ta.t...g.. .....a.... .......... .........a ......a...
APSR  .c.a..t... .....a..a. .aa..... a......... ...aca.... .a..ct.... ta.t...g.. .....a.... .......... .........a ......a...
 SUC  t.ca...a.. ....t.t... .aat..... a......... ...ac.a... .a...t.... ta.t...g.. .....aa... .......... .........a .gac..a..t
 INS  t..t...a.. g...t.t... .att..... a......... ...ac.a.c. .a...t.... ta.t...g.. .....aa... .......t.. .........a ......a..t
 NEV  .t.a...a.. ....t.t... .ctt..... a......... ...acaa... .a...t.... .a.t...g.. .....a..a. .t.....t.. .........a .........t
 PEN  ...a...a.. ....t.t... .att..... a........t ...acaa... .a...t.... ta.t...g.. .......... .t........ .........a ..........
 SON  ...a...a.. ....t.t... .att..... g......... ...acaa... .a...t.... ta.t...g.. .......... .t........ .........a ..........
 FER  .g.a...a.. ....t.t... .att..... a........t ...acaa... .a...t.... .a.t...g.. .....a.... .t.....t.. .........a ..........
CALI  .g.a...a.g ....t.t... .att..... a........t ...acaa... .a...t.... .a.t...g.. .....a.... .t........ .........a ..........
CALO  ...a...a.g ....t.t... .att..... a........t ...acaa... .a...t.... .a.t...g.. .....a.... .t........ .......c.a ..........
CALA  ...a...a.g ....t.t... .att..... a........t ...acaa... .a...t.... .a.t...g.. .....a.... .t........ .......c.a ..........
 APP  tt.a...a.. ....t.t... .att..... a......... ...acaa... .a..tt.... .a.t...g.. .....a.... ....t..... .........a .........t
 LAP  tt.a...a.. ....t.t... ..at..... a......... ...acaa... .a...t.... .a.t...g.. ........a. .t........ .........a .........t
 CRO  ...a...a.. ..c.t.t... .aat..... a......... ...ac.a... .a...t.... .a.t...g.. ......a.c. .......... .......c.a ........ct
 GRI  ...a...... ...gt.t... .ctt..g.a. ......c... ...ac.a.c. .a...t.... .a.t...g.. .....c.... .t..t..... .........a ...c....ct
MORO  .t.a...c.. ..c..ct.a. .cat..... a......... ...ac.a... .a...t.... g..t...g.. .....ca.c. .......t.. .........a ..........
MORU  .t.a...c.. ..c..ct.a. .cat..... a....c.... ...ac.a... .a...t.... g..t...g.. ..g..ca.c. .......t.. .........a ..........
 IMP  t..a...a.. ....t.t.a. .att..... a......... ...ac.a... .a...t.... .a.t...g.a .....aa... .t..c..... .........a ........ct
BNEM  ...a...a.. ....t.t... .att..... a........c ...acaa... .a...t.... .a.t...g.a .....c.... .c........ c..c...c.a ........ct
BNEH  ...a...a.. ....t.t... .att..... a........c ...acaa... .a...t.... .a.t...g.a .....c.... .c........ c..c...c.a ........ct
 VOS  t..a...a.. ..cgt.t.a. .att..... a......... ...ac.a... .a...t.... .a.t...g.a .....a.... .t..t...g. a.........a .........t
 TRN  t..a...a.. ....t.t.a. .att..... a......... ...ac.a... .a...t.... .a.t...g.a .......... .t..t..... .........a ..........
 HUN  t..a...a.. ....t.t.a. .att..... .......... ...ac.a... .a...t.... .a.t...g.a .......... .t..t..... .........a ..........
 SYL  t..a...a.. ....t.t.a. .aat..... a......... ...acca... .a...t.... .a.t...g.a ......a... .t..t..... .........a .........t
 MEL  t..a...a.. ....t.t.a. .aat..... a......... ...ac.a... .a...t.... .a.t...g.a .....aa... .t........ ...c...c.a .........t
 VGN  t..a...a.. ....t.t.a. ..at..... a.....c..t ...ac.a... .a...t.... ta.t...g.a .....aa... .t..t..... .........a .........t
 CLG  t..a...a.. ....t.t.a. ..at..... a........t ...ac.a... ....tt.... .a.t...g.a .....aa... .t..t..... .........a .........t
 CEN  t..a...a.. ..c.t.t.a. ..at..... a......... ...ac.a... .a...t.... .a.t...g.a .....aa... .c..t..... .........a .........t
 FFL  t..a...a.. ....t...a. ..at..... a..c.....t ...ac.a... .a..ct.... .a.t...g.a .....aa... .t..t..... c.........a .........t
SITN  t..a...a.. ....t.t.a. ..at..... a..c.....t ...ac.a... .a..ct.... .a.t...g.a .....aa... .t..t..... .........a .........t
SITB  t..a...a.. ....t...a. ..at..... a..c.....t ...ac.a... .a..ct.... .a.t...g.a .....aa... .t..t..... .........a .........t
 MXE  ...a...a.. ...gtat... .aa..... a......... ...ac.a... .a..tt.... .a.t...g.. ......a... .t........ .........a ........ct
 MXC  ...a...a.. ....t.t.a. .aag..... a......... ...acaa... .a...t.... ta.t...g.a ..c...a... .t........ .........a ........ct
 TRS  ...a..ta.. ...gtat... ..a..a.... a.....c... ...acaa.c. .....t.... .a.t...g.. .....aa... ...ata.... .......c.a ..ca.....t
 FRK  ...a...a.. ...gtat.a. .aa..... a.....c... ...ac.a... .a...t.... .a.t...g.. .....aa... .t........ .........a .........t
 MOD  ...a...a.. ...gt.t... ..a..... a......... ...ac.a... .a...t.... .a.t...g.. .....aa... .t........ .........a .........t
 TRC  .......a.. ...gtat... .aa..... a......... ...ac.a... .a...t.... .a.t...g.. ......a.c. .t........ .........a ........ct
OCNC  ...a...a.. ...gt.t.a. .aat..... a......... ...acaa... .a...t.... .a.t...g.. ......a.c. .t........ .........a .........t
OCNA  ...a...a.. ...gtat... .aa..... a......... ...ac.a... .a..tt.... .a.t...g.. ......a... .t........ .........a ........ct
 OCF  ...a...a.. ...gtat... .aa..... a......... ...ac.a... .a..tt.... .a.t...g.. ......a... .t........ .........a ........ct
 OCN  ...a...a.. ...gtat... .aa..... a......... ...ac.a... .a..tt.... .a.t...g.. ......a... .t........ .........a ........ct
 OCW  ...a...a.. ...gtat... .aa..... a......... ...ac.a... .a..tt.... .a.t...g.. ......a... .t........ .........a ........ct
```

Figure 4.3 (continued)

```
 CAR  gactacgtcc cggagtttat tttggacaat gttcagaaat ctgtggtata aatcatagtt ttatgccaat tgtattagaa agaacttcat atgattttta aataaattga
APS1  ..aa...... a...a...t. .....t.... .......... t......... ........a. ....a..... .a..a.t... tc........ t.c.a.a..t tt........
APS2  ..aa...... a...a...t. .....t.... .......... t......... ........a. ....a..... .a..a.t... tc........ t.c.a.a..t tt........
APSR  ..aa...... a...a...t. .....t.... .......... t......... ........a. ....a..... .a..a.t... tc........ t.c.a.a..t tt........
 SUC  .tt....a.. a...a...t. .......... ....t..... t.....g... ........a. ....a..t.. .a........ ......aat. ..at.aa..t ct........
 INS  .tt....a.. a...a...tc ..c..t.... ....t..... t......... .....c..a. ....a..t.. .a..c..... .......at. ..atcaa..t c..t......
 NEV  .ta.t..a.. ....t.a... .......... .......... t.....a... ........a. .c..a..... .a.g..... .....a.... ..a..aa..t t.........
 PEN  ..a.t..... a...a..... .....t.... .......... t.....a... ........a. ....a..... .a........ .......... ..a..aa..t tc........
 SON  ..a.t..... a...a..... .....t.... .......... t.....a... ........a. ....a..... .a........ .......... ..a..aa..t tc........
 FER  ..a.t..... a...a..... .....t.... .......... t......... ........a. ....a..... .a.ga..... .......... ..a..aa..t tt........
CALI  ..a.t..... a...a..... .....t.... .......... t......... ........a. ....a..... .a.ga..... .......... ..a..aa..t tt........
CALO  ..a.t..... a...a..... .....t.... .......... t......... ........a. ....a..... .a.ga..... .......... ..a..aa..t tt........
CALA  ..a.t..... a...a..... .....t.... .......... t......... ........a. ....a..... .a.ga..... .......... ..a..aa..t tt........
 APP  .ca.t..a.. t...a..... .......... .......... t.....a... ........a. ....a..t.. .a........ .......tt. ..a..aa..t tt..g.....
 LAP  .ta.c..... t...a.c... .....t.... ....t..... ......a... ........a. ....a..t.. .a..c..... ........t. ..a..a.a.t t.........
 CRO  .ta.c..a.. t...a.c..c .....t.... .c........ t......... ........a. ....a..... ca..c..... .......at. ..a..aca.t ct........
 GRI  .ca.t..a.. t...a..... .....t.... .......... t......... .....c.... ....a..... .a........ .....a.at. ..a..a.a.t t..t......
MORO  .ta.t..a.. ....a.c... .....t.... .......... t.....a... ..c.....a. ....a..... .a........ .......tc. ..a...ca.t ct........
MORU  .ta.t..a.. ....a.c... .....t.... .......... t.....a... ..c.....a. ....a..... .a........ .......tc. ..a...ca.t ct........
 IMP  .ta.t..a.. t...a..... .....t.... .c..t..... t.....a... ........a. ....a..t.. .a........ ........t. ....a..a.t tt........
BNEM  .ta.c..a.. a...a..... .....g.... ....t..... t.....g... .....c..a. ....a..t.. .a........ ........t. ....a..a.t tt.......g
BNEH  .ta.c..a.. a...a..... .....g.... ....t..... t.....g... .....c..a. ....a..t.. .a........ ........t. ....a..a.t tt.......g
 VOS  .ta.t..... a...a..... .....t.... ....t..... t......... .....c..a. ....a..t.. .a........ ........t. ....a.ca.t t.........
 TRN  cta.t..... a...a..... .......... ....t..... t..c..a... ........a. .c..a..t.. .a........ ........t. ....a..a.t t.........
 HUN  cta.t..... a...a..... .......... ....t..... t..c..a... ........a. .c..a..t.. .a........ ........t. ....a..a.t t.........
 SYL  .ta.t..a.. t...a..... .......... .c..c..... ......a... .....c..a. ....a..... .a........ ..t.....t. ....a.ca.t tt........
 MEL  .ta.t..a.. t...a..... .....t.... ....t..... ......a... .....c..a. ....t..... .a........ ........ca .a..a...a.t tt........
 VGN  .ta.t..a.. t...a..... .......... ....t..... t.....a... ........a. ....a..t.. .a........ ..t.....t. ....a..a.t tt........
 CLG  .ta.t..a.. a...a..... .......... ....t..... t......... ........a. ....a..t.. .a........ ..t.....t. ....a....t tt........
 CEN  .ta.t..a.. a...a..... .......... ....t..... t......... ........a. ....a..t.. .a........ ..t.....t. ....a..a.t tt........
 FFL  .ta.t..a.. a...a..... .......... ....t..... t......... ........a. ....a..t.. .a........ ..t.....t. ....a..a.t tt........
SITN  .ta.c..a.. a...a..... .......... ....t..... t......... ........a. ....a..t.. .a........ ..t.....t. ....a..a.t tt........
SITB  .ta.c..a.. a...a..... .......... ....t..... t..c...... ........a. ....a..t.. .a........ ..t.....t. ....a....t tt........
 MXE  .ta.t..... t...a..... .....t.... ....t..... t.....g... ........a. .c..a..t.. .a........ ........t. ....a..a.t ctc.......
 MXC  .ca.t..a.. t...a..... .......... ....t..... t.....g... ........a. .c..a..t.. .a........ .....a..t. ..a....a.t tt........
 TRS  .ta.t..... t...a..... .....t...c ....t..... t.....a... .......... ....a..... .a........ ........t. ....a..a.t ttc....c.g
 FRK  .ta.t..c.. t...a..... .....t.... .......... t..c..a... .......... .c..a..... .a........ ........t. ....a..a.t ttc.......
 MOD  .ta.t..... t...a..... .....t.... ....t..... t.....a... .......... .c..a..... .a........ ........t. ....a..a.t ttc.......
 TRC  .ta.t..... t...a....c .....t.... ....t..... t.....a... .......... ....a..... .a.g..... ........t. ....a..a.t ttc.......
OCNC  .ta.t..a.. t...a..... .....t.... ....t..... t.....g... ........a. .c..a..t.. .a........ ........t. ....a..a.t ctc.......
OCNA  .ta.t..a.. t...a..... .....t.... ....t..... t.....g... .......... ....a..... .a........ ........t. ....a..a.t ctc.......
 OCF  .ta.t..... t...a..... .....t.... ....t..... t.....g... ........a. .c..a..t.. .a........ ........t. ....a..a.t ctc.......
 OCN  .ta.t..... t...a..... .....t.... ....t..... t.....g... ........a. .c..a..t.. .a........ ........t. ....a..a.t ctc.......
 OCW  .ta.t..... t...a..... .....t.... ....t..... t.....g... ........a. .c..a..t.. .a........ ........t. ....a..a.t ctc.......
```

Figure 4.3 (continued)

```
 CAR  attaaaaaaa caaat---at t------taa ---attagtt aa
APS1  g.a..t...c a.---..... c......... aaa....... ..
APS2  g.a..t...c a.---..... c......... aaa....... ..
APSR  g.a..t...c a.---..... c......... aaa....... ..
 SUC  .......... t.---..... c.......g. aaa....... ..
 INS  .......... t.---..... ........g. aaa....... ..
 NEV  ......tc.. a...ctaa.. .tttataa.. aaa....... ..
 PEN  ...g....t. a.---..... .......... aaag...... ..
 SON  ...g....t. a.---..... .......... aaag...... ..
 FER  ........t. a.---..... .......... aaag...... ..
CALI  ........t. a.---..... .......... aaag...... ..
CALO  ........t. a.---..... .......... aaag...... ..
CALA  ........t. a.---..... .......... aaag...... ..
 APP  .......t.. a.---..... .......... aaa....... ..
 LAP  .........t ..---..... ........g. aaa....... ..
 CRO  ........t. a.---..... ........g. aaa....... ..
 GRI  ........t. a.---...-- -ttaatt.g. aaa....... ..
MORO  .......tc. a.---...-- -ttaatt.g. aaa....... ..
MORU  .......tc. a.---...-- -ttaatt.g. aaa....... ..
 IMP  .......... a.---..... attaata.g. aaa....... ..
BNEM  ........t. a.---..... a...att.g. aaa....... ..
BNEH  ........t. a.---..... a...att.g. aaa....... ..
 VOS  ........t. a.---..... ........g. aaa....... ..
 TRN  ........t. a.---..... ........g. aaa....... ..
 HUN  ........t. a.---..... ........g. aaa....... ..
 SYL  .......... a.---..... ........g. aaa....... ..
 MEL  .......... a.---..... ........g. aaa....... ..
 VGN  .......... a.---..... ........g. aaa....... ..
 CLG  .......... a.---....a ........g. aaa....... ..
 CEN  .......... a.---..... ........g. aaa....... ..
 FFL  .......... at---..... ........g. aaa....... ..
SITN  .......... a.---..... ........g. aaa....... ..
SITB  .......... a.---..... ........g. aaa....... ..
 MXE  .......... a.---..... a...ata.g. aaa....... ..
 MXC  .......... t.---..... atat....g. aaa....... ..
 TRS  .......... tt---..... a...ata.g. aaa....... ..
 FRK  .......... a.---..... a...ata.gt aaa....... ..
 MOD  .......... a.---..... a...ata.g. aaat...... ..
 TRC  .......... t.---...t. a...ata.g. aaat...... ..
OCNC  .......... a.---..... a...ata.g. aaat...... ..
OCNA  .......... a.---..... a...ata.g. aaat...... ..
 OCF  .......... a.---..... a...ata.g. aaat...... ..
 OCN  .......... a.---..... a...ata.g. aaat...... ..
 OCW  .......... a.---..... a...ata.g. aaat...... ..
```

Figure 4.3 (continued)

Table 4.3 The constant sites, variable sites, and parsimony informative sites at each codon position, all nucleotide base positions and amino acid residues of the COII gene of bumblebees (outgroup was not included).

	Total	Constant sites	Variable sites	Parsimony informative sites
First codon	234	146	88	61
Second codon	234	183	51	29
Third codon	234	55	179	146
Entire sequence	702	383	319	236
Amino acid sequences	234	131	103	64

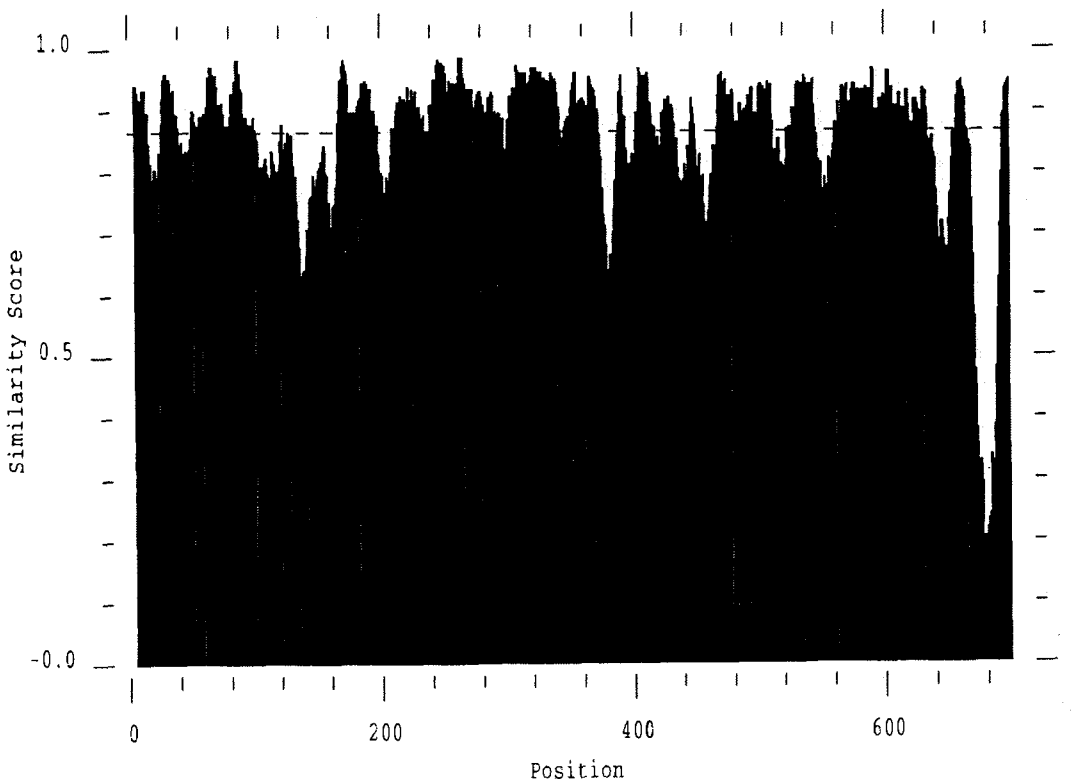


Figure 4.4 The similarity of overall nucleotide sequences of the COII gene. The dashed line represents the average similarity (86%). The window size is 10 basepairs.

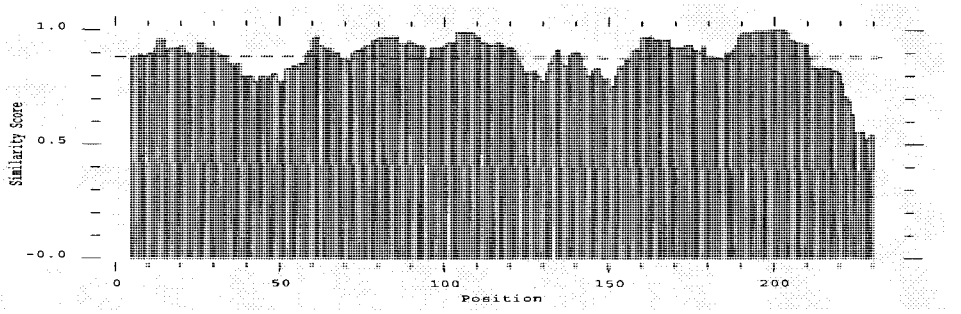


Figure 4.5a The similarity of DNA sequences at the first codon position of the COII gene. The dashed line is the average similarity of the sequences (88%). The window size is10 base pairs.

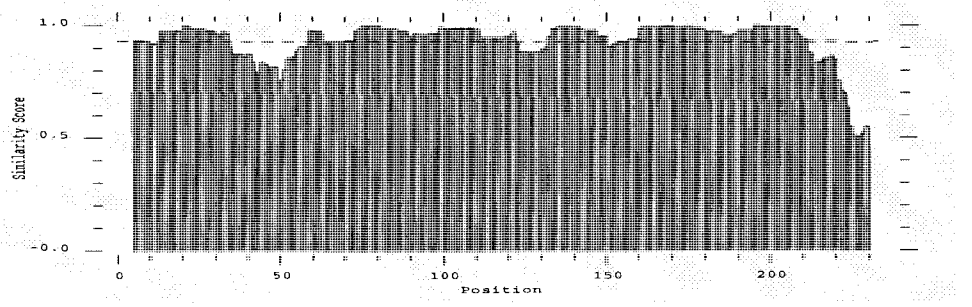


Figure 4.5b The similarity of DNA sequences at the second codon position of the COII gene. The dashed line is the average similarity of the sequences (92%).The window size is 10 base pairs.

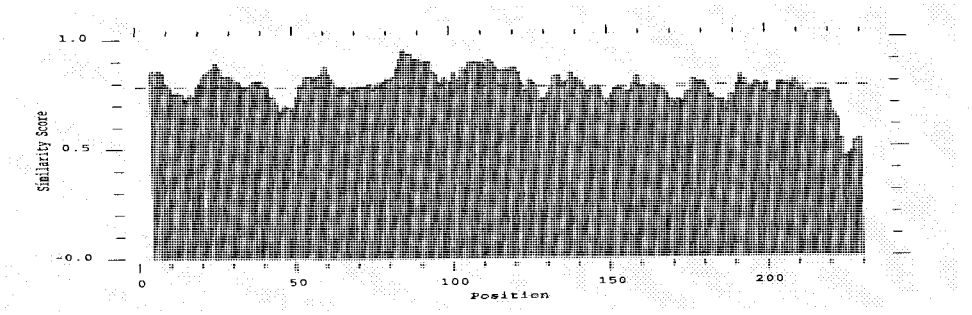


Figure 4.5c The similarity of DNA sequences at the third codon position of the COII gene. The dashed line is the average similarity of the sequences (78%). The window size is 10 base pairs.

The similarity of the amino acid residues was about 84% (Figure 4.6) and the range of variation was 45% to 18% (the high variation at the 3' end was not counted because this variation occurred by insertion of gaps when the sequences were aligned).

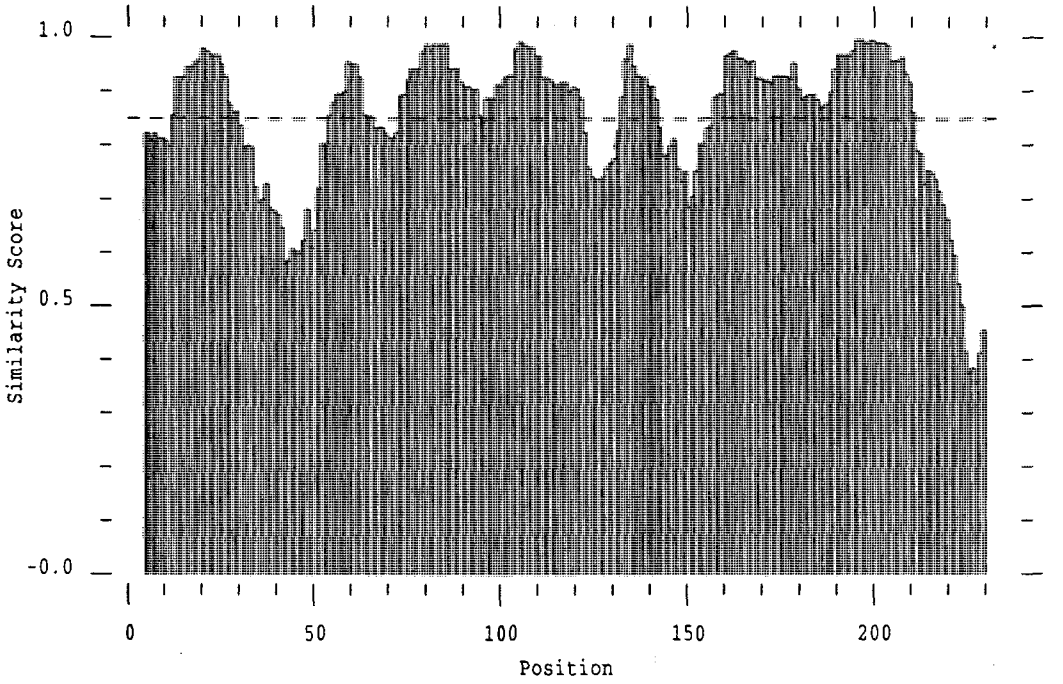


Figure 4.6 The similarity of the amino acid sequences of the COII gene. The dashed line is the average similarity of the sequences (84%) and each bar represents 10 amino acid residues.

The COII gene sequences of bumblebees were A+T rich with very low G+C content (Table 4.4). The base composition at the first and second codon position was similar: Their percent of A+T was very high (1st codon is 75% and 2nd codon is 71%) but the percent G+C was low (1st codon is 24% and 2nd codon is 28%). In the third codon position, the average G content was only 0.7% and average C content was only 3.7% while A+T content was high up to 95.6%. (Table 4.4)

Table 4.4 The percent base composition by codon position and overall (outgroup was not counted)

	A	C	G	T
First codon	41.40	11.88	12.57	34.16
Second codon	30.09	17.21	10.91	41.79
Third codon	43.92	3.75	0.69	51.63
All positions	38.50	10.97	8.10	42.44

The range of transition and transversion rates for pairs of nucleotide bases between bumblebee taxa are presented in Table 4.5. At most positions, CT transitions exceeded AG transitions, and the highest CT transitions occurred at the third codon position. AT transversions exceeded those of AC, CG and GT, and the highest rate was found at the third codon position. GC and GT transversions rarely occurred.

The COII gene was rich in isoleucine residues (2,309 residues) (Table 4.6), and most of them (61%) were translated from the ATT codon. Phenylalanine, asparagene and leucine were also found in very high amounts, translated from TTT,

Table 4.5 The range of transition and transversion rates for the bumblebee COII gene by codon position and overall

	Transition		Transversion			
	AG	CT	AT	AC	CG	GT
First position	0-13	0-11	0-21	0- 4	0-1	0-3
Second position	0- 4	0-10	0-11	0- 6	0-3	0-2
Third position	0- 8	0-37	0-48	0-19	0-1	0-5
All position	0-18	0-49	0-75	0-13	0-5	0-7

Table 4.6 The codon frequencies of COII genes of bumble bee in this study

Amino acid	Codon	Number	Composite X100000	Amino acid	Codon	Number	Composite X100000
Gly	GGG	15	148	Trp	TGG	11	108
Gly	GGA	148	1459	End	TGA	284	2801
Gly	GGT	95	937	Cys	TGT	153	1509
Gly	GGC	10	99	Cys	TGC	14	138
Glu	GAG	4	39	End	TAG	0	0
Glu	GAA	408	4023	End	TAA	57	562
Asp	GAT	342	3372	Tyr	TAT	513	5059
Asp	GAC	13	128	Tyr	TAC	33	325
Val	GTG	1	10	Leu	TTG	4	39
Val	GTA	88	868	Leu	TTA	824	8125
Val	GTT	91	897	Phe	TTT	668	6587
Val	GTC	0	0	Phe	TTC	105	1035
Ala	GCG	0	0	Ser	TCG	3	30
Ala	GCA	29	286	Ser	TCA	435	4290
Ala	GCT	49	483	Ser	TCT	315	3106
Ala	GCC	5	49	Ser	TCC	24	237
Arg	AGG	0	0	Arg	CGG	3	30
Arg	AGA	86	848	Arg	CGA	177	1745
Ser	AGT	61	602	Arg	CGT	42	414
Ser	AGC	0	0	Arg	CGC	1	10
Lys	AAG	4	39	Gln	CAG	1	10
Lys	AAA	390	3846	Gln	CAA	228	2248
Asn	AAT	791	7800	His	CAT	203	2002
Asn	AAC	42	414	His	CAC	19	187
Met	ATG	18	177	Leu	CTG	0	0
Ile	ATA	798	7869	Leu	CTA	82	809
Ile	ATT	1420	14003	Leu	CTT	58	572
Ile	ATC	91	897	Leu	CTC	3	30
Thr	ACG	0	0	Pro	CCG	1	10
Thr	ACA	204	2012	Pro	CCA	245	2416
Thr	ACT	267	2633	Pro	CCT	146	1440
Thr	ACC	10	99	Pro	CCC	9	89

AAT, and TTA respectively. Six codons were not found in the COII gene: GTC, GCG, AGG, AGC, ACG, TAG, and CTG.

Phylogenetic Analysis

Since different phylogenetic analytical methods have different assumptions (Hillis *et al.*, 1994 and Swofford *et al.*, 1996), we used a variety of methods to reconstruct phylogenetic trees. The resulting trees were occasionally not consistent. Therefore, we used congruence between the results to suggest the most reliable tree and explain the different branch patterns of the trees.

We compared our clusters of bumblebees, represented in the resulting phylogenetic trees, to the traditional section and subgeneric system. In this study, we used the system proposed by Stephen (1957), which is similar to that of Frison (1927), and Richards (1968), as a reference to the grouping of bumblebee species from the resulting tree. This in turn was compared to another, rather different system proposed by Milliron (1961).

Distance Analysis.

For neighbor-joining analysis, we performed two types of correction: the Kimura-two parameter correction and Log-Det transformation. Resulting trees were identical except for the placement of *B. o. nigroscutatus* type C in subgenus *Bombus* group and *B. crotchii* in subgenus *Separatobombus* Frison group.

Neighbor joining analysis with the Kimura two-parameter correction inferred a phylogenetic tree that divided bumblebees in this study into many groups (Figure 4.7). The genus *Psithyrus* was monophyletic (100% bootstrap support) and formed a sister group to the genus *Bombus*, which is also monophyletic group (79% bootstrap support). The genus *Bombus* formed two lineages.

The first lineage was composed of two subgenera, *Bombias* and *Fervidobombus*, forming sister groups. The subgenus *Bombias* was represented by only one species, *B. nevadensis*. The subgenus *Fervidobombus* consisted of 4 species clustered together with very high bootstrap value (99%) providing very strong support for this subgenus. *Bombus pennsylvanicus* and *B. sonorus* formed a group with 100% support, as did *B. californicus* and *B. fervidus*. Two subspecies of *B. californicus*, *B. c. californicus* F. Smith and *B. c. consanguineus* Handlirsch, were grouped together and displayed very little difference in branch length. A polymorphic *B. californicus* (intermediate) found at Hell's Canyon, which has been considered to be intermediate between *B. fervidus* and *B. c. consanguineus* based on morphological evidence (Stephen, 1957), clustered tightly with *B. californicus*, with 97% bootstrap support.

The second lineage, supported by a bootstrap value of 60%, was composed of 6 subgenera: *Subterraneobombus* Vogt, *Melanobombus* Dalla Torre, *Separatobombus*, *Crotchiibombus* Franklin, *Pyrobombus*, and *Bombus*.

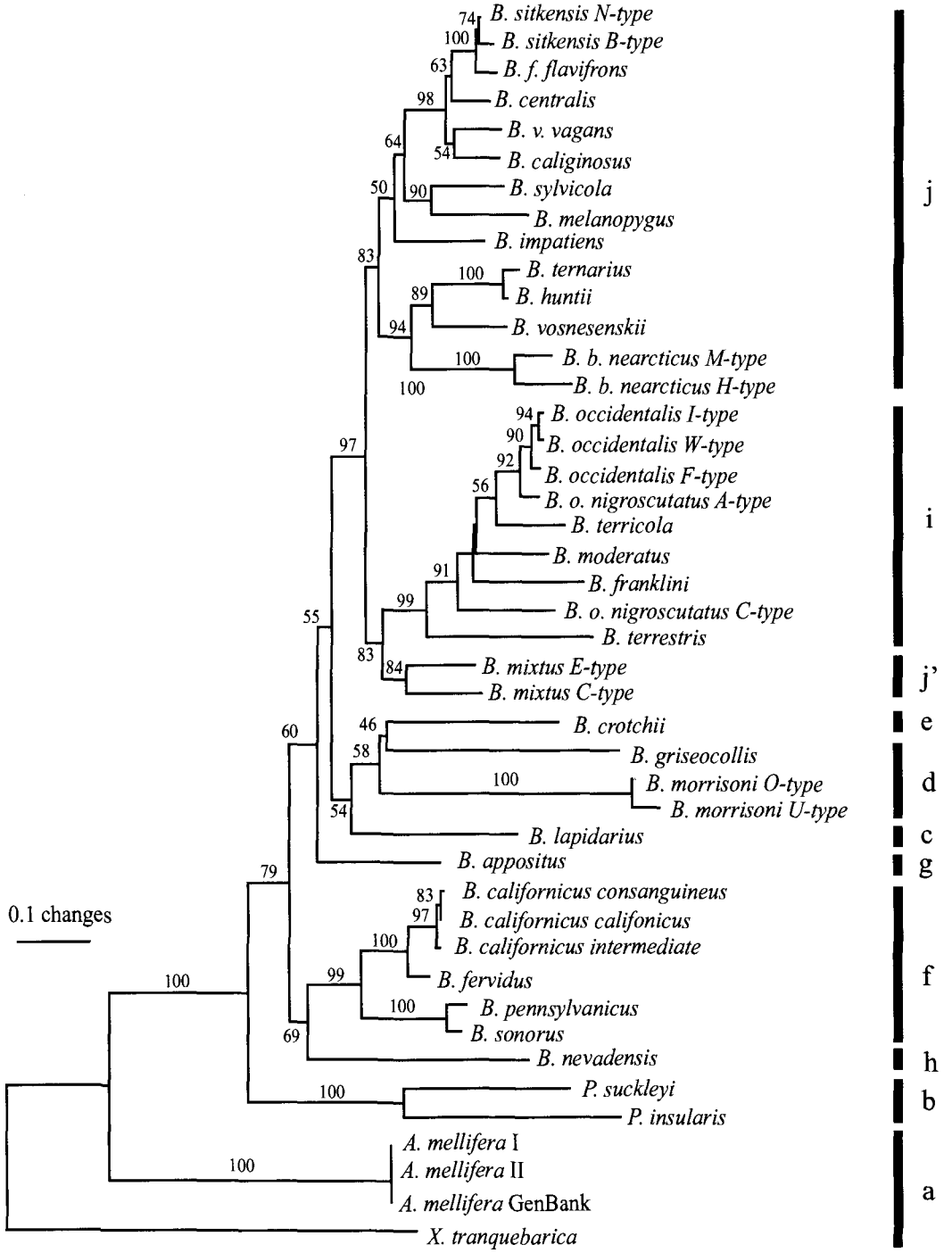


Figure 4.7 A phylogenetic tree reconstructed from COII gene by the neighbor-joining method using the Kimura 2-parameter correction, the numbers on the branch are bootstrap branch support values. (a=outgroup, b=*Psithyrus*, c=*Melanobombus*, d=*Separatobombus*, e=*Crotchiibombus*, f=*Fervidobombus*, g=*Subterraneobombus*, h=*Bombias*, i=*Bombus*, j=*Pyrobombus*)

The subgenus *Subterraneobombus*, represented by *B. appositus* Cresson, formed a separate lineage, while the subgenera *Melanobombus* and *Crotchiibombus*, represented by *B. lapidarius* and *B. crotchii* respectively, clustered with the subgenus *Seperatobombus*, represented by *B. griseocollis* Degeer and *B. morrissoni*. In this cluster, two specimens of *B. morrissoni* were grouped together with 100% bootstrap support, although they were collected from different places, central Oregon and Utah, some differences in their COII sequences were detected.

The subgenus *Bombus* formed a monophyletic group with a high bootstrap value (99%). The Palearctic species, *B. terrestris*, formed a sister group with the Nearctic species of *Bombus* in the PNW with strong bootstrap support (91%). Within the Nearctic species, the cluster of all polymorphic *B. o. occidentalis* (including *B. o. nigroscutatus* type A) was confirmed by a high bootstrap value (92%). *Bombus o. nigroscutatus* type C was not included in this cluster but rather was placed close to *B. terrestris*. However, the branch support values among *B. terricola*, *B. franklini*, and *B. moderatus*, were very low and if this branch were collapsed, *B. o. nigroscutatus* type C would be placed closer to the others *B. occidentalis* subspecies.

Bombus mixtus, classified as a member of the subgenus *Pyrobombus* (Stephen, 1957), clustered with the members of the *Bombus* subgenus, with a relatively high bootstrap support (83%). The other members of subgenus *Pyrobombus* formed a monophyletic lineage (bootstrap 83%). Within the *Pyrobombus* lineage, there were two groups. The first group, with 94% bootstrap

support, consisted of *B. ternarius*, *B. huntii*, *B. vosnesenskii*, and two forms of *B. b. nearcticus*. The second group was supported by low bootstrap value (50% and 64% respectively). Thus this part of the tree was poorly resolved, with the exception of two groups: *Bombus sylvicola* was grouped with *B. melanopygus* with 90% branch support and formed a sister group to the last group, consisting of *B. v. vagans*, *B. caliginosus*, *B. centralis*, *B. f. flavifrons*, and two forms of *B. sitkensis* (98% support).

The neighbor joining method with Log-Det transformation provided an almost identical phylogenetic tree as the neighbor-joining method with Kimura two-parameter correction (Figure 4.8). The exceptions were the lineages for the subgenera *Bombus* and *Separatobombus*. With the log-determinant transformation method, the Nearctic species of subgenus *Bombus* were clustered together and separated from the Palearctic species with 89% branch support. The Nearctic species potentially formed 2 groups. The first group was composed of *B. moderatus*, *B. terricola*, *and B. franklini* with very low branch support. The second group was composed of all of the *B. occidentalis* subspecies, forming a monophyletic group which included *B. o. nigroscutatus* type C (bootstrap support 85%). In the subgenus *Separatobombus*, *B. crotchii* clustered with *B. morrissoni* rather than *B. griseocollis* but very low bootstrap value.

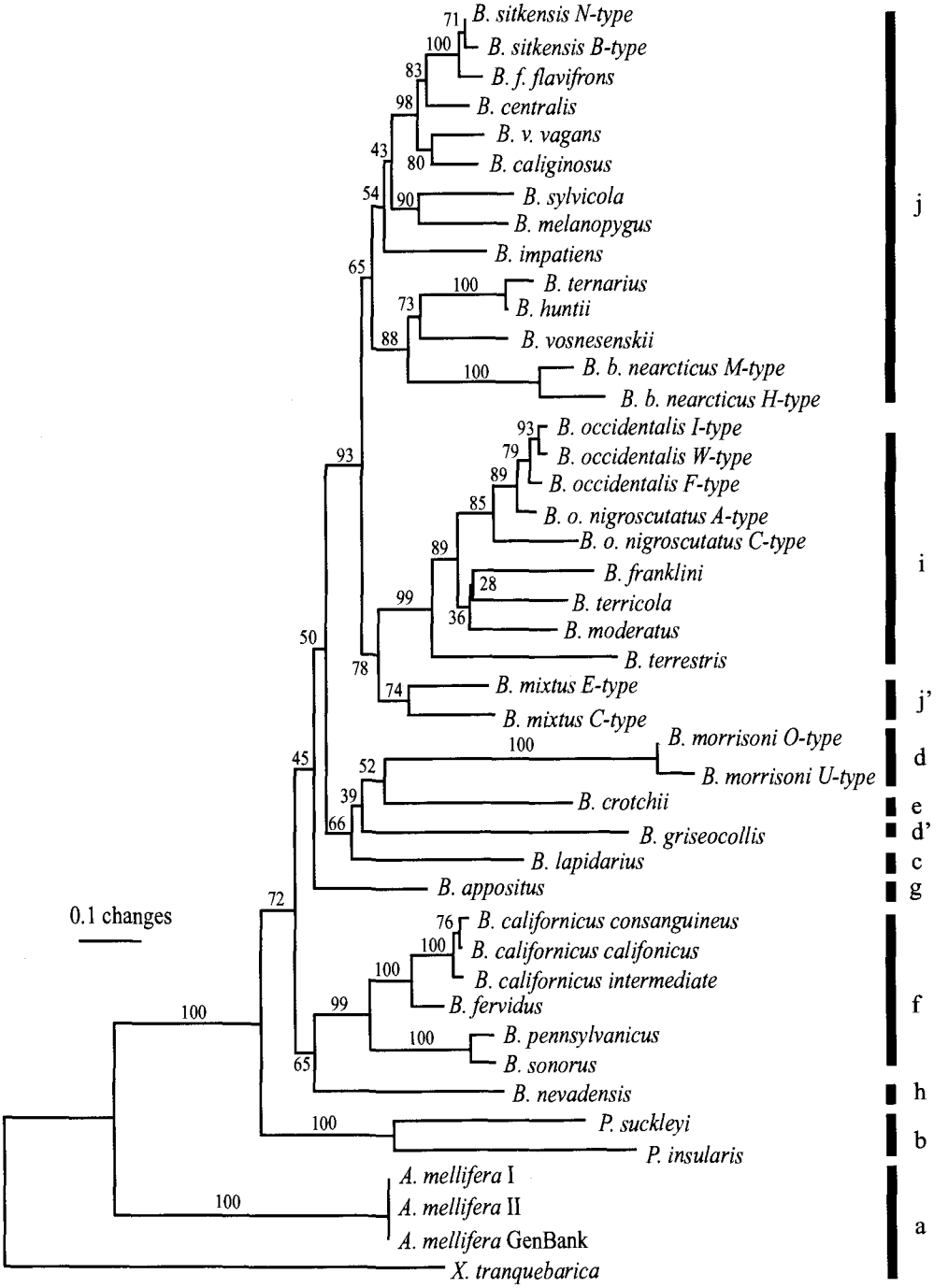


Figure 4.8 A phylogentic tree reconstructed from COII gene by the neighbor-joining method using Log-Det transformation. The numbers on the branch are bootstrap branch support values.(a=outgroup, b=*Psithyrus*, c=*Melanobombus*, d=*Separatobombus*, e=*Crotchiibombus*, f=*Fervidobombus*, g=*Subterraneobombus*, h=*Bombias*, i=*Bombus*, j=*Pyrobombus*)

Maximum Parsimony Analysis.

After 4,855,412 arrangements were tried, thirty-two parsimonious trees with 1,092 steps (CI=0.458, RI=0.679, RC=0.311, HI=0.542) were recoverd. Although the information in some interbranches was lost when those thirty-two parsimonious trees were combined, the remaining branch orders still resulted in a tree that is almost identical to the one reconstructed using the neighbor joining method with Log-Det transformation (Figure 4.9). The *B. mixtus* clade, however, was moved to cluster with subgenus *Pyrobombus*, with very low branch support value.

Although bootstrap analysis of the maximum parsimony tree provided high support for most of the main clusters, the bootstrap values in many branches were much lower than those from the distance analysis (Figure 4.9). After the data set was weighted with a rescaled consistency index, the resulting phylogenetic analysis produced a single tree identical to the consensus tree except for a clearer branch pattern at the end terminal and the very weak clustering of the *B. mixtus* clade. *Bombus mixtus* type E groups with the subgenus *Bombus,* whereas *B. mixtus* type C forms a branch outside the *Bombus* and *Pyrobombus* clade with very low support (data were not shown)

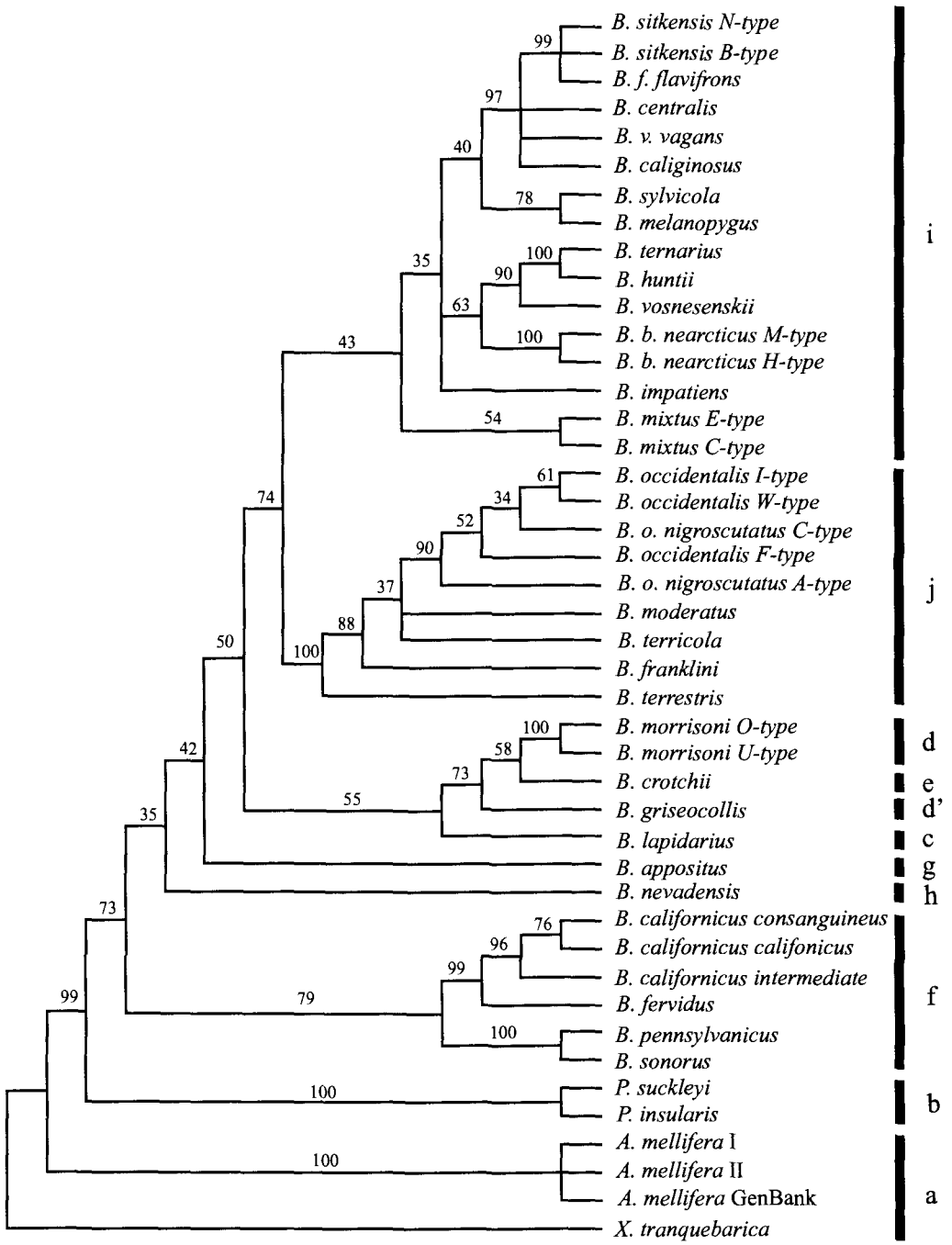


Figure 4.9 A consensus tree of COII gene reconstructed from 32 most parsimonious trees resulting from maximum parsimony analysis, after 4,855,412 tried, with 1,092 tree length, CI=0.458, RI=0.679, RC=0.311, HI=0.542 (a=outgroup, b=*Psithyrus*, c=*Melanobombus*, d=*Separatobombus*, e=*Crotchiibombus*, f=*Fervidobombus*, g=*Subterraneobombus*, h=*Bombias*, i=*Bombus*, j=*Pyrobombus*)

DISCUSSION

Nucleotide Composition Bias.

The COII genes of bumblebees in this study were biased for a high percent AT, with a very low amount of G on the coding strand. The first, second and third codon positions had A, T and T respectively, at the highest-frequency base. Since ATT codes for isoleucine (I), the ATT richness in the COII gene reflected the high amount of isoleucine (1,420 residues) in the data set. Furthermore, the second codon position not only had T as the most frequent nucleotide, but displayed very low nucleotide variation (only 8%). This evidence suggests preferential usage of hydrophobic amino acids such as phenylalanine (F), isoleucine (I), leucine (L), methionine (M), and valine (V). However, since an average of 30% of second codon positions were A's, hydrophilic amino acids such as lysine (AAA) and uncharged amino acids such as asparagine (AAT) and tyrosine (TAT) also occur. The extremely high bias against G (0.6%) at the third codon position suggests that codons such as GCG, AGG, TAG, CTG, GAG, GTG, AAG, TTG, TCG, CGG, CAG, and CCG rarely occurred in this COII gene. The AT bias possibly happened via directed mutation pressure (Osawa *et al.*, 1992), which has been commonly found in mtDNA such as cytochrome *b* of plant and animal (Jermiin *et al.*, 1994). Under mitochondrial conditions of exposure to free radical mutagens, mutations may occur and accumulate because of inefficient repair mechanisms. Mutations at

the third codon position would be especially likely to survive because most of them would be silent substitutions that would not change the amino acid residue.

Because of the AT compositional bias in the COII gene of bumblebee, the conventional distance estimation for phylogenetic analysis may not be appropriate. Most conventional models assume that the process of nucleotide substitution is approximately stationary over time and across the lineage (Lockhart *et al.*,1992); e.g., the models of Jukes and Cantor (1969), Kimura (1980), and Tajima and Nei (1984). When nucleotide frequency bias occurs, the distance estimated from these models can be misleading because they tend to group together similar nucleotide sequences which share bias in base composition, especially at the third codon position. However, a correction method was developed and proposed to correct the substitution rates of sequences with non-stationary nucleotide substitution or with base compositional bias (Lockhart *et al.*, 1994; Steel, 1994). To correct for the base frequency bias problem, we applied Log-Det transformation to determine the evolutionary distance and to reconstruct the phylogenetic tree of bumblebees. This allowed us to compare the conventional Kimura method and Log-Det method in order to determine the effect of nucleotide frequency bias on bumblebee mitochondrial cytochrome oxidase II gene phylogeny.

Phylogeny of Bumblebee in the Pacific Northwest.

Psithyrus vs. Bombus

All phylogenetic trees reconstructed by various methods consistently placed the two *Psithyrus* species together with 100% bootstrap support. This genus formed a monophyletic group that was a sister group to the genus *Bombus*. Since the genetic and evolutionary basis of the large phenotypic variation in bumblebees is poorly known, phylogenetic relationship of *Psithyrus* and *Bombus* could not be solved by morphological methods alone (Richards, 1927) and has been long disputed (Pekkarinen *et al.*, 1979). However, the single cluster of *Psithyrus* species in this study is additional evidence to support the hypothesis that *Psithyrus* is a monophyletic group. This agrees with interpretations derived from numerical taxonomic analysis of wing venation (Plowright and Stephen, 1973), allozyme studies (Pekkarinen *et al.*, 1979; Pamilo *et al.*, 1981; Pamilo *et al.*, 1987), morphological investigation (Milliron, 1970), cladistic analysis based on morphological data (Williams, 1985) and also molecular phylogenetic analysis based on mitochondrial cytochrome oxidase subunit I gene sequences (Pedersen, 1996). Like the study of Pekkarinen and co-workers (1979), our phylogenetic analysis of *Psithyrus,* based on COII gene sequences, not only places the two *Psithyrus* species in a monophyletic cluster, but also places them as a sister group to the genus *Bombus.*

The color resemblances between the species of *Psithyrus* and their *Bombus* hosts has been suggested to imply a genetic relationship between them (Path, 1922). Richards (1927) inferred similar evolution using this kind of evidence for parasitic sand wasps of the genus *Nysson* and hypothesized that *Psithyrus*, as parasites of bumblebees, diverged from the same or closely related species of their bumblebee hosts. If true, *Psithyrus* species would have polyphyletic origins, and each species would be most closely related to its host species (also Pouvreau, 1973). However, the relationship between *Psithyrus* and *Bombus* presented by the phylogenetic trees in this study are not in agreement with those hypotheses. The resemblance of many *Psithyrus* species to their hosts is more likely the result of convergence via Müllerian mimicry. Although some morphological resemblances have been found in the Nearctic region, such as between *P. insularis* F. Smith and *B. californicus*, *B. caliginosus*, or *B. vosnesenskii* (Thorp *et al.*, 1983), *P. insularis* here seems to be less host specific than those in the Palaearctic region (Richards, 1975) and it does not share a recent ancestor with any of those *Bombus* species. Moreover, *Psithyrus*, in this study, is separated from the entire group of *Bombus* with a very long branch connection; therefore, these phylogenetic results support the hypothesis that *Psithyrus* is separate from *Bombus* and should be considered as a distinct genus.

Sections within the genus Bombus.

There are many available classifications of the genus *Bombus* that arrange subgenera into sections or groups, such as those developed by Sladen (1899), Frison (1927), Stephen (1957), and Milliron (1961). The clusters of the *Bombus* species in this study provides a grouping system that closely parallels to the classification presented by Stephen (1957), who in turn followed the classification system defined by Radoszkowski (1884), Vogt (1911), Krüger (1917), Krüger (1920), Franklin (1913), and Frison (1927). Stephen's classification divides the genus *Bombus* into 3 sections: Odontobombus Krüger (subgenera *Fervidobombus* and *Subterraneobombus*), Anodontobombus Krüger (subgenera *Bombus, Pratobombus* or *Pyrobombus* and *Melanobombus*), and Boopobombus Frison (subgenera *Bombias, Separatobombus* and *Cullumanobombus*) (Table 4.7).

In our COII phylogenetic trees, most species separate into those sections with the exception of 3 small subgenera each of which were represented by only one species in our analysis: subgenera, *Melanobombus* (*B. lapidarius*), *Bombias* (*B. nevadensis*) and *Subterraneobombus* (*B. appositus*). Even though the bootstrap support values for two problematic subgenera, *Melanobombus* (*B. lapidarius*), and *Subterraneobombus* (*B. appositus*), were low, their branching order presented by all the COII phylogenetic trees, was consistent enough to arrange them in a unique place. The subgenus *Bombias* (*B. nevadensis*) was clustered with subgenus *Fervidobombus* in all the neighbor-joining analysis with 69%and 65% bootstrap support but not in the parsimony tree.

Table 4.7 The positions of the subgenera of *Bombus* resulting from the COII gene phylogenetic trees compared to the classification system proposed by Frison (1927), Stephen (1957), and Richards (1968).

Stephen (1957), Frison (1927), and Richards (1968)	Species clusters as subgenus from this study's phylogenetic trees
Odontobombus	Cluster 1
Subterraneobombus	*Fervidobombus*
Fervidobombus	
Boopobombus	Cluster 2
Separatobombus	*Separatobombus*
*Crotchiibombus**	*Crotchiibombus*
Bombias	*Melanobombus*‡
Cullumanobombus†	
Anodontobombus	Cluster 3
Bombus	*Bombus*
Pratobombus or Pyrobombus	*Pyrobombus*
Melanobombus	
	Problematic group‡
	Subterraneobombus(Cluster 2 and 3)
	Bombias (Cluster 1 or 2 and 3)

* included in *Separatobombus* in Stephen's classification
† not included in this study
‡ the arrangement of subgenera based on their representative species on the resulting trees are not in agreement with the reference system.

The first problematic subgenus was *Melanobombus* (*B. lapidarius*) that grouped with cluster two (Boopobombus) rather than cluster three (Anodontobombus) in all phylogenetic trees. This arrangement was not found in any other classification. The closest classification system to this arrangement was suggested by Milliron (1961) (Table 4.8) in which *Melanobombus* was placed with *Crotchiibombus, Separatobombus* and *Pyrobombus* under the genus *Pyrobombus*. However, *Melanobombus* under Milliron's classification is more closely related to *Pyrobombus* than *Separatobombus*. Although the branch of the subgenus

Melanobombus in all our trees had low bootstrap support, even if the branch was collapsed, this subgenus still would be placed far from *Pyrobombus*. We, therefore, kept this subgenus *Melanobombus* clustered with section Boopobombus, based on COII gene sequence data until there is more evidence to clarify its status.

Table 4.8 The positions of the subgenera of *Bombus* resulting from the COII gene phylogenetic trees compared to the classification of Bombinae proposed by Milliron (1961)

The classification suggested by Milliron (1961) (from Plowright and Stephen, 1973)			**Species clusters from the phylogenetic trees**
Genus	**Subgenus**	**Species -groups**	
Megabombus	*Bombias*	*Bombias*	*Bombias**
	Megabombus	*Alpinobombus*	*Fervidobombus*
		Fervidobombus	*Subterraneobombus**
		Thoracobombus	
		Megabombus	
		Kallobombus	
		Subterraneobombus	
		Rhodobombus	
Pyrobombus	*Cullumanobombus*	*Fraternobombus*	*Crotchiibombus*
		Crotchiibombus	*Separatobombus*
		Separatobombus	*Melanobombus*
		Cullumanobombus	
	Pyrobombus	*Pyrobombus*	*Pyrobombus*
		Melanobombus	
Bombus		*Bombus* s.s.	*Bombus.*

* unresolved subgenus

The second problematic subgenus, *Subterraneobombus* (*B. appositus*), was consistently placed closer to cluster two (Boopobombus) and cluster three (Anodontobombus) in all phylogenetic analyses with low bootstrap support (42%-60%). This is not in agreement with the reference subgeneric system which

considers this subgenus to be closely related to *Fervidobombus* in the section Odontobombus.

The third problematic subgenus is *Bombias* that grouped with cluster one (Odontobombus) in two out of the three phylogenetic analyses rather than cluster two (Boopobombus). In the parsimony analysis, however, its position was as sister group to cluster two (Boopobombus) and cluster three (Anodontobombus) with low bootstrap support.

When considering the potential grouping of the second and third problematic subgenera with *Fervidobombus* and named as a new genus, *Megabombus*, based on the classification system proposed by Milliron (1961) (Table 4.8), the result remained unclear because this grouping did not agree with their branch order, presented on the phylogenetic trees. Milliron's classification arranged *Bombias* as independent subgenus and grouped *Subterraneobombus* and *Fervidobombus* together within subgenus Megabombus; however, in our result, neighbor-joining trees group *Bombias* with *Fervidobombus* and parsimony tree place *Bombias* between *Fervidobombus* and *Subterraneobombus*. Until there is more evidence, we therefore kept these two problematic subgenera as unresolved.

In the Anodontobombus section, the COII trees agreed with the classification of Sladen (1899), Krüger (1917, 1920), Frison (1927), and Stephen (1957) in that the subgenus *Bombus* was more closely related to subgenus *Pyrobombus*.

Subgenus Fervidobombus

Bombus sonorus and *B. pennsylvanicus* were grouped together with high support and there was additional evidence such as the same size PCR product, and $tRNA^{lue}$ overlapping with the COII gene from this study to support grouping these two species closely together,. Evidence from their COII gene sequences, which differed by 7 bp, combined with geographic evidence, in which no intergradation occurs, and a rearing experiment by Thorp *et al.* (1983), supports the conclusion that these bumblebees are separate species.

Bombus fervidus is most closely related to *B. californicus*, although there is some questions as to their specific status. Milliron (1973a) and Labogule (1990) classified them as co-specific, whereas most others (Thorp *et al.* ,1983; Poole, 1996) considered them as separate species. Since these two species are recently diverged, the intermediate forms support a continuity connecting them (Stephen,1957). Various intermediate forms, from southern British Columbia, western Oregon and Hell's Canyon exhibit characteristics which appear to grade between *B. californicus* and *B. fervidus.* In this study, typical forms of both species and all intermediate forms cited by Stephen (1957) were analyzed. The results clearly show that all intermediate forms cluster close to *B. californicus* rather than *B. fervidus,* with very high bootstrap support. The COII sequence of *B. fervidus* was at least 10 bp different from *B. californicus* whereas the differences among the forms of *B. californicus* were only a few basepairs. This result supports classifying these bumblebees as independent species.

Subgenus Separatobombus and Crotchiibombus. Do we need to separate them into 2 subgenera?

Our phylogenetic trees from all analyses show *B. crotchii* to cluster with the two members of subgenus *Separatobombus* although its specific relationships within subgenus *Separatobombus* were not resolved. These results are not in agreement with traditional classifications that place this species in the separate subgenus, *Crotchiibombus,* based on the differences in color pattern, clypeus, frons, flagellomere, and geographic distribution.

However, there is significant evidence to support our results. The structure of male genitalia of *B. crotchii* is very close to *B. morrisoni* Cresson, which along with other traits prompted Stephen (1957) causing this species to place it in the subgenus *Separatobombus*. Numerical taxonomic analysis of wing venation also placed this species very close to *B. morrisoni* in the *Separatobombus* clade (Plowright and Stephen, 1973). We therefore suggest that these two subgenera could be reduced to a single subgenus, using the name *Separatobombus* as in the classification presented by Stephen (1957).

Subgenus Pyrobombus and Bombus

The close relationship between the subgenera *Pyrobombus* and *Bombus* is supported with a high bootstrap value. This relationship agrees with the classification of many taxonomists (e.g. Sladen (1899), Krüger (1917), Frison (1927), Stephen (1957), Plowright and Stephen (1973), and Pamilo *et al.* (1987)).

However, in neighbor-joining trees, *B. mixtus* is placed in the *Bombus* group with fairly strong bootstrap support (83 and 78%) whereas in the parsimony tree, this species clustered as the deepest-branching member of subgenus *Pyrobombus,* with weak bootstrap support (43%). The latter relationship is in agreement with other evidence such as morphology and numerical taxonomic analysis of wing venation.

The phylogenetic analysis of the subgenus *Pyrobombus* and *Bombus* based on the complete COII gene sequences were not only similar to those based on the partial COII gene sequences (see chapter 2 and 3) but also inferred significant relationships among species within these subgenera, with higher bootstrap support. The subgenus *Pyrobombus* was divided into 5 clades.

The first clade was composed of *B. sitkensis, B. f. flavifrons, B. centralis, B. v. vagans,* and *B. caliginosus.* The inclusion of *B. caliginosus* within this clade, supported by high bootstrap values, indicates that the similarity of the color pattern between *B. caliginosus* and *B. vosnesenskii* is a result of mimicry, as suggested by Thorp *et al.* (1983). Furthermore, *B. centralis* was more closely related to *B. f. flavifrons* than *B. melanopygus,* which had been suggested by phylogenetic analysis of COI gene sequences (Chapter 3)

The second clade was composed of 2 species, *B. sylvicola* and *B. melanopygus,* and the third clade comprised *B. impatiens.* The fourth clade was composed of *B. ternarius, B. huntii, B. vosnesenskii* and *B. b. nearcticus* (94%, Fig. 4.7; 88% Fig. 4.8; 63% Fig. 4.9). There was strong bootstrap support for the placement of *B. b. nearcticus* in the fourth clade rather than the first clade, resulting

from the COI phylogenetic tree (Chapter 3). This placement was supported by other evidence (e.g. Plowright and Stephen (1973), Thorp *et al.* (1983) and Koulianos (1999)).

In the parsimony analysis (Fig. 4.9), *B. mixtus* comprised the fifth clade in subgenus *Pyrobombus*. Both neighbor-joining analyses placed these sequences as the deepest branch within the *Bombus* cluster with 83% and 78% bootstrap support. Neither of these results is supported by others systematists. For example, Stephen (1957) suggests that *B. mixtus* is closely related to *B. sitkensis.* Thorp *et al.* (1983) suggest that this species belongs to the group of *B. edwardsii*, *B. melanopygus*, *B. sitkensis* and *B. sylvicola.* Although the branch order of the *B. mixtus* clade connecting to the subgenus *Pyrobombus* had low support and was unstable in the maximum parsimony tree, compared to the neighbor-joining trees, it may provide a better explanation of the relationship of this species, since the result is more congruent to morphological evidence and to the numerical analysis of Plowright and Stephen (1973). The low bootstrap support of this cluster in the maximum parsimony tree may be caused by sampling error since there are very few characters used to infer the position of group in the tree. Clearly, more evidence is needed to clarify the placement of the *B. mixtus* group.

Phylogenetic analysis of the whole COII gene resulted in clear relationships among the species in the subgenus *Bombus,* with higher support value than the partial COII gene (Chapter 2) and confirmed with very strong support (99-100%), that this subgenus forms a monophyletic clade. The European species, *B. terrestris*,

was separated from the PNW species with high support (88-91%) suggesting that *B. terrestris* is distantly related to *B. occidentalis*. *Bombus occidentalis* formed its own clade, separated from *B. franklini, B. moderatus* and *B. terricola,* with very high support value (85-90%). This confirmed the classification, suggested by Stephen (1957), Thorp (1970), Thorp *et al.* (1983) and Scholl (1992), that these should be separate species not related to *B. occidentalis*. Within the *B. occidentalis* clade, the long branch formed by *B. o. nigroscutatus* California type in the analysis of partial COII gene sequences in Chapter Two was resolved when the longer sequence was used for analysis with the appropriate analysis model (neighbor-joining with Log-Det transformation and maximum parsimony). In addition, the bootstrap values in this analysis were higher than in the partial COII analysis. This result also confirms that *B. o. nigroscutatus* in California and Alaska are distantly related and possibly should be classified as different subspecies.

CONCLUSIONS

The objective of this study was to infer the phylogenetic relationships of bumblebees in the PNW by comparing nucleotide sequences of the mitochondrial COII gene. However, to retrieve this gene required amplification of a large portion of mitochondrial DNA, from $tRNA^{leu}$ to $tRNA^{asp}$. The by-product of this amplification is the intergenic region, located between $tRNA^{leu}$ and the start codon of the COII gene. This region was detected in most species of bumblebee in the PNW with the exception of two subgenera, *Fervidobombus* and *Bombias*. This

region could potentially form a secondary structure with loop and stem. The origin of this region is still unclear; however, in the honey bee, Cornuet *et al.* (1991) suggested that it occurrs from DNA duplication of the COI, COII, and $tRNA^{leu}$ gene followed by DNA insertions and deletions which make this region variable in size and nucleotide base composition. Indeed, since this region provides high variation in term of size and sequence, it should be suitable to clarify organismal relationships at the species or lower level. However, it could not be use to infer phylogenetic relationships of bumblebees because some species did not have this region. In addition, the size of this region among bumblebee species was extremely variable, making alignment of the sequences impossible. Therefore, the phylogeny of bumblebees in this study was based on only the COII gene, as it was more homologous and could be easily aligned with the aid of the translated amino acid sequences of the COII gene.

Our results show that the COII gene does contain a significant phylogenetic signal to allow the examination of the relationship among species of bumblebees in the PNW. Phylogenetic trees not only clarified the generic status between the genera *Psithyrus* and *Bombus*, but also provided clear relationships within the genus *Bombus* at many taxonomic levels from subgenus to species. COII gene sequences clarified the phylogenetic relationships among many subspecies and also populations of bumblebees in the PNW. Furthermore, the comparisons of these results with other phylogenetic analyses, taxonomic data, and geographic distribution provides an understanding of the evolution of bumblebee in the PNW.

Finally, the analysis of the polymorphic species, e.g. *B. occidentalis, B. mixtus, B. b. nearcticus, B. sitkensis,* and *B. californicus,* provided the basis for future studies at the population level that will allow a clearer understanding of the processes of adaptation and evolution and explore the effect of ecological change.

ACKNOWLEDGEMENTS

I would like to thank J. Cane who kindly sent specimens of *B. impatiens* and *B. pennsylvanicus* from Alabama, R.W. Thorp who sent me *B. crotchii* and *B. sonorus*, and B. Schricker who provided *B. terrestris* and *B. lapidarius* from Germany, and M. L. Carlson who provided *B. occidentalis nigroscutatus* from Alaska. Special thanks you to Dr. Lynn Royce for her help and support, and kindly collected *B. sitkensis and B. mixtus.*

REFERENCES

Brower, A.V.Z. (1994). Phylogeny of *Heliconius* butterflies inferred from mitochondrial DNA sequences (Lepidoptera: Nymphalidae). *Mol. Phylogenet. Evol.* 3: 159-174.

Brown, W. M. (1985). The mitochondrial genome of animals. *In* "Molecular Evolutionary Genetics" (R. J. MacIntyre, Ed.), pp. 95-130. Plenum, New York.

Clary, D. O., and Wolstenholme, D. R. (1985). The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. *J. Mol. Evol.* 22: 252-271.

Cornuet, J. M., Garnery, L., and Solignac, M. (1991). Putative origin and function of the intergenic region between COI and COII of *Apis mellifera* L. mitochondrial DNA. *Genetics* 1128: 393-403.

Crozier, R. H., Crozier, Y. C., and Mackinlay, A. G. (1989). The CO-I and CO-II region of honeybee mitochondrial DNA: evidence for variation in insect mitochondrial evolutionary rates. *Mol. Biol. Evol.* 6: 399-411.

Estoup, A., Solignac, M., Cornuet, J. M., Goudet, J., and Scholl, A. (1996). Genetic differentiation of continental and island populations of *Bombus terrestris* (Hymenoptera: Apidae) in Europe. *Molec. Ecol.* 5: 19-31.

Farris, J. S. (1969). A successive approximations approach to character weighting. *Syst. Zool.* 18: 374-385.

Farris, J. S. (1989). The retention index and the rescaled consistency index. *Cladistics* 5: 417-419.

Franklin, H. J. (1913). The Bombidae of the New World. *Trans. Am. Entomol. Soc.* 38: 177-486.

Frison, T. H. (1927). A contribution to our knowledge of the relationships of the Bremidae of America north of Mexico (Hymenoptera). *Trans. Am. Entomol. Soc.* 53: 51-78.

Garnery, L., Vautrin, D., Cornuet, J. M., and Solignac, M. (1991). Phylogenetic relationships in the genus *Apis* inferred from mitochondrial DNA sequence data. *Apidologie* 22: 87-92.

Heinrich, B. (1994). "Bumblebee Economics," Harvard Univ. Press, London.

Hillis, D. M., Huelsenbeck, J. P., and Cunningham, C. W. (1994). Application and accuracy of molecular phylogenies. *Science* 264: 671-677.

Hillis, D. M., Mable, B. K., Larson, A., Davis, S. K., and Zimmer, E. A. (1996). Nucleic acids IV: Sequencing and Cloning. *In* "Molecular Systematics" (D. M. Hillis, C. Moritz, and B. K. Mable, Eds.), pp. 321-381. Sinauer Associates Inc., Sunderland, Massachusetts.

Hobbs, G. A. (1964). Phylogeny of bumble bees based on brood-rearing behaviour. *Can. Entomol.* 96: 115-116.

Hoy, M. A. (1994). "Insect Molecular Genetics," Academic Press, USA.

Jermiin, L. S., Graur, D., Lowe, R. M., and Crozier, R. H. (1994). Analysis of directional mutation pressure and nucleotide content in mitochondrial cytochrome *b* genes. *J. Mol. Evol.* 39: 160-173.

Jukes, T. H., and Cantor, C. R. (1969). Evolution of protein molecules. *In* "Mammalian Protein Metabolism" (H. N. Munro, Ed.), pp. 21-123. Academic Press, New York.

Kimsey, L. S. (1984). The re-evaluation of the phylogenetic relationships in the Apidae (Hymenoptera). *Syst. Entomol.* 9: 435-441.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16: 111-120.

Koulianos, S. (1999). Phylogenetics of the bumblebee subgenus *Pyrobombus* (Hymenoptera: Apidae) inferred from mitochondrial cytochrome B and cytochrome oxidase I sequences. *Ann. Entomol. Soc. Amer.* 92: 355-358.

Krüger, E. (1917). Zur Systematik der mitteleuropäischen Hummein (Hym.). *Ent. Mitt.* 6: 55-66.

Krüger, E. (1920). Beiträge zur Systematik und Morphologie der mitteleuropäischen Hummein. *Zoöl. Jahrb., Abt. Syst.* 42: 289-464.

Labogule, J. M. (1990). *Bombus* of Mexico and Central America (Hymenoptera, Apidae). *Univ. Kan. Sci. Bull.* 54: 35-73.

Lockhart, P. J., Howe, C. J., Bryant, D. A., Beanland, T. J., and Larkum, A. W. (1992). Substitutional bias confounds inference of cyanelle origins from sequence data. *J. Mol. Evol.* 34: 153-162.

Lockhart, P. J., Steel, M. J., Hendy, M. D., and Penny, D. (1994). Recovering evolutionary trees under a more realistic model of sequence evolution. *Mol. Biol. Evol.* 11: 605-612.

Michener, C. D. (1974). "The Social Behavior of The Bees," Harvard Univ. Press, Massachusetts.

Michener, C. D., and Grimaldi, D. A. (1988). The oldest fossil bee: Apoid history, evolutionary status, and antiquity of social behavior. *Proc. Natl. Acad. Sci. USA* 85: 6424-6426.

Milliron, H. E. (1961). Revised classification of the bumblebees. A synopsis (Hymenoptera: Apidae). *J. Kans. Entomol. Soc.* 34: 49-61.

Milliron, H. E. (1971). A monograph of the Western Hemisphere bumblebees (Hymenoptera: Apidae; Bombini). I. The genera *Bombus* and *Megabombus* subgenus *Bombias*. *Mem. Entomol. Soc. Can.* 82: 1-80.

Milliron, H. E. (1973a). A monograph of the Western Hemisphere bumblebees (Hymenoptera: Apidae; Bombini). II The genus *Megabombus* subgenus *Megabombus*. *Mem. Entomol. Soc. Can.* 89: 81-237.

Milliron, H. E. (1973b). A monograph of the Western Hemisphere bumblebees (Hymenoptera: Apidae; Bombini). III The genus *Pyrobombus* subgenus *Cullumanobombus*. *Mem. Entomol. Soc. Can.* 91: 238-333.

Osawa, S., Jukes, T. H., Watanabe, K., and Muto, A. (1992). Recent evidence for evolution of the genetic code. *Microbiol. Rev.* 56: 229-264.

Owen, R. E., and Plowright, R. C. (1980). Abdominal pile color dimorphism in the bumblebee, *Bombus melanopygus*. *J. Hered.* 71: 241-247.

Owen, R. E., and Plowright, R. C. (1988). Inheritance of metasomal pile colour variation in the bumblebee *Bombus rufocinctus* Cresson (Hymenoptera: Apidae). *Can. J. Zool.* 66: 1172-1178.

Pamilo, P., Pekkarinen, A., and Varvio-Aho, S. L. (1981). Phylogenetic relationships and the origin of social parasitism in Vespidae and in *Bombus* and *Psithyrus* as revealed by enzyme genes. *In* "Biosystematics of Social Insects" (P. E., Howse, J. L., Clement, Eds.), pp. 37-47. Academic Press, New York.

Pamilo, P., Pekkarinen, A., and Varvio-Aho, S. L. (1987). Clustering of bumblebee subgenera based on interspecific genetic relationships (Hymenoptera, Apidae: *Bombus* and *Psithyrus*). *Ann. Zool. Fennici.* 24: 19-27.

Pedersen, B. V. (1996). A phylogenetic analysis of cuckoo bumblebees (*Psithyrus*, Lepeletire) and bumblebees (*Bombus*, Latreille) inferred from sequences of the mitochondrial gene cytochrome oxidase I. *Mol. Phylogenet. Evol.* 5: 289-297.

Pekkarinen, A., Varvio-Aho, S. L. , and Pamilo, P. (1979). Evolutionary relationships in northern European *Bombus* and *Psithyrus* species (Hymenoptera, Apidae) studied on the basis of allozymes. *Ann. Entomol. Fennici.* 45: 77-80.

Plath, O. E. (1922). Notes on *Psithyrus*, with records of two new American hosts. *Biol. Bull.* 43: 23-44.

Plowright, R. C., and Stephen, W. P. (1973). A numerical taxonomic analysis of the evolutionary relationships of *Bombus* and *Psithyrus* (Apidae: Hymenoptera). *Can. Entomol.* 105:733-743.

Plowright, R. C., and Owen, R. E. (1980). The evolution significance of bumble bee color patterns: a mimetic interpretation. *Evolution* 34: 622-637

Poole, R. W. (1996). "Nomina Insecta Nearctica [sic], a checklist of the insects of North America. Volume 2: Hymenoptera, Mecoptera, Megaloptera, Neuroptera, Rhaphidioptera, Tricoptera," Rockville, Maryland.

Pouvreau, A. (1973). Les enemies des bourdons. *Apidologie* 4: 103-148.

Radoszkowski, O. (1884). Revision des armures copulatrices des mêles du genre *Bombus*. *Bull. Soc. Imp. Nat., Moscou* 59: 51-92.

Richards, K. W. (1975). Population ecology of bumblebees in southern Alberta. Ph.D. dissertation, Univ. Kansas, Lawrence, 117pp.

Richards, O. W. (1927). The specific characters of the British bumblebees (Hymenoptera). *Trans. R. Ent. Soc. Lond.* 75: 233-268.

Richards, O. W. (1968). The subgeneric divisions of the genus *Bombus* Latreille (Hymenoptera: Apidae). *Bull. Br. Mus. (Nat. Hist.) Entomol.* 22:209-276.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstruction phylogenetic trees. *Mol. Biol. Evol.* 4: 406-425.

Sambrook, J., Fritsch, E., and Maniatis, T. (1989). "Molecular Cloning. A Laboratory Manual," Cold Spring Harbor Laboratory Press, New York.

Scholl, A., Thorp, R. W., and Obrecht, E. (1992). The genetic relationship between *Bombus franklini* (Frison) and other taxa of the subgenus *Bombus* s. str. (Hymenoptera: Apidae). *Pan-Pacific Entomol.* 68: 46-51.

Sladen, F. W. L. (1899). Bombi in captivity, and habits of *Psithyrus*. *Entomol. Monthly Mag.* 10: 230-234.

Smith, J. J., and Bush, G. L. (1997). Phylogeny of the genus *Rhagoletis* (Diptera: Tephritidae) inferred from DNA sequences of mitochondrial cytochrome oxidase II. *Mol. Phylogenet. Evol.* 7: 33-43.

Staden, R., Bonfield, J., and Kathryn, B. (1997). "Staden computer package (version 0.99.2)," Medical Research Council, Laboratory of Molecular Biology, Cambridge, UK.

Steel, M. A. (1994). Recovering a tree from the leave colourations it generates under a Markov model. *Appl. Math. Lett.* 7: 19-23.

Stephen, W. P. (1957). Bumblebees of Western America (Hymenoptera: Apoidae). *Tech. Bull. Oreg. Agric. Exp. Stn.* 40.

Stephen, W. P., and Cheldelin, I. R. (1973). Phenetic grouping in bees of the tribe Bombini based on the enzyme α- glycerophosphate dehydrogenase. *Biochem. Sys.* 1: 69-76.

Sutcliffe, G. H., and Plowright, R. C. (1988). The effects of food supply on adult size in the bumble bee *Bombus terricola* Kirby (Hymenoptera: Apidae). *Can. Entomol.* 120: 1051-1058.

Swofford, D. L. (1999). "PAUP*4: Phylogenetic Analysis Using Parsimony, Version 4d65," Sinauer Associated, Inc. Publishers, Massachusetts.

Swofford, D. L., Olsen, G. J., Wadell, P. J., and Hillis, D. M. (1996). Phylogenetic inference. *In* "Molecular Systematics" (D. M. Hillis, C. Moritz, and B. K. Mable, Eds.), pp. 407-514. Sinauer Associates Inc., Sunderland, Massachusetts.

Tajima, F., and Nei, M. (1984). Estimation of evolutionary distance between nucleotide sequences. *Mol. Biol. Evol.* 1: 269-285.

Thorp, R. W. (1970). The type locality of *Bombus franklini* and notes on putative Arizona records of other Bombini. *Pan-Pacific Entomol.* 46: 177-180.

Thorp, R. W., Horning, D. S. Jr., and Dunning, L. L. (1983). Bumblebees and cuckoo bumblebees of California. *Bull. Calif. Insect Surv.* 23.

Vogler, A. P., and Pearson, D. L. (1996). A molecular phylogeny of the tiger beetles (Cicindelidae): congruence of mitochondrial and nuclear rDNA data sets. *Mol. Phylogenet. Evol.* 6: 321-338.

Vogt, O. (1911). Studien über das Artproblem. Über das Variieren der Hummelm. 2 Teil. *Sber. Ges. Naturf. Freunde., Berl.* pp. 31-74.

Williams, P. H. (1985). A preliminary cladistic investigation of relationships among the bumble bees (Hymenoptera, Apidae). *Syst. Entomol.* 10: 239-255.

Williams, P. H. (1991). The bumble bees of the Kashmir Himalaya (Hymenoptera: Apidae, Bombini). *Bull. Br. Mus. (Nat. Hist.) Entomol.* 60: 1-204.

Williams, P. H. (1994). Phylogenetic relationships among bumble bees (*Bombus* Latr.): a reappraisal of morphological evidence. *Syst. Entmol.* 19: 327-344.

Williams, P. H. (1998). An annotated checklist of bumblebees with an analysis of patterns of description (Hymenoptera: Apidae, Bombini). *Bull. Br. Mus. (Nat. Hist.) Entomol.* 67: 79-152.

Willis, L. G., Winston, M. L., and Honda, B. M. (1992). Phylogenetic relationships in the honeybee (Genus *Apis*) as determined by the sequence of the cytochrome oxidase II region of mitochondrial DNA. *Mol. Phylogenet. Evol.* 1: 169-178.

Winston, M. L. (1987). "The Biology of the Honey Bee," Harvard Univ. Press, Cambridge.

Wisconsin Package version 10. (1998). "Genetics Computer Group (GCG)," Madison, WI.

CHAPTER 5

CONCLUSIONS

The objectives of this research were to clarify the taxonomic status of bumblebees in the PNW and explore their relationships via phylogenetic analysis using nucleotide-sequencing data from the mitochondrial cytochrome oxidase genes. We started by using the partial COII gene and secondary structure of the intergenic region between $tRNA^{leu}$ and the COII gene to study the subgenus *Bombus*. The objectives of this study were to clarify the relationships between the Nearctic species and the Palearctic species, the specific status of *Bombus* species in the PNW, and the genetic variation of *B. occidentalis*. The phylogenetic trees reconstructed by neighbor-joining and maximum parsimony, as well as the secondary structure of the $tRNA^{leu}$/COII intergenic region, provided evidence to separate *Bombus* species between the PNW and Europe. *Bombus terrestris* was distantly related to *B. occidentalis*. *Bombus moderatus* was not synonymous to *B. lucorum*. In the PNW group, although some branch orders of the trees were weakly supported, topologies from all analytical methods were consistent and provided the resolution to explain phylogenetic relationships and to clarify most of the conflicting species. We confirmed that *B. franklini* and *B. terricola* were separate species, not closely related to *B. occidentalis*. The trees clearly showed that *B. occidentalis* with ferruginous tip, found in southern Oregon, belonged with the

species *B. occidentalis* rather than *B. franklini* although these bees look alike and occupy the same habitat. The branch order resulting from the analysis of individuals identified as *B. o. occidentalis* suggested that this species may be composed of three subspecies rather than only two: *B. o. occidentalis* and *B. o. nigroscutatus.* The *B. o. nigroscutatus* found in California appeared to be distantly related to the one found in Alaska. The difference between these two bees was detected by the COII gene and secondary structure of intergenic region.

Based on the results using subgenus *Bombus*, the partial COII gene showed remarkable characteristics for phylogenetic analysis of bumblebees. We used this region again to analyze the phylogenetic relationship of *Pyrobombus* species. We also did a phylogenetic analysis of this subgenus using the partial COI region, allowing us to compare and combine the results from these two different genes. The phylogenetic trees resulting from the partial COI gene were not congruent with those from the partial COII gene. Even when the partial COII was re-analyzed by adding the intergenic region, differences still occurred, but the trees were improved with higher resolution and stronger branch support. When the data sets from these two genes were combined and tested by partition homogeneity analysis, we found that these two genes have different phylogenetic history, demonstrated by three conflicting taxa. When these three taxa were removed and the new combined data sets were re-analyzed, all reconstructed trees were congruent. Of course, the phylogenetic information of these three species was lost. We solved this problem

by adding them to the congruent tree, basing their position on morphology and other molecular phylogeny.

The final phylogenetic trees not only explained the relationship of many confusing species, but also provided support of radiation in many species, by comparing branch patterns with ecological and geographical evidence, for example, in *B. sitkensis*, *B. mixtus*, and *B. b. nearcticus*.

Finally, we analyzed the phylogenetic relationship of bumblebees in the PNW based on complete COII gene sequences. Resulting phylogenetic trees presented the generic status between *Psithyrus* and *Bombus,* confirmed the traditional subgeneric classification system and clarified the confusing taxonomic status of many *Bombus* species. Furthermore, the comparisons of these results with others such as others phylogenetic analyses, taxonomic data, and geographic distribution provided many valuable insights to the understanding of the evolution of bumblebees in the PNW. For example, differences among individuals of many polymorphic species, such as *B. occidentalis, B. mixtus, B. b. nearcticus, B. sitkensis,* and *B. californicus*, can be detected by the COII gene, enabling us to better understand the process of species radiation. Finally, these results give us new avenues for future studies among populations of bumblebees that will allow us to explore and understand the effects of ecological changes on the process of organismal adaptation.

BIBLIOGRAPHY

Ahnert, F. (1998). "Introduction to Geomorphology," Arnold, London.

Alford, D. V. (1969). A study of the hibernation of bumblebees in southern England. *J. Anim. Ecol.* 38: 149-170.

Alford, D. V. (1975). "Bumblebees," Davis-Poynter, London.

Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, J. E., Reeb, C. A., and Saunders, N. C. (1987). Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annu. Rev. Ecol. Syst.* 18: 489-522.

Ballard, J. W., Olsen, G. J., Falth, D. P., Odgers, W. A., Rowell, D. M., and Atkinson, P. W. (1992). Evidence from 12S ribosomal RNA sequences that Onychophorans are modified arthropods. *Science* 258: 1345-1348.

Barrett, M., Donoghue, M. J., and Sober, E. (1991). Against consensus. *Syst. Zool.* 40: 486-493.

Borror, D. J., Triplehorn, C. A., and Johnson, N. F. (1992). "An Introduction to the Study of Insects," Saunders College Publishing, Philadelphia, USA.

Brower, A. V. Z., and DeSalle, R. (1994). Practical and theoretical considerations for choice of a DNA sequence region in insect molecular systematics, with a short review of published studies using nuclear gene regions. *Ann. Entomol. Soc. Amer.* 87: 702-716.

Brower, A.V.Z. (1994). Phylogeny of *Heliconius* butterflies inferred from mitochondrial DNA sequences (Lepidoptera: Nymphalidae). *Mol. Phylogenet. Evol.* 3: 159-174.

Brown, W. M. (1985). The mitochondrial genome of animals. *In* "Molecular Evolutionary Genetics" (R. J. MacIntyre, Ed.), pp. 95-130. Plenum, New York.

Bull, J. J., Huelsenbeck, J. P., Cunningham, C. W., Swofford, D. L., and Waddell, P. J. (1993). Partitioning and combining data in phylogenetic analysis. *Syst. Biol.* 42: 384-397.

Clary, D. O., and Wolstenholme, D. R. (1985). The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. *J. Mol. Evol.* 22: 252-271.

Cornuet, J. M., Garnery, L., and Solignac, M. (1991). Putative origin and function of the intergenic region between COI and COII of *Apis mellifera* L. mitochondrial DNA. *Genetics* 1128: 393-403.

Crozier, R. H., and Crozier, Y. C. (1993). The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organization. *Genetics* 133: 97-117.

Crozier, R. H., Crozier, Y. C., and Mackinlay, A. G. (1989). The CO-I and CO-II region of honeybee mitochondrial DNA: Evidence for variation in insect mitochondrial evolutionary rates. *Mol. Biol. Evol.* 6: 399-411.

Daly, H. V. (1991). Systematics and identification of africanized honey bees. *In* "The African Honey Bee," (M. Spivak, D. J. C. Fletcher, and M. D. Breed, Eds.), pp. 13-44. Westview Press, Boulder.

de Queiroz, A. (1993). For consensus (sometimes). *Syst. Biol.* 42: 368-372.

de Queiroz, A., Donoghue, M., and Kim, J. (1995). Separate versus combined analysis of phylogenetic evidence. *Annu. Rev. Ecol. Syst.* 26: 657-681.

Doyle, J. A., Donoghue, M. J., Zimmer, E. A. (1994). Integration of morphological and rRNA data on the origin of angiosperms. *Ann. Mo. Bot. Gard.* 81: 419-450.

Estoup, A., Solignac, M., Cornuet, J. M., Goudet, J., and Scholl, A. (1996). Genetic differentiation of continental and island populations of *Bombus terrestris* (Hymenoptera: Apidae) in Europe. *Molec. Ecol.* 5: 19-31.

Estoup, A., Solignac, M., Harry, M., and Cornuet, J. M. (1993). Characterization of $(GT)_n$ and $(CT)_n$ microsatellites in two insect species: *Apis mellifera* and *Bombus terrestris*. *Nucleic Acids Res.* 21: 1427-1431.

Farris, J. S. (1969). A successive approximations approach to character weighting. *Syst. Zool.* 18: 374-385.

Farris, J. S. (1989). The retention index and the rescaled consistency index. *Cladistics* 5: 417-419.

Farris, J. S., Källersjö, M., Kluge, A. G., and Bult, C. (1994). Testing significance of incongruence. *Cladistics* 10: 315-319.

Felsenstein, J. (1995). "PHYLIP computer package program (version 3.5c)," University of Washington, Seattle.

Franklin, H. J. (1913). The Bombidae of the New World. *Trans. Am. Entomol. Soc.* 38: 177-486.

Frison, T. H. (1927). A contribution to our knowledge of the relationships of the Bremidae of America north of Mexico (Hymenoptera). *Trans. Am. Entomol. Soc.* 53: 51-78.

Garnery, L., Vautrin, D., Cornuet, J. M., and Solignac, M. (1991). Phylogenetic relationships in the genus *Apis* inferred from mitochondrial DNA sequence data. *Apidologie* 22: 87-92.

Harrison, R. G. (1989). Animal mtDNA as a genetic marker in population and evolutionary biology. *Trends Ecol. Evol.* 4: 6-11.

Heinrich, B. (1994). "Bumblebee Economics," Harvard Univ. Press, London.

Hillis, D. M., Huelsenbeck, J. P., and Cunningham, C. W. (1994). Application and accuracy of molecular phylogenies. *Science* 264: 671-677.

Hillis, D. M., Mable, B. K., Larson, A., Davis, S. K., and Zimmer, E. A. (1996). Nucleic acids IV: Sequencing and Cloning. *In* "Molecular Systematics" (D. M. Hillis, C. Moritz, and B. K. Mable, Eds.), pp. 321-381. Sinauer Associates Inc., Sunderland, Massachusetts.

Hobbs, G. A. (1964). Phylogeny of bumble bees based on brood-rearing behaviour. *Can. Entomol.* 96: 115-116.

Hobbs, G. A. (1967). Ecology of species of *Bombus* (Hymenoptera: Apidae) in southern Alberta. VI. Subgenus *Pyrobombus*. *Can. Entomol.* 99: 1272-1292.

Howland, D. E., and Hewitt, G. M. (1995). Phylogeny of Coleoptera based on mitochondrial cytochrome oxidase I sequence data. *Insect Mol. Biol.* 4: 203-215.

Hoy, M. A. (1994). "Insect Molecular Genetics," Academic Press, USA.

Huelsenbeck, J. P., and Hillis, D. M. (1993). Success of phylogenetic methods in the four-taxa case. *Syst. Biol.* 42:267-264.

Huelsenbeck, J. P., Bull, J. J., and C. W. Cunningham. (1996). Combining data in phylogenetic analysis. *Trends Ecol. Evol.* 11:152-157.

Jermiin, L. S., Graur, D., Lowe, R. M., and Crozier, R. H. (1994). Analysis of directional mutation pressure and nucleotide content in mitochondrial cytochrome *b* genes. *J. Mol. Evol.* 39:160-173.

Jukes, T. H., and Cantor, C. R. (1969). Evolution of protein molecules. *In* "Mammalian Protein Metabolism" (H. N. Munro, Ed.), pp. 21-123. Academic Press, New York.

Kimsey, L. S. (1984). The re-evaluation of the phylogenetic relationships in the Apidae (Hymenoptera). *Syst. Entomol.* 9: 435-441.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16: 111-120.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X. and Wilson, A. C. (1989). Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. USA* 86: 6196-6200.

Koulianos, S. (1999). Phylogenetics of the bumblebee subgenus *Pyrobombus* (Hymenoptera: Apidae) inferred from mitochondrial cytochrome B and cytochrome oxidase I sequences. *Ann. Entomol. Soc. Amer.* 92: 355-358.

Krüger, E. (1917). Zur Systematik der mitteleuropäischen Hummein (Hym.). *Ent. Mitt.* 6: 55-66.

Krüger, E. (1920). Beiträge zur Systematik und Morphologie der mitteleuropäischen Hummein. *Zoöl. Jahrb., Abt. Syst.* 42: 289-464.

Labogule, J. M. (1990). *Bombus* of Mexico and Central America (Hymenoptera, Apidae). *Univ. Kan. Sci. Bull.* 54: 35-73.

Lockhart, P. J., Howe, C. J., Bryant, D. A., Beanland, T. J., and Larkum, A. W. (1992). Substitutional bias confounds inference of cyanelle origins from sequence data. *J. Mol. Evol.* 34: 153-162.

Lockhart, P. J., Steel, M. J., Hendy, M. D., and Penny, D. (1994). Recovering evolutionary trees under a more realistic model of sequence evolution. *Mol. Biol. Evol.* 11: 605-612.

Lui, H., and Beckenbach, A. T. (1992). Evolution of the mitochondrial cytochrome oxidase II gene among 10 orders of insects. *Mol. Phylogenet. Evol.* 1:41-52.

Lunt, D. H., Zhang, D.-X., Szymura, J. M., and Hewitt, G. M. (1996). The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. *Insect Mol. Biol.* 5: 153-165.

May, B. (1992). Starch gel electrophoresis of allozymes. *In* "Molecular Genetic Analysis of Populations: A Practical Approach" (A. R. Hoelzel Ed.) pp. 1-27. IRL Press, Oxford.

Michener, C. D. (1944). Comparative external morphology, phylogeny, and a classification of the bees (Hymenoptera). *Bull. Amer. Mus. Nat. Hist.* 82: 157-326.

Michener, C. D. (1974). "The Social Behavior of The Bees," Harvard Univ. Press, Massachusetts.

Michener, C. D., and Grimaldi, D. A. (1988). The oldest fossil bee: Apoid history, evolutionary status, and antiquity of social behavior. *Proc. Natl. Acad. Sci. USA* 85: 6424-6426.

Milliron, H. E. (1961). Revised classification of the bumblebees. A synopsis (Hymenoptera: Apidae). *J. Kans. Entomol. Soc.* 34: 49-61.

Milliron, H. E. (1971). A monograph of the Western Hemisphere bumblebees (Hymenoptera: Apidae; Bombini). I. The genera *Bombus* and *Megabombus* subgenus *Bombias. Mem. Entomol. Soc. Can.* 82: 1-80.

Milliron, H. E. (1973a). A monograph of the Western Hemisphere bumblebees (Hymenoptera: Apidae; Bombini). II The genus *Megabombus* subgenus *Megabombus. Mem. Entomol. Soc. Can.* 89: 81-237.

Milliron, H. E. (1973b). A monograph of the Western Hemisphere bumblebees (Hymenoptera: Apidae; Bombini). III The genus *Pyrobombus* subgenus *Cullumanobombus. Mem. Entomol. Soc. Can.* 91: 238-333.

Moritz, C., Dowling, T.E., and Brown, W. M. (1987). Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Annu. Rev. Ecol. Syst.* 18: 269-292.

Nei, M. (1987). "Molecular Evolutionary Genetics," Columbia Univ. Press, New York.

Olmstead, R. G., and Sweere, J. A. (1994). Combining data in phylogenetic systematics: an empirical approach using three molecular data sets in the Solanaceae. *Syst. Biol.* 43: 467-481.

Osawa, S., Jukes, T. H., Watanabe, K., and Muto, A. (1992). Recent evidence for evolution of the genetic code. *Microbiol. Rev.* 56: 229-264.

Owen, R. E. (1983). Chromosome numbers of 15 North America bumblebee species (Hymenoptera, Apidae, Bombini). *Can. J. Genet. Cytol.* 25: 26-29.

Owen, R. E. (1985). Difficulties with the interpretation of patterns of genetic variation in the eusocial Hymenoptera. *Evolution* 39: 201.

Owen, R. E., and Plowright, R. C. (1980). Abdominal pile color dimorphism in the bumble bee, *Bombus melanopygus*. *J. Hered.* 71: 241-247.

Owen, R. E., and Plowright, R. C. (1988). Inheritance of metasomal pile colour variation in the bumblebee *Bombus rufocinctus* Cresson (Hymenoptera: Apidae). *Can. J. Zool.* 66: 1172-1178.

Owen, R. E., Mydynski, L. J., Packer, L., and McCorquodale, D. B. (1992). Allozyme variation in bumblebees (Hymenoptera: Apidae). *Biochem. Genet.* 30: 443-453.

Pamilo, P., Pekkarinen, A., and Varvio-Aho, S. L. (1981). Phylogenetic relationships and the origin of social parasitism in Vespidae and in *Bombus* and *Psithyrus* as revealed by enzyme genes. *In* "Biosystematics of Social Insects" (P. E., Howse, J. L., Clement, Eds.), pp. 37-47. Academic Press, New York.

Pamilo, P., Pekkarinen, A., and Varvio-Aho, S. L. (1987). Clustering of bumblebee subgenera based on interspecific genetic relationships (Hymenoptera, Apidae: *Bombus* and *Psithyrus*). *Ann. Zool. Fennici.* 24: 19-27.

Pamilo, P., Varvio-Aho, S. L., and Pekkarinen, A. (1978). Low enzyme variability in Hymenoptera as a consequence of haplodiploidy. *Hereditas* 88: 93.

Pamilo, P., Varvio-Aho, S. L., and Pekkarinen, A. (1984). Genetic variation in bumblebees (*Bombus, Psithyrus*) and putative sibling species of *Bombus lucorum*. *Hereditas* 101: 245.

Pedersen, B. V. (1996). A phylogenetic analysis of cuckoo bumblebees (*Psithyrus*, Lepeletire) and bumblebees (*Bombus*, Latreille) inferred from sequences of the mitochondrial gene cytochrome oxidase I. *Mol. Phylogenet. Evol.* 5: 289-297.

Pekkarinen, A., Varvio-Aho, S. L., and Pamilo, P. (1979). Evolutionary relationships in northern European *Bombus* and *Psithyrus* species (Hymenoptera, Apidae) studied on the basis of allozymes. *Ann. Entomol. Fennici.* 45: 77-80.

Plath, O. E. (1922). Notes on *Psithyrus*, with records of two new American hosts. *Biol. Bull.* 43: 23-44.

Plath, O. E. (1934). "Bumblebees and Their Ways," The Macmillan Company, New York.

Plowright, R. C., and Owen, R. E. (1980). The evolution significance of bumble bee color patterns: a mimetic interpretation. *Evolution* 34: 622-637

Plowright, R. C., and Stephen, W. P. (1973). A numerical taxonomic analysis of the evolutionary relationships of *Bombus* and *Psithyrus* (Apidae: Hymenoptera). *Can. Entomol.* 105: 733-743.

Plowright, R. C., and Stephen, W. P. (1980). The taxonomic status of *Bombus franklini* (Hymenoptera: Apidae). *Can. Entomol.* 112: 475-479.

Poole, R. W. (1996). "Nomina Insecta Nearctica [sic], a checklist of the insects of North America. Volume 2: Hymenoptera, Mecoptera, Megaloptera, Neuroptera, Rhaphidioptera, Tricoptera," Rockville, Maryland.

Pouvreau, A. (1973). Les enemies des bourdons. *Apidologie* 4: 103-148.

Radoszkowski, O. (1884). Revision des armures copulatrices des mêles du genre *Bombus*. *Bull. Soc. Imp. Nat., Moscou* 59: 51-92.

Randi, E. (1996). A mitochondrial cytochrome B phylogeny of the *Alectoris* partridges. *Mol. Phylogenet. Evol.* 6: 214-227.

Richards, K. W. (1975). Population ecology of bumblebees in southern Alberta. Ph.D. dissertation, Univ. Kansas, Lawrence, 117pp.

Richards, O. W. (1927). The specific characters of the British bumblebees (Hymenoptera). *Trans. R. Ent. Soc. Lond.* 75: 233-268.

Richards, O. W. (1968). The subgeneric divisions of the genus *Bombus* Latreille (Hymenoptera: Apidae). *Bull. Br. Mus. (Nat. Hist.) Entomol.* 22: 209-276.

Rodrigo, A. G., Kelly-Borges, M., Bergquist, P. R., and Bergquist, P. L. (1993). A randomisation test of the null hypothesis that two cladograms are simple estimates of a parametric phylogenetic tree. *N. Z. J. Bot.* 31: 257-268.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstruction phylogenetic trees. *Mol. Biol. Evol.* 4: 406-425.

Sambrook, J., Fritsch, E., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press, New York.

Scholl, A., Obrecht, E., and Owen, R. E. (1990). The genetic relationship between *Bombus moderatus* Cresson and the *Bombus lucorum* auct species complex (Hymenoptera: Apidae). *Can. J. Zool.* 68: 2264-2268.

Scholl, A., Thorp, R. W., and Obrecht, E. (1992). The genetic relationship between *Bombus franklini* (Frison) and other taxa of the subgenus *Bombus* s. str. (Hymenoptera: Apidae). *Pan-Pacific Entomol.* 68: 46-51.

Scholl, A., Thorp, R. W., Owen, R. E., and Obrecht, E. (1995). Specific distinctiveness of *Bombus nevadensis* Cresson and *B. auricomus* (Robertson) (Hymenoptera: Apidae) enzyme electrophoretic data. *J. Kans. Entomol. Soc.* 65: 134-140.

Simon, C. (1991). Molecular systematics at the species boundary: exploiting conserved and variable regions of the mitochondrial genome of animals via direct sequencing from amplified DNA. *In* "Molecular Techniques in Taxonomy" (G. M. Hewitt, A. W. B. Johnston, and J. P. W. Young, Eds.), pp.33-71, Springer Verlag, Berlin.

Sladen, F. W. L. (1899). Bombi in captivity, and habits of *Psithyrus*. *Entomol. Monthly Mag.* 10: 230-234.

Sladen, F. W. L. (1919). Notes on the Canadian representatives of British species of bees. *Can. Entomol.* 51: 124-130.

Smith, J. J., and Bush, G. L. (1997). Phylogeny of the genus *Rhagoletis* (Diptera: Tephritidae) inferred from DNA sequences of mitochondrial cytochrome oxidase II. *Mol. Phylogenet. Evol.* 7: 33-43.

Smithz, J., and Moritz, R. F. A. (1996). Molecular phylogeny of Vespidae (Hymenoptera) and the evolution of sociality in wasps. *Mol. Phylogenet. Evol.* 9: 183-191.

Snyder, T. P. (1974). Lack of allozyme variability in three bee species. *Evolution* 28: 687.

Staden, R., Bonfield, J., and Kathryn, B. (1997). "Staden computer package (version 0.99.2)," Medical Research Council, Laboratory of Molecular Biology, Cambridge, UK.

Steel, M. A. (1994). Recovering a tree from the leave colourations it generates under a Markov model. *Appl. Math. Lett.* 7: 19-23.

Stephen, W. P. (1957). Bumblebees of Western America (Hymenoptera: Apoidae). *Tech. Bull. Oreg. Agric. Exp. Stn.* 40.

Stephen, W. P., and Cheldelin, I. R. (1973). Phenetic grouping in bees of the tribe Bombini based on the enzyme α- glycerophosphate dehydrogenase. *Biochem. Sys.* 1: 69-76.

Sutcliffe, G. H., and Plowright, R. C. (1988). The effects of food supply on adult size in the bumble bee *Bombus terricola* Kirby (Hymenoptera: Apidae). *Can. Entomol.* 120: 1051-1058.

Swofford, D. L. (1993). "PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1," Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

Swofford, D. L. (1999). "PAUP*4: Phylogenetic Analysis Using Parsimony, Version 4d65," Sinauer Associated, Inc. Publishers, Massachusetts.

Swofford, D. L., Olsen, G. J., Wadell, P. J., and Hillis, D. M. (1996). Phylogenetic inference. *In* "Molecular Systematics" (D. M. Hillis, C. Moritz, and B. K. Mable, Eds.), pp. 407-514. Sinauer Associates Inc., Sunderland, Massachusetts.

Szymura, J. M., Lunt, D. H., and Hewitt, G. M. (1996). The sequence and structure of the meadow grasshopper (*Chorthippus parallelus*) mitochondrial srRNA, ND2, COI, COII, ATPase8 and 9 tRNA genes. *Insect Mol. Biol.* 5: 127-139.

Tajima, F., and Nei, M. (1984). Estimation of evolutionary distance between nucleotide sequences. *Mol. Biol. Evol.* 1: 269-285.

Thorp, R. W. (1970). The type locality of *Bombus franklini* and notes on putative Arizona records of other Bombini. *Pan-Pacific Entomol.* 46: 177-180.

Thorp, R. W., Horning, D. S. Jr., and Dunning, L. L. (1983). Bumblebees and cuckoo bumblebees of California. *Bull. Calif. Insect Surv.* 23.

Tkalcu, B. (1974). Ergebnisse der 1. Und 2. mongolisch-tschechoslowakischen entomologisch-botanischen Expedition in der Mongolei. Nr. 29. Hymenoptera, Apidae, Bombinae. *Sb. Faun. Praci Entomol. Odd. Eleni.* 15: 25-58.

Vogler, A. P., and Pearson, D. L. (1996). A molecular phylogeny of the tiger beetles (Cicindelidae): congruence of mitochondrial and nuclear rDNA data sets. *Mol. Phylogenet. Evol.* 6: 321-338.

Vogt, O. (1911). Studien über das Artproblem. Über das Variieren der Hummelm. 2 Teil. *Sber. Ges. Naturf. Freunde., Berl.* pp. 31-74.

Williams, P. H. (1985). A preliminary cladistic investigation of relationships among the bumble bees (Hymenoptera, Apidae). *Syst. Entomol.* 10: 239-255.

Williams, P. H. (1991). The bumble bees of the Kashmir Himalaya (Hymenoptera: Apidae, Bombini). *Bull. Br. Mus. (Nat. Hist.) Entomol.* 60:1-204.

Williams, P. H. (1994). Phylogenetic relationships among bumble bees (*Bombus* Latr.): a reappraisal of morphological evidence. *Syst. Entmol.* 19: 327-344.

Williams, P. H. (1998). An annotated checklist of bumblebees with an analysis of patterns of description (Hymenoptera: Apidae, Bombini). *Bull. Br. Mus. (Nat. Hist.) Entomol.* 67: 79-152.

Willis, L. G., Winston, M. L., and Honda, B. M. (1992). Phylogenetic relationships in the honeybee (Genus *Apis*) as determined by the sequence of the cytochrome oxidase II region of mitochondrial DNA. *Mol. Phylogenet. Evol.* 1: 169-178.

Winston, M. L. (1987). "The Biology of the Honey Bee," Harvard Univ. Press, Cambridge.

Wisconsin Package version 9.0. (1996). "Genetics Computer Group (GCG)," Madison, WI.

Wisconsin Package version 10. (1998). "Genetics Computer Group (GCG)," Madison, WI.

Wolstenholme, D. R. (1992). Animal mitochondrial DNA: structure and evolution. *Int. Rev. Cytol.* 141: 173-216.