ABSTRACT

Phylogenetics and Systematics of the Millipede genus *Brachycybe* Wood, 1864

(Platydesmida: Andrognathidae)

by

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The genus *Brachycybe* Wood, 1864 (Platydesmida: Andrognathidae) belongs to an ancient millipede lineage that has persisted since the Miocene. The group displays a Holarctic distribution with species in the eastern Nearctic, western Nearctic, and eastern Palaearctic. Like many millipede groups, its taxonomy to date has been based solely on morphology, which may under represent species richness by ignoring cryptic species. Here we present the first phylogenetic analyses for *Brachycybe* based on molecular data obtained from the mitochondrial genes *cytochrome c oxidase 1* and *cytochrome b*. Standard phylogenetic methods were used to reconstruct the evolutionary relationships of the group. Using the internal phylogenetic framework, we evaluated geographic associations among clades; species boundaries were evaluated using the cohesion species concept. The application of molecular phylogenetics, phylogeographic methods, and the cohesion species concept to the genus *Brachycybe* is used to evaluate its species diversity.

The mitochondrial genome of *Brachycybe lecontii* Wood, 1864 (Platydesmida: Andrognathidae) is also presented here along with the phylogeny of Myriapoda, produced using the amino acid sequences from the entire mitochondrial genome. The complete mitochondrial genome sequence of *Brachycybe lecontii* comprises 15,465 base pairs and includes 13 protein-encoding genes, 21 tRNA genes, two rRNA genes, and two large non coding regions that are approximately 436 and 464 bp in length. The genome is uncharacteristic of typical Diplopods and varies dramatically from other sequenced members of the Arthropoda. The unique gene synteny is novel among others because of the rearrangement of two protein coding genes (nad1 and nad5) along with their retained transcriptional polarity. The phylogeny produced from these data recover the monophyly of the Myriapoda classes. However, support values within the Diplopoda are low, possibly due to the inclusion of atp8 in the analysis. This will have important implications for taxonomic research because it will expand the growing bank of complete millipede mitochondrial genomes, allow more robust phylogenetic analyses, and aid in the development of more genetic markers and consequently larger nucleotide datasets.

Phylogenetics and Systematics of the Millipede genus *Brachycybe* Wood, 1864 (Platydesmida:

Andrognathidae)

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by

Chad Lee Spruill

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INTRODUCTION

The class Diplopoda, millipedes, are an extremely diverse group of terrestrial arthropods that play an important role in forest ecosystems as detritivores (Hopkin and Read, 1992). They represent a large part of the terrestrial biodiversity, with 12,000 described species and an estimated 80,000 extant species (Hoffman *et al*., 2002; Shelley and Marek, 2005). Such a high level of diversity within the class may be attributed to a number of the group's attributes. Diplopoda is the oldest known terrestrial animal group (Wilson *et al*., 2004), and they have specific niche requirements and exhibit low vagility, characteristics that tend to result in population isolation (Bond and Stockman, 2008; Sierwald and Bond, 2007; Walker et al, 2009). However, despite their high diversity and prominent role as detritivores in terrestrial ecosystems, they remain surprisingly understudied.

Many major diplopod groups are in need of taxonomic and systematic revision as few genera and species have been evaluated in a rigorous, phylogenetic manner (Sierwald and Bond, 2007). Although morphological approaches to evaluating classification schemes and species boundaries have been instrumental in laying the early evolutionary framework for many groups, a total evidence phylogenetic approach that employs both molecular and morphological character sets is currently considered one of the more robust approaches to addressing evolutionary history and building natural classifications (Glass 1976; Marek and Bond, 2006; Sierwald and Bond, 2007).

Problems with diplopod classification are not only limited to higher level groupings but also exist at the species level, as many species boundaries have not been evaluated in a rigorous manner (Bond and Sierwald, 2003; Enghoff, 1995; Marek and Bond, 2006; Tanabe, 2002).

Traditionally, species delineation in millipedes has focused on males, specifically on gonopodal characters (Sierwald and Bond, 2007); females are rarely described and specimens were often not even maintained in collections (Sierwald and Bond, 2007). In addition, behavioral or ecological characters have rarely been explored, and molecular characters have only recently begun to be considered for millipedes (Sierwald and Bond, 2007). Based on the few molecular examinations to date, certain millipede species appear to be incredibly subdivided at the population level (Marek and Bond, 2006; Sierwald and Bond, 2007) and consequently many nominal diplopod taxa may mask numerous cryptic species (But see Marek (2010) for an example of where morphological species are consistent with molecular species limits).

Cryptic species provide a great challenge to species delimitation, (Bond et al., 2001; Hedin and Wood, 2002; Sinclair et al., 2004; Boyer et al., 2007), not only because of the innate morphological vs. molecular conundrum but also due to the ever-increasing documentation of this phenomenon in other arthropod groups. Cryptic species present a challenge to morphological and molecular species delimitation alike; they just manifest themselves in different ways. A strict morphological approach grossly underestimates the amount of diversity (Bond et al., 2001; Bickford et al., 2006) whereas a sequence-based approach could potentially result in inflation (Bickford et al., 2006). Also, as demonstrated by Bailey et al. 2010 (in press), cryptic species are not entirely endemic to morphological constructs- that is, species paraphyly (Funk and Omland, 2003) and sexual selection by female choice or sexual conflict (Bond et al., 2001) can mask species boundaries in molecular data sets. Confounding this is the fact that species are the fundamental units of many biological studies and without this framework every ecological, evolutionary, phylogenetic, behavioral, physiological, comparative, and conservation related study would suffer (Bailey et al., 2010). These genetically divergent, yet

morphologically homogeneous organisms become problematic when using lineage based approaches to delimit species because populations may have diverged genetically long before they would be recognized as species phenotypically (Bond and Stockman, 2008). A number of authors have sought to abrogate problems of species delimitation by framing the species problem as a testable hypothesis. One such framework is the cohesion species concept proposed by Templeton (1989).

The cohesion species concept defines a species as an evolutionary lineage, where boundaries arise from the forces that create reproductive communities (Templeton, 1998b). Species cohesion is maintained through demographic processes like ecological interchangeability and genetic exchangeability. Populations are ecologically interchangeable if they occupy the same type of niche, and genetically exchangeable if there are no factors limiting gene flow. Gene flow can be limited by geographical barriers (mountains, rivers, etc.), isolation by distance, incompatible genitalia, and post-zygotic isolating mechanisms. Evaluating gene trees in a geographical context allows tests of genetic exchangeability by identifying potential physical barriers to gene flow. Ecological interchangeability is commonly tested by evaluating the amount of morphological differentiation or by niche-based distribution modeling by applying GIS environmental data and determining the amount of distribution model overlap (see Stockman and Bond, 2007).

As noted, species delimitation in millipedes has traditionally relied on differences in somatic morphological characters (Gardner, 1974), and the order Platydesmida is no exception. Primary divisions between families are typically based on sternal plate variation and the presence or absence of a sternal process (Gardner, 1974). Further divisions are based on differentiation in other somatic characteristics (e.g. shape of paranota, tubercules, etc.). The use of somatic

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characters is not commonplace in millipedes; rather, most millipedes are described based on differences in sexual characters, specifically gonopod (modified appendages used for mating) morphology (Gardner, 1974). The main reason andrognathid (and all other millipedes in the order Platydesmida) species descriptions are not based on gonopod morphology is due to their small size. For example, Gardner (1975) believed that although andrognathid gonopods appear generally primitive in structure, their apical processes (tip of gonopod, used for insemination) might possess distinctive, species specific shapes. Unfortunately, these characteristics were too difficult to discern using a 500x compound microscope. Differences in sexual characters are often good indicators of reproductive isolation (Coyne and Orr, 2004); thus, further examination of andrognathid genitalia using light and scanning electron microscopy could greatly enhance our knowledge of andrognathid species.

Study Taxon

The genus *Brachycybe* is a member of the Andrognathidae, the larger of two families in the order Platydesmida. Twelve andrognathid genera are recognized; these include *Andrognathus, Brachycybe, Dolistenus, Fioria, Gosodesmus, Ischnocybe, Mitocybe, Plutodesmus, Pseudodesmus, Sinocybe, Symphyopleurium,* and *Yamasinaium.* The second family, Platydesmida contains just two genera, *Platydesmus* and *Desmethus*. The Platydesmida are widespread geographically, occurring in temperate and tropical regions (Gardner, 1975). The Andrognathidae have a more extensive distribution and occur in North America, Europe, Japan, China, Sumatra, and the Malay Peninsula (Gardner, 1975). This widespread distribution indicates they are an ancient species and within the andrognathids, the genus *Brachycybe* is considered to be among the oldest, dating to at least the Miocene (Gardner, 1975).

Brachycybe Wood, 1864 is composed of small, nonvagile millipedes that are no more than 4-5 centimeters in length. The genus is diagnosable by a broad, dorsoventrally flattened body (with a variable number of tubercles on its dorsal side), alate paranota and lack of ocelli (Fig. 1.1). Species are usually reddish but vary over the geographic range. Millipedes in this genus generally inhabit moist areas on the underside of fallen debris where fungi are often present. Indeed, the diet is presumed to be fungi, though only one study has documented this phenomenon (Gardner, 1975). Andrognathids are usually associated with decaying wood, living on or within it, and all are thought to consume fungus and are specifically drawn to fungi that grow on decaying oak wood (Gardner, 1974). Many andrognathids share similar morphological characteristics, which may be due to habitat similarities. Of all the andrognathid genera, *Gosodesmus* appears to be the most closely related to *Brachycybe* because of its tuberculate collum and relatively well developed paranotal flanges (Gardner, 1974). These millipedes also occur in sympatry (personal observation). Based on their high relative abundances in old growth forests (personal observation), this is likely the preferred habitat for members of these genera, where they almost always occur in aggregations that vary in number (Fig. 1.2). These specific habitat requirements, along with the group's low vagility, likely allow populations to become rapidly isolated.

Fig. 1.1. Image illustrating anatomy of *B. lecontii*.

Fig. 1.2. Image of *B. lecontii* congregation in optimal habitat, feeding on lichen.

Brachycybe comprises seven known species and possibly an eighth in Taiwan (Shelley *et al.*, 2005). Five species occur in the United States (see localities collected, Fig. 1.3A), whereas the other two inhabit Japan, South Korea, and China. Members of the genus that occur in the eastern United States (Fig. 1.3B) are *B. lecontii* Wood, 1864, and *B. petasata* Loomis, 1939. *Brachycybe lecontii* is extensively distributed throughout eastern Texas and Oklahoma, Missouri, Arkansas, Louisiana, Mississippi, Georgia, Alabama, South Carolina, Tennessee, Kentucky, Southern West Virginia, Virginia, and North Carolina. However, *B. petasata* is restricted to the Blue Ridge including western North Carolina, eastern Tennessee, northern Georgia, and northeastern Alabama. *Brachycybe lecontii and B. petasata* occur sympatrically in the Blue Ridge mountain area (personal observation).

Brachycybe rosea Murray, 1877*, B. producta* Loomis, 1939*,* and *B. picta* Gardner, 1975, occupy the western United States and are endemic to California and southern Oregon. *Brachycybe producta* is the most widespread and abundant of the three species, occurring in both California and Oregon, sometimes occurring in sympatry with both *B. rosea* and *B. picta. Brachycybe picta* is characterized by small populations limited to two counties (Marin and Mendocino) on the California coast. To date only 24 specimens have been documented, indicating that *B. picta* may be rare and possibly threatened (Shelley *et al.*, 2005).

Brachycybe nodulosa Verhoeff, 1935, was thought to be endemic to Japan; however, one specimen has been collected in South Korea. *Brachycybe cooki* Loomis, 1942, is known only from the type locality in Guling Province, China. The distribution gaps present on the islands of Japan, as well as South Korea and China, are likely the result of insufficient geographic sampling.
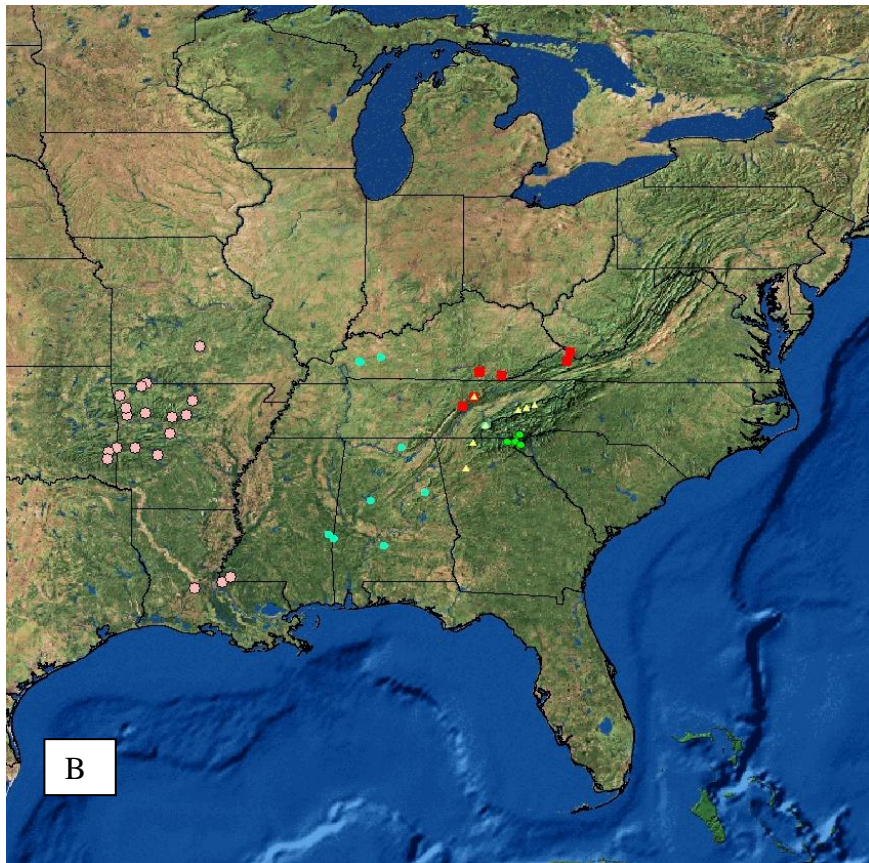
Fig. 1.3. Localities of collected *Brachycybe*. Figure A displays areas sampled across the

United States. Figure B shows localities of *B. petasata* (yellow triangles) and *B. lecontii* clades (other colored shapes).

The disjunct holarctic distribution of *Brachycybe,* with species occupying the eastern Palaearctic as well as the eastern and western Nearctic, combined with the long terrestrial presence of this millipede group, suggests that many geographic barriers and geological processes may have influenced the diversity and distribution of this genus. Hendrixson and Bond (2007), Sanmartin *et al*. (2001), and Wen (1999) have proposed a number of hypotheses regarding the disjunct Asia and North American distributional pattern, although a more recent hypothesis supports a direct connection between Asia and North America (McCarthy, 2005).

Project Objectives

The primary objectives of this study are: 1) to sequence the cytochrome b and cytochrome c oxidase I gene regions for all species (and multiple populations) of *Brachycybe*; 2) evaluate a morphological data set for all species; 3) reconstruct the phylogeny of *Brachycybe* using the two mitochondrial genes; 4) employ a phylogenetic approach to evaluate biogeography of the genus; 5) evaluate species boundaries within the genus for species that were sampled sufficiently. The approach outlined herein will provide the phylogenetic framework necessary to construct a natural classification for the genus and to evaluate species boundaries for further taxonomic classification, allowing proper species delimitation.

MATERIALS AND METHODS

Collection of fresh tissue primarily focused on the eastern nearctic species (*B. lecontii*

and *B. petasata)* and secondarily on the three species in the western nearctic (*B. rosea, B. picta, and B. producta),* with the primary aim to cover the entire range of species. Several eastern Palearctic specimens were also obtained from Japan. Sampling involved capturing 3-5 specimens per location (following the collection protocol of Wiens and Penkrot, 2002); however, due to the poor habitat suitability of some areas, fewer were often collected. Live specimens were transported back to the laboratory for DNA extraction and preserved in ethanol or RNAlater (QIAGEN).

The mid region of specimens were removed using a razor blade or forceps for DNA extraction. Preservation of the anterior region is necessary due to the density of diagnostic characters in that body area. All important morphological features, such as the gonopods and column, were preserved in 80% ethanol for further morphological analyses. Tissue used for DNA extraction was preserved in RNAlater (QIAGEN) at -80 degrees C. Total genomic DNA was extracted using the QIAGEN DNEasy Tissue kit. Standard polymerase chain reaction (PCR) protocols and reagents were used to extract approximately 1400 bp of mtDNA from the cytochrome b (Cytb) and cytochrome c oxidase subunit 1 (CO1) protein coding regions using primers BRAC_CObA (5'-ACAGGATTATTTTTATCYATWC-3') and BRAC_CObR (5'-GGGCATGATCCAATTCAAGT-3') for Cytb and Cl-J-1718 (5'-GGAGGATTTGGAAATTGATTAGTTCC-3') and CO1rev (5'-GCAATATCTAAAGATGAATTTGCT-3') for CO1. Amplified fragments were cleaned by using ExoSAP-IT (USB) and sequenced on an ABI Prism 3130 automated DNA sequencer (Applied Biosystems).

Morphological characteristics were assessed using a Quanta 200 Mark 1 (FEI) scanning electron microscope. Specimens (preserved in 80% ethanol) were moved to 100% ethanol for at

least an hour and then chemically dried by submersion in 1,1,1,3,3,3 hexamethyl disilizane (Acros Organics). After 1-2 hours in hexamethyl disilizane, the specimens were left to dry overnight under a fume hood. Specimens were then mounted onto stubs and sputter coated for approximately 4 minutes at 15 milliAmps using an Anatech LTD sputter coater.

Phylogenetic Analysis

Clustal X (Thompson *et al*., 1997) and Muscle (Edgar, 2004) were used to initially align DNA sequences. Bayesian analyses were conducted on datasets partitioned by codon for each gene. A concatenated matrix comprising CO1 and Cytb were constructed using Mesquite version 2.72 (Maddison and Maddison, 2009). The program Kakusan 3 (Tanabe, A. S., 2007) was used to determine the appropriate model of DNA substitution for the Bayesian analyses. MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) was set up to run at least 2,000,000 generations with four Markow Chain Monte-Carlo (MCMC) chains; generation number was increased until convergence was reached (standard deviation of split frequencies <0.01). The *sump* and *sumt* commands along with Tracer v3.1 (Rambaut and Drummond, 2005) were used to summarize and investigate the convergence of parameters. A majority rule consensus tree was produced by discarding all trees prior to log likelihood stabilization (burn-in) by the contype=allcompat command.

In order to evaluate node support, parsimony and maximum likelihood analyses were performed using the programs PAUP* (Swofford, 2002) and Garli 0.951 (Zwickl, 2006). Heuristic searches were performed using 100 random addition sequences and 1,000,000 rearrangements with tree bisection-reconnection (TBR) branch swapping. One tree was held at each step during stepwise addition; all characters were equally weighted and gaps were treated as

missing. Node support was further evaluated by non-parametric bootstrapping with the number of replicates set to 1000 for the parsimony analysis and 100 in the ML analysis. Analyses were run on CO1 and Cytb datasets separately, together, and with the exclusion of the third codon position. Bootstrap analyses were run using only the concatenated dataset along with the dataset that excluded the third position.

Evaluation of Species Boundaries

Species status and boundaries were tested using the cohesion species concept. A population may qualify as a cohesion species if they are *derived from a single evolutionary lineage* (i.e., must share a common ancestor), and they must be *genetically exchangeable and/or ecologically interchangeable* (Templeton, 2001). The validity of nominal species was verified using these criteria followed by the evaluation of possible cryptic species using the same standards.

To determine whether populations sampled are from a single evolutionary lineage, a basal lineage starting point must be established. If the focal taxon is genealogically exclusive, then the first hypothesis is supported and the second can be evaluated. For nominal species that are confirmed in this manner, candidate species—cryptic species—were designated based on geographic concordance of monophyletic lineages. These candidate species were then evaluated for genetic exchangeability and ecological interchangeability before they are considered for elevation to species status.

Genetic exchangeability is assessed as a sister group comparison of the daughter lineages based on the previously selected basal lineages. If the lineages are geographically disjunct and populations are absent from the intervening area, then gene flow is not possible. In other cases

lineages may be parapatric with or without geographic barriers. Ecological interchangeability will then need to be evaluated to determine if the lineages should be treated as cohesion species. Non-genetically exchangeable populations are then evaluated for ecological interchangeability using niche based distribution modeling (Stockman and Bond, 2007) and assessing habitat differences by evaluating adaptive divergence within a phylogenetic framework (Bond and Stockman, 2008). Distribution models were created in MAXENT (Phillips *et al.*, 2006) and include environmental variables thought necessary for the survival of *Brachycybe*. All of these data were combined and analyzed in a Geographic Information System (GIS).

Niche-Based Distribution Modeling

Niche-based distribution models were used to yield projections of a species' potential habitat using geographical presence data along with a set of environmental (predictor) variables. The distribution models produced by MAXENT are based on 19 bioclimatic variables that can be downloaded from the WorldClim website (http://www.worldclim.org/bioclim). The distribution models (DM) were created using MAXENT, which is considered to be one of the better comparative analysis programs (Elith et al. 2006; Pearson et al. 2006; Phillips et al. 2006). MAXENT is a machine-learning method that is based on finding the probability distribution of maximum entropy (i.e., closest to uniform) to estimate a target probability distribution (Stockman and Bond, 2007). MAXENT offers many advantages, which include: 1) requiring only species presence data, utilization of both continuous and categorical data, and 2) an efficient deterministic algorithm that allows for convergence on the optimal probability distribution (Phillips et al. 2006). Information output from MAXENT as an Ascii layer is combined and further analyzed in ArcGis (ESRI).

RESULTS

Summary of Sequence Data

Four hundred and eight millipedes were collected from 66 locations in the eastern and western United States of which 167 were sequenced. Following sequence alignment, identical haplotypes were collapsed, yielding 115 unique haplotypes.

The sequence divergence (uncorrected p) average for the CO1 dataset within the in-group is 11%, with a minimum divergence of 0.1% and a maximum of 26%. The sequence divergence average for the Cytb dataset is 13%, with a minimum of 0.1% and a maximum of 29%. The base frequency Chi square value for the CO1 dataset was 281.31 with 321 degrees of freedom and a p = 0.95, indicating that the frequencies are homogeneous. However, the Chi square value for the Cytb dataset was 615.59 with 327 degrees of freedom and a p < 0.0001, indicating that base frequencies are not homogeneous.
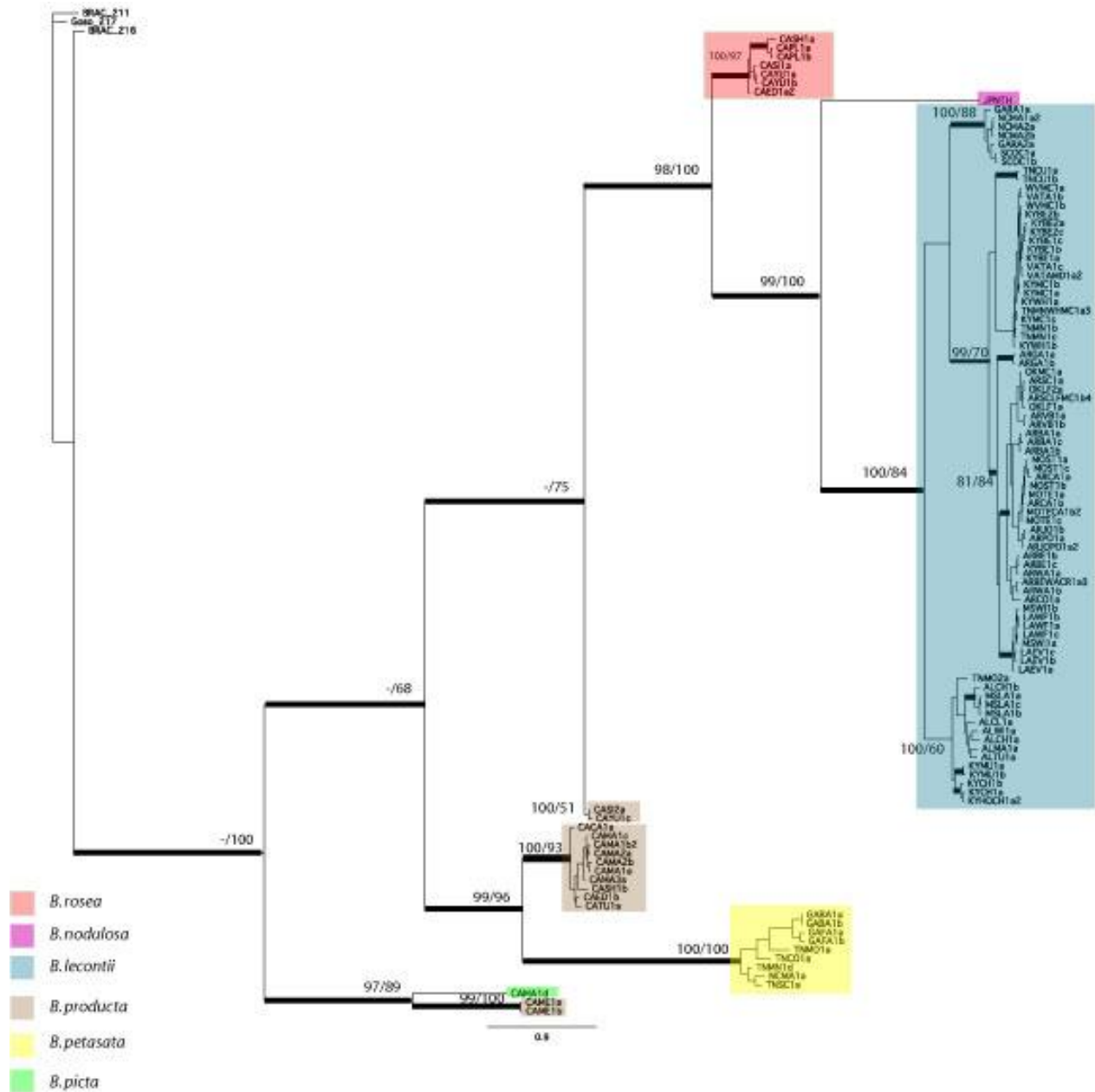
Phylogenetic Analyses

DNA substitution models obtained from Kakusan 3 (Tanabe, 2007) for each partitions were: CO1 and Cytb Bayesian analysis (HKY85+Γ), the models for the CO1 concatenated set are: first codon position (GTR+Γ), second (F81+Γ), and third (HKY85+Γ). The models used for the Cytb partition are: first and second, (GTR+Γ) and the third (HKY85+Γ) (Table 1.1). All model determinations are based on the Bayesian Information Criterion. The combined Bayesian analysis ran for 6 million generations discarding the first 4 million, CO1 ran for 9 million discarding the first 3 million, and Cytb ran for 4 million generations discarding the first half. The corresponding likelihood values for the arithmetic harmonic means are: -11994.23 and -12085.27, -5852.96 and   -5941.13, -6209.07 and -300.35.

Phylogenetic trees produced from Bayesian, Parsimony, and Maximum Likelihood are highly congruent. All nominal species except *B. producta* were genealogically exclusive (monophyletic) and recovered by > 98% posterior probabilities (Fig. 1.4, darkened lines). These clades have overwhelming Bayesian posterior probability and bootstrap support, as well as, large divergence estimates (Fig. 1.4). The Bayesian tree defines a minimum of four clades/lineages comprising *Brachycybe lecontii* and three *Brachycybe produta* clades, all have high associated support values.

Table 1.1: Summary of Bayesian analyses.

| Partitions	Codon position	Likelihood values (Arithmetic, harmonic)
CO1	1: HKY85+Γ	-5852.96, -5941.13
	2: HKY85+Γ	
	3: HKY85+Γ	
Cytb	1: HKY85+Γ	-6209.07, -6300.35
	2: HKY85+Γ	
	3: HKY85+Γ	
CO1 concatenated	1: GTR+Γ	-11994.23, -12085.27
	2: F81+Γ	
	3: HKY85+Γ	
Cytb concatenated	1: GTR+Γ	-11994.23, -12085.27
	2: GTR+Γ	
	3: HKY85+Γ	

Fig. 1.4.   Inferred mtDNA haplotype genealogy for *Brachycybe* and outgroups using Bayesian inference.   The data set used was partitioned by codon position and concatenated (Cytb and CO1).   Dark lines represent > 98% posterior probability support.   Numbers at nodes are parsimony (1st) and maximum likelihood bootstrap values.   Nominal species are represented

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by color.

Niche Models

Distribution models were produced with MAXENT using bioclim climatic variables, from Worldclim (Hijmans et al. 2005). These variables along with the known localities of each clade were used to identify possible niche variation from the data. Models were produced only for *B. lecontii* and *B. producta*. *Brachycybe lecontii* was the only species that was further subdivided by clade for subsequent niche-based distribution modeling in MAXENT.
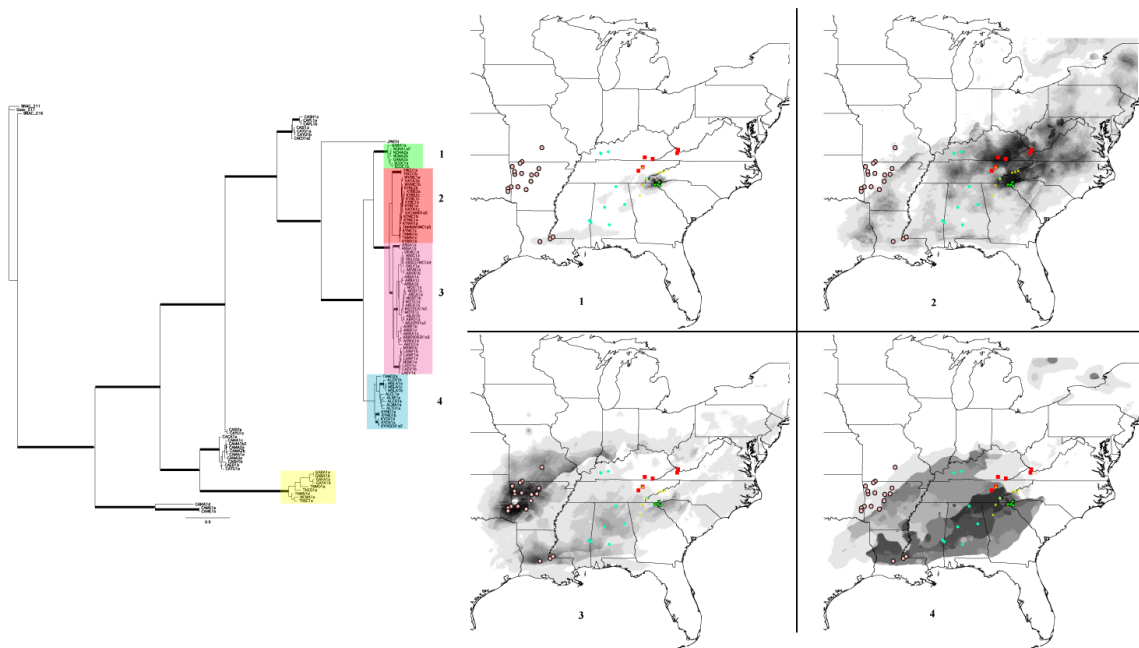
Fig. 1.5. Distribution models of all *B. lecontii* clades 1-4. Each *Brachycybe* clade is colored for tree to map correspondence. Clade 1 is green: the corresponding distribution model is labeled with a 1, clade 2 is red: the corresponding distribution model is labeled with a 2, clade 3 is pink: the corresponding distribution model is labeled with a 3, and clade 4 is blue: the corresponding distribution model is labeled with a 4.

The distribution models for the *B. lecontii* clade 1 and clade 3 (Fig. 1.5) were the only models that projected habitat exclusivity from all other clades. It is also worthwhile to note that in all DM's, except the clade 3 model, this clade was almost entirely excluded. This is also true for the *B. petasata* DM (Fig. 1.6) that completely excludes clade 3. Many of the represented clades were not optimal for this type of analysis because of the paucity of specific specimens sampled and the large geographic area that they cover.
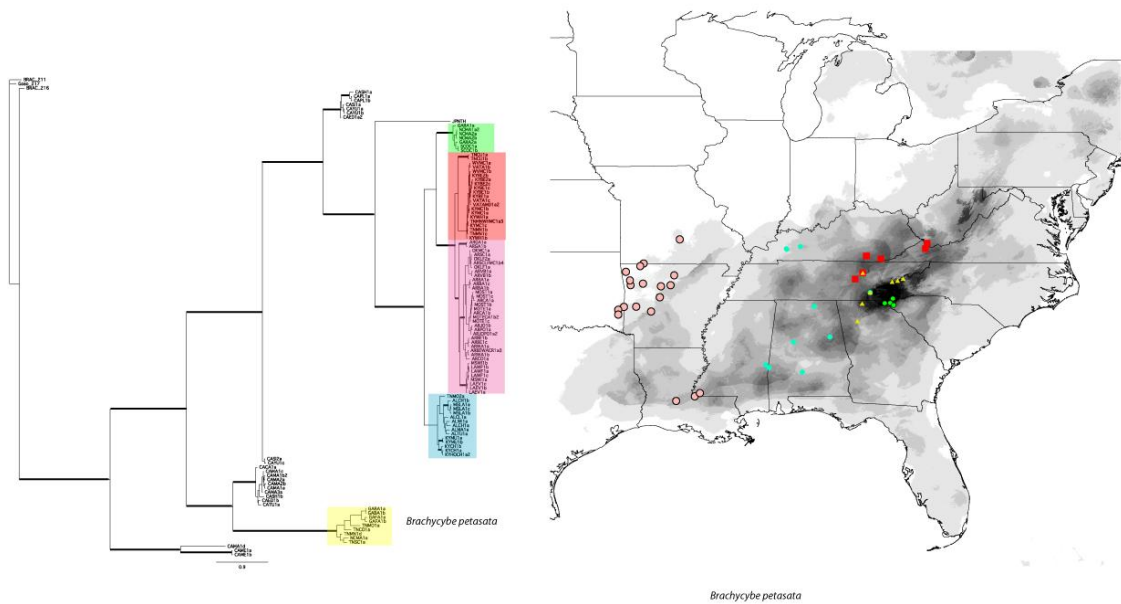
Fig. 1.6. Distribution model of *B. petasata* (yellow).

Morphology

Scanning electron microscopy was used to visualize potential morphological divergence between nominal species and possible cryptic species that were identified to evaluate further (*a posteriori*) by the phylogenetic inference. Because differences in gonopod morphology are often useful as a metric of species diversity, the gonopods were thoroughly examined (Figs. 1.7 and 1.8). While the focus was on gonopod morphology, images of other areas were recorded, such

as, dorsal turbercules, paranota, and the collum covering the head. We were unable to quantify gonopodal disparity. Figure 1.7 (A-D) illustrates gonopods from two *B. lecontii* clades. Figrues 1.7A and 1.7B are from clade one (Fig. 1.5), whereas 1.7C and 1.7D are images recorded for individuals from clade 4 (Fig. 1.5). Although these clades exhibit high genetic divergence the gonopods appear to be homogenous. Although we would expect morphological homogeneity of gonopods from individuals drawn from the same nominal species, we would alternatively expect some species specific variation. Figure 1.7 (E, F) shows *B. petasata,* the other species that can be found in sympatry with *B. lecontii*. Although members of this species are readily diagnosable from *B. lecontii* on the basis of other somatic features, no qualitative differences can be observed in their genitalia structures. Figure 1.8 represents images from two western American and one Japanese species: *B. rosea* (A and B)*, B. producta* (C and D)*,* and *B. noduloso* (E and F). Figure 1.4 displays the large amount of genetic variation between these groups but, likewise, no qualitative differences seem apparent in their genitalic structures (Figs. 1.7 and 1.8). It was unknown whether there would be apical process (finger-like projections at tip of gonopod) variation between species because they were too small to be seen using light microscopes. It is evident now that there are no gonopodal differences among *Brachcybe* species. The only morphological variation present involved the already known somatic differences previously mentioned.
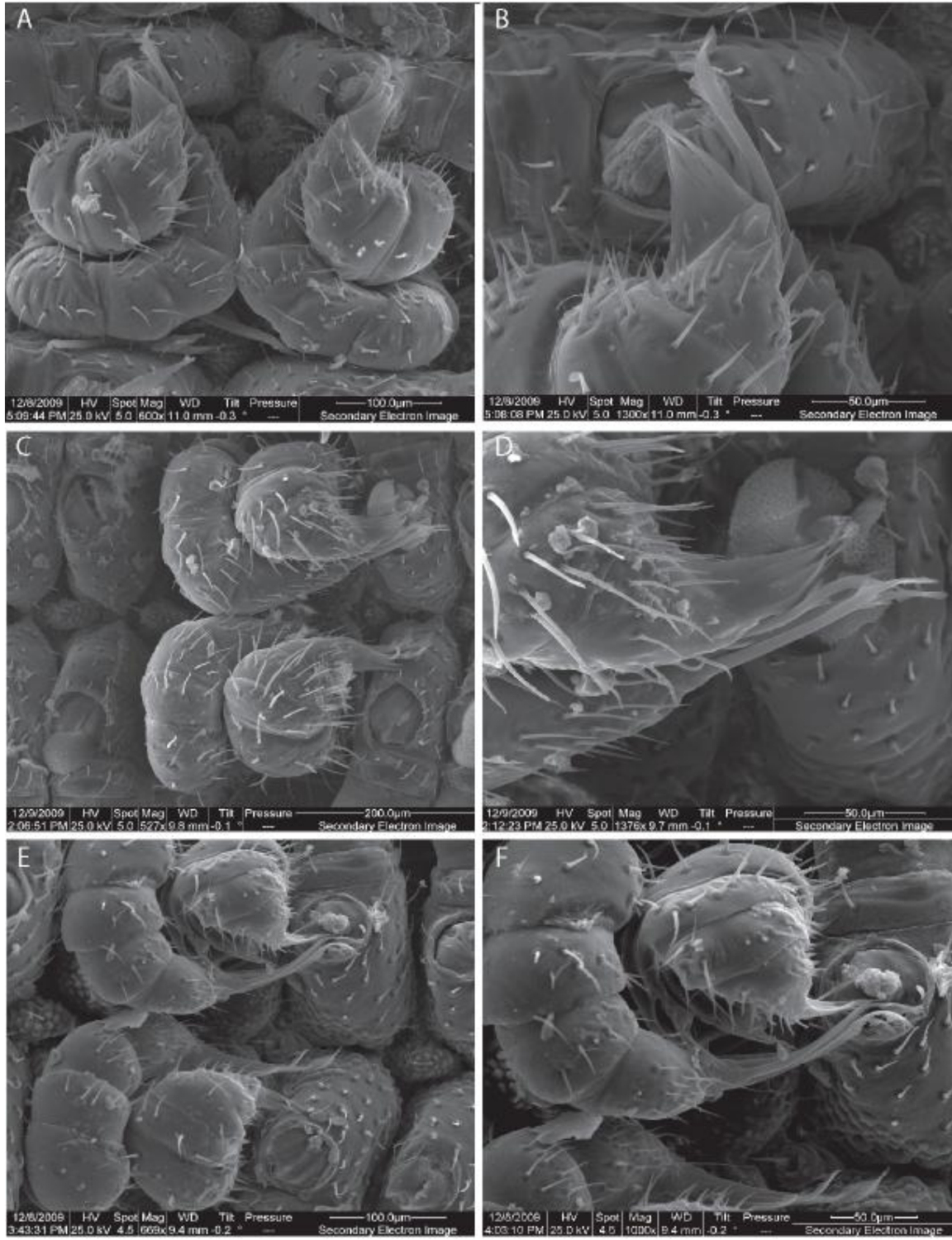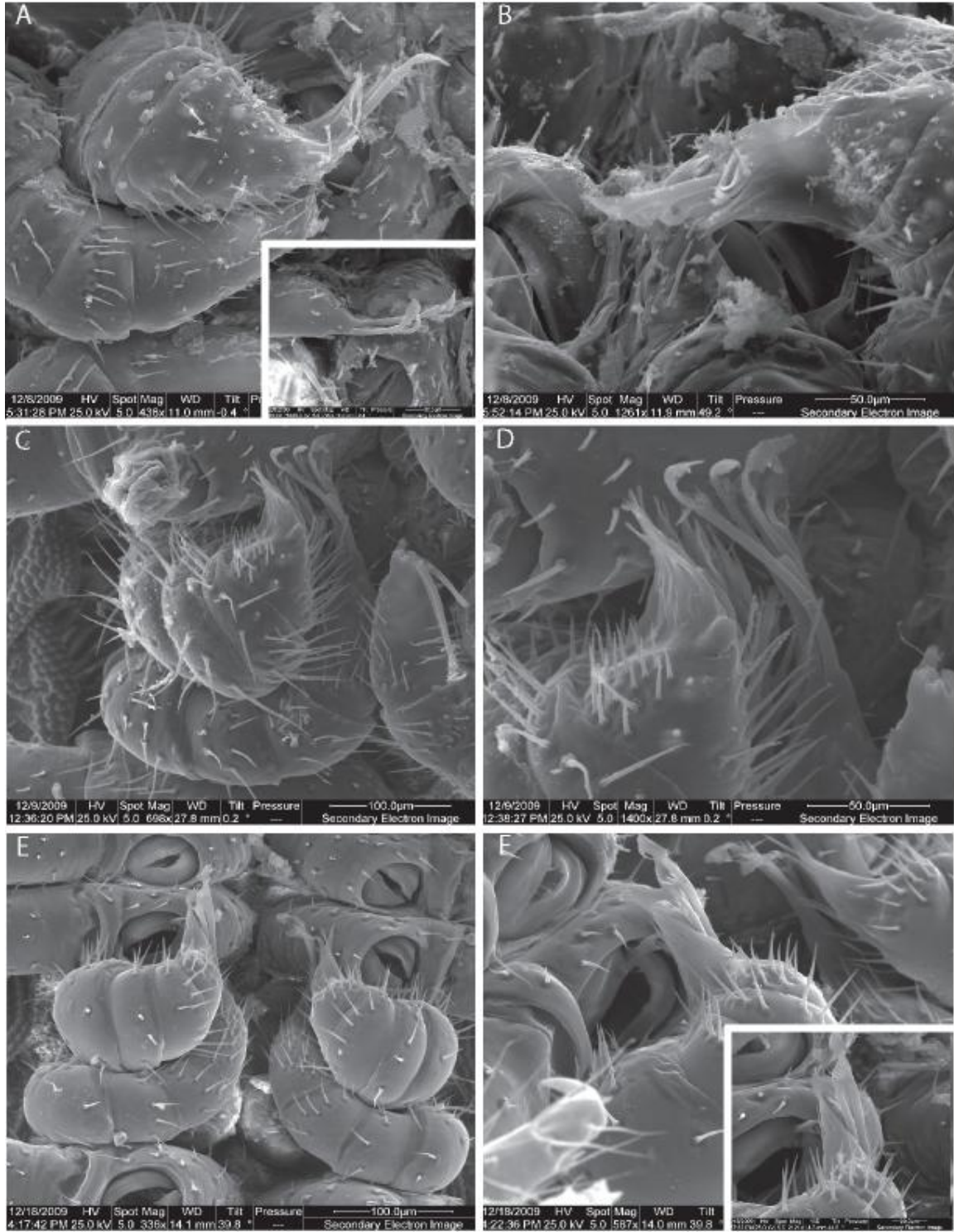
Fig. 1.7. **Images of *Brachycybe* gonopods.** Images A and B are from the *B. lecontii*_83-clade1,

C and D are from *B. lecontii*_356-clade 4, E and F are from *B. petasata*_77.

Fig. 1.8. Images of *Brachycybe* gonopods. Images A and B are from *B. rosea*_169, C and D from *B. producta*_173-clade 2, E and F from *B. noduloso*_418

Species delimitation in the genus *Brachycybe* is problematic due to the group's small size and homogenous morphology. Gonopodal heterogeneity, one of the more important diagnostic characteristics in millipedes, is absent within *Brachycybe*. The only morphological differences present involve certain somatic characters that most inexperienced individuals would be hard pressed to recognize and evaluate. Conversely, members of this genus and some populations within nominal species appear to be quite divergent at a molecular genetic level, and thus morphology may belie the actual evolutionary diversity contained within *Brachycybe*. A lineage based approach to species delimitation is desired to evaluate species beyond a visual based approach. However, organisms that exhibit strong population structuring without morphological differentiation present a challenge for lineage based approaches (Hedin and Wood 2002, Sinclair *et al*. 2004). Under a strict lineage based approach almost all population groups are independent lineages (Agapow et al., 2004; Hickerson et al., 2006) because delimitation based solely on sequence information would yield roughly 20 species. In order to correctly identify species, multiple lines of evidence are needed for delimitation (de Queirox, 2005, Will *et al*., 2005). Here the use of the cohesion species concept to delimit species boundaries is imperative due to its systematic nature.

Evaluation of species boundaries for eastern clades. - Species status and boundaries were tested specifically for the two eastern species of *Brachycybe* for which collections were more robust. The first step was to determine if the two species are *derived from a single evolutionary lineage* (sharing a common ancestor). Figures 1.5 and 1.6 illustrate the concordance between haplotype trees and the associated locality information for each taxon. Each nominal species is indeed genealogically exclusive, satisfying the first hypothesis of the cohesion species concept.

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Monophyly of the nominal species suggests lack of gene flow and, therefore, non-genetic exchangeability. Non-genetically exchangeable populations were evaluated for ecological interchangeability using niche based distribution modeling (Stockman and Bond, 2007) and assessing habitat differences by evaluating adaptive divergence within a phylogenetic framework. Following the cohesion species delimitation schematic from Bond and Stockman (2008) we failed to reject the hypothesis that *B. petasata* comprises a single species. All individuals sampled are derived from a single evolutionary lineage and each population was genetically exchangeable and ecologically interchangeable. Alternatively, based on the combined Bayesian analysis, *B. lecontii* can be split into 4 separate clades (Fig. 1.5). Although, there are no morphological differences between clades 1-4 of *B. lecontii*, the high levels of genetic divergence is indicative of cryptic species. Following the same cohesion species protocol, each potential species was evaluated for genetic exchangeability and ecological interchangeability.

All clades of *B. lecontii* are allopatric and separated by geographic barriers to gene flow, therefore, reinforcing the fact that these lineages are not genetically exchangeable. But are the clades ecologically interchangeable? Figure 5 shows the niche based distribution models produced for each clade. All models, except for the clade 3 model, suggest that populations are ecologically interchangeable, given the amount of niche overlap in the models. In this case, we are unable to reject hypothesis 2 (lineages are genetically and/or ecologically exchangeable) for clades 1, 2, and 4 (see Table 1.2). However, the distribution model for clade 3 does not overlap with the models produced for all other clades. Likewise, the distribution models produced for each of the other clades also exclude clade 3. All clades of *B. lecontii*, except for 3, were parapatric geographically but were retained as cohesion species because they appeared to be

ecologically interchangeable (sensu Crandall et al., 2000). It is interesting to note that geographically, clade 3 is the most disjunct.

Table 1.2: Results of species delimitation. The first column represents *B. lecontii* clades depicted in figure 5; G_E refers to genetic exchangeability and E_I refers to ecological interchangeability.

Clades	G_E	E_I	Inference
1	No	Yes	Noncohesive clade: niche conservatism considered
2	No	Yes	Noncohesive clade: niche conservatism considered
3	No	No	Noncohesive clade: geographical discordance
4	No	Yes	Noncohesive clade: niche conservatism considered

Many of the clades show extreme genetic divergence but have little to no ecological divergence, a pattern indicative of niche conservatism. Niche conservatism is characterized by the organisms' low vagility and specific habitat requirements. They share similar ecological habitats but are separated by many geographical barriers that have attributed to an absence of gene flow and increase in genetic divergence (Peterson et al. 1999, Raxworthy et al. 2003, Wiens and Graham 2005). Based on the data presented here, we hypothesize that clade 3 should be elevated to species level.

Summary

Systematics has two major goals: 1) discover and describe species and 2) determine the phylogenetic relationships of these species (Wiens, 2007). Species are the fundamental units of biology and many now agree that they can be defined as lineages (e.g., de Queiroz, 2007; Knowles and Carstens, 2007; Shaffer and Thomson, 2007; Raxworthy et al., 2007; Rissler and Apodaca, 2007, Wiens, 2007). But how do we delimit those lineages? We know that applying any single approach haphazardly can result in failure, by either overinflating or under inflating "true" species numbers. The application of the cohesion species concept to the genus *Brachycybe* provides a more accurate representation of species number, regardless of their cryptic nature. Our evaluation is by no means perfect or complete, but we consider it to be a step in the right direction.

Biodiversity is often measured by total numbers of species and is only one of the reasons why it is necessary to appropriately describe each one. With such fragile and specific habitat requirements *Brachycybe* is in danger of becoming extinct in heavily populated areas. Concern has been expressed for (*B. picta* and *B. petasata*) the two species with enlarged flabellate colla that are considered to be the oldest and most primitive morphologically (Shelley *et al*., 2005). *Brachcybe picta* does appear to be among the older species based on its basal location on the phylogenetic tree. It is important to note that only approximately 25 specimens (1 of 25 personally collected) have been collected, despite extensive sampling efforts north of San Francisco Bay. It is unfortunate that there are not, and may never be enough specimens to adequately support life history questions within this species.

CHAPTER 2: Complete Mitochondrial Genome of *Brachycybe lecontii* Wood, 1864

(Platydesmida: Andrognathidae) and Myriapod Phylogeny

INTRODUCTION

The class Diplopoda is a megadiverse group and include the oldest known terrestrial organisms (Wilson *et al.*, 2004). As such, this group has served as an integral part of terrestrial ecosystems for millions of years. Millipede diversity comprises 12,000 described species with upper estimate of over 80,000 (Hoffman *et al.*, 2002; Marek and Shelley, 2005). Despite being extremely diverse and widespread millipedes remain despairingly understudied and have received little systematic attention at higher phylogenetic levels. The use of complete mitochondrial genomes has shown promise in reconstructing the evolutionary history of a variety of taxa, including arthropods (e.g. Boore, 1999; Curole *et al.*1999; Hassanin, 2006; Hwang *et al.*, 2001; Hwang and Kim, 1999; Lim *et al.*, 2006) due to their low recombination and rearrangement rate (Boore, 1999; Boore and Brown, 1998). Genome evolution can be studied more efficiently using mitochondrial systems because of their smaller size and lack of complexity when compared to nuclear genome systems (Boore, 1999). The ability to sequence and analyze total mitochondrial genomes will likely play a larger part in phylogenetic reconstruction at all phylogenetic levels.

To date, only three diplopod mitochondrial genomes have been sequenced. Lavrov *et al.* (2002) presented the mitochondrial genomes of two millipedes, *Narceus annularus* (Rafinesque, 1820) (Spirobolida: Spirobolidae) and *Thyropygus sp.* (Pocock, 1894) (Spirostreptida: Harpagophoridae), and Woo *et al.* (2007) sequenced *Antrokoreana gracilipes* (Verhoeff, 1938) (Juliformia: Julida). These organisms all share similar gene arrangements with the exception of some transfer RNA gene positions that differed in *A. gracilipes*. Animal mitochondrial genomes

are known to evolve rapidly, yet gene arrangement is relatively unchanged over long periods of evolutionary time (Boore, 1999). This identical gene arrangement is said to be common within major groups but variable between them (Boore, 1999). With such an extreme amount of diversity within the phylum Arthropoda, large amounts of differentiation are likely. The complete mitochondrial genome of *Brachycybe lecontii* (Diplopoda: Platydesmida: Andrognathidae) is presented here with comments on its incredibly unique gene synteny.

MATERIALS AND METHODS

A section of the mid-region of the trunk was excised from the body of *Brachycybe lecontii* specimens using a razor blade and forceps, stored in RNAlater (Qiagen Inc., Valencia, CA) at -80˚C, and used for DNA extractions. Total genomic DNA was extracted using the QIAGEN DNEasy Tissue kit (Qiagen Inc., Valencia, CA). The 16S mtDNA region was amplified and sequenced using the universal arthropod primers LR-J 12887 (5'-CCG GTC TGA ACT CAG ATC ACG T-3') and LR-N 13398 (5'-CGC CTG TTT ATC AAA AAC AT-3') (Simon *et al.* 1995). Nested primers were designed for *Brachycybe* specimen 0040 using previously generated 16S data following Hwang *et al.* (2001a): HPKSaa_brachy (5'-ATG CTA CCT TCG TAC AGT TAA TAT ACT GCA AC) and HPK16Sbb_brachy (5'-CAT ATT GAT AAA TAA GTT TGT GAC CTC GAT GTT-3'). These nested primers were used along with Takara LA Taq polymerase to amplify the remainder of the mitochondrial genome. The following 50 µL PCR mixture was used: 24.5 µL ultra pure water, 8 µL 2.5mM dNTP mixture, 5 µL 10X LA PCR buffer, 5 µL of each 2.5 µM or 10 µM primer, 0.5 µL Takara LA Taq, and 2 µL genomic DNA. The thermal cycle parameters described by Hwang *et al.* (2001) were used, and this product was purified using the ExoSAP-IT procedure.

Cloning techniques were used to shotgun sequence the non-16S regions of the mitochondrial genome followed by primer walking to fill in gaps. A Qiagen Repli-g Genome Amplification Kit was used to obtain enough concentrated DNA for the cloning procedure. Purified mtDNA was digested using the enzymes *Rsa1* (5' GTAC 3') and *Alu1* (5' AGCT 3') in order to get appropriately sized fragments. All cloned amplification products were cleaned using ExoSAP-IT (USB). Purified PCR products were then gel extracted, cloned using an Invitrogen Zero Blunt Non-Topo kit (Invitrogen, Carlsbad, CA), and the resulting clones were harvested and amplified with the primers T7 (5'-TAATACGACTCACTATAGGG-3') and M13 (5'-CAGGAAACAGCTATGAC-3') reverse. Amplified mtDNA fragments were cleaned using Sephadex (Sigma- Aldrich) and sequenced with an ABI 3130x Automated DNA Sequencer (Applied Biosystems). The sequences were edited, cleaned of vector contamination, and annotated using the computer program Sequencher 4.8 (Gene Codes, Ann Arbor, MI).

A second round of cloning was done to fill-in and reinforce the previously produced mitochondrial genome template. In order to get appropriately sized fragments and proper spread, the genome was nebulized instead of digested using the previously mentioned restriction endonucleases. Again, using a Zero Blunt PCR Cloning Kit (Invitrogen, Carlsbad, CA), DNA was inserted into vectors and transformed into *E. coli* cells, which were then grown overnight on kanamycin agar plates. Isolated colonies, each containing a cloned *B. lecontii* mitochondrial region, were amplified and sequenced using the primers M13 Reverse and T7 from the cloning kit. A 25 µL PCR reaction was done using the following mixture: 12.5 µL Promega GoTaq Green Master Mix (Promega, Madison, WI), 9.5 µL ultra pure water, 1.5 µL of each 2.5 µM or 10 µM primer, and 1 swab of an isolated colony. The following thermal cycle parameters were used: initial denaturation at 94°C for 10 min; 30 cycles of denaturation at 94°C for 1 min,

annealing at 56°C for 1 min, and extension at 72°C for 1 min; and final extension at 72°C for 10

min. PCR products were cleaned up with ExoSAP-IT and sequenced using the same protocol

described above. Sequences were viewed, edited, and assembled in the program Sequencher 4.8

(Gene Codes, Ann Arbor, MI).

Genome Annotation

Most tRNA genes were identified using tRNAscan-SE 1.21 (Lowe and Eddy, 1997) using

a cove cutoff score set to 2, which is the recommend setting to detect as many potential genes as

possible. Additional tRNA genes were found by cross comparisons with other known arthropod

genomes, specifically those of the millipede *Thyropygus*, and by the identification of tRNA

specific anticodons. However, even the latter approaches failed to locate the Histidine tRNA

sequence. The tRNA Histidine was placed in the area expected be based on examination of other

Diplopod genomes. Ribosomal RNA genes were aligned with other known millipede sequences

in order to reinforce the location of each because rRNA's are not displayed in an open reading

frame (ORF) finding program. Protein-coding genes were identified by using the NCBI ORF

finder and by running a BLAST search against all millipede DNA sequences available in

Genbank. Non-coding regions were easily identified because sequencing was difficult in these

areas, where there were many base repeats. After each gene and tRNA was found the location of

non-coding regions were highlighted.

Phylogenetic Analysis

The program MAFFT (Katoh 2002) was used to align all 13 protein-encoding genes. The

genes for each species were concatenated prior to phylogenetic analysis. The program RAxML

(Stamatakis *et al.* 2008) was used to produce a phylogentic tree of the included myriopod groups (Fig. 2.2) using the MTMAM+I+G substitution model. Maximum likelihood analysis consisted of 100 tree search replicates, and the best tree had nodal support accessed via 100 bootstrap replicates. The resulting tree was left unrooted because no appropriate outgroup taxon was included in the analysis.

The maximum likelihood (ML) analysis was performed using amino acid sequences from all 13 protein-encoding genes for each myriapod class except the Pauropoda. However, atp8 is not usually included in these analyses because of the high degree of sequence variability and short length (Woo *et al.* 2007). Therefore, the addition of atp8 (101 bps) to the analysis may influence the topology of the resulting phylogenetic tree. In order to determine the amount of variation within atp8 the program Paup (Swofford, 2002) was used to quantify the number of variable characters in the nucleotide and associated amino acid data sets. The percentages of variable characters within both sets were equal at 82.8%.

RESULTS AND DISCUSSION

The complete mitochondrial genome sequence of *Brachycybe lecontii* comprises 15,465 base pairs. It includes 13 protein-encoding genes, 21 tRNA genes (cannot find histidine, anticodons found for the other 2), two rRNA genes, and two large non coding regions that are approximately 436 and 464 bp in length respectively (Fig. 2.1 and Table 2.1). Anticodons were found in the areas where the missing tRNA's should be located. However, it would not be unusual if the tRNA's were lost; for example, some cnidarians have a tRNA deficit, having gained some genes not found in other mtDNA's (Beagley *et al.* 1995; Beagley *et al.* 1996; Beagley *et al.* 1998; Beaton *et al.* 1998; Pont-Kingdon *et al.* 1998; Wolstenholme 1992). Other

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variations in mitochodrial genome composition include some nematodes that lack atp8 (Keddie *et al.* 1998; Okimoto *et al.* 1991; Okimoto *et al.* 1992) and a bivalve that lacks atp8 and contains an extra tRNA (Hoffmann *et al.* 1992). The composition and size of the *Brachycybe* genome is comparable to all other sequenced millipedes. The two non-coding regions are perhaps diagnostic of the class Diplopoda.

**Table 2.1: Table illustrating gene positions, along with size, and strand direction.**

Feature	Gene Product	Position	Size (bp)	Strand
cr1	Control region 1	1-464	464	N/A
nad4	NADH dehydrogenase subunit 4	465-1802	1338	–
nad4L	NADH dehydrogenase subunit 4L	1796-2080	284	–
trnT	tRNA Threonine	2087-2150	63	+
trnL2	tRNA Leucine 2	2157-2220	61	-
16S	Large subunit ribosomal RNA	2221-3528	1309	–
trnV	tRNA Valine	3529-3596	66	–
12S	Small subunit ribosomal RNA	3597-4325	701	–
cr2	Control region 2	4326-4761		N/A
trnY	tRNA Tyrosine	4762-4823	61	+
trnP	tRNA Proline	4824-4888	64	
nad1	NADH dehydrogenase subunit 1	4863-5804	941	-
trnL1	tRNA Leucine 1	5799-5861	1510	
trnI	tRNA Isoleucine	5859-5920	61	
trnM	tRNA Methionine	5920-5982	62	

nad2	NADH dehydrogenase subunit 2	5944-6993	1054	+
trnH	tRNA Histidine	?		
trnC	tRNA Cysteine	6992-7052	60	
trnW	tRNA Tryptophan	7058-7120	62	
cox1	Cytochrome c oxidase subunit 1	7123-8658	1535	+
cox2	Cytochrome c oxidase subunit 2	8662-9339	678	+
trnK	tRNA Lysine	9341-9407	66	
trnD	tRNA Aspartic Acid	9406-9469	62	
atp8	ATP synthase F0 subunit 8	9487-9624	101	+
atp6	ATP synthase F0 subunit 6	9612-10283	671	+
cox3	Cytochrome c oxidase subunit 3	10287-11129	842	+
trnG	tRNA Glycine	11071-11137	66	
nad3	NADH dehydrogenase subunit 3	11135-11503	368	+
trnA	tRNA Alanine	11490-11558	65	
trnR	tRNA Arginine	?		
trnN	tRNA Asparagine	11637-11702	68	
trnS1	tRNA Serine 1	?		
trnE	tRNA Glutamic Acid	11757-11821	64	
nad6	NADH dehydrogenase subunit 6	11807-12301	494	+
cob	apoytochrome b	12303-13415	1112	+
trnS2	tRNA Serine 2	13417-13483	65	
trnQ	tRNA Glutamine	13513-13578	64	
trnF	tRNA Phenylalanine	13753-13814	61	
nad5	NADH dehydrogenase subunit 5	13712-15437	1724	–

The mitochondrial genome of *Brachycybe* can be broken up into two gene clusters ((nad1-nad5) and (12S rRNA-nad4)) separated by the two aforementioned non-coding regions. In other sequenced millipede mitochondrial genomes these clusters have opposite transcriptional directions; however, the mitochondrial genome of *Brachycybe* is dramatically different in that the cluster containing the rRNA genes has been shortened by the movement of the nad1 and nad5 genes, along with their associated tRNA's (Fig. 2.1) to the other opposing gene cluster. These translocated genes retain their original transcriptional polarity, which is strange considering that no promoter for transcription initiation has been found. These observations are novel among other sequenced members of this class and deviate from other mitochondrial genomes, as the majority of other animal mitochondrial genomes are transcribed from a single strand (Shadel and Clayton, 1997) or arranged in clusters with alternating polarity and have a single non-coding region (Lavrov et *al* 2002 and Boore 1999).

The gene cluster (nad1-nad5) includes the majority of genes in the mitochondrial genome of *Brachycybe* and consists of 38.0% adenine, 15.8% cytosine, 8.5% guanine, and 37.8% thymine. The composition of the cluster (12S rRNA-nad4) containing the rRNA genes is 36.6% adenine, 6.2% cytosine, 14.3% guanine, and 42.9% thymine. Together the A+T concentration is 77.6%, which is not unusual for arthropod mitochondrial genomes that usually range from 60.4% *in Tigriopus japonicas* (Crustacea) to 86.7% in *Melipona bicolor* (Hymenoptera) (Woo *et al.* 2007). Alternatively, the A+T concentration in *Brachycybe* is much higher than that of other millipedes (*N. annularis* 63.7%, *T. sp* 67.8%, *A. gracilipes* 62.1%).
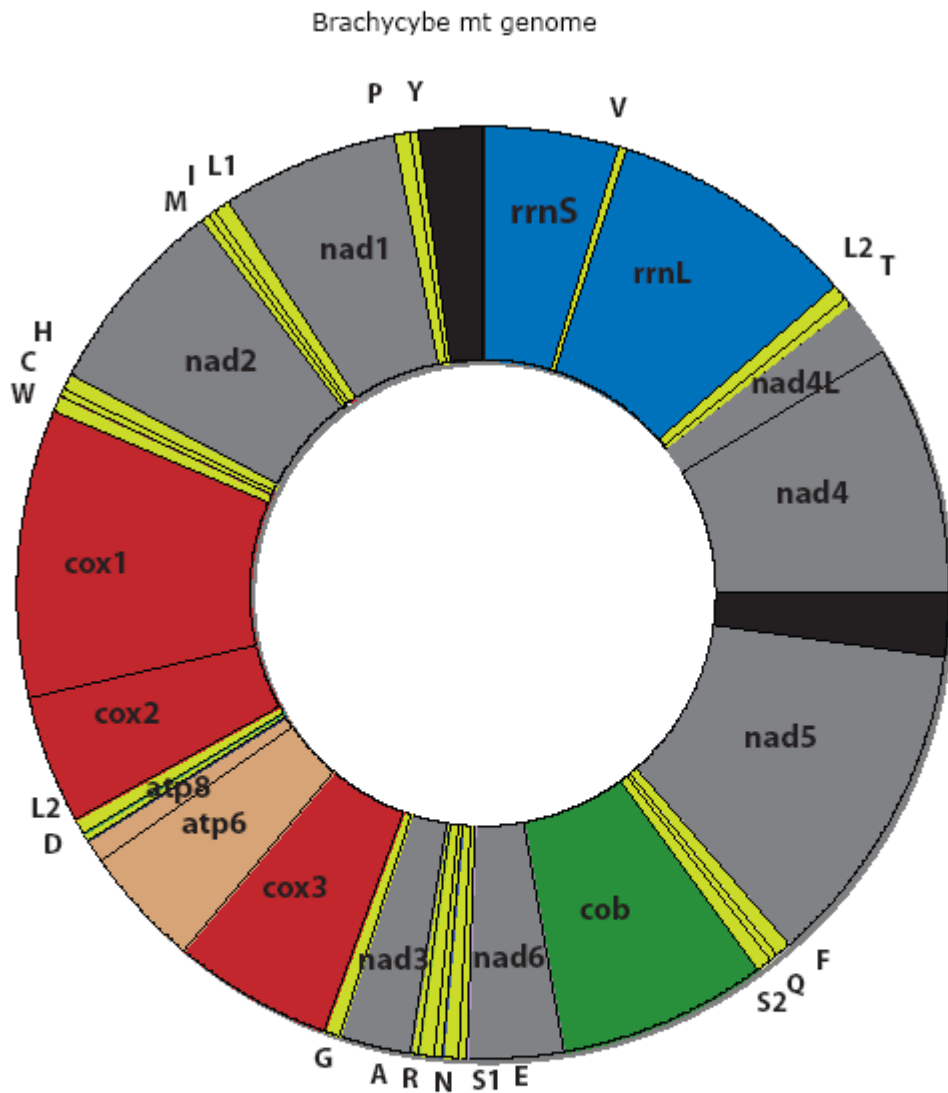
Fig. 2.1: Complete mitochondrial genome of *Brachycybe lecontii*. Gene regions are color coded by type: blue represents rRNA genes, gray represents NADH dehydrogenase, green represents the only apocytochrome b gene, red represents cytochrome c oxidase genes, brown represents ATP synthase genes, and yellow represents all tRNA genes.

It is difficult to determine how the nad1 and nad5 rearrangement occurred. It is likely that the two regions were duplicated and lost many times throughout this millipede's long history, as they are thought to have persisted since the Miocene (Gardner, 1975). The rearrangement of gene order within mitochondrial genomes is still a debatable issue and is explained by one of two models. The first is known as the duplication/random loss model. In this model rearrangements occur and accrue randomly. Alternatively, in the non-random loss model rearrangements occur in a predictable/mechanistic manner (Lavrov *et al.* 2002). Lavrov *et al.* 2002 explains that the non-random model can predict the pattern of gene loss, therefore, the resulting gene order based on the genes' transcriptional polarities and their positions in the genome. This model also would also explain why there are two non-coding regions.

**Phylogenetic Analysis of the Myriapoda**

The phylogenetic analysis performed suggests each represented class (Diplopoda, Chilopoda, and Symphyla) of the subphylum Myriapoda is monophyletic. The clades comprising the myriapod classes each have high bootstrap support at greater than or equal to 97%. The best tree recovered from the RAxML search had a −log likelihood value of 63059.82. The placement of *Brachycbe*, as sister to *Appalachioria* is not well supported with a bootstrap value of 46%. All associations within the Diplopoda have weak support (Fig. 2.2); however, the class as a whole has strong support (bootstrap value = 100) (Fig. 2.2).
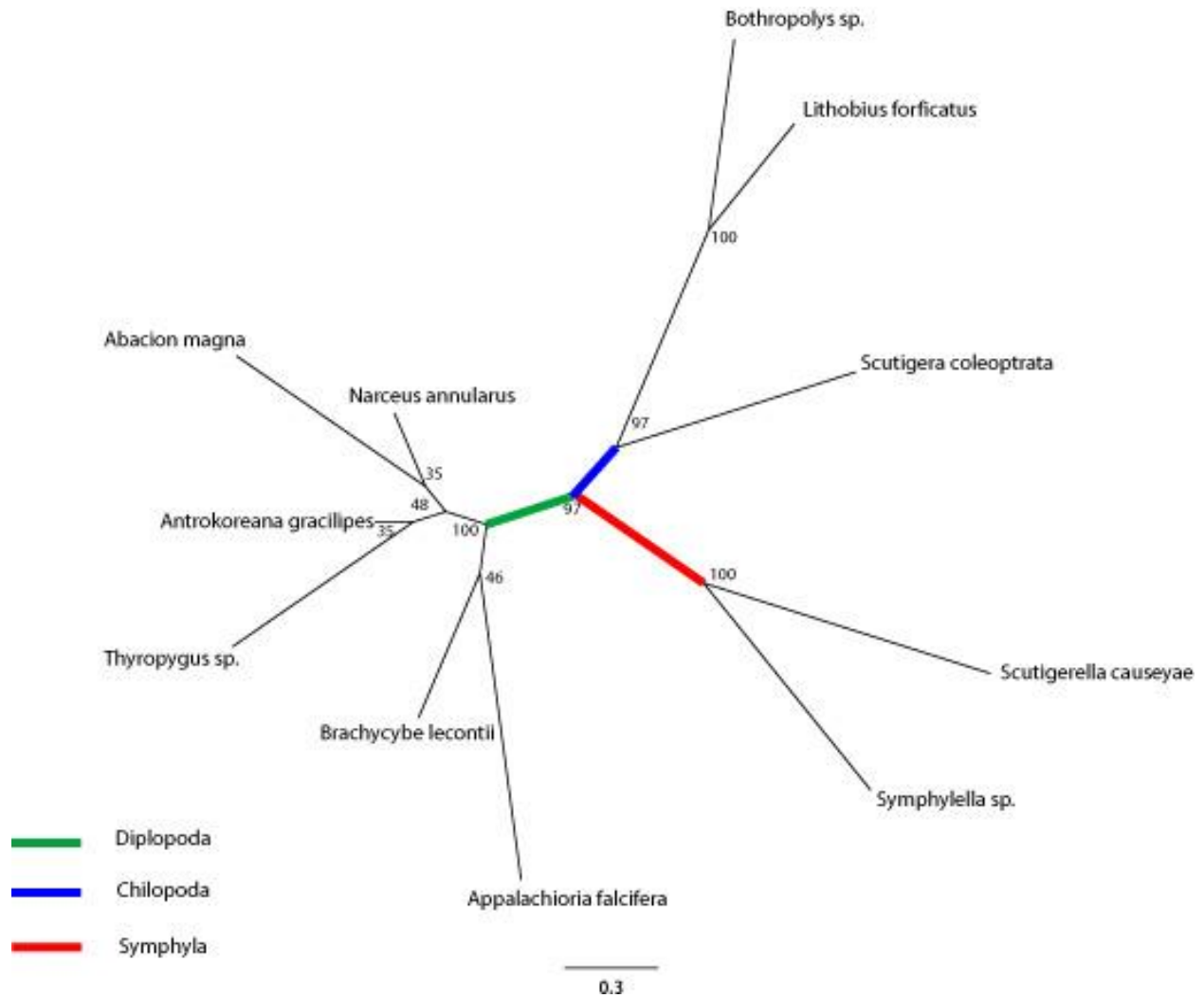
Fig. 2.2: Maximum Likelihood tree of all Myriapods (excluding class Pauropods) produced using amino acids. Classes are designated by color on the tree.

If mitochondrial genome comparisons are capable of indicating evolutionary relatedness then *Narceus* and *Thyropygus*, which are almost identical in gene composition, should have high phylogenetic support. The most recent total evidence phylogeny of all diplopod orders was produced by Sierwald and Bond 2008. Based on their analysis and others (e.g. Woo *et al.* 2007) spirobolids and spirostreptids should be closely related (Fig. 2.3). However, based on the ML analysis produced here (Fig. 2.2) these two genera are not closely related. The spirostreptid

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(*Thyropygus*) is more closely related to a member of the order Julida (*Antrokoreana*).
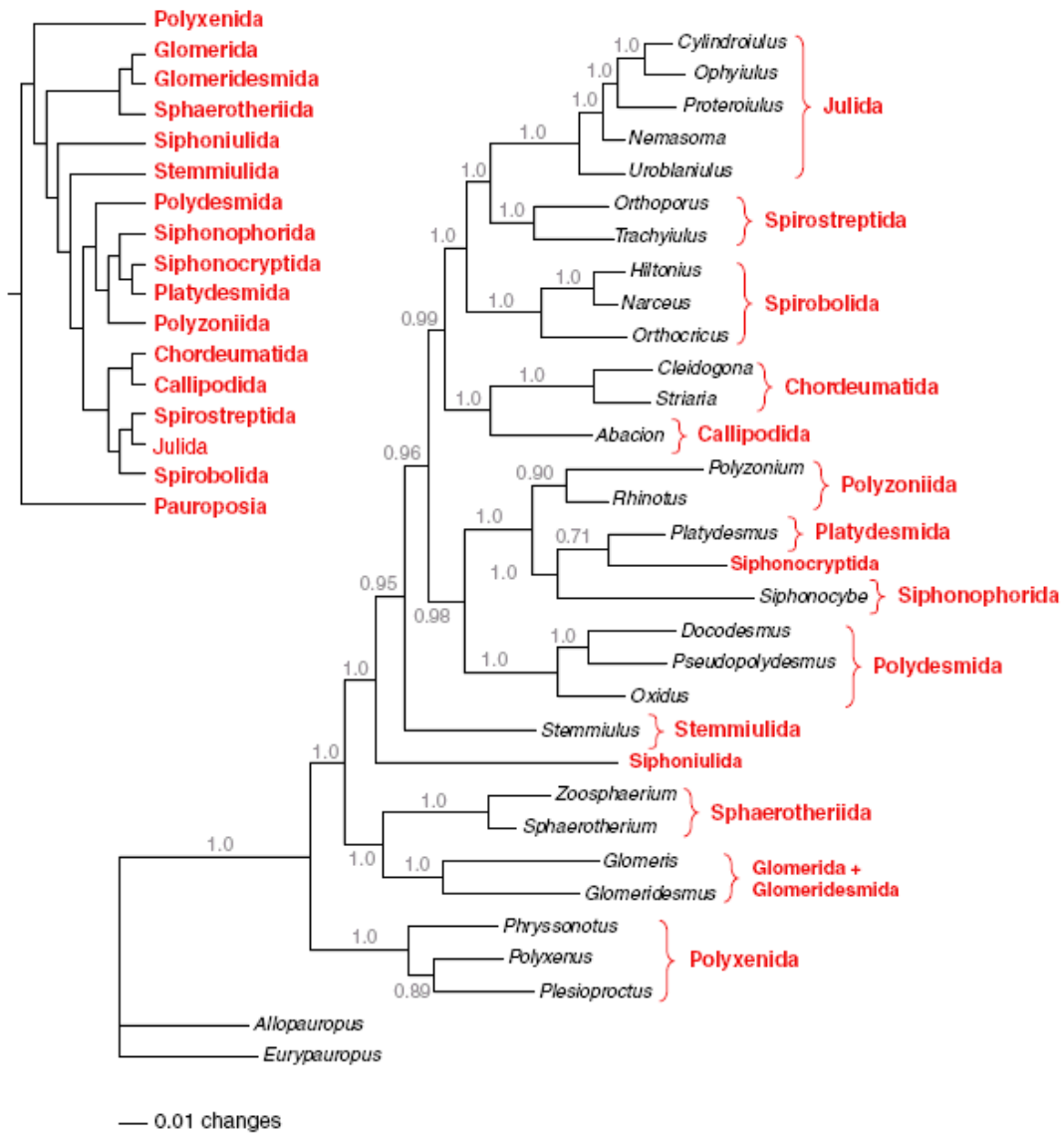
Fig. 2.3: Phylogenetic hypothesis of diploid orders based on total evidence using Bayesian inference (from Sierwald and Bond 2007).

As previously discussed atp8 was used in this analysis. Considering that this region is highly variable and short, the use of it here may have lead to an erroneous hypothesis of relationship. The atp8 region is often disregarded in phylogenetic reconstructions of deep relationships because of its high degree of sequence variability (Woo *et al.* 2007). Indeed, when analyzed the amount of variation among the groups for the atp8 region was dramatic at 82.8%. This indicates that within the nucleotide data set at least 2 of 11 taxa vary at 159 of 192 and within the amino acid data set at least 2 of 11 vary at 48 of the 50 characters. If the intent was to address recent evolutionary events the inclusion of atp8 would be warranted, however, when inferring deep level relationships these regions should probably be disregarded.

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