RATE OF DIVERSIFICATION IN CRICKETS (ORTHOPTERA: ENSIFERA) AND A POSSIBLE ROLE OF F SUPERGROUP *WOLBACHIA* IN BUSH CRICKETS

by

KANCHANA PANARAM

Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF TEXAS AT ARLINGTON

August 2007

ACKNOWLEDGEMENTS

I would not have reached my highest education that I can possibly reach without help and support from so many people. I must thank my parent and siblings for all supports that have giving me until this very day. My fiancé, Vinod Krishnan always encourages me that I can accomplish everything if I have a will and he has given me the will power I need.

Conducting research and publication preparation are highly challenging tasks for me, and I would not have accomplished this far with out help from my mentors. I greatly am greatly indebted to my major advisor, Dr. Jeremy L. Marshall who has provided me research resources and knowledge, motivated me and lead me to this final stage of my study, my committee members Drs. Esther Betrán, Paul Chippindale, Elena de La Casa-Esperon, James V. Robinson and Graduate Advisor, Dr. Daniel Formanowicz for taking time to help me going through graduate school processes. In addition, I am very thankful for receiving great training in vector biology research and help from Drs. Jaroslaw and Elzbieta Krzywinski during my last year at UTA.

Friends are one of the important factors of my happiness during my 4 years at UTA. I especially thank Nicole Grose and Ellen Stringer for being such great support. Gloria Burlingham, Linda Taylor, Belinda Zollotuchen, Morris Anthony and Jane Pugh have been wonderful to me and made my life less complicated.

My graduate study was sponsored by the Development and Promotion of Science and Technology Talents Project (DPST) under the supervision of the Royal Thai Government. My doctoral research was funded by a grant from the Advanced Research Program of Texas to JLM (ARP 003656-0067-2001).

July 26, 2007

ABSTRACT

RATE OF DIVERSIFICATION IN CRICKETS (ORTHOPTERA: ENSIFERA)

AND A POSSIBLE ROLE OF F SUPERGROUP *WOLBACHIA*

IN BUSH CRICKETS

Publication No. _____

Kanchana Panaram, PhD.

The University of Texas at Arlington, 2007

Supervising Professor: Jeremy L. Marshall, Ph.D.

Part I: Rates of speciation can tell us more than how many species have survived over a period of time. They indicate whether there are some characteristics of organisms or biogeography scenarios facilitate or hinder speciation. Many groups of ensiferan insects (e.g. Hawaiian sword-tailed, North American ground and field crickets) have been reported to have rapid speciation rates. To investigate whether the pattern of rapid speciation is common in crickets, we estimated overall diversification rates as a whole and within the clade. Ensifera as a whole does not appear to have particularly rapid diversification rates compared to other insect suborders. In addition,

some ensiferan clades are unexpectedly species rich given divergence times. The cause of elevated species richness remains unknown.

Part II: *Wolbachia pipientis*, an intracellular, α-proteobacterium, is commonly found in arthropods and filarial nematodes. Most infected insects are known to harbor strains of *Wolbachia* from supergroups A or B, whereas supergroups C and D occur only in filarial nematodes. Here, we present molecular evidence from two genes (*ftsZ* and *16S* rDNA) that 2 Orthopterans (the bush cricket species *Orocharis saltator* and *Hapithus agitator*; Gryllidae: Eneopterinae) are infected with *Wolbachia* from the F supergroup. Additionally, a series of PCR tests revealed that these bush cricket specimens did not harbor nematodes, thus indicating that our positive results were not a by-product of nematodes being present in these cricket samples. Patterns of molecular variation suggest that: (1) strains of F supergroup *Wolbachia* exhibit less genetic variation than the nematode-specific C and D supergroups but more than the A and B supergroups found in arthropods and (2) that there is no evidence of recombination within F supergroup strains. The above data support that horizontal transfer of F supergroup *Wolbachia* has likely occurred recently between these diverse taxonomic groups. Moreover, the limited genetic variation and lack of recombination in the F supergroup suggest it has radiated relatively rapidly with either (1) little time for recombination to occur or (2) selection against recombination as occurs in the mutualistic C and D strains of *Wolbachia* – both of which remain to be explored further.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

CHAPTER 1

RATE OF DIVERSIFICATION IN CRICKETS (ORTHOPTERA: ENSIFERA)

1.1 Introduction

The key to understand the evolution of species diversity among lineages is to understand the variation in diversification rates (Barraclough and Vogler, 2002). A diversification rate we observed today is a net rate resulted from subtracting extinction rate from speciation rate. The fluctuation in either speciation or extinction rates over a period of time result in changes in the net diversification rates, which can reveal plausible causes of the present species diversity. For examples, prior studies of flowing plants have shown increased diversification rates of several angiosperm clades compared to the sister clades as a result of so-called "key-innovations", biological novelties elevating frequency of speciation events in new adaptive zones (Doyle and Donoghue, 1986; Bond, 1989; Sanderson and Donoghue, 1994; Hagen and Kadereit, 2003; Ree, 2005). Sexual selection is often presumed to result in rapid speciation, as evidenced in the classic example of rapid speciation in African lake cichlids (e.g. Fryer and Iles, 1972; Turner and Burrows, 1995; McCune and Lovejoy, 1998, Turner, 1999, Knight and Turner, 2004). Adaptive radiation is shown to yield initially high rates of speciation that decrease as niches become filled (Simpson, 1953; Schluter 2000), such

as the North American wood-warbler genus *Dendroica* (MacArthur, 1958; Mayr, 1963; Morse, 1989; Lovette and Bermingham, 1999).

The Ensifera is one of the two suborders of Orthoptera. The suborder includes crickets, katydids (long-horned grasshoppers) and wetas (Jerusalem crickets). The characters of these ensiferan insects that differentiate them from the other suborder Caelifera (or short-horned grasshoppers) are their long, thread-like antennae, needle-like ovipositors and musical mating calls (usually males). Ensifera is considered one of the oldest groups of insects. The first ensiferan-like fossils are from the Late Carboniferous (about 300 million years ago) and the split between the Caelifera and the Ensifera is not more recent than the Permo-Triassic boundary (about 250 million years ago, Zeuner, 1939). Many ensiferan insects have been model organisms in a variety of research, including such as acoustic communication, behavioral and evolutionary processes, especially Tettigoniidae and Gryllidae (e.g. Otte, 1992; Hammond and Bailey 2003; Broughton and Harrison, 2003 and etc.).

More than 13,000 species of ensiferan insects have been described worldwide in a variety of habitats, especially in the tropics (Eades, Otte, Naskrecki, 2006; Alexander 1968). The family Tettigoniidae (katydids) alone contains over 6,400 species. The species diversity in Ensifera is only about 0.13 percent of the global insect diversity (10 million or fewer species, Gaston and Hudson 1994). The combination of an old lineage and a small percentage of the species diversity seem to suggest that Ensifera have diversified at low rates and experienced high levels of extinction. However, rapid speciation rates in ensiferan insects have been reported in several studies. For examples,

Hawaiian crickets in the genus *Laupala* have been estimated to have the fastest speciation rate (4.17 species/million years) among arthropods (Mendelson and Shaw 2005). Marshall (2004) suggested that a species complex of North American ground crickets, *Allonemobius*, has a rate of speciation of 3 species per 3,000-30,000 years, i.e. approximately 100 species/million years. Phylogenetic analyses of mitochondrial and nuclear genes of another group of North American crickets, *Gryllus spp.* (field crickets) also suggested rapid speciation of at least 3 species since the Pliocene/Pleistocene (about 1.8 million years ago, Harrison and Bogdanowicz, 1995; Broughton and Harrison, 2003). These estimated diversification rates exceed the average estimated rate of arthropod speciation (0.16 species/million years, Coyne and Orr, 2004) and the average diversification rates of insect orders (0.008 to 0.06 species/million years, Mayhew, 2002). Even though the cricket studies have shown very rapid speciation rates compared to average diversification rates in arthropods and insect orders, the rates were estimated from species level data within particular geographic regions, i.e. they do not represent Ensifera as a whole. Therefore, the diversification rate of the Ensifera as a whole, whether rapid or not, is still unknown.

The present study estimated diversification rates in crickets as a whole and within major groups of crickets that are often studied. We investigated whether the pattern of rapid speciation as seen in prior studies of North American crickets holds true for all ensiferan clades (Harrison and Bogdanowicz, 1995; Broughton and Harrison, 2003; Marshall, 2004; Mendelson and Shaw, 2005). An intermediate approach is used to avoid overestimating diversification rates of Ensifera (as a whole and within) using

method of moment estimator (Magallón and Sanderson 2001), considering extinction rates given divergence time estimated from a multiple fossil calibrated molecular phylogeny. Fossil records are often fragmented and incomplete. The time estimated from incomplete fossil records can lead to inaccurate divergence times (Marshall, 1990). With an appropriate method, molecular divergence time estimation can provide credible estimates. In addition to diversification rate estimation, we also tested a hypothesis on species diversity of the group whether a clade contains more of fewer species than expected if it diversify at an average diversification rate of Ensiferan as a whole under a specific level of extinction after a time interval.

1.2 Materials and Methods

1.2.1 DNA isolation, PCR and Sequencing

The crickets from family Gryllidae were collected from United States localities. The specific names and collecting locations were shown on figure 1.1 (with black dots and State's name abbreviations). Several specimens were provided by Bill Brown (tree cricket, *Oecanthus quedripunotatus,* SUNY) and Rick Brandenburg (NCST, two mole crickets, *Scapteriscus vicinus* and *S. borellii*). Total genomic DNA was isolated from either whole crickets or one half the body using DNeasy™ Tissue Kit (QIAGEN). The DNA samples were stored at -20ºC. The rest of the body was stored at -80ºC.

The *18S* rDNA gene, a nuclear gene often used in insect phylogenetic reconstruction at interspecific levels and higher (Caterino et al. 2000), was chosen because we focused on resolving evolutionary relationships at family and subfamily levels. The undiluted isolated genomic DNA was used to amplify partial *18S* rDNA

gene using a pair of primers: 18sF_20 (5'GCG GCT TAA TTT GAC TCA AC) and 18sR_490 (5'CGG TAG TAG CGA CGG GCG GT). These primers were designed based on conserved regions among *Allonemobius socius* (AF514507), *Neoamusia shawae* (AF514658), *Pteronemobius ohmachii* (AF514517) and *Homogryllus sp.* (AF514649). Each PCR mix (50 µl-reaction) contained 0.4 µM of each primer, 0.2 mM PCR nucleotide mix (Promega), 2.5 mM $MgCl_2$, 2 units of Taq Polymerase (in buffer A, Promega) and 20-120 ng of genomic DNA in 1x thermophilic DNA Polymerase buffer A (Promega, containing 10mM Tris-HCl pH 9.0 at 25°C, 50mM KCl and 0.1% Triton®X-100). The PCR mix was prepared on ice and amplified on a GenAmp® PCR system 9700 thermocycler (AB Applied Biosystems) using the following temperatures; 2 min 94ºC, 25 cycles of 2 min 94ºC, 30 sec 45ºC, 1 min 72ºC, and 4 min 72ºC. The annealing temperature (45ºC) increase by 0.4 ºC every cycle and the extension period was also extended 15 seconds each cycle. The PCR products were visualized on 1% agarose gel and the target bands (~ 500 base pair fragments) were purified using QIAquick® Spin Kit (QIAGEN). The purified PCR products were sequenced directly using ABI technology with the primers used in DNA amplification (18sF_20 and 18sR_490).

1.2.2 Sequence Alignment and Phylogenetic Reconstruction

The obtained DNA sequences were aligned with homologous sequences available on the public database using Sequencher, version 4.2 (Gene Codes) and ClustalX 1.73 (Thompson *et al*., 1997). Four species of the Caelifera and a cockroach were chosen as outgroups. The accession numbers of the DNA sequences are listed in

table 1.1. DNA sequence alignments were edited manually with a reference of ribosomal RNA secondary structure (Kjer 1995, 2004) in Bioedit 7.0.5.3 (Hall, 1999). The nucleotide substitution model GTR+I+G was chosen using Akaike Information Criterion (AIC) in ModelTest 3.6 (Posada and Crandall, 1998). Phylogenetic analyses were performed on the 403 base pair alignment of *18S* rDNA sequences using maximum parsimony (MP), maximum likelihood (ML) in PAUP* 4.0b 10 (Swofford, 2002) and Bayesian inference analyses in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Maximum parsimony and maximum likelihood optimal criteria were performed using the heuristic search with 10 random taxon addition replicates, and tree-bisection and reconnection (TBR) branch swapping. All characters were equally weighted and gaps were treated as missing data. Bootstrap analyses (100 replicates) on the Maximum parsimony and maximum likelihood trees were also performed to determine the robustness of the nodes via heuristic search.

As for Bayesian analyses, the analysis consisted of 4,200,000 generations and four chains, using MrBayes version 3.0B4 (Ronquist and Huelsenbeck, 2003). Trees were sampled every 100 generations, resulting in 42,001 total trees. The likelihood values were plotted against number of generations to determine if the likelihood values of the trees reached a plateau before the designated 'burnin'. The first 8,401 trees (20 %) of total trees were considered 'burnin' and discarded. Bayesian posterior probabilities (BPP) were estimated from the 50% majority rule consensus of the remaining 33,600 trees.

To determine the most appropriate divergence time estimation method for the data set, the assumption of a molecular clock was tested by comparing the log likelihood of the maximum likelihood tree of rate-constant and a rate-variable model of nucleotide substitution in PAUP* 4.0b10. The likelihood ratio statistic was compared to X^2 -distribution, with the degree of freedom equal to the number of terminal sequences minus 2. The null hypothesis (H_0) assumes that all lineages share the same, constant nucleotide substitution rate of evolution (molecular clock fashion).

1.2.3 Fossils of the Ensifera

We obtained fossil records of the earliest specimens deposited in the Orthoptera Species File Online 2.4 (OSF Online 2.4, see table1.2) that were classified into modern ensiferan families or subfamilies. The ages of fossils were given as geologic time periods and they were translated to absolute ages (in million years) with reference of the Geologic Time Scale 2004 (Gradstein *et al.* 2004). The midpoint value of each stratum was used as an absolute fossil age estimate for divergence time estimation analyses. The absolute fossil ages were used as minimum age constrained points in divergence time estimation. Each fossil age estimate was placed on the internal node between members of the clade and the sister group of that clade that share the most recent common ancestor.

1.2.4 Determination of Fossil Age Inconsistency by Fossil Cross-validation Method and Estimation of Divergence Times

The ages estimated from fossil records, which are often incomplete, might not be accurate and result in errors if used to calibrate divergence time on a molecular

phylogeny (Marshall, 1990). Inconsistent fossils that give significantly different age estimation must be removed before performing the divergent time estimation analysis. We followed the three-step fossil cross-validation method developed by Near, *et al.* (2005) with penalized likelihood method implemented in r8s (Sanderson, 2002; 2003). Fossil ages were used as minimum age constrained calibration points of internal nodes using command CONSTRAIN MIN_AGE in r8s. We were able to assign fossils of the ensiferan crickets to 8 internal nodes of the cricket phylogeny as listed in table 1.2. The reconstructed *18S* rDNA maximum likelihood phylogeny was assumed to be the true phylogeny of the Ensifera because the tree was reconstructed using the most appropriate nucleotide substitution model to the data set. After removing an inconsistent fossil (Stenopelmatidae: *Triassophyllum leopardii*) from the analysis, the remaining 7 fossil ages were used to constrain internal nodes on the inferred cricket phylogeny in the divergence time estimation.

Due to discovery of significant deviation from constant rate model of the data set, each divergence time estimation analysis was performed using penalized likelihood method (Sanderson, 2002) implemented in r8s1.71 (Sanderson, 2003) with the optimal smoothing factor and a fixed age of 251.4 million years before present at the root of the tree, which is the split between the Caelifera and the Ensifera is not more recent than the Permo-Triassic boundary (about 250 million years, Zeuner, 1939). Nonparametric rate smoothing method (NPRS) provides more accurate divergence time estimation when 1) sequence lengths are sufficiently long, 2) rates are truly non-clocklike and 3) rates are moderately to highly autocorrelated in time (Sanderson, 1997). In our case, the

DNA sequence length is not long and the data did not follow molecular clock fashion. Penalized likelihood method provides an intermediate approach between molecular clock and a nonparametric method of divergence time estimation and seems to be appropriate for the data.

The divergence time estimates were then used in the diversification rate estimation of ensiferan clades that are included in the reconstructed phylogeny. The divergence time estimates were considered as stem group ages as defined in Magallón and Sanderson (2001). Stem group is defined as the most inclusive group of taxa that contain all extant and extinct members of the clade (Magallón and Sanderson, 2001).

1.2.5 Estimating Rates of Diversification in the Ensifera

We assumed that speciation and extinction of lineages follows a stochastic birth-and-death process (Bailey, 1964). Specifically, speciation rate (?) and extinction rate (μ) are assumed to occur at constant rates. The true values of ? and μ are not known, but diversification rate (*r)* and relative extinction rate (e), simple transformations of ? and μ, could be assumed based on observed data. First, diversification rate (*r)* is defined as r = ? – μ. This parameter r can range from negative to positive infinity. The value is negative when extinction rate is greater that speciation rate, which means that the lineage extinction probability is 100 %. Second, relative extinction rate (e) is defined as e = μ/?. The relative extinction rate, e, can range from zero to positive infinity. If the extinction rate is less than speciation rate, the e will be less than 1 (e <1). The probability of the lineages going extinct is less than 1. If

extinction rate is equal or greater than speciation rate (e = 1), the probability of extinction is 1 (the lineages are destined to extinction).

Because extinction events occur, yet at unknown rates, it is unrealistic to estimate diversification rates using a maximum likelihood estimator. The maximum likelihood estimator for r is defined as $r = (\log n) / t$, considering clades no extinction or e = 0). In absence of extinction in the estimate, diversification rates could be overestimated if lineages were old and have experienced extinctions. Therefore, the method of moment estimator that account for relative extinction rate e would be more appropriate for such an old clade like Ensifera.

We calculated diversification rates of the ensiferan lineages using ages estimated from the molecular divergence time estimates followed equation (6) in Magallón and Sanderson (2001) for stem group clades when n = number of extant lineages in the clade, and t = time or age of the clade.

$$r = \frac{1}{t} \log[n(1 - e) + e]$$

The value of e, relative extinction rate, is unknown. However, we used e = 0 and 0.9 as done in Magallón and Sanderson (2001). The values were chosen to cover the two extremes: 1) when there is no extinction (e = 0) and 2) when extinction rate is relatively high (e = 0.9). For each clade, two diversification rates were estimated as $r_{0.0}$ and $r_{0.9}$, when there was no extinction and relatively high extinction rate, respectively. The diversification rates $r_{0.0}$ and $r_{0.9}$ of the Ensifera as a whole were estimated using the time of the split between Caelifera and Ensifera (251.4 million years before present).

All numbers of species of representative ensiferan clades on our phylogeny, including that of Ensifera as a whole, were obtained from Orthoptera Species File Online (OSF 2.4: Eades, Otte, Naskrecki, 2006). The numbers include only valid species deposited on the database. The time t of each ensiferan clade was divergence time estimate from out divergence time analysis.

1.2.6 Hypothesis Tests on Species Diversity

In addition to estimating diversification rates, we asked a question about species diversity of the ensiferan lineages whether an observed number of extant species of a clade is unexpectedly species rich or species poor, given a background diversification rate. A species rich clade contains higher number of species than expected after a period of time t since its origin, given a specific background diversification rate (r) and a relative extinction rate (e). A species poor clade is the opposite. In order to answer the question, we followed the hypothesis test on diversity in Magallón and Sanderson (2001). To test this hypothesis of species richness, an exact expected number of species of each lineage is not necessary; instead, we calculated a 95% confidence interval of expected a number of species. An observed number of species that falls outside the interval is considered significantly different from the expected at 95% confidence level.

We defined an expected number of species (k) as a number of species of a hypothetical clade having diversified at a background diversification rate r under a specific relative extinction rate e a time interval t. As stated previously, specific values of k are not required, but approximate values at time t since the origins are essential. For each clade, a 95% confidence interval of the expected number of species at time t after

it originated with the diversification rate r of the Ensifera as a whole (also called a background diversification rate) was calculated in absence of extinction and under high relative extinction rate e ($r_{0.0}$ or $r_{0.9}$). We calculated an upper bound k_u and a lower bound k_l of a 95% confidence interval for every divergence time of ensiferan clades on the inferred phylogeny using equation [2] and [10] in Magallón and Sanderson (2001). The equations are shown as follow:

$$ß_r = \frac{(e^{rt} - 1)}{(e^{rt} - e)} \qquad [2]$$

upper bound k_u : $P[N(t) = k] = ß_r^{k-1}$ [10a]

lower bound k_l: $P[N(t) < k] = 1 - ß_r^{k-1}$. [10b]

The values of upper limits (k_u) and the lower limits (k_l) of 95% confidence intervals can be obtained by solving the above equations with probability equal to 0.025. Observed numbers of species of ensiferan clades that fall above k_u or below k_l of the confidence intervals are considered species rich and poor, respectively.

1.3 Results

1.3.1 Phylogeny of Ensifera

All phylogenetic analysis methods resulted in a similar topology (figure 1.1 and 1.2), especially at family and subfamily levels. The topology of gryllid subfamilies is consistent in all methods. The separations among families were not resolved. Grylloid families (Gryllidae and Gryllotalpidae) were not placed as a sister group in the present study, whereas the other families (Tettigoniidae, Stenopelatidae, Rhaphidophoridae,

Gryllacrididae, Schizodactylidae and Anostostomatidae) were grouped together and placed as a sister group of Gryllidae instead of Gryllotalpidae. This topology is different from all previous phylogenetic hypotheses of the ensiferan relationships (Ander, 1939; Zeuner, 1939; Judd, 1948; Ragg, 1955; Sharov, 1968; Gwynne, 1995; Desutter-Grandcolas, 2003; Jost and Shaw, 2006). Tettigoniidae is not monophyletic and grouped with Anostostomatidae. Rhaphidophoridae is grouped with Schizodactylidae and Prophalangopsidae (sometimes called Haglidae). This is similar to the phylogenetic hypothesis of Ander (1939). Stenopelmatidae is grouped with Gryllacrididae. Within the family Gryllidae, our phylogeny yields some similarity with Gwynne's (1995) phylogenetic hypothesis. Nemobiinae (ground crickets) is a sister group to Trigonidiinae (sword-tailed crickets). Eneopterinae (bush crickets) is not monophyletic , but is position closely related to the subfamily Gryllinae (field crickets) and Oecanthinae (tree crickets). Mogoplistinae is basal to other Gryllidae subfamilies. However, the result differs from Gwynne's by the position of Myrmecophilinae, which belongs to family Gryllidae. It is placed as a paraphyletic group of Gryllidae regardless of methods.

The maximum parsimony analysis resulted in 2,044 trees and a 50% majority rule consensus was calculated. The maximum parsimony phylogenetic tree was presented as a consensus tree in figure1.1 with percentages of consensus topology under branches. This resulted a polytomy between Tettigonoidea group and Gryllidae. Bootstrap support values of the shortest trees range between 53% and 100% and are indicated on the branches. The maximum likelihood analysis resulted in a single most

likely phylogenetic tree. The bootstrap supports of the maximum likelihood tree range between 55% and 100% and are indicated on branches of the tree in figure 1.2. The posterior probabilities from Bayesian inference phylogenetic tree were between 57% and 100% and shown under the branches of the maximum likelihood phylogeny in bold letters (figure 1.2). We used the maximum likelihood tree to represent the ensiferan phylogeny for our divergence time estimation analyses.

1.3.2 Divergence Time Estimation

Divergence time estimates from multiple fossil calibrated penalized likelihood analysis are shown in table 1.2. The estimated ages are also shown on the calibrated maximum likelihood tree (figure 1.3). The oldest divergence time estimate of Ensifera (the imaginary most common ancestor of ensiferan clades) is 250.30 million years before present or around early Cretaceous. Oecanthinae, the youngest clade age we observed, was estimated to have a divergence time of 1.46 million years before present or between the Lower and Middle Pleistocene.

1.3.3 Diversification Rate of Ensifera

Based on the number of species classified as ensiferan members in OSF 2.4 online and the age of the split between Caelifera and Ensifera (about 251 million years ago, Zeuner, 1939), we obtained a net diversification of the Ensifera as a whole. In absence of extinction (e = 0), the estimated ensiferan diversification rate is 0.0163 species per million years ($r_{0.0}$ = 0.0163, table 1.3) and the diversification rate under relatively high extinction rate (e = 0.9) is 0.0123 ($r_{0.9}$ = 0.0123, table 1.3). The obtained

diversification rates of Ensifera as a whole in the absence of extinction and under a high relative extinction rate were used as background diversification rates of the Ensifera.

1.3.4 Diversification Rates of Ensiferan Clades

Diversification rates in the absence of extinction ($r_{0.0}$) and under high relative extinction rates ($r_{0.9}$) of 8 subfamilies of Gryllidae and 7 ensiferan families (including Gryllidae) are shown in table 1.3. The values of $r_{0.0}$ range between 0.0051 and 1.5134 events per million years and the values of $r_{0.9}$ range between 0.0016 and 0.8445 species/million years (table 1.3). The family Tettigonidiidae (katydids) has the highest diversification rate among the ensiferan families studied here ($r_{0.0}$ = 0.0171, and $r_{0.9}$ = 0.0126) and it is higher than the diversification rate of the Ensifera as a whole. Rhaphidophoridae is only the other ensiferan family in the present study to exceed the background diversification rate or the diversification rates of the Ensifera as a whole. Among the subfamilies of Gryllidae (true crickets), the Oecanthinae (tree crickets) has the highest diversification rates ($r_{0.0}$ = 1.5134, and $r_{0.9}$ = 0.8445). Oecanthinae, Mogoplistinae, Trigonidiinae, and Gryllinae have higher diversification rates than the background diversification rates in both levels of relative extinction, whereas Nemobiinae has higher rate only in the absence of extinction.

1.3.5 Hypothesis Tests on Species Diversity

The 95% confidence intervals of expected numbers of species for all ensiferan clades under the background diversification rates or ($r_{0.0}$ = 0.0163 and $r_{0.9}$ = 0.0123) are shown in table 1.4. None of the ensiferan clades fell below the lower limit of the 95% confidence intervals. This means that the observed ensiferan clades are not species poor.

Among ensiferan families, only Gryllidae, Rhaphidophoridae and Tettigonidiidae are species rich regardless of relative extinction rates. Five of eight subfamilies of Gryllidae are species rich, including Oecanthinae, Mogoplistinae, Trigonidiinae, Gryllinae and Nemobiinae.

1.4 Discussion

1.4.1 Diversification Rates of Ensiferan Insects

Our estimated diversification rates of Ensifera as a whole under reasonable ($r_{0.0}$ = 0.0163 and $r_{0.9}$ = 0.0123) relative extinction rates are not extraordinarily high as the speciaiton rates reported in prior studies of crickets (*Lapaula* 4.17 species/million years, Mendelson and Shaw 2005; *Allonemobius* 100 species/million years, Marshall, 2004; *Gryllus* 1.67 species/million years, Harrison and Bogdanowicz, 1995, and Broughton and Harrison, 2003). The large differences in diversification rates between our estimates and the rapid speciation rate studies suggest that the rapid speciation might have involved in specific clades later on in ensiferan diversification, not at the origin of Ensifera. Biogeographic scenarios could also explain the rapid speciation the some organisms, especially in Hawaiian Islands and North America. On the other hand, the species concept used to identify ensiferan insects in some specimens might have caused the slow diversification rates. Many groups of crickets are very cryptic. They can be distinguished from each other only from calling songs, behavioral and ecological characters or combinations of these characters. Therefore, there is a high possibility that many current single species are actually species complexes and undescribed. For examples, 376 of 492 species of Australian crickets were newly described by Otte and

Alexander (1983) using calling songs combining with other morphological, behavioral and ecological characters and the majority of the new species was described by calling songs. Four sibling species of the North American ground crickets *Pictonemobius* were considered as a single species *P. ambitiosus* (Gross *et al.*1989) and described based on the differences of calling songs, morphology and habitats. This concern remains in question and to be explored further.

Are the estimated diversification rates of Ensifera as a whole in this study particularly higher or lower than other groups of organisms? We compared our estimate of diversification rate in absence of extinction to a study of hexapod speciation that applied the approach similar to our study (Mayhew, 2002) and found that diversification rate of the order Orthoptera was estimated to be 0.034 species/million, twice as much as our estimate. This is reasonable considering the other orthopteran suborder Caelifera, containing approximately equal number of species as Ensifera, was included in the estimate (Eades, Otte and Naskrecki, 2006). Additionally, the diversification rates of Ensifera as a whole are about 10 times lower than the average estimated rate of arthropod speciation (0.16 species/million years, Coyne and Orr 2004) and 3 times lower than that of Insecta (0.0417 species/million years, Mayhew, 2002). On the other hand, our estimates of diversification rates as a whole do not appear to be exceptionally low compared to the average diversification rates of hexapod orders (0.008 to 0.06 species/million years, Mayhew, 2002). From Mayhew's (2002) study, Grylloblattaria was the only insect order that has lower diversification rate than our estimate at

comparable age. Other insects having higher diversification rates than the Ensifera at a comparable taxonomic level such are shown in table 1.5.

Half of the ensiferan families and subfamilies of the Gryllidae have higher diversification rates than that of Ensifera as a whole (background diversification rate). This might suggest that these lineages have evolved some characteristics that promote speciation as previously found in other insect groups, such as phytophagy in Diptera, Coleoptera, Hymenoptera and lepdoptera (Mitter *et al.*, 1988) or secondary sexual traits (calling songs in crickets: Mendelson and Shaw, 2005). Another plausible explanation for elevated diversification rates in certain ensiferan clades is that these clades have been under spotlight. Our estimates relied solely on the compiled data from OSF Online database (Eades, Otte and Naskrecki, 2006) and deposited ensiferan specimen records were lineages that are often studied, including Tettigonidae and Gryllidae (i.g. Otte, 1992; Forest, 2001, and Gwyne, 2001).

1.4.2 Hypothesis Test on Species Diversity

Species rich clades do not necessarily have rapid diversification rates. Having high species richness may be a result of old age, not rapid diversification rate. Low diversification rates are a result of having few species, or old age, or both. Slow diversifying clades can be detected only when the clades are old because at young age, either fast or slow diversifying clades will have few species. McPeek and Brown (2007) suggested that clade age, not diversification rate, explains species richness among animal taxa. The study found that longevity of a clade correlates with species richness,

i.e., old clades have more time to accumulate species and that results in higher level of species richness than younger clades.

Our diversification rate estimates are based on few assumptions, including accurate divergence time estimation, and constant birth-death stochastic model diversification rates. Any violation will result in errors of the diversification rate estimates and that also affects the result of the hypothesis test of species richness. Overestimating divergence time will result in lower diversification rates than real values and also place clades below the lower limits of expected numbers of species or making clades falsely species poor.

Five of eight subfamilies of Gryllidae, including Oecanthinae, Mogoplistinae, Gryllinae, Trigonidiinae and Nememobiinae, and the family Tettigoniidae are unexpectedly species rich. The former four clades also have higher diversification rates than the background. The unexpectedly high species richness might be the result of 1) these ensiferan clades are under a spotlight, 2) they might share characteristics that promote speciation such as calling songs as stated earlier in section 1.4.1 or a combination of both.

We traced species richness of ensiferan clades onto the phylogeny to see whether there is species richness has evolutionary relatedness or it is simply a random pattern (figure 1.4). If the elevated species richness were a random event, we would expect at least 5% of ensiferan clades to be unexpectedly species rich. However, half of the subfamilies of the Gryllidae are unexpectedly species rich. It is possible that gryllid subfamilies have evolved traits that lead to high species richness and lost in 2 clades (in

Phalangopsinae and Eneopterinae). The similar scenario might have occurred among the families Stenopelmatidae, Rhaphidophoridae and Schizodactylidae. Another issue that we wanted to point out is that the species rich clades are also have elevated diversification rates (rates higher than that of Ensifera as a whole). This pattern needs to be investigated further.

1.5 Conclusion

The estimated diversification rates of Ensifera as a whole under reasonable relative extinction rates are not unusually high or low. In addition, the unexpectedly high diversity coupled with higher-than-background speciation rates of ensiferan clades might be products of the clades having characters that elevate speciation, or bias in species number data due to method of species identification among ensiferan lineages and undescribed species. The obvious causes remain unknown. Sexual selection by secondary reproductive traits such as calling song is a good candidate as suggested by Mendelson and Shaw's (2005) Hawaiian cricket study.

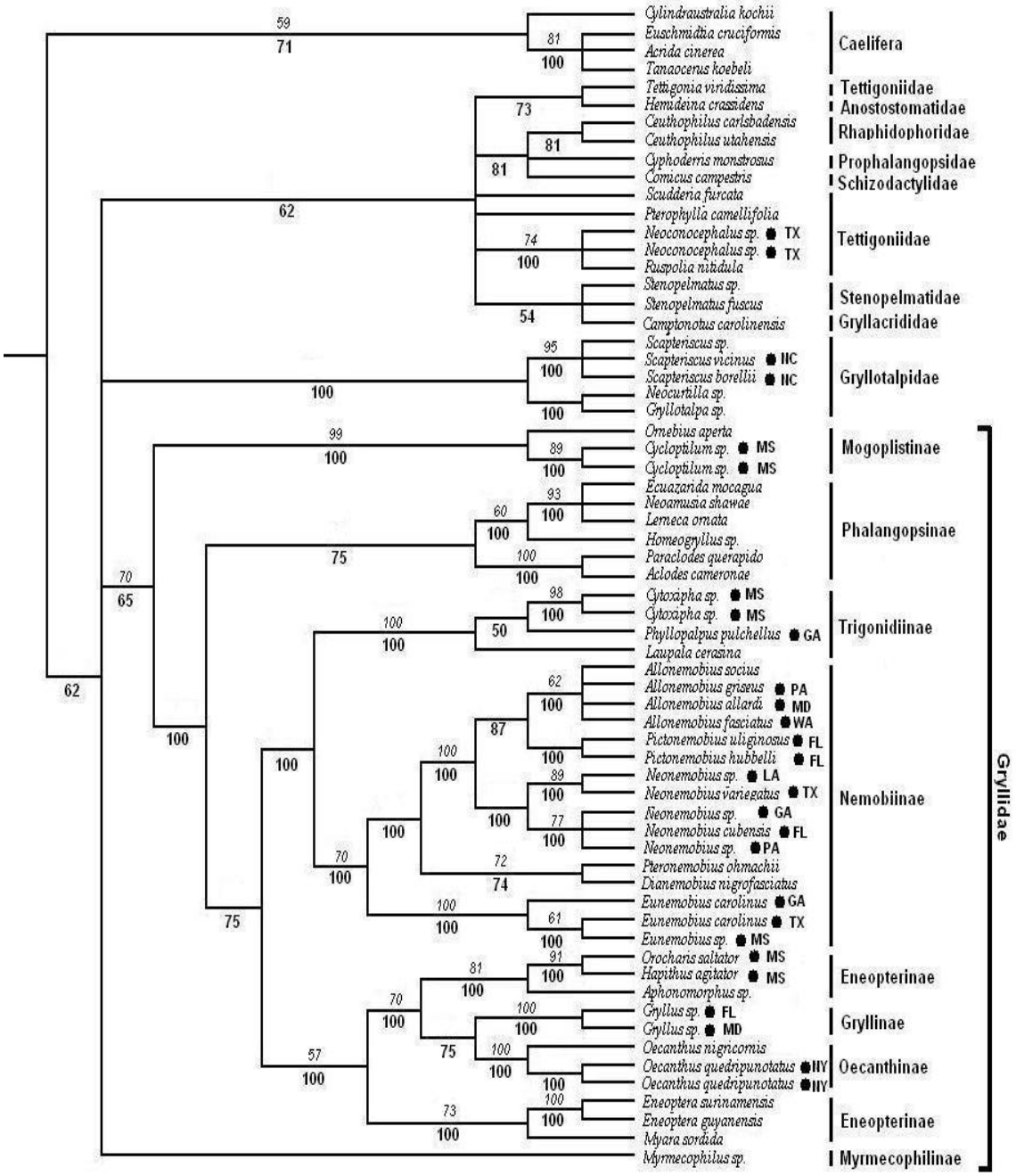


Figure 1.1 Maximum parsimony tree consensus of Ensifera based on *18S* rDNA gene. Branch lengths are not to scale. Bootstrap support values are indicated above branches in italic numbers. The bold numbers under branches are 50% majority consensus supports of 2,044 trees. The DNA sequences generated in this study are indicated by black dots. Cricket specimens collected from USA localities in this study are indicated in State's name abbreviations following the taxon names.

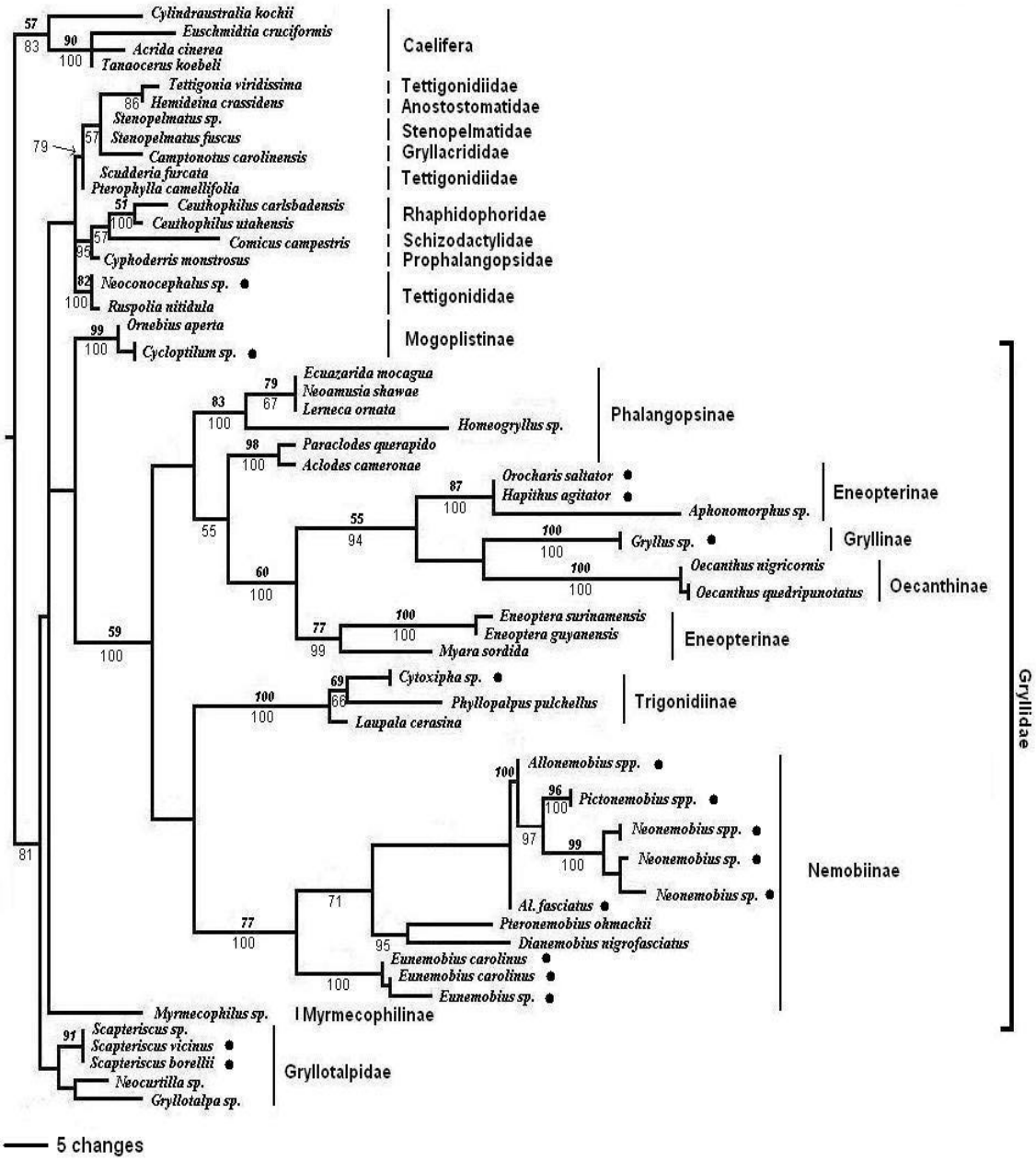


Figure 1.2 Maximum likelihood tree of Ensifera based on *18S* rDNA gene. Bootstrap support values are indicated over branches in bold italic numbers. The numbers under branches are Bayesian posterior probabilities from the 50% majority rule consensus of the remaining Bayesian inference 33,600 trees. The DNA sequences generated in this study are indicated by black dots. The phylogeny was reconstructed from the same DNA sequence alignment in maximum parsimony analysis (figure 1.1). Note that the zero-branch-length species were reduced to one exemplar.

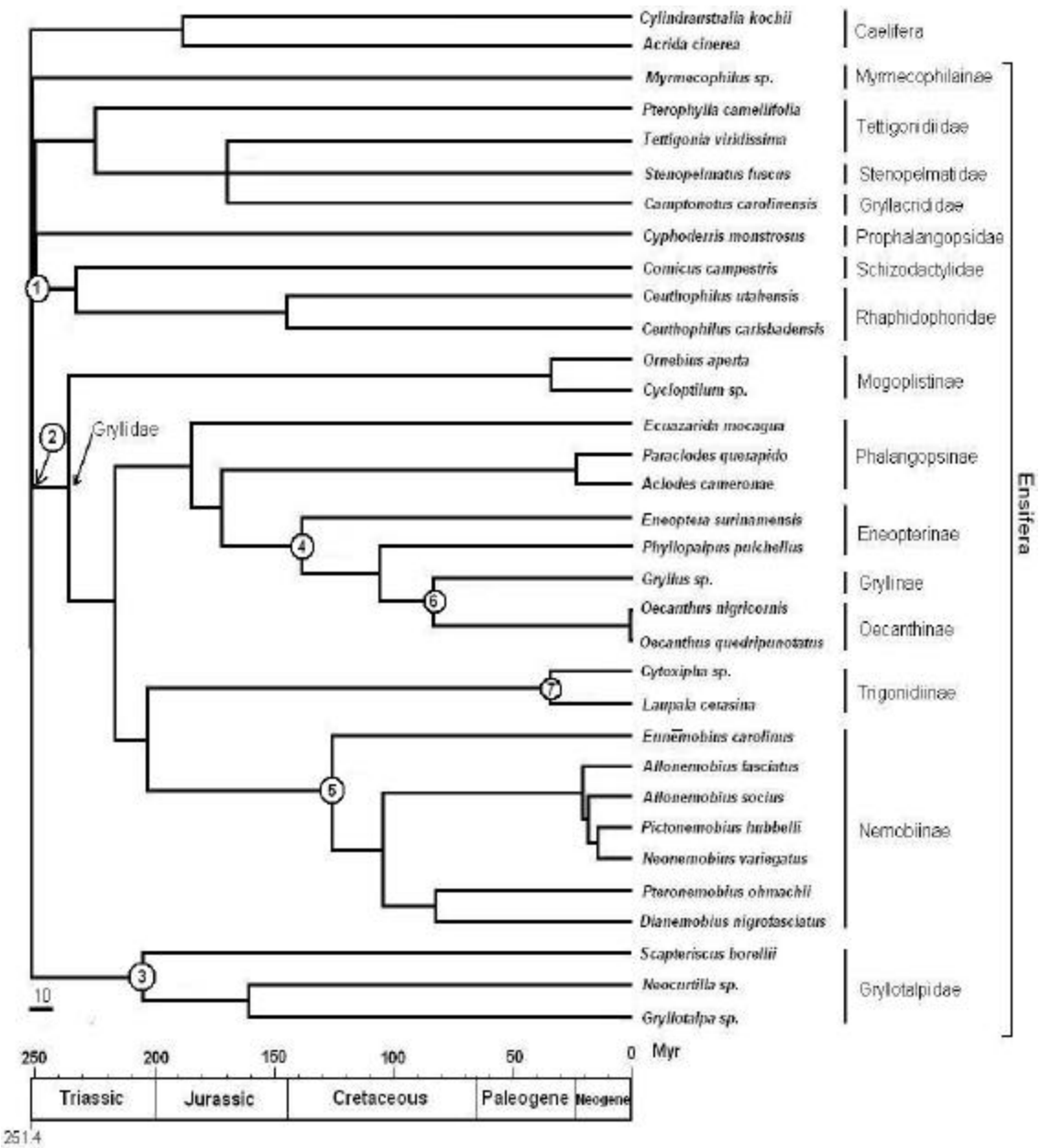


Figure 1.3 Divergence time calibrated phylogeny of Ensifera using 7 fossils as calibration points. The fossil calibrated internal nodes are numbered in circles as listed in table 1.2.

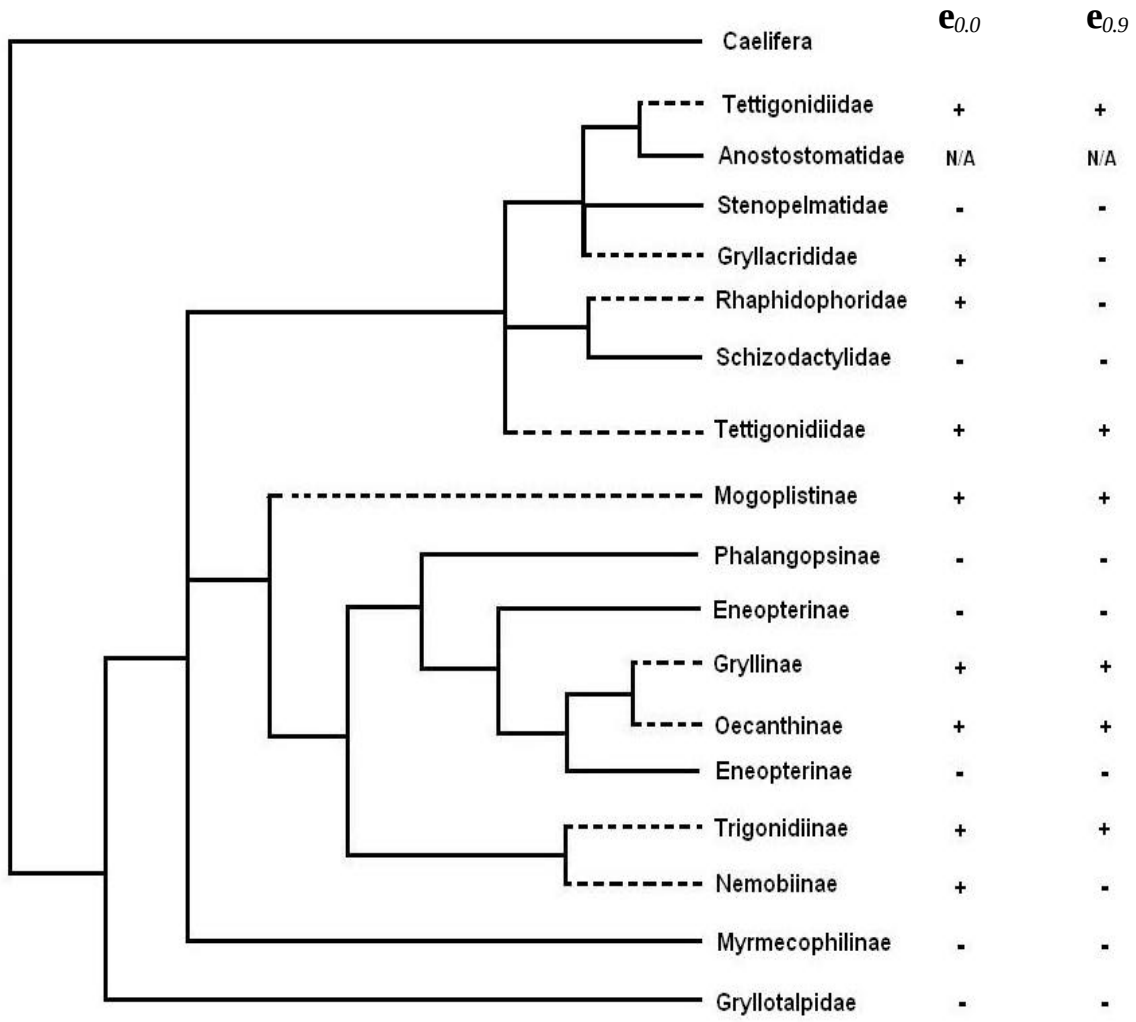


Figure 1.4 Cladogram showing species richness and diversification rates of ensiferan clades. The dash lines represent species rich clades and the solid lines are ensiferan clades that have numbers of species as expected. Clades with plus (+) signs indicate that they have diversification rates higher than that of Ensifera as a whole at 2 levels of relative extinction rates ($e_{0.9}$ and $e_{0.9}$) and minus signs indicate clades with diversification rates lower that that of Ensifera as a whole.

Table 1.1 List of accession numbers of nucleotide sequences used in phylogenetic reconstruction.

Suborder	Family	Subfamily	Species	Accession no.
Caelifera	Acrididae	Acridinae	*Acrida cinerea*	Z97560
	Cylindrachetidae		*Cylindraustralia kochii*	Z97565
	Euschmidtiidae	Euschmidtiinae	*Euschmidtia cruciformis*	Z97567
	Tanaoceridae	Tanaocerinae	*Tanaocerus koebeli*	Z97567
Ensifera	Anostostomatidae	Deinacridinae	*Hemideina crassidens*	Z97570
	Gryllotalpidae	Gryllotalpinae	*Neocurtilla sp.*	AF514522
	Gryllotalpidae	Gryllotalpinae	*Scapteriscus sp.*	AF514523
	Gryllotalpidae	Gryllotalpinae	*Gryllotalpa sp.*	AF514521
	Gryllidae	Eneopterinae	*Eneoptera surinamensis*	AF514645
	Gryllidae	Eneopterinae	*Eneoptera guyanensis*	AY905331
	Gryllidae	Eneopterinae	*Myara sordida*	AY905339
	Gryllidae	Mogoplistinae	*Ornebius aperta*	AF514515
	Gryllidae	Myrmecophilinae	*Myrmecophilus sp.*	AF514657
	Gryllidae	Nemobiinae	*Dianemobius nigrofasciatus*	AF514618
	Gryllidae	Nemobiinae	*Pteronemobius ohmachii*	AF514517
	Gryllidae	Nemobiinae	*Allonemobius socius*	AF514507
	Gryllidae	Oecanthinae	*Oecanthus nigricornis*	AF514514
	Gryllidae	Phalangopsinae	*Homeogryllus sp.*	AF514649
	Gryllidae	Phalangopsinae	*Neoamusia shawae*	AF514658
	Gryllidae	Phalangopsinae	*Ecuazarida mocagua*	AF514644
	Gryllidae	Phalangopsinae	*Paraclodes querapido*	AF514659
	Gryllidae	Phalangopsinae	*Aclodes cameronae*	AF514507
	Gryllidae	Phalangopsinae	*Lerneca ornata*	AF514654
	Gryllidae	Podoscirtinae	*Aphonomorphus sp.*	AF514640
	Gryllidae	Trigonidiinae	*Laupala cerasina*	AF514651
	Gryllacrididae	Gryllacridinae	*Camptonotus carolinensis*	AY521876
	Prophalangopsidae	Cyphoderinae	*Cyphoderris monstrosus*	AF514518
	Rhaphidophoridae	Ceuthophilinae	*Ceuthophilus utahensis*	AY521870
	Rhaphidophoridae	Ceuthophilinae	*Ceuthophilus carlsbadensis*	Z97563
	Stenopelmatidae	Stenopelmatinae	*Stenopelmatus fuscus*	AY121145
	Stenopelmatidae	Stenopelmatinae	*Stenopelmatus sp.*	AF514528
	Schizodactylidae	Comicinae	*Comicus campestris*	Z97564
	Tettigoniidae	Conocephalinae	*Ruspolia nitidula*	Z97582
	Tettigoniidae	Phaneropterinae	*Scudderia furcata*	AF514542
	Tettigoniidae	Tettigoniinae	*Tettigonia viridissima*	Z97587
	Tettigoniidae	Pseudophyllinae	*Pterophylla camellifolia*	AF423804

Table 1.2 Fossil ages used in divergence time estimation are shown. Ages are the midpoint values of the time periods from Geologic Time Scale (Gradstein et al. 2004). Seven fossils were used as calibration points on internal nodes of the maximum likelihood phylogeny and indicated in numbers as seen on the phylogeny.

Family / Subfamily	Species	Time period	Age (Myr)	Internal node no.
Nemobiinae	*Nemobius ornatipes*	Eocene	45.00	5
Gryllinae	*Gryllus oligocaenicus*	Oligocene, Lower Oligocene	30.75	6
Eneopterinae	*Cearagryllus previstus*	Cretaceous, Lower Cretateous	125.00	4
Trigonidiinae	*Trigonidium dominica*	Dominican amber inclusion	20.00	7
Myrmecophilinae	*Araripemyrcophilops gracilis*	Cretateous	105.85	2
Gryllotalpidae	*Merchandia magnifica*	Cretaceous, Lower Cretaceous	125.00	3
Haglinae	*Protshorkuphlebia triassica*	Lower Triassic	248.95	1
Stenopelmatidae	*Triassophyllum leopardii*	Triassic	225.80	N/A

Table 1.3 Divergence time estimates from penalized likelihood method with multiple fossil calibrations and diversification rate estimates of ensiferan clades. The divergence times are shown in million years before present unit. The net diversification rates of ensiferan clades in absence of extinction and under high relative extinction rates are shown as $r_{0.0}$ and $r_{0.9}$, respectively. The net diversification rates in bold letters indicate higher that the ensiferan rate as a whole (background diversification rates).

Family / Subfamily	No. of species	Estimated divergence time (million years)	Net diversification rate	
			$r_{0.0}$	$r_{0.9}$
Ensifera	12,528	251.40	0.0163	0.0123
Gryllidae	3,753	250.08	0.0143	0.0103
Oecanthinae	162	1.46	**1.5134**	**0.8445**
Mogoplistinae	303	34.07	**0.0728**	**0.0439**
Trigonidiinae	503	34.76	**0.0777**	**0.0492**
Gryllinae	880	83.29	**0.0354**	**0.0234**
Nemobiinae	285	125.68	**0.0195**	0.0117
Eneopterinae	136	138.07	0.0155	0.0084
Phalangopsinae	133	184.09	0.0073	0.0035
Myrmecophilinae	71	250.08	0.0074	0.0036
Gryllacrididae	679	168.49	**0.0168**	0.0109
Rhaphidophoridae	542	144.44	**0.0189**	0.0121
Stenopelmatidae	40	168.49	0.0095	0.0041
Gryllotalpidae	96	204.09	0.0097	0.0050
Tettigoniidae	6,412	223.14	**0.0171**	**0.0126**
Schizodactylidae	15	232.40	0.0051	0.0016
Prophalangopsidae	66	248.95	0.0079	0.0040

Table 1.4 The observed numbers of species of ensiferan clades from OSF online 2.4 (Eades, Otte, and Naskrecki, 2006) and the expected numbers of species considered the clades evolved at diversification rates of Ensifera as a whole after a time *t* since the origins in absence of extinction ($r_{0.0}$) and under high relative extinction rates ($r_{0.9}$). The upper limits (k_u) and lower limits of 95 % confidence intervals of expected species numbers are shown. The numbers of species in bold letter indicate that the numbers are higher than expected upper limits of 95% confidence intervals of expected species numbers k_u in absence of extinction and under high relative extinction rates. Note that no ensiferan clade is species poor.

Family / Subfamily	**Number of species (*n*)**	**Divergence time estimate *t* (million years)**	**Expected no. of species at $r_{0.0}$**		**Expected no. of species at $r_{0.9}$**	
			k_u	k_l	k_u	k_l
Gryllidae	**3,753**	250.08	*216.54*	2.48	752.29	6.16
Oecanthinae	**162**	1.46	27.73	*1.18*	*2.97*	*1.01*
Mogoplistinae	**303**	34.07	13.40	*1.09*	*21.87*	*1.14*
Trigonidiinae	**503**	34.76	34.14	*1.23*	*22.34*	*1.15*
Gryllinae	**880**	83.29	5.40	*1.03*	*68.13*	*1.46*
Nemobiinae	**285**	125.68	216.54	*2.48*	*137.60*	*1.94*
Eneopterinae	136	138.07	1.98	*1.01*	*165.70*	*2.13*
Phalangopsinae	133	184.09	101.87	*1.69*	316.70	3.17
Myrmecophilinae	71	250.08	56.63	1.38	752.29	6.16
Gryllacrididae	**679**	168.49	5.32	1.03	255.76	2.75
Rhaphidophoridae	**542**	144.44	73.29	*1.50*	*181.89*	*2.24*
Stenopelmatidae	40	168.49	5.32	1.03	255.76	2.75
Gryllotalpidae	96	204.09	212.57	*2.45*	413.93	3.83
Tettigoniidae	**6,412**	223.14	37.98	*1.25*	*531.50*	*4.64*
Schizodactylidae	15	232.40	162.11	2.11	599.34	5.11
Prophalangopsidae	66	248.95	139.28	1.95	741.49	6.08

Table 1.5 Diversification or speciation rates of other insects

Taxon	Speciation or diversification rates (species/million years)	References
Coleoptera (beeteles)	0.025-0.204	Wilson, 1983
Hymenoptera (7 families)	0.104-0.25	Wilson, 1983
Formicidae (ants)	0.106	Coyne & Orr, 2004
Diptera (22 families)	0.037-0.233	Wilson, 1983

CHAPTER 2

F SUPERGROUP *WOLBACHIA* IN BUSH CRICKETS: WHAT DO PATTERNS OF SEQUENCE VARIATION REVEAL ABOUT THIS SUPERGROUP AND HORIZONTAL TRANSFER BETWEEN NEMATODES AND ARTHROPODS?

2.1 Introduction

The bacterial endosymbiont *Wolbachia pipientis* is associated with arthropods and filarial nematodes. In general, *Wolbachia* has been shown to influence host reproductive biology with such effects enhancing the spread and maintenance of *Wolbachia* within populations (Werren, 1997; Weeks et al. 2002). For example, arthropod-specific strains have been shown to alter host reproductive biology by inducing cytoplasmic incompatibility, parthenogenesis, feminization, or male killing (Werren, 1997; Weeks et al., 2002), while nematode-specific strains appear to play a mutualistic role in development and reproduction (Bandi et al., 1999; Langworthy et al., 2000; Casiraghi et al., 2002). The profound reproductive effects of *Wolbachia*, in combination with its widespread occurrence in invertebrates (see Lo *et al.*, 2002; Casiraghi et al., 2005), have made understanding its dynamics and evolutionary history a central focus of evolutionary biologists (e.g., Boyle et al., 1993; Zhou et al., 1998; Shoemaker et al., 1999; Bordenstein et al., 2001; Jiggins et al., 2002; Hurst and Jiggins, 2005).

As for strain diversity, *Wolbachia* is currently divided into 8 supergroups (Vanderkerckhove et al., 1999, Lo et al., 2002; Rowley et al., 2004, Bordenstein and Rosengaus, 2005). Supergroups A and B are found only in arthropods, whereas supergroups C and D are found only in filarial nematodes and supergroup E is currently found only in Collembola (springtails) (Vanderkerckhove et al., 1999; Lo et al., 2002; Czarnetzki and Tebbe, 2004). Supergroup F is found in both arthropods and nematodes, including termites (*Microcerotermes* sp. and *Kalotermes flavicollis*), filarial nematodes (*Mansonella* spp.), a weevil (*Rhinocyllus conicus*) and two cimicids (Lo et al., 2002; Rasgon and Scott, 2004; Casiraghi et al., 2005). Supergroup G is found in Australian spiders (Rowley et al., 2004) and Supergroup G is found in termites (*Zootermopsis* spp.) (Bordenstein and Rosengaus, 2005).

Based on *16S* rDNA phylogenetic analyses, the F supergroup occurs in the widest array of host types, including insects from Coleoptera, Isoptera and Hemiptera and nematodes of genus *Mansonella*. Here, our molecular phylogenetic analysis confirms that two additional insect host species harbor *Wolbachia* strains from the F supergroup (the North American bush crickets, *Hapithus agitator* and *Orocharis saltator*, Gryllidae, Eneopterinae).

Although much attention has been paid to the A, B, C, and D supergroups of *Wolbachia*, relatively little work has been conducted on the F supergroup (Casiraghi et al., 2001b; Rasgon and Scott, 2004; Casiraghi et al., 2005). Interestingly, the wide host range of the F supergroup raises several questions. First, is there any evidence to suggest that F supergroup *Wolbachia* found in these diverse hosts is not a by-product of

them harboring nematodes (Casiraghi et al., 2005), thus relegating this supergroup as being nematode specific? Second, do patterns of molecular variation indicate a history of limited recombination as occurs in nematode-specific strains of *Wolbachia* or is there evidence of rampant recombination as is the case for the arthropod specific strains of *Wolbachia* (Jiggins, 2002; Baldo et al., 2006)? Third, and finally, do patterns of molecular variation, phylogenetic relationships, and host range tell us anything about the occurrence of horizontal transfer and the evolutionary history of this little studied *Wolbachia* supergroup? These questions were the focus of this study.

2.2 Materials and methods

2.2.1 Specimens

Bush crickets were collected from two locations: *Hapithus agitator* Uhler 1864 from Gulf Shores, Alabama and *Orocharis saltator* Uhler 1864 from Biloxi, Mississippi. Other crickets known to be infected by A and B supergroup *Wolbachia* (*Allonemobius walkeri* and *Neonemobius variegatus*, respectively) were used as controls for *Wolbachia* infection (Marshall, 2004). A filarial nematode specimen, *Dirofilaria immitis*, infected by C supergroup *Wolbachia* provided by Drs. Claudio Bandi and Maurizio Casiraghi (Università di Milano) was used as a positive control for the presence of filarial nematode genes. All specimens were stored in 95% ethanol at room temperature or –80ºC before DNA extraction.

2.2.2 DNA Extraction, PCR, and Sequencing

Genomic DNA was extracted using a DNA extraction kit (DNeasy tissue kit, QIAGEN). For each cricket specimen, the DNA was extracted from half of the head, thorax, abdomen and legs. We isolated the nematode DNA from different parts for the body to include reproductive tissues containing *Wolbachia*. The remaining parts of all specimens were stored at -80ºC.

We tested for the presence of *Wolbachia* in the *D. immitis* and cricket specimens by amplifying a fragment of *16S* rDNA and the cell-cycle gene *ftsZ*. For both genes, we used general primers which amplify *Wolbachia* genes from all supergroups (Casiraghi et al., 2001a). PCRs for *16S* rDNA and *ftsZ* genes were conducted in a 50µl volume under the condition described in Bandi et al., 1994, with the extension time of 2 min (Casiraghi et al., 2001). Approximately 1,000 and 800bp fragments of *16S* rDNA and *ftsZ* genes, respectively, were successfully amplified. All PCR products were purified (using QIAquick gel extraction kit, Qiagen) and directly sequenced on an ABI Prism 377 DNA Sequencer following general protocols (Marshall, 2004). Forward and reverse sequences for each fragment were aligned in Sequencher™ 3.1 (Gene Codes Corporation) and edited by eye to yield a consensus sequence. The successfully amplified *Wolbachia* sequences from the bush cricket specimens were deposited in GenBank.

2.2.3 Sequence Assemblage and Phylogenetic Analysis

The *Wolbachia 16S* rDNA and *ftsZ* gene sequences obtained from the bush crickets were aligned with homologous sequences of both genes that were deposited in

GenBank and used by Lo et al. 2002 (supergroups A-F), Rowley et al. 2004 (supergroup G) and Bordenstein and Rosengaus 2005 (supergroup H). Accession numbers for *Wolbachia sequences* used in this study are shown in Table 2.1. All sequences were aligned in ClustalX 1.83 (Thompson et al. 1997) and variable base pair positions were checked for reading and editing errors in BioEdit 7.0.5.3 (Hall, 1999). Straightforward alignments without large gaps were generated.

Because our samples have small overlapping regions with the two recently found supergroups G and H, we generated 2 sets of alignments for each gene in order to confirm the placement of *Wolbachia* from the bush crickets with both the presence and absence of all supergroups. The first set included all available sequences in the database that have short overlaps with our bush cricket *Wolbachia* and the other included only the supergroups containing the longest overlapping parts with the bush cricket *Wolbachia*. For *16S* rDNA, the total of 365 bp (all supergroups) and 852 bp (largest overlap) and for and *ftsZ* gene, 485 bp (all supergroups) and 718 bp (largest overlap) were used to reconstruct phylogenetic trees using maximum parsimony (MP) and Bayesian inference (BI) methods. For MP analyses, we subjected the data matrices to maximum parsimony, branch-and-bound search using gaps as a fifth base, 10 random taxon addition replicates, and tree-bisection and reconnection (TBR) branch swapping. All characters were equally weighted. Bootstrap analyses (100 replicates) on the shortest length trees via branch-and-bound search were also performed to determine the robustness of the nodes in the phylograms (except for the short fragment of 16S rDNA, we used heuristic search instead due to the computational limitation). All phylogenetic

analyses were performed in PAUP 4.0b 10 (Swofford, 2002). All trees were unrooted.

As for Bayesian analyses (BI), the analysis for each gene consisted of 5,000,000 generations and four chains, using MrBayes version 3.0B4 (Ronquist and Huelsenbeck, 2003). The appropriate models of sequence evolution for each data set were chosen via Akaike Information Criterion (AIC) in ModelTest 3.6 (Posada and Crandall, 1998). The selected models were as following: HKY+I +G for 365 bp fragment of *16S* rDNA; TrN+I+G for 852 bp fragment of *16S* rDNA; TrN+I+G for 485 bp fragment of *ftsZ* and TrN+I for 718 bp fragment of *ftsZ* genes. Trees were sampled every 100 generations, resulting in 50,000 total trees. The likelihood values were plotted against number of generations to determine if the likelihood values of the trees reached a plateau before the designated 'burnin'. The first 5,000 trees (10 %) of total trees were considered 'burnin' and discarded. Bayesian posterior probabilities (BPP) were estimated from the 50% majority rule consensus of the remaining 45,000 trees. Since MP and Bayesian analyses resulted in topologically similar phylogenies, only MP trees are reported with MP bootstrap support values and Bayesian posterior probabilities on the branches. The phylogenetic trees reconstructed from shorter fragments of *16S* rDNA and *ftsZ* genes were used to better understand the placement of *Wolbachia* from the bush crickets on the trees among all eight supergroups

2.2.4 Verification of the Presence/Absence of Filarial Nematodes in Bush Crickets

We conducted two PCR experiments to test if the *Wolbachia* obtained from the bush cricket samples came from a filarial nematode inside the bush crickets or from the

bush crickets themselves. First, lacking physical evidence of nematode parasites in the bush crickets, we used PCR to amplify a specific gene for filarial nematodes. The specific *12S* rDNA primers used were 12s-nem-F (5' AACTGGATTAGTACCCAGGT 3') and 12s-nem-R (5' CTAAACAATCAT ACATGCACC 3'). We designed these primers based on conserved regions among available filarial nematode *12S* sequences from GenBank, including *Brugia pahangi* (AJ544842), *B. malayi* (AJ544843), *Dirofilaria immitis* (AJ544831), *D. repens* (AJ544832), *Onchocerca gibsoni* (AJ544837), *O. gutturosa* (AJ544838), *O. volvulus* (AJ544840), *Setaria tundra* (AJ544834) and *Wuchereria bancrofti* (AJ544844). These nematode sequences cover the host genetic diversity in which C, D and F supergroup *Wolbachia* occur (Casiraghi et al., 2004), as well as filarial nematodes in general. PCR was conducted in a 50µL volume using the following reaction chemistry: 2.5 mM $MgCl_2$, 1x thermophilic bufferA (Promega), 0.2 mM PCR nucleotides, 1 unit of Taq polymerase (in buffer A, Promega), and 0.4µM each of forward and reverse primers. The thermocycler profile was: 94ºC for 5 min, followed by 25 cycles of 94ºC for 15 sec, 50ºC for 15 sec, 72ºC for 30 sec, ending with 72ºC for 7 min and stored at 4ºC. The expected product was approximately 150 bp. DNA isolates from several ground crickets infected with *Wolbachia* (supergroups A and B) were used as a series of negative controls, while a specimen of *D. immitis* was used as a positive control. The amplified product from the nematode was purified (using QIAquick gel extraction kit, Qiagen) and directly sequenced using ABI technology to verify its identity.

Second, the possible occurrence of filarial nematodes in these bush crickets was also confirmed by another region of *12S* rDNA conserved in both crickets and nematodes. The universal primers, 12s-F (5' AAGAGCGACGGGCGATGTGT 3') and 12s-R (N-14588 from Richard Harrison's lab 5'AAA CTA GGA TTA GAT ACC CTA TTA T 3') were used to amplify *12S* rDNA from all samples. In crickets, an ≈ 350 bp fragment should be amplified, while an ≈ 450 bp fragment should be recovered from nematodes. PCR was performed in a 25µl volume under the following conditions: 2.5 mM $MgCl_2$, 1x thermophilic bufferA (Promega), 0.4 µM for each primer, 0.2 mM PCR nucleotide, 1 unit of DNA polymerase (in buffer A, Promega). The thermocycler profile was: 94ºC for 2 min, 35 cycles of 94ºC for 15 sec, 45ºC for 15 sec 72ºC for 30 sec, ending with 72ºC for 7 min and stored at 4ºC until used. Once again, infected ground crickets and *D. immitis* were used as controls. The PCR products were visualized as before.

2.2.5 Patterns of Molecular Variation

Patterns of molecular variation within each supergroup, including numbers of polymorphic sites, nucleotide diversity (Pi), average number of pairwise nucleotide differences (k), and Fu and Li's D were conducted separately on both genes using DnaSP 3.53 (Rozas and Rozas, 1999). Additionally, patterns of recombination in each supergroup and in various combinations of supergroups were assessed for the *ftsZ* gene with two programs (MaxChi and Chimera) implemented in RDP version 2 (Martin and Rybicki, 2000). MaxChi (based on the procedure outlined by Maynard Smith, 1992) has been shown to perform well at detecting recombination under a wide variety of

circumstances (Posada, 2002). Chimera is based on a deviation of the MaxChi procedure and although more false positives occur, it is more likely to detect recombination when recombination is present (Posada and Crandell, 2001; Posada, 2002).

2.3 Results

2.3.1 Phylogenetic Analysis and Comparison of 16S rDNA and ftsZ Gene Sequences

Partial *16S* rDNA (853 bp) and *ftsZ* gene (729 bp) sequences from *Wolbachia* in the two bush crickets (GenBank Accession numbers: *H. agitator*, *16S* rDNA DQ536098, *ftsZ* DQ536100; *O. saltator*, *16S* rDNA DQ536097, *ftsZ* DQ536099) we used in this study showed high nucleotide sequence similarity (99 and 97% homology, respectively) to those from *K. flavicollis* and *Microcerotermes* sp. which were classified as F supergroup in Lo et al. (2002). Moreover, the *Wolbachia* gene sequences from our bush crickets were almost identical even though the bush crickets belong to different genera. Specifically, of 872 bp of *16S* rDNA sequence only one transition occurred and no differences were identified in 735 bp of *ftsZ* nucleotide sequence.

For the *16S* rDNA 852 bp fragments, 117 characters were variable and 73 were parsimony-informative and for the *16S* rDNA 365 bp fragments, 33 of 52 variable characters were parsimony-informative. For the *ftsZ* 718 bp fragments, 218 were variable and 163 were parsimony-informative and for the *ftsZ* 485 bp fragments, 111 of 149 variable characters were parsimony-informative.

Both maximum parsimony and Bayesian inference (BI) trees based on the longer fragments of *16S* rDNA and *ftsZ* genes showed that *Wolbachia* from our bush cricket samples are closely related to other F supergroup strains (Fig. 2.1 and 2.2). Both phylogenetic trees also have congruent topologies at the supergroup level with high bootstrap values [*16S* rDNA (852 bp): 54-100 %; *ftsZ* (718 bp): 57-100 %] and Bayesian posterior probabilities [*16S* rDNA (852 bp): 71-100 %; *ftsZ* (718 bp): 96-100 %], and they are also consistent with previous phylogenies (Lo et al., 2002; Czarnetzki and Tebbe, 2004; Casiraghi et al., 2005). The placement of *Wolbachia* from the two bush crickets in the F supergroup is confirmed in both *16S* rDNA and *ftsZ* phylogenies with high support values (*16S* rDNA: bootstrap = 71, BPP = 86; *ftsZ*: bootstrap = 95, BPP = 100; Figs.2.1 and 2.2).

Even though the shorter fragments of both genes from all *Wolbachia* supergroups were analyzed, the placement of *Wolbachia* from our bush cricket samples was in F supergroup with good support (*16S* rDNA: bootstrap = 55, BPP = 75; *ftsZ*: bootstrap = 96, BPP = 100, phylogenies not shown). With fewer parsimony-informative sites, the topology of the trees at a supergroup level remained consistent with previous phylogenies (Lo et al., 2002; Czarnetzki and Tebbe, 2004; Casiraghi et al., 2005) and with the trees of longer fragments in this study.

2.3.2 Verification of the Absence of Filarial Nematodes in Bush Crickets

PCR amplification of the nematode *12S* rDNA gene using *12S* nematode specific primers gave an expected DNA fragment (~ 150 bp) from *D. immitis*, whereas the same PCR condition did not amplify any DNA fragment from cricket specimens

(figure 2.3a). Furthermore, the PCR test with *12S* rDNA universal primers amplified a larger size fragment (~ 450 bp) from *D. immitis* than those from crickets (figure 2.3b). All crickets had only one band of ~ 350 bp without a second band from nematodes. These two PCR tests suggest that filarial nematodes were not present in the bush crickets used in this study.

2.3.3 Patterns of Molecular Variation

Our analysis of within supergroup molecular variation for the A, B, C, D, and F supergroups of *Wolbachia* suggests that the F supergroup has less nucleotide variation than either C or D, but more than either A or B (Table 2.2). Additionally, no evidence of recombination was found within the F supergroup or for the combined C-D-F clade (Table 2.2), although evidence of recombination was found for the B supergroup and the combined analysis of both A and B supergroups. This is consistent with previous findings utilizing different methods and sequences (e.g., Jiggins, 2002; Baldo et al., 2006).

2.4 Discussion

Our phylogenetic analysis indicates that the *Wolbachia* genes amplified from these two bush crickets belong to the F supergroup. Additionally, the absence of filarial nematode genes in the DNA samples of these bush crickets suggests that *Wolbachia* found in both of these species is not a by-product of them harboring filarial nematodes – a result also found for termites that harbor strains of F supergroup *Wolbachia* (Casiraghi et al. 2005). These results, in combination with previous findings (e.g., Lo et al., 2002; Rasgon and Scott, 2004; Casiraghi et al. 2005), suggest that the F supergroup is

monophyletic and has the broadest host range (i.e., nematodes and arthropods) of any of the *Wolbachia* supergroups.

Despite the wide host range of the F supergroup, there is relatively little nucleotide variation among the strains of this supergroup in comparison to the C and D supergroups (Table 2.2). These data suggest that horizontal transfer between nematodes and arthropods has occurred recently relative to the initial divergence of arthropod and nematode strains – a suggestion supported by Casiraghi et al. (2005). Moreover, the combination of wide host range and limited genetic variation indicate a recent radiation of the F supergroup via horizontal transfer from host to host.

There is a lack of detectable recombination among F supergroup strains, even when analyzed in combination with other supergroups. Low recombination levels are in stark contrast to the rampant recombination found within and among strains of the A and B supergroups of *Wolbachia* – which are mostly parasitic on their hosts (see also Jiggins, 2002 and Baldo et al., 2006). Instead, a lack of recombination is consistent with findings for the mutualistic C and D supergroup strains harbored by nematodes. One possibility is that the low level of nucleotide variation hampers the ability to detect recombination, but the F supergroup has higher levels of nucleotide variation than the B supergroup of *Wolbachia*, within which numerous recombination events were detected.

An intriguing possibility is that strains of the F supergroup behave as mutualists in their hosts, much like strains of C and D *Wolbachia* do in their nematode hosts (e.g., Bandi et al., 1999; Langworthy et al., 2000; Casiraghi et al., 2002). This lifestyle may result in strong purifying selection against recombination, not just limited opportunities

for horizontal transfer as appears to be the case in nematodes. However, this latter possibility is only a hypothesis and needs to be considered with some caution. For example, the lack of recombination could be a consequence of a recent, rapid spread of the F supergroup among hosts, thus resulting in little time and opportunity for divergence and recombination. In the end, the lifestyle of F supergroup strains of *Wolbachia*, whether mutualistic or parasitic, remains an unresolved question, although curing experiments using populations of the bush crickets studied here may help provide some insights into the lifestyle of this little studied supergroup of *Wolbachia*.

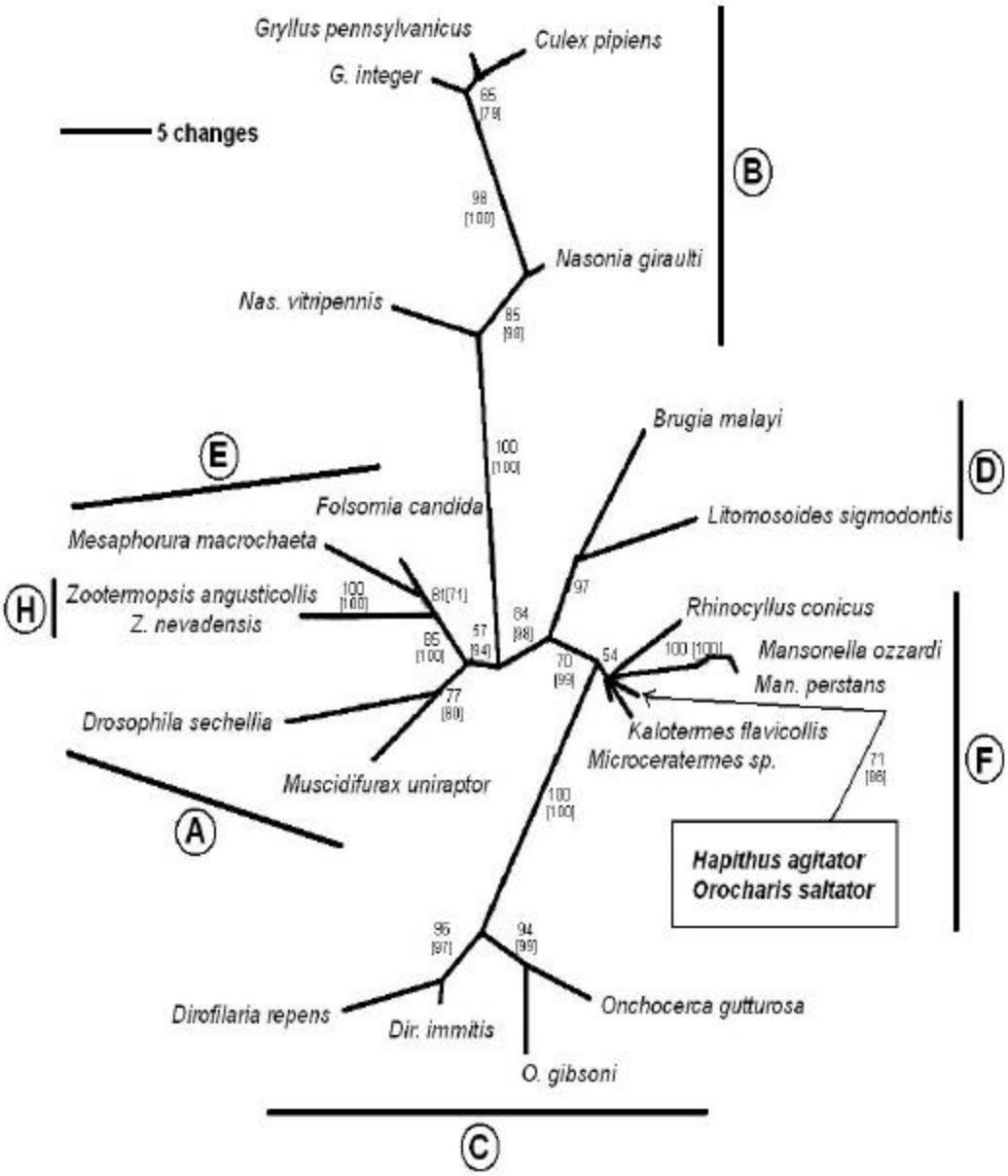


Figure 2.1 Unrooted phylogeny of *16S* rDNA gene of *Wolbachia pipientis* reconstructed using the maximum parsimony method. The letters in circles indicate *Wolbachia* supergroups based on Vanderkerckhove et al. (1999), Lo et al. (2002), Rowley et al. (2004) and Bordenstein and Rosengaus (2005). The names of taxa represent host names (accession numbers are shown in Table 1). The bootstrap values for braches of MP tree are shown above posterior probabilities from a 45,000-tree 50% majority-rule consensus in Bayesian analysis shown in parentheses. The bush cricket samples are indicated in box with bold font.

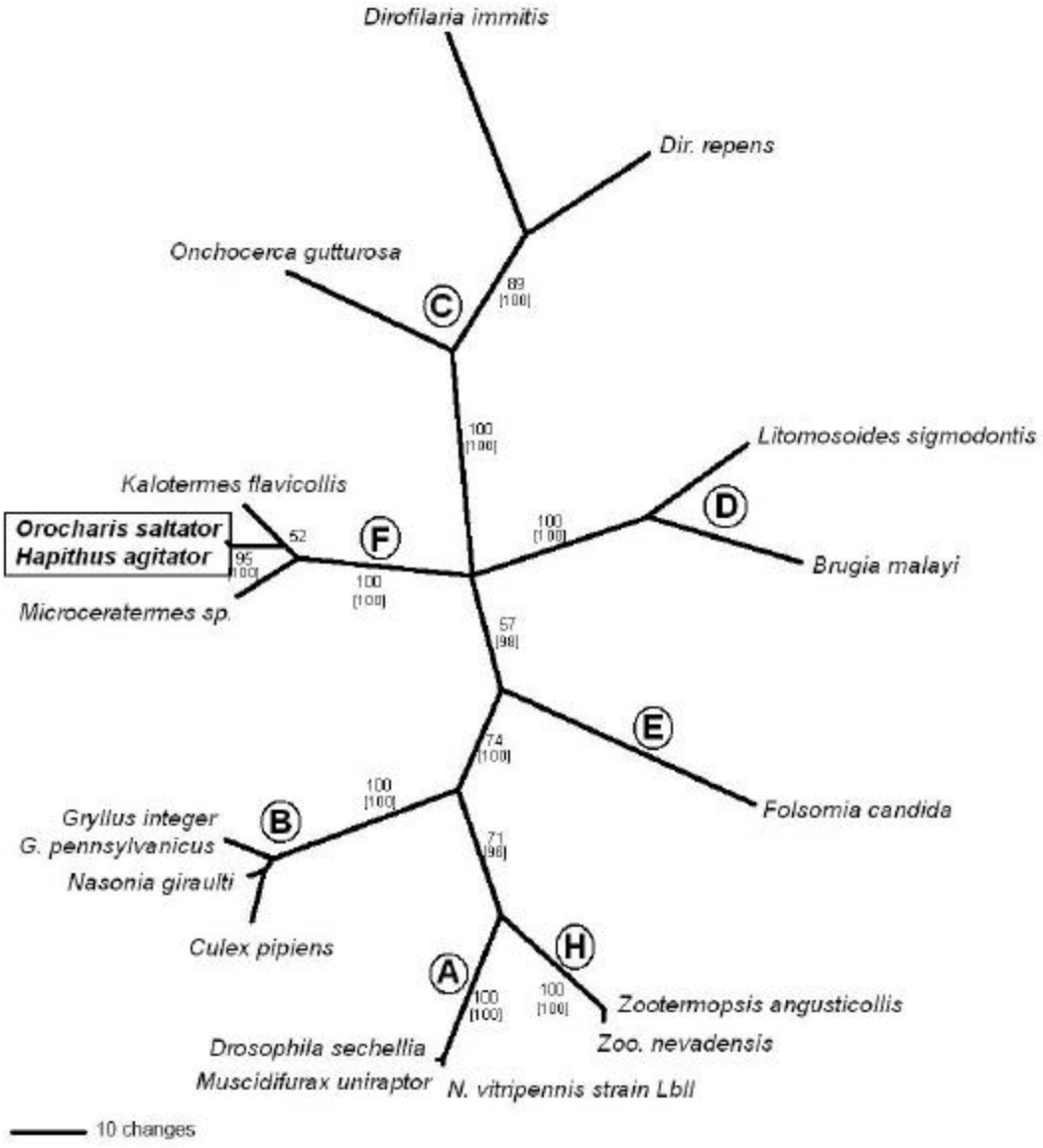
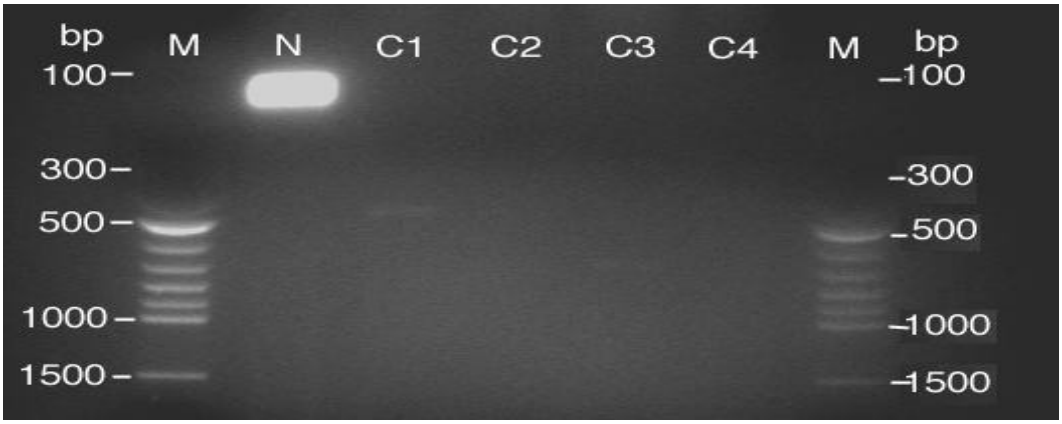


Figure 2.2 Unrooted phylogeny of *ftsZ* gene of *Wolbachia pipientis* reconstructed using the maximum parsimony method. The letters in circles indicate *Wolbachia* supergroups based on Vanderkerckhove et al. (1999), Lo et al. (2002), Rowley et al. (2004) and Bordenstein and Rosengaus (2005). The names of taxa represent host names (accession numbers are shown in table 1). The bootstrap values for braches of MP tree are shown above posterior probabilities from a 45,000-tree 50% majority-rule consensus in Bayesian analysis shown in parentheses. The bush cricket samples are indicated in box with bold font.

a.



b.

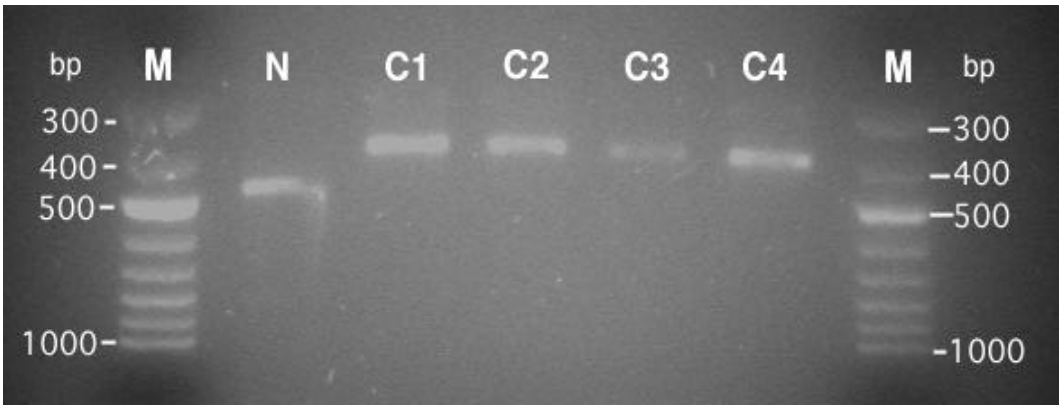


Figure 2.3 Agarose gel electrophoresis of PCR products from a) *12S rDNA* nematode specific, and b) *12S rDNA* universal primers. M represents DNA marker, ranging from 300 to 1,000 base pair in length. *Dirofilaria immitis* is used as a positive control for nemotode infection in cricket samples (N). Lanes C1 to C4 are cricket specimens: *Hapithus* agaitator, *Orocharis saltator*, *Neonemobius variegatus* and *Allonemobius socius*, respectively. The latter two species of crickets are used as amplification positive control.

Table 2.1 List of accession numbers of *Wolbachia* sequences used in the phylogenetic analyses in this study.

Host species	Host order	*Wolbachia* supergroup	Accession no.	
			16S rDNA	*ftsZ*
Diaea circumlita wDiacir1	Araneae	A	AY486071	-
Drosophila sechellia	Diptera	A	U17059	U28179
Muscidifurax uniraptor	Hymenoptera	A	L028821	U28186
Nasonia vitripennis strain *LbII*	Hymenoptera	A	-	U28188
Culex pipiens	Diptera	B	X61768	U28209
Nasonia giraulti	Hymenoptera	B	M84689	U28203
Nasonia vitripennis	Hymenoptera	B	M84687	-
Gryllus integer	Orthoptera	B	U83094	AJ011269
Gryllus pennsylvanicus	Orthoptera	B	U83090	U28195
Dirofilaria immitis	Spirurida	C	AF487892	AJ010272
Dirofilaria repens	Spirurida	C	AJ276500	AJ010273
Onchocerca gibsoni	Spirurida	C	AJ276499	-
Onchocerca gutturosa	Spirurida	C	AJ276498	AJ010266
Brugia malayi	Spirurida	D	AJ051145	AJ010269
Litomosoides sigmodontis	Spirurida	D	AF069068	AJ010271
Folsomia candida	Colembola	E	AF179630	AJ344216
Mesaphorura macrochaeta	Colembola	E	AJ422184	-
Rhinocyllus conicus	Coleoptera	F	M85267	-
Cimex lectularius	Hemiptera	F	AY316361	AY316362
Oeciacus vicarius	Hemiptera	F	AY091456	AY091457
Kalotermes flavicollis	Isoptera	F	Y11977	AJ292345
Microceratermes sp.	Isoptera	F	AJ292347	AJ292346
Hapithus agitator	Orthoptera	F	DQ536098	DQ536100
Orocharis saltator	Orthoptera	F	DQ536097	DQ536099
Mansonella ozzardi	Spirurida	F	AJ279034	-
Mansonella perstans	Spirurida	F	AY278355	-
Diae acircumlita wFiacir3	Araneae	G	AY486073	-
Diaea sp. R1 wDiaspp2	Araneae	G	AY468070	-
Zootermopsis angusticollis	Isoptera	H	AY764279	AY764283
Zootermopsis nevadensis	Isoptera	H	AY744280	AY764284

Table 2.2 Molecular variation for 663 base pairs of *ftsZ* within the A, B, C, D and F supergroups of *Wolbachia.*

			Nucleotide Variation			# of Recombination Events	
Supergroup	n	# polymorphic sites	*k*	Pi	Fu and Li D	MaxChi	Chimera
A	22	32	8.84	0.0133	-1.329^{ns}	0	0
B	25	79	16.73	0.0249	-0.121^{ns}	30	26
A-B	47	115	35.22	0.0532	0.259^{ns}	88	6
C	9	98	42.72	0.0646	0.374^{ns}	0	0
D	8	51	20.39	0.0312	0.548^{ns}	0	0
F	5	43	19.80	0.0299	-0.452^{ns}	0	0
C-D-F	22	171	60.68	0.0931	0.727^{ns}	0	0

REFERENCES

Alexander, R. D., 1968. Life cycle origins, speciation, and related phenomena in crickets. Q. Rev. Biol. 43: 1-41.

Bailey, N. T. J., 1964. The elements of stochastic processes with applications to natural sciences. Wiley, New York.

Baldo, L., S. Bordenstein, J. J. Wernegreen, & J. H. Werren, 2006. Widespread recombination throughout *Wolbachia* Genomes. Mol. Bol. Evol. 23:437-449.

Bandi, C., T. J. C. Anderson, C. Genchi & M. L. Blaxter, 1998. Phylogeny of *Wolbachia* in filarial nematodes. Proc. R. Soc. Lond. Ser. B 265: 2407-2413.

Bandi, C., J. W. McCall, C. Genchi, S. Corona, L. Venco, & L. Sacchi, 1999. Effects of tetracycline on the filarial worms *Brugia pahangi* and *Dirofilaria immitis* and their bacterial endosymbionts *Wolbachia*. Int. J. Parasitol. 29: 357–364.

Bandi, C., A. J. Trees & N. W. Brattig, 2001. *Wolbachia* in Filarial nematodes: evolutionary aspects and implications for the pathogenesis and treatment of filarial diseases. Vet. Parasitol. 98: 215-238.

Barraclough, T. G. & A. P. Vogler, 2002. Recent diversification rates in North American tiger beetles estimated from a dated mtDNA phylogenetic tree. Mol. Biol. Evol. 19(10):1706-1716.

Bond, W. J., 1989. The tortoise and the hare: ecology of angiosperm dominance and gymnosperm persistence. Biol. J. Lin. Soc. 36: 227-249.

Bordenstein, S. R., F. P. O'Hera, & J. H. Werren, 2001. *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. Nature 409: 707–710.

Bordenstein, S. R. & R. B. Rosengaus, 2005. Discovery of a novel *Wolbachia* supergroup in Isoptera. Curr. Microbiol. 51: 393-398.

Boyle, L., S. L. O'Neill, H. M. Robertson, & T. L. Karr, 1993. Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila*. Science 260: 1796–1799.

Broughton, R. E. & R. G. Harrison, 2003.Nuclear gene genealogies reveal historical, demographic and selective factors associated with speciation in field crickets. Genetics, 163: 1389–1401.

Casiraghi, M., T. J. C. Anderson, C. Bandi, C. Bazzocchi, & C. Genchi, 2001a. A phylogenetic analysis of filarial nematodes: comparison with the phylogeny of *Wolbachia* endosymbionts. Parasitol. 122: 93-103.

Casiraghi, M., O. Bain, R. Guerrero, C. Martin, V. Pocacqua, S. L. Gardner, A. Franceschi, & C. Bandi, 2004. Mapping the presence of *Wolbachia pipientis* on the phylogeny of filarial nematodes: evidence for symbiont loss during evolution. Intern. J. Parasitol. 34: 191-203.

Casiraghi, M., S. R. Bordenstein, L. Baldo, N. Lo, T. Beninati, J. J. Wernegreen, J. H. Werren & C. Bandi, 2005. Phylogeny of *Wolbachia pipientis* based on *gltA*,

groEL and *ftsZ* gene sequences: clustering of arthropod and nematode symbionts in the F supergroup, and evidence for further diversity in the *Wolbachia* tree. Microbiol. 151: 4015-4022.

Casiraghi, M., G. Favia, G. Cancrini, A. Bartoloni, & C. Bandi, 2001b. Molecular identification of *Wolbachia* from the filarial nematode Mansonella ozzardi. Parasitol. Res. 87:417-420.

Casiraghi, M., J.W. McCall, L. Simoncini, L.H. Kramer, L. Sacchi, C. Genchi, J.H. Werren & C. Bandi, 2002. Tetracycline treatment and sex-ratio distortion: a role for *Wolbachia* in the moulting of filarial nematodes? Internat. J. Parasitol. 32: 1457-1468.

Caterino, M. S., S. Cho, & F. A. H. Sperling, 2000. The current state of insect molecular systematics: 'A thriving tower of Babel'. Ann. Rev. of Entomol. 45: 1-54.

Czarnetzki, A. B. & C. C. Tebbe, 2004. Detection and phylogenetic analysis of *Wolbachia* in Collembola. Environ. Microbiol. 6(1): 35-44.

Coyne, J. R. & H. A. Orr, 2004. Speciation. Sinauer, Sunderland, MA.

Desutter-Grandcolas, L., 2003. Phylogeny and the evolution of acoustic communication in extant Ensifera (Insecta, Orthoptera). Zool. Scr. 32(6): 525-561.

Doyle, J. A. & M. J. Donoghue, 1986. Seed plant phylogeny and the origin of angiosperms: an experimental cladistic approach. Bot. Rev. 52: 321-431.

Eades, D. C., Otte, D. & Naskrecki, P., 2006. Orthoptera Species File Online. Version 2.4 [10/10/06]. <http://osf2.orthoptera.org>.

Felsenstein, J., 2005. PHYLIP (Phylogeny Inference Package) version 3.6. University of Washington, Seattle.

Forrest, T.G., 1991. Power output and efficiency of sound production by crickets. Behav. Ecol. 2: 327-338.

Fryer, G. & T. D. Iles, 1972. The cichlid fishes of the Great Lakes of Africa: their biology and evolution. Oliver and Boyd, Edinburgh.

Gaston, K. J. & E. Hudson, 1994. Regional patterns of diversity and estimates of global insect species richness. Biodiversity and Conservation, 3(6): 493-500.

Gradstein, F. M., J. G. Ogg, & A. G. Smith, 2004. A geologic time scale 2004. Cambridge University Press, Cambridge, UK

Gross, S. W., D. L. Mays & T. J. Walker, 1989. Systematics of *Pictonemobius* ground crickets (Orthoptera: Gryllidae). Trans. Amer. Entomolol. Soc. 115(4): 433-456.

Gwynne, D. T., 1995. Phylogeny of Ensifera (Orthoptera): a hypothesis supporting multiple origins of acoustic signaling, complex spermatophores and maternal care in crickets, katydids, and weta. J. Orthoptera Res. 4: 203-218.

Gwynne D. T., 2001. Katydids and bush-crickets, reproductive behavior and evolution of the Tettigoniidae. Ithaca, NY: Cornell Univerisity Press.

Hall, T. A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41: 95-98.

Hammond, T. J. & W. J. Bailey, 2003. Eavedropping and defensive auditory masking in an Australian bush cricket, Caedicia (Phaneropterinae: Tettigoniidae: Orthoptera). Behaviour 149: 79-95.

Hurst, D. D. & F. M. Jiggins, 2005. Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. Proc. Roy. Soc. Lond. Ser. B 272: 1525-1534.

Jiggins, F. M., 2002. The rate of recombination in *Wolbachia* bacteria. Mol. Biol. Evol. 19:1 640-1643.

Jiggins, F.M., G. D. D. Hurst, & Z. Yang, 2002. Host-symbiont conflicts: positive selection on an outer membrane protein of parasitic but not mutualistic Rickettsiaceae. Mol. Biol. Evol.19: 1341-1349.

Jost, M. C. & K. L. Shaw, 2006. Phylogeney of Ensifera (Hexapoda: Orthoptera) using three ribosomal loci, with implications for the evolution of acoustic communication. Mol. Phylogen. Evol. 38: 510-530.

Judd, W., 1948. A comparative study of the prebentriculus of orthopteroid insects, with reference to its use in taxonomy. Can. J. Res. D 26: 93-161.

Kjer, K. M., 1995. Using rDNA secondary structure in phylogenetic studies to identify homologous positions: an example of alignment and data presentation from frogs. Mol. Biol. Evol. 4(3): 314-330.

Kjer, K. M., 2004. Aligned *18S* and Insect Phylogeny. Syst. Biol. 53(3): 506–514.

Knight, M. E. & G. F. Turner, 2004. Laboratory mating trials indicate incipient speciation by sexual selection among populations of the cichlid fish pseudotropheus zebra from Lake Malawi. Proc. R. Soc. Ser. B 271(1540): 675-680.

Langworthy, N. G., A. Renz, U. Mackenstedt, K. Henkleduhrsen, M. B. De Bronsvoort, V. N. Tanya, M. J. Donnelly, & A. J. Trees, 2000. Macrofilaricidal activity of tetracycline against the filarial nematode *Onchocerca ochengi*: elimination of *Wolbachia* precedes worm death and suggests a dependent relationship. Proc. R. Soc. Lond. Ser. B 267: 1063–1069.

Lo, N., M. Casiraghi, E. Salati, C. Bazzocchi & C. Bandi, 2002. How many *Wolbachia* supergroups exist? Mol. Biol. Evol. 19: 341-346.

Lovette, I. J. & E. Bermingham , 1999. Explosive speciation in the new world *Dendroica* warblers. Proc. R. Soc. Lond. B 266: 1629-1636.

MacArthur, R. H., 1958. Population ecology of some warblers of northeastern coniferous forests. Ecology 39: 599-619.

Magallón, S. & M. J. Sanderson, 2001. Absolute diversification rates in angiosperm clades. Evolution 55(9): 1762-1780.

Marshall, C. R., 1990. The fossil record and estimating divergence times between lineages: maximum divergence times and the importance of reliable phylogenies. J. Mol. Evol. 30: 400-408.

Marshall, J. L., 2004. The *Allonemobius-Wolbachia* host-endosymbiont system: Evidence for rapid speciation and against reproductive isolation driven by cytoplasmic incompatibility. Evolution 58(11): 2409–2425.

Martin, D. & E. Rybicki., 2000. RDP: detection of recombination amongst aligned sequences. Bioinformatics 16: 562-563.

Mayhew, P. J., 2002. Shifts in hexapod diversification and what Haldane could have said. Proc. R. Soc. Lond. Ser. B. 269: 969-974.

Maynard Smith, J., 1992. Analyzing the mosaic structure of genes. J. Mol. Evol. 34: 126–129.

Mayr, E., 1963. Animal species and evolution. Cambridge, MA: Harvard University Press.

Morse, D. H., 1989. American Warbler, an ecological and behavioral prospective. Cambridge, MA: Harvard University Press.

McPeek, M. A. & J. M. Brown, 2007. Clade age and not diversification rate explains species richness among animal taxa. Am. Nat. 169: 97–106.

Mendelson, T. C. & K. L. Shaw, 2005. Rapid speciation in an arthropod. Nature, 433: 375-376.

Near, T. J., P. A. Meylan & H. B. Shaffer, 2005. Assessing concordance in fossil calibration points in molecular clock studies: an example using turtles. Am. Nat. 165(2): 137-146.

Otte, D. & R. D. Alexander, 1983. The Australian crickets (Orthoptera: Gryllidae). Philadelphia, PA: Academy of Natural Science of Philadelphia.

Otte, D., 1992. Evolution of cricket songs. J. Orthopt. Res., 1: 25-49.

Posada, D., 2002. Evaluation of methods for detecting recombination from DNA sequences: empirical data. Mol. Biol. Evol. 19: 708–717.

Posada, D. & K. A. Crandall, 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817-818.

Posada D. & K. A. Crandall, 2001. Evaluation of methods for detecting recombination from DNA sequences: Computer simulations. *Proc. Natl. Acad. Sci. USA,* 98**:** 13757–13762.

Rasgon, J. L. & T. W. Scott, 2004. Phylogenetic characterization of *Wolbachia* symbionts infecting *Cimex lectularius* L. and *Oeciacus vicarius* Horvath. (Hemiptera: Cimicidae). J. Med. Entomol. 41: 1174-1178.

Ree, R. H., 2005. Detecting the historical signature of key innovations using stochastic models of character evolution and cladogenesis. Evolution 59(2): 257–265.

Ronquist, F. & J. P. Huelsenbeck, 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574.

Rowley, S. M., R. J. Raven & E. A. McGraw, 2004. *Wolbachia pipientis* in Australian spiders. Curr. Microbiol. 49: 208-214.

Rozas, J. & R. Rozas, 1999. DnaSP version 3.0: An integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics 15: 174-175.

Sanderson, M. J., 1997. A nonparametric approach to estimating divergence times in the absence of rate constancy. Mol. Biol. Evol. 14(12): 1218-1231

Sanderson, M. J., 2002. Estimating absolute rates of Molecular Evolution and Divergence Times: A penalized likelihood approach. Mol. Biol. Evol. 19(1): 101–109.

Sanderson, M. J., 2003. r8s: inferring absolute rates of molecular evolution and divergence times in the absence of a molecular clock Bioinform. Application Notes 19(2): 301–302.

Sanderson, M. J. & J. A. Doyle, 2001. Sources of error and confidence intervals in estimating the age of angiosperms from rbcL and *18S* rDNA data. Am. J. Bot. 88: 1499-1516.

Schluter, D., 2000. The Ecology of Adaptive Radiation. Oxford University Press, Oxford.

Sharov, A.G., 1968. In: Rodendorf, B.B. (Ed), Phylogeny of the Orthopteroidea. Akad. A. Nauk. SSSR Trudy Paleo. Inst., Vol. 188. Israel Program for Scientific Translations, Jerusalem.

Shoemaker, D. D., V. Katju, & J. Jaenike, 1999. *Wolbachia* and the evolution of reproductive isolation between *Drosophila recens* and *Drosophila subquinaria*. Evolution 53: 1157–1164.

Simpson, G. G., 1953. The major features of evolution. New York: Columbia University Press.

Swofford, D. L., 2002. PAUP, Phylogenetic Analysis using Parsimony (*and Other Methods):Version 4. Sinauer Associates, Sunderland, MA.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin & D. G. Higgins, 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 24: 4876-4882.

Turner, G. F. & M. T. Burrows, 1995.A model of sympatric speciation by sexual selection. Proc. Biol. Sci. 260(1359): 287-292.

Vandekerckhove, T. M. T., S. Watteyne, S. Willems, J. G. Swings, J. Mertens & M. Gillis, 1999. Phylogenetic analysis of the 16s rDNA of the cytoplasmic bacterium *Wolbachia* from the novel host *Folsomia candida* (Hexapoda, Collembola) and its implications for Wolbachia taxonomy. FEMS Microbiol. Let. 180**:** 279-286.

von Hagen, K. B. & J. W. Kadereit, 2003. The diversification of Halenia (Gentlanaceae): ecological opportunity versus key innovation. Evolution 57(11): 2507–2518.

Weeks, A. R., K. T. Reynolds, & A. A. Hoffmann, 2002. *Wolbachia* dynamics and host effects: what has (and has not) been demonstrated?. Tre. Ecol. Evol. 17: 257–62.

Werren, J. H., 1997. Biology of *Wolbachia*. Annu. Rev. Entomol. 42: 587-609.

Werren, J. H. & D. M. Windsor, 2000. *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? Proc. R. Soc. Lond. B 261: 1277-1285.

Wilson, M. V. H., 1983. Is there a characteristic rate of radiation for the insects? Paleobiol. 9(1): 79-85.

Zhou, W. M., F. Rousset, & S. O'Neill, 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. Proc. R. Soc. Lond. B 265: 509–515.

Zeuner, F. E., 1939. Fossil Orthoptera Ensifera. London: British Museum Natural History.

BIOGRAPHICAL INFORMATION

Kanchana Panaram is a Thai scholar. She received B.S. in Biology from Mahidol University, Bangkok, Thailand in 2001. In the same year, she received a scholarship to study overseas and pursued her M.S. in Biology at New York University. During 2 years in New York, she studied genetic variation in the Mexican blind cavefish, *Astyanax mexicanus*. She transferred to University of Texas at Arlington in 2003 to work on her research in insect phylogenetics and species interaction between insects and their parasites with Dr. J. L. Marshall. After receiving doctoral degree in Quantitative Biology, Kanchana plans to return to Thailand and work for a Royal Thai Government research institution.