RADIATION OF THE BARK LOUSE GENUS *KILAUELLA* ACROSS THE HAWAIIAN ISLANDS

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Entomology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2015

Urbana, Illinois

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ABSTRACT

Island systems serve as important models for studies of evolutionary processes and speciation. The Hawaiian Island group is one of the most isolated island chains in the world, and many groups of insects have undergone dramatic diversification within these islands. Studies of adaptive radiation on Hawaii could promote understanding of the evolutionary process underlying diversification patterns, but studies of Hawaiian taxa from a systematics standpoint are limited. The bark louse genus *Kilauella* (Psocoptera: Elipsocidae) represents one of the most abundant genera of insects across all islands of the Hawaiian chain, and is a prime candidate for a phylogenetic study. This work aims to explore the diversification pattern of these bark lice across the modern high islands. *Kilauella* specimens were collected from the islands of Kauai, Oahu, Molokai, Maui Nui, and Hawaii to create a phylogeny exploring the speciation patterns of the genus. Our results show evidence of forward 'stepping stone' radiation across the Hawaiian Islands with a potentially significant level of within island radiation, but resolution in the phylogeny is a problem for elucidation of an exact pattern. Molecular dating estimates show that genus *Kilauella* may be a relatively young radiation, with an origin at approximately 6.74 mya (95% confidence interval 9.48 to 4.38 mya), corresponding roughly with the uplift of the island of Nihoa at 7.2 mya.

ACKNOWLEDGEMENTS

I would like to thank my advisor Kevin P. Johnson for all of his support on this work. He has been a great advisor over the last two years for not only this project – which started out at a slow and difficult pace, but for all the opportunities I've had for growth as a student in his lab.

Thank you to all the members of the Johnson Lab for their time and support during my time there. Thanks to fellow graduate student Drew Sweet for the countless hours spent in the lab troubleshooting PCR reactions, thanks to post doctoral students Julie Allen and Bret Boyd for their help with analyses and looking forward to career paths, and Therese Catanach and Jen Nowak for their advice and help on other parts of my lab work. I'd like to thank Emilie Bess for all the collection of specimens for this project, and for its original proposal. Lastly, thank you to my committee members Chris Dietrich and Jim Whitfield for all of your suggestions on how to make this project a better publication and thesis.

Thanks to Dr. Johnson for the funding for the sequencing and analysis for this project, and thank you to the National Science Foundation IGERT program for the personal funding for the last two years.

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INTRODUCTION

The importance of island systems for understanding the historical process of diversification has long been appreciated due to the inherent biogeographic simplicity of islands, adding to the ease of modeling ecological and evolutionary processes (Darwin, 1859; Johnson, Adler, & Cherry, 2000; Jordan, Simon, & Polhemus, 2003; Losos & Ricklefs, 2009; MacArthur & Wilson, 1967). The Hawaiian Islands serve as an ideal model for study of speciation and diversification processes. Hawaii is the most isolated chain of islands in the world, (Fleischer, McIntosh, & Tarr, 1998; Simon, 1987) and of its complete fauna, a significantly large fraction is endemic species. For instance, total arthropod diversity on Hawaii is made up of 99% endemic species (Bennett & O'Grady, 2012; Wagner & Funk, 1995). Ecological and genetic studies have shown that the modern Hawaiian terrestrial arthropod diversity of 8,000 to 10,000 species was generated by approximately 350-400 colonization events (Gillespie, Claridge, & Roderick, 2008; Howarth & Mull, 1992). Therefore, diversification processes within the island chain must have generated this large endemic fauna. High diversification rates are enabled by multiple potential factors, such as the steep climate gradients on the islands. Ecosystems on Hawaii range from highland cloud forests to lowland beach stands, all present on a relatively small scale and in a remote location (Simon, 1987; Wagner & Funk, 1995). The islands have been shown to possess the fastest speciation rates on the planet for arthropods such as the *Laupala* cricket (Mendelson & Shaw, 2005). Diversification processes on Hawaii must involve some unique factors in order to have generated the present day biodiversity, since fauna present on most islands are simple allopatric isolates of nearby mainland taxa (Lande, 1980; Mayr, 1970).

The geologic history of this volcanic island chain is well documented with precise dates of individual island uplift. The formation of the entire Hawaiian chain occurred by the movement of Pacific plates over a volcanic hot spot that resulted in linear and chronological formation of islands (Carson & Clague, 1995; Neall & Trewick, 2008). The oldest northwestern island in the Hawaiian chain is Kure Atoll dated to 29 million years old, and the youngest southeastern island is Hawaii dated to 0.43 million years old (Cowie & Holland, 2008). This pattern of formation provides a unique opportunity for exploring evolutionary processes leading to speciation. The uplift of islands in a chronological order such as this has been called a "stepping stone" pattern (Kimura & Weiss, 1964), with new islands appearing continuously. This provides continual colonization opportunities through time. Extreme rates of erosion occur as the islands age, and lineages of a given organism must colonize newly emerging islands or face extinction (Price & Clague, 2002). This erosion of older islands complicates estimation of the ages of common ancestors, because no fossil record is preserved prior to 0.12 million years ago. A fossil calibration node is a typical method by which dated phylogenetic analyses are performed, as it provides the minimum age of a given node. All lineage ages and divergence times must be estimated solely by the dates of island emergence and rates of molecular evolution here (Fleischer et al., 1998). Island uplift times provide the maximum age of a given node, as individuals present here could not be any older than the existence of the island (Bess, Catanach, & Johnson, 2014; Drummond, Suchard, Xie, & Rambaut, 2012; Fleischer et al., 1998). Despite these issues, the 'stepping stone' formation of the islands provides a clear system for studying diversification processes. A time calibrated analysis using the age of each of the islands as calculated by K-Ar dating for the maximum ages of well supported nodes should give a confident estimate of the history of a group (Fleischer et al., 1998).

There are several possible evolutionary patterns through which generation of diversity of an endemic could occur on the Hawaiian Islands (Figure 1). They include forward 'stepping stone' colonization (Hennig, 1965; Kimura & Weiss, 1964), back-and-forth colonization, and within island radiation with forward 'stepping stone' colonization. Forward stepping stone involves simple allopatric isolation of lineages between newly emergent and older islands. This pattern has been observed in both birds (Vanderwerf, Young, Yeung, & Carlon, 2010) and moths (Rubinoff, 2008). The second pattern, back-and-forth colonization, involves forward stepping stone processes but also backwards stepping stone processes, in which a lineage re-colonizes an older island. This pattern has been observed in crane flies (Nitta & O'Grady, 2008) and bees (Magnacca & Danforth, 2006). It is also possible that within island adaptive radiation could be combined with a forward stepping stone process, generating additional diversity within each island lineage. This last pattern is notable in that many species appear over a relatively small time scale (Cowie & Holland, 2008). This pattern has been observed in *Drosophila* (Carson & Kaneshiro, 1976).

Bark lice (Insecta: Psocoptera) in the genus *Kilauella* provide a potentially strong model for study of the process of diversification on the Hawaiian Islands. These members of the bark lice family Elipsocidae are an extremely diverse endemic group of the Hawaiian Islands. *Kilauella's* type specimen was described as *Elipsocus erythrostictus* (Perkins, 1899), which was later split off into the genus *Kilauella* with 7 other members (Enderlein, 1913). Described species in the genus are *K. debilis, K. erythrostictus, K. frigida, K. inaequifusca, K. micramaura, K. psylloides* (Perkins, 1899) and *K. vinosa* (McLachlan, 1883). The members of the genus are separated by a wing character from *Elipsocus*, with a unmerged medial vein and radial sector that is connected by a strong cross vein. No other characters are listed in the original description

besides color characteristics of two newly described species (Enderlein, 1913). However, this genus has been estimated to have over 200 species based on museum collections and preliminary morphological examination (Thornton, 1984). Thornton redefined the genus using genital characters in 1990, differentiating the genus from relatives *Elipsocus* and *Palistreptus* by the male phallosome ring and the female subgenital plate, but this work remains unwritten and unpublished (Emilie Bess, personal observation). *Elipsocus* remains the closest known relative of *Kilauella* from molecular studies, but the phylogeny this study is based on only implemented one gene (Yoshizawa & Johnson, 2014).

In addition to the lack of taxonomic work, there are currently no published phylogenetic studies on this large group despite being among the most abundant insect genera on the Hawaiian Islands. *Kilauella* and other bark lice make up a substantial amount of the insect biomass in middle and high elevation forests in Hawaii (Gagne & Howarth, 1981). The ecological diversity of these lice across the island chain is extreme, ranging from dead leaf specialists to bark dwellers, with morphological diversity in pigmentation patterns rivaling that family level diversity of other Psocoptera (K.P. Johnson and E. Bess, personal observation). Collection localities were not precise enough to assign the different specimens used in this study to different niche habitats, but it is known that color pattern correlates with ecological in *Kilauella* and close relative *Palistreptus*. For instance, the common white and black colored morphs are known to primarily reside in tree branches (E. Bess, personal observation; Thornton, 1984). Other common morphs include primarily yellow, pink and brown colored individuals, but their ecological specializations are unknown (P. Gero, personal observation). Although the species involved in each ecological role are currently unknown, *Kilauella* are notable members of Hawaiian trophic

networks as fungus and detritus feeders, as well as prey items for other arthropods and birds due to their abundance (Baldwin, 1953; Thornton, 1984).

Species of *Kilauella* appear to have undergone dramatic radiation across the surveyed Hawaiian Islands. The age of the original island chain colonization is unknown, but these small and winged insects have a high potential for colonization of islands as transported by high altitude eastward wind currents (Gillespie & Roderick, 2002). With this high dispersion potential, we might expect more inter-island diversification, but within island radiation may have played a dramatic role in generation of the ecological diversity discussed above (Cowie & Holland, 2008). Establishment of few colonists with low lineage diversity provides significant opportunity for niche diversification, as seen in silverswords, spiders and leafhoppers (Bennett & O'Grady, 2012; Gillespie, 2004; Purugganan & Robichaux, 2005). With 'stepping stone' and within island radiation, different niche specialists and ecomorphs residing on an island will be more related to one another than to similar specialists from other islands. It is also possible that within island radiation in *Kilauella* has resulted from an increased role of sexual selection due to lower predatory selective pressure on newly colonized islands, resulting in a higher speciation rate. This pattern has been observed in *Drosophila* (Carson & Kaneshiro, 1976). This scenario may be relatively unlikely however, as the unpublished taxonomic work by Thornton indicated high conservation in genital characters relative to body size, coloration, and other ecologically significant characters (E. Bess, personal observation).

MATERIALS AND METHODS

Sample Collection and Extraction

Specimens were collected on the islands of Kauai, Oahu, Molokai, Maui Nui, and Hawaii during collecting trips in 2007 and 2008. At each locality, *Kilauella* were collected in large numbers due to their abundance. Most samples collected were documented with a general locality and a set of GPS coordinates, but at the least the origin island was documented. All information on the specimens collected is listed in Table 1. Specimens were later chosen from these large samples in an effort to maximize the morphological diversity represented, and to evenly represent the 5 islands for the purposes of this biogeographic study. Due to the lack of available sampling locations on Oahu, this island has less representation in this study than the other 4 islands. Each specimen represented here was documented with a photograph taken through a dissecting microscope camera and later identified to an ecomorph category to attempt assignment of ecological roles. Voucher photographs and additional representatives of each morphotype are deposited in the Johnson Lab at the University of Illinois Urbana-Champaign. *Elipsocus*, a bark louse genus with a global distribution, is currently identified as the closest relative to *Kilauella*, and was used as the outgroup in this study (Johnson & Mockford, 2003; Yoshizawa & Johnson, 2014). Extraction of DNA from each sample was performed with a Qiagen DNeasy® Blood and Tissue Kit with a modified procedure for small body size. Samples were ground with a sterile disposable plastic tissue grinder, and tissue lysis was run for 2 days to allow maximum DNA extraction. A total of 77 extractions were performed in addition to the outgroup taxa extractions previously performed by Emilie Bess. After individuals were removed

for sequencing issues later in the project, a total of 96 individuals are present in the analyses for this study.

DNA Amplification and Sequencing

DNA fragments were amplified by polymerase chain reaction (PCR) with the New England BioLabs® Taq 5X Master Mix and Bioline (Meridian Life Science®) MyTaq™ kit. Genes were chosen for the study based on the availability of PCR primers for the lice, and which genes would amplify in trial PCRs. The genes surveyed included COI (cytochrome oxidase c subunit 1), 12S, BR50, Wingless and EF1α (Elongation Factor 1 alpha). These genes are commonly used for studies on lice for their informativeness and ease of amplification (Bess et al., 2014; Yoshizawa & Johnson, 2014). Amplification was exceedingly difficult for many of the extractions for this study, and no PCR bands were obtained for BR50 or Wingless across a test of 15 extractions. These genes were abandoned. Segments of COI, 12S and EF1α were amplified across the included 77 extractions, creating a data set of two mitochondrial genes and one nuclear gene. Primers used for each gene segment are listed in Table 2. PCR product was purified with Affymetrix ExoSAP-IT®, and sequencing reactions were performed with Life Technologies BigDye® Terminator v3.1 Cycle Sequencing Kit. Sequences were generated at the University of Illinois Urbana-Champaign High-Throughput Sequencing and Genotyping Unit on Applied Biosystems 3730xl DNA Analyzers. Final gapless gene lengths were 446 base pairs of COI, 419 base pairs of 12S and 693 base pairs of EF1α. Approximately 10 individuals in each gene alignment had a missing sequence due to alignment difficulties. Any taxa missing more than two thirds of the data set were removed from all final analyses for the project.

Sequence Alignment and Phylogenetic Analysis

Sequences were visualized, trimmed, edited and aligned in Geneious version R7 and R8 (Kearse et al., 2012). Alignments were optimized by eye, especially for EF1α, due to highly divergent areas in the gene. Sequences were checked against annotated phthirapteran louse genomes to confirm that all sequence was coding exon, and submitted to BLAST to verify sequence identity (K. Johnson Lab, unpublished data). Model testing for each gene was run in jModelTest2 (Darriba, Taboada, Doallo, & Posada, 2012; Guindon & Gascuel, 2003). Highest likelihood models were determined as GTR + I + G for both COI and EF1a, and GTR + G for 12S. The number of parsimony informative sites for each gene partition, and average pairwise divergences for COI (Table 3) were calculated in MEGA 6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) in order to gauge how informative the sequence data is. 142 parsimony informative sites (PIS) were found in COI, 89 PIS in 12S and 146 PIS in EF1α.

Due to a large degree of alignment uncertainty, the program Bali-Phy was implemented in order to co-estimate each individual gene alignment simultaneously with the phylogeny (Redelings & Suchard, 2005; Suchard & Redelings, 2006). The COI alignment was left fixed, because this alignment had less variability and was easily aligned by eye. 12S and EF1a alignments were modeled for indels by Bali-Phy and allowed to vary. It has been shown that using this approach to tree estimation reduces errors due to alignment ambiguity, and may be more accurate than other Bayesian approaches like MrBayes (Redelings, 2014). With two uncertain gene alignments, this approach seemed likely to increase confidence of phylogenetic estimation for downstream analyses. Four chains of approximately 7,000 iterations were run, and confirmed to have converged by the Bali-Phy post processing script statreport (Suchard & Redelings, 2006) and the BEAST program Tracer v1.6 (Rambaut, Suchard, Xie, & Drummond,

2014). The estimated alignments were confirmed based on resolution and support of the resulting Bali-Phy phylogeny, and then used for additional maximum likelihood and Bayesian analyses. Garli 2.0 (Zwickl, 2006) was used to generate maximum likelihood phylogenetic trees, and MrBayes 3.2.5 (Ronquist & Huelsenbeck, 2003) was used to generate Bayesian phylogenetic trees following the same model parameters discussed above. Individual maximum likelihood and Bayesian analyses were run for each gene partition in order to confirm that there were no topology conflicts. After comparing the 6 resulting phylogenetic trees and confirming that no conflicts existed between the partitions, the three genes were combined into one alignment for concatenated analyses. A maximum likelihood phylogeny with 100 bootstraps generated in Garli and a converged Bayesian phylogeny generated in MrBayes and verified in Tracer were combined with the Bali-Phy topology. This final combined phylogeny is shown in Figure 2, with support values annotated on the tree as Bali-Phy / MrBayes / Garli. The phylogeny is divided into 'groups' A, B and C for the ease of discussion.

Using the alignments and phylogeny generated by the above methods, a series of dated phylogenetic trees were generated in BEAST v1.8.2 (Drummond et al., 2012). Several different combinations of calibration nodes were used to compare the effects on the node dates across the tree. The differences between these analyses are discussed below. Due to the lack of resolution for most multiple island relationships in the phylogeny presented in Figure 2, the calibration of the final dated analysis was kept highly conservative. A total of three different nodes were dated for the analysis by identification of monophyletic groups that clearly represented a colonization from the Maui Nui complex to Hawaii (Bess et al., 2014; Fleischer et al., 1998). These monophyletic clades were found in Bali-Phy, MrBayes and Garli analyses of the data set shown in Figure 2. Resolution problems in older island relationships made it difficult to determine the

polarity of colonization events. For instance, one analysis used 'group' A from Figure 2 as a calibration node, because this clade may represent a radiation from the island of Kauai through the other 4 islands. However, it cannot be conclusively determined that it is not the case that this is a back colonization from the younger to the older islands because of the lack of structure in the clade (Bellemain & Ricklefs, 2008; Johnson et al., 2000). Thus, only potential Maui-Nui to Hawaii colonization nodes were considered for the final analysis. Due to the relatively recent age of the uplift of the island of Hawaii, it is unlikely that these nodes would represent a back colonization from Hawaii to Maui Nui. Hence, these nodes were calibrated as having a maximum age of the uplift of the island of Hawaii at 0.43 mya.

Two separate analyses were run in BEAST for each set of tested calibrations, with standard deviations of calibrated node ages set at 0.1 and 0.01. All analyses were run for 30 million generations each and sampled every 1,000 generations (Bess et al., 2014). Clock rates were set following Tajima's Rate Tests in MEGA, with strict clocks set for COI and 12S partition and a relaxed clock set for the EF1a partition (Tajima, 1993). All resulting distributions were analyzed in Tracer v1.6 (Rambaut et al., 2014) to ensure proper convergence. The resulting 30,000 tree sets were summarized in TreeAnnotator v1.8.2. The final dated phylogeny is shown in Figure 3.

RESULTS

Phylogenetic Analysis

Our phylogenetic analysis shows a potentially complex history of evolution of these bark lice across the Hawaiian Islands, with several possible independent lineages. No matching sequences (confirmed in genetic distance tests for Table 3) across all sampled individuals and genes were found, so this study may represent up to 95 distinct species of *Kilauella*. However, species limits would need to be evaluated by an extensive morphological revision as well as dense genetic sampling to make this conclusion.

Rather than exemplifying one of the possible evolutionary trajectories identified in Figure 1, there is marginal evidence for each possible hypothesis in our data. Maximum likelihood and Bayesian analyses using the gene alignments produced by estimation in Bali-Phy did not show any topological conflicts, but had highly variable levels of resolution. Maximum likelihood analysis does not seem to handle this data set well, and showed the lowest support levels. Little structure exists in the backbone of the phylogeny in phylogenetic analyses. Both Bayesian approaches offered moderate to high support throughout the phylogeny. Disparity between the Bali-Phy support values and the other methods most likely exist because Bali-Phy models the indels and incorporates probabilities for each base in the alignments. As discussed previously, the Bali-Phy support values may be more reliable in this case (Redelings, 2014). Despite support differences, tip level relationships in each topology remained consistent throughout all analyses performed, and most were highly supported across the three different methods. With some extraneous examples, taxa on each island appear to group together with high confidence within their respective clades.

Evidence for several independent lineages of *Kilauella* is present in the phylogeny shown in Figure 2. Three different 'groups' of taxa, designated by letters A, B and C, are labeled for ease of discussion. Group A is the largest monophyletic group in the phylogeny with high support levels. It includes all five islands, with a high support Kauai group sister to a large polytomy comprising species from the other four islands. Additional Kauai taxa group with the highly supported individuals with much weaker support, but still appear to be closely related. It is likely there is not sufficient signal to resolve the exact relationships here. Many small groups within this clade have high support across all three methods. As mentioned before, islands appear to group together well within their respective 'groups' or clades. Two of these nodes, with sister Hawaii and Maui Nui individuals, were used as calibration points for the tested calibrations and the final highly conservative BEAST analysis presented in Figure 3 (Drummond et al., 2012). 'Group' A could possibly represent a forward colonization event down the island chain, but the direction of this colonization cannot be determined with this level of taxon sampling (Bellemain & Ricklefs, 2008; Johnson et al., 2000). As discussed above, it is difficult to determine if this was a forward colonization or a reverse colonization without well supported phylogenetic structure.

'Group' B has the poorest backbone support in the entire phylogeny, and is essentially a large polytomy. Analysis in Bali-Phy indicated some supported deeper structure, but this is not supported by the other methods. The majority of the variability in support values and topology occurred here when different analysis methods were compared, with maximum likelihood analyses struggling the most to find structure. For instance, a group of 3 Maui Nui individuals moves around within this 'group' because of low support. Without these three individuals, 'group' B appears monophyletic in the BEAST analysis presented in Figure 3. It is likely that

these individuals and this 'group' would be much more resolved with more information, either by sequence or taxon sampling. Despite these problems, shallow and tip level relationships are highly supported, similar to 'group' A. Islands group together with relatively high confidence, most notably the large Molokai / Maui Nui group and the several Hawaiian groups. An additional terminal node with sister Maui Nui and Hawaii individuals was used as a calibration point in this group. This node was used in all calibration tests, including the final highly conservative set of nodes presented in Figure 3.

'Group' C contains several highly supported tip groups, but does not have a supported backbone, similar to 'group' B. Unlike 'group' B however, C is consistently derived across all analyses and partitions. This group contains short tip branches on long deep branches and represents all five islands in the chain. This 'group' may represent a poorly sampled lineage of *Kilauella*. Similar to the other 'groups', it is likely that these relationships would become clearer with additional sequence data or sampling.

Dated Phylogeny

The final highly conservative dated phylogeny was generated in BEAST (Drummond et al., 2012) using three calibration points identified in phylogenetic analyses discussed above. These nodes represent terminal sister pairings of Hawaii and Maui Nui. These nodes are shown highlighted in yellow on Figure 2 and the resulting Figure 3. As discussed in the Methods section, the three clades set as calibration points are the most likely candidates for a colonization event from the island of Maui Nui to the island of Hawaii, and were dated at the age of the island of Hawaii at 0.43 mya (Bellemain & Ricklefs, 2008; Bess et al., 2014; Carson & Clague, 1995; Fleischer et al., 1998; Johnson et al., 2000). Only these points were used for calibration in order

to minimize the assumptions of assigning calibration points.. Due to the lack of high support and taxon sampling, it is difficult to establish the direction of colonization for the relationships among older islands. Support value problems are similar in the BEAST analysis and phylogenetic analyses presented in Figure 2. The support levels are shown in Figure 3 as a color gradient on the branches in order to highlight each of the node ages. For the analysis presented in Figure 3, the islands of Maui Nui and Molokai are undifferentiated. Previous studies on bark lice have shown that taxa on these islands behave as a single unit, since these islands were connected by a land bridge until only 0.2 mya (Bess et al., 2014). It would likely be difficult to differentiate them in a dating analysis.

The highly conservative dated analysis shows an origin of the *Kilauella* genus in Hawaii at approximately 7.84 mya (95% confidence interval 11.21 to 5.27 mya), and a split from the sister genus *Elipsocus* at approximately 12.97 mya. This establishment approximately coincides with the uplift of the island of Nihoa, the youngest island in the northwestern Hawaiian island chain, dated at 7.2 million years (Price & Clague, 2002). Analysis including less conservative data points indicated a much older age for *Kilauella*, showing the origin at approximately 23 mya (95% confidence interval 30.5 to 17.43 mya). This analysis implemented 'group' A as a major calibration point, in which Kauai is sister to the major group of the other four islands. The node was set at a maximum age of the island of Kauai, 5.1 mya. However, as discussed before, this calibration is likely not reliable because the direction of colonization cannot be readily established. The conservative analysis dates this node at 3.16 mya (95% confidence interval 4.5 to 2.08 mya), a full 2 million years younger than the less conservative calibration. This possible origin age approximately coincides with the origin of Lanyan island at 20.7 mya, but its wide 95% confidence interval covers a range of old northwestern islands. Other analyses with minor

tip calibration changes found origins in between this range of values, but the highly conservative calibration indicated the youngest age of 7.84 million years.

These dates may represent the range of possibilities for the establishment of the *Kilauella* most recent common ancestor (MRCA) on Hawaii, and diversification occurred from there forward down the island chain. Analysis with a complete taxon set would be required for a strong origin conclusion, as the importance of sampling potential origin sites has been noted for accuracy of determining the origin point of a given group (Cowie & Holland, 2008). Although it is likely that *Kilauella* represents a single origin on the Hawaiian Islands based on analyses with additional outgroups (these were not included in final results due to branch length issues), higher sampling would be necessary to infer that this genus is not composed of distinct monophyletic lineages (Cowie & Holland, 2008). *Kilauella* appears to be monophyletic based on its endemism on Hawaii and this study, and thus may represent a single point origin, but this conclusion would require sampling on the old northwestern Hawaiian Islands.

From the establishment of *Kilauella* on the Hawaiian chain, there appears to have been multiple independent radiations. Major splits appear to occur on the older islands of Kauai and Oahu (with dates in the range of 6.18 to 3.03 mya) with modern ancestors appearing on all five islands in the major clades. The oldest two major splits are dated at 6.18 mya (95% confidence interval 9.28 to 3.81 mya), which represents the MRCA of 'group C' and 5.58 mya (95% confidence interval 7.98 to 3.82 mya), which represents the MRCA of 'groups' A and B. Groups A and B become monophyletic lineages in this analysis. With the exception of a branch containing three individuals from Maui Nui, denoted in Figure 3 by a dotted line, 'groups' A and B are both monophyletic clades. This Maui Nui group was noted earlier for changing positions slightly in different analyses due to low support, so it is not certain where it belongs. Without

this branch, the origin of clade A is dated at 3.16 mya (95% confidence interval 4.5 to 2.08 mya) and the origin of clade B is dated at 3.41 mya (95% confidence interval 4.77 to 2.39 mya). Both of these dates and ranges coincide with the uplift of the island of Oahu at 3.7 mya.

Within monophyletic 'group' A, major branch splits are dated between 1.92 and 0.58 mya. This 'group' contains the majority of the Maui Nui complex and Hawaii individuals present in the study. The structure here is weakly supported and, as seen in Figure 2, is essentially a large polytomy. Note that the terminal nodes are highly supported (mostly blue), similar to the phylogeny in Figure 2. The large group of Kauai individuals remains sister to this radiation, and also remains split with moderate support. After its origin around the uplift of Oahu at approximately 3.16 mya, 'group' A appears to have diversified heavily on the complex of Maui Nui during its uplift between 1.9 and 1.6 mya. Note that two of the calibration nodes occur in this clade, highlighted in yellow.

'Group' B, also monophyletic in this analysis, has major branch splits dated between 3.03 and 1.54 mya. This is significantly older than the splits present in 'group' A, and covers a range prior to the uplift of the Maui Nui complex. These splits have low support, as expected from the branch supports in 'group' B in Figure 2. It is unlikely that many of these represent actual evolutionary divergence, so the significantly red branches will not be considered. It is difficult to conclude where diversification of this lineage occurred with this data. However, if splits were reinforced with additional data, this clade must have diversified mostly on the older islands of Kauai and Oahu. These split times are all dated before the uplift of the Maui Nui complex at 1.9 mya, with the exception of three shallow nodes dated at 1.83, 1.63 and 1.54 mya. These nodes may represent splits when colonization of new islands occurred, as the terminal individuals are all present on the islands of Maui Nui and Hawaii.

The structure of ‘group’ C is identical to the phylogeny in Figure 2, with few taxa on long branches that have high support at the tips. Although the origin of this ‘group’ has a wide 95% confidence interval, it appears to be older than the ‘new’ islands examined in this study. In all tested calibrations, the lineage is dated before the emergence of Kauai at 5.1 mya. This clade may represent an older and poorly sampled lineage. The longer branches diverge at nodes with recent dates of 1.68 mya (95% confidence interval 2.55 to 1.02 mya) and 0.44 mya (95% confidence interval 1.57 to 0.02 mya). These nodes provide evidence for recent diversification with the uplift of the Maui Nui complex and the island of Hawaii, both represented in this clade. Additional sampling may provide more evidence for the history of this lineage, similar to ‘group’ B.

DISCUSSION

Multiple Radiations throughout History

Our evidence shows that there may be a complex evolutionary history of the *Kilauella* complex on the Hawaiian Islands. There is no clear evidence for the three hypothetical evolutionary patterns shown in Figure 1. However, there is evidence for forward colonization from the oldest to youngest islands, along with recent within island radiation on each island. Although support levels are not high enough to provide the structure necessary to identify a forward 'stepping stone' model of diversification, all five islands are present in each of the major clades identified as A, B and C. In nearly all cases, the older islands of Kauai and Oahu are dated at older splits than the younger islands of Maui Nui and Hawaii. This relationship is prevalent in 'group' A, and is weakly supported in 'group' B. Evidence for within island radiation is notable in the large polytomy present in 'group' A in both Figure 2 and Figure 3, which show evidence for a large and recent expansion of taxa on the islands of Maui Nui and Hawaii. It may be the case that this radiation is too recent to derive the exact structure in the case of 'group' A, and this is why the clade presents as a large polytomy of individuals from both Maui Nui and Hawaii.

With this evidence, it is possible that each of the major clades identified potentially spread from the older islands and radiated throughout the younger four islands, and the data suggests that within island radiation may be a factor, especially in the newest and largest islands. This sort of forward 'stepping stone' with within island radiation scenario, as shown in Figure 1, is more probable for small winged insects like *Kilauella*, which could easily traverse the island chain on the dominant westerly winds in the Pacific (Gillespie & Roderick, 2002). A forward 'stepping stone' with within island radiation pattern was first demonstrated in similarly small and

winged drosophilid flies (Carson & Kaneshiro, 1976), and has been frequently found in other Hawaiian lineages (Wagner & Funk, 1995). With our unresolved phylogeny, there are many potential colonization routes for the patterns observed in the phylogeny in Figure 2. These potential routes are interpreted in Figure 4 (Holland & Hadfield, 2004)

Kilauella on Maui Nui and Hawaii seem to have radiated relatively recently, as evidenced by the short branch lengths for the majority of individuals on these islands in Figure 2, and the recent dates on nodes in Figure 3. Given the large land area and rapid uplift of the Maui Nui complex followed by the main island of Hawaii, this may be a result of diversification into a wide array of different niche habitats. Morphtypes of extracted specimens were examined in order to add evidence to this hypothesis, but the morphotypes appear to be completely randomized on the phylogeny in Figure 2. An in depth examination of the morphological diversity in *Kilauella* would be necessary to add evidence to this hypothesis, especially with examination of Thornton's unpublished work on species in *Kilauella*. However, the habitat diversity hypothesis suggests that increased diversification on the new large islands may be warranted given the size and ecological diversity of the two islands, two traits that are often correlated (Gillespie & Roderick, 2002; Whittaker, 1998). There is a comparative lack of available habitat and collecting localities on the older island of Oahu relative to Maui Nui and Hawaii, part of why Oahu is the lowest represented island in this study (K. Johnson, personal observation).

Confidence of Phylogenetic Reconstruction

Although the topology in Figure 2 and Figure 3 shows evidence for a forward 'stepping stone' and within island radiation across the Hawaiian Islands, several factors have been noted in

other island biogeography studies that could significantly bias the final results. Extinction is likely a problem in reconstruction of older lineages of *Kilauella* and other taxa, such as the long branches present in 'group' C with few terminal taxa (Cowie & Holland, 2008). This could account for the poor support seen in the backbone of Figure 2, especially considering that *Kilauella* may represent a single origin with highly rapid rates of diversification such as those seen in 'group' A. The restricted distribution necessitated by the relatively tiny landmass of Hawaiian Islands implies that population sizes are similarly restricted, and this likely leads to higher extinction rates. The rapid erosion of older islands that necessitates movement of species to new islands could additionally be a source of extinction. A pattern of old lineage extinction has been observed in the Hawaiian land snail *Succinea caudua* that contributed to difficulty of phylogenetic reconstruction (Holland & Cowie, 2009). Higher extinction rates have been demonstrated in comparisons of larger north Atlantic islands and smaller Pacific islands such as the Hawaiian Islands (Sadler, 1999).

Resolution problems in the phylogeny may also be due to the relatively poor documented taxonomy of the species of *Kilauella*. With the unpublished descriptions of *Kilauella* species written by Thornton numbering nearly 200, and this study representing less than 100 individuals, there are certainly taxon sampling problems here. This problem, in conjunction with the possibility of old lineage extinction described above, could explain the poor resolution for 'group' B and the long branches in 'group' C. It is currently impossible to determine the influence of missing taxa in this study due to the lack of availability of Thornton's descriptions, and the lack of characters in Enderlein's original description of genus *Kilauella*. However, the morphological diversity appears to be high on examination of voucher photographs and the

genetic diversity also appears to be high due to the lack of matching sequences throughout the data set.

The robustness of genetic and phylogenetic signal may also bias the topology in Figure 2 and 3 and the resulting conclusions. Although two mitochondrial markers and one nuclear marker are employed, it is clear that the phylogenetic signal is relatively weak at deep levels due to the low support values. The backbone of our phylogeny in Figure 2 is weakly supported at best in several cases. The analysis in Bali-Phy seems to help compensate for alignment and phylogenetic signal problems by it's method of modeling indels, and shows higher support than MrBayes and Garli at most nodes. In previous Hawaiian studies, such as those on *Laupala* crickets (Shaw, 2002), informativeness of markers used has been problematic in generating strong conclusions on speciation processes such as what is attempted here (Cowie & Holland, 2008; Rubinoff & Holland, 2005). However, our data shows approximately a quarter of the sites in each gene alignment are parsimony informative sites, as discussed above. There also seem to be relatively high sequence distances between clades as shown in Table 3. It is more likely that the noise obstructing the phylogenetic structure is due to extinction and significant numbers of missing taxa as discussed above. Additionally, phylogenetic noise may be present due to the amplification difficulties discussed in the Methods section. This resulted in a final alignment with some missing data that may also influence support values, but any taxa with less than two-thirds of the data set were removed from the final analysis, and missing data was modeled in Bali-Phy analyses.

FIGURES AND TABLES

Figure 1: Maps and Diversification Scenarios for *Kilauella*. A map of the Hawaiian Island chain is shown with the established K-Ar ages of uplift of each island. Three idealized models, with corresponding colors, are shown to hypothesize how *Kilauella* may have diversified across the island chain.

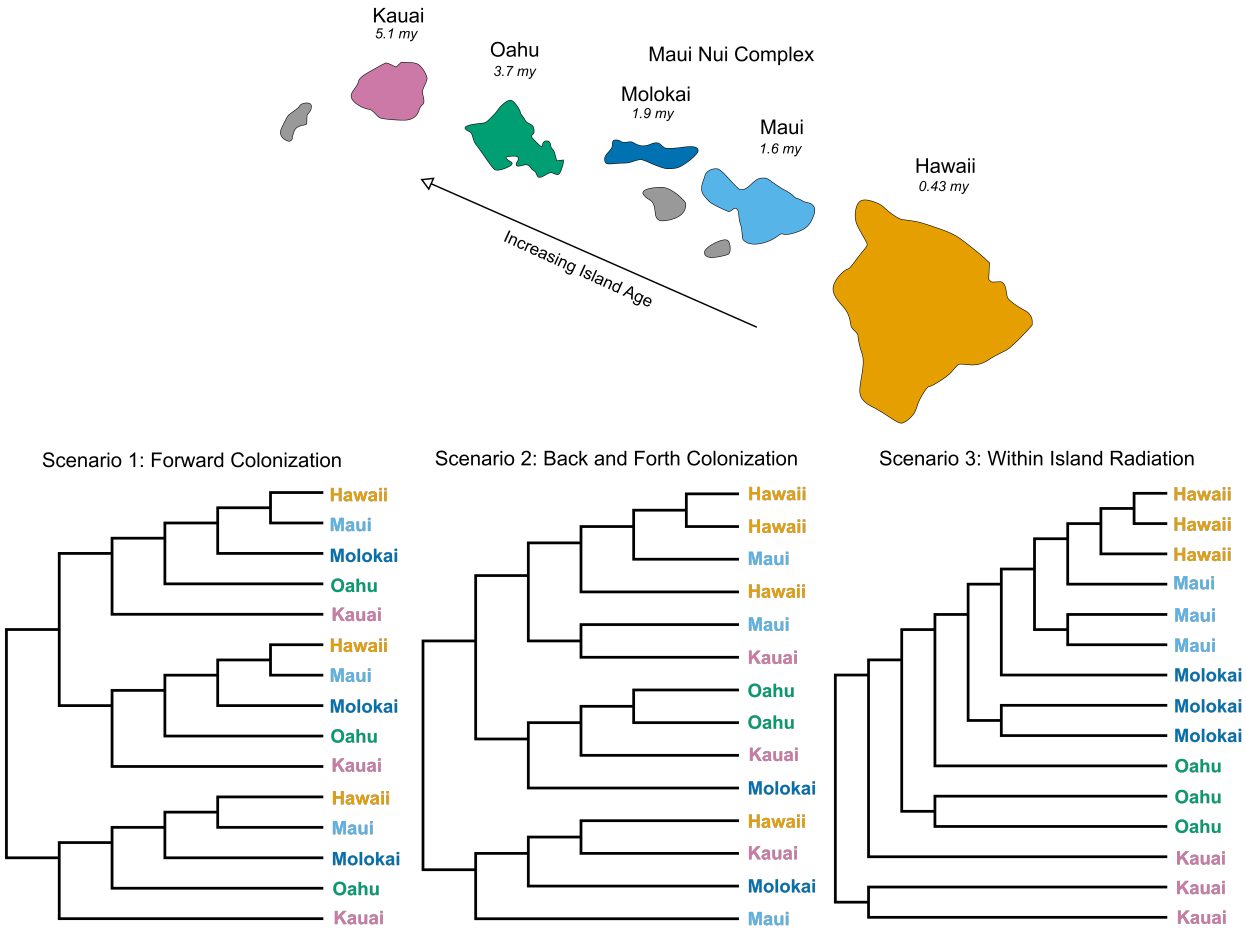

Table 1: Collection information and distribution of the *Kilauella* specimens used in this study. The specimens are ordered by the sequence in which they were extracted. If previously extracted by Emilie Bess for the original proposal for this project, the specimen is denoted with an 'E'. All available locality information is included, including islands collected on and GPS coordinates if they were available.

Ex #	Locality Information	GPS Coordinates	Date Collected
1	Molokai, Kamakou Preserve	21.1184N, 156.9027W	21-Jul-08
2	Molokai, Kamakou Preserve	21.11818N, 156.90814W	22-Jul-08
3	Molokai, Molokai Forest Preserve	21.13067N, 156.92191W	20-Jul-08
4	Molokai, Kapu Ranch	Unknown	23-Jul-08
5	Molokai, Kamakou Preserve	21 07.130"N, 156 56.126"W	24-Jul-08
6	Maui, Haleakala National Park	20.7557N, 156.2227W	30-Mar-08
7	Unknown	Unknown	Unknown
8	Maui, Haleakala National Park	20.7596N, 156.2307W	30-Mar-08
9	Molokai, Molokai Forest Preserve	21.13300N, 156.93242W	20-Jul-08
10	Molokai, Kamakou Preserve	21 06.922"N, 156 56.122"W	24-Jul-08
11	Hawaii, Hawaii Volcanoes N. Park	19.36524N, 155.21608W	7-Aug-08
12	Hawaii, Hawaii Volcanoes N. Park	19.49215N, 155.38599W	9-Aug-08
13	Hawaii, Honomolino Preserve	19.20613N, 155.82213W	8-Aug-08
14	Hawaii, Honomolino Preserve	19.21520N, 155.77654W	8-Aug-08
15	Hawaii, Kahilipali Preserve	19.10392N, 155.62238W	8-Aug-08
16	Hawaii, Mauna Kea Forest Reserve	19.80861N, 155.39743W	4-Aug-08
17	Hawaii, Mauna Kea Forest Reserve	19.80861N, 155.39743W	4-Aug-08
18	Hawaii, Kohala Mt. Rd.	20.04793N, 155.73683W	5-Aug-08
19	Hawaii, Makaula-Ooma Mauka Tract	19.72203N, 155.94734W	3-Aug-08
20	Hawaii, Kona Region	19.70770N, 155.92415W	3-Aug-08
21	Hawaii, Pu'u O Umi	20.07374N, 155.72264W	5-Aug.08
22	Hawaii, Honomolino Preserve	19.20613N, 155.82213W	8-Aug-08
23	Oahu, Koolau Mountains	21.31500N, 157.74301W	2-Aug-08
24	Oahu, Honouliuli Preserve	21.41117N, 158.09944W	15-Jul-08
25	Oahu, Koolau Mountains	21.31997N, 157.74257W	2-Aug-08
26	Oahu, Koolau Mountains	21 18'58"N, 157 44'39"W	2-Aug-08
27	Oahu, Ka'ala	21.50649N, 158.14442W	27-Jul-08
28	Oahu, Keaiwa	21.40822N, 157.87662W	16-Jul-08
29	Oahu, Honouliuli Preserve	Unknown	17-Jul-08
30	Oahu, Honouliuli Preserve	21.41117N, 158.09944W	15-Jul-08
31	Kauai, Hulea River Valley	21.9353N, 159.40053W	12-Apr-08
32	Kauai, Kalalau Valley	22.15539N, 159.64973W	10-Apr-08
33	Maui, Ulupalakua Ranch	20.65224N, 156.35519W	20-Jan-07
34	Hawaii, Kolaoa	19.70761N, 155.92398W	3-Jan-07
35	Kauai, Walmea Canyon	22.05137N, 159.66002W	7-Apr-08
36	Maui, Waihee Ridge Trail	20.94956N, 156.53618W	31-Mar-08
37	Hawaii, Saddle Road	19.67562N, 155.37579W	10-Jan-07
38	Maui, Haleakala National Park	20.7726N, 156.23627W	23-Mar-08
39	Kauai, Kalalau Valley	22.15539N, 159.64973W	10-Apr-08

Table 1 (continued)

40	Maui, Ulupalakua Ranch	20.65224N, 156.35519W	20-Jan-07
41	Hawaii, Kolaoa	19.70761N, 155.92398W	3-Jan-07
42	Kauai, Walmea Canyon	22.05137N, 129.66002W	7-Apr-08
43	Maui, Waihee Ridge Trail	20.94956N, 156.53618W	31-Mar-08
44	Hawaii, Hawaii Volcanoes N. Park	19.43796N, 155.30083W	5-Jan-07
45	Kauai, Koke'e SP, Kalalau Valley	22.15539N, 159.64973W	10-Apr-08
46	Hawaii, Kaloko Drive	19.70761N, 155.92398W	3-Jan-07
47	Hawaii, Saddle Road	19.67562N, 155.37579W	10-Jan-07
48	Hawaii, Hawaii Volcanoes N. Park	19.43798N, 155.30083W	5-Jan-07
49	Maui, Haleakala National Park	20.7726N, 156.23627W	23-Mar-08
50	Oahu, Nahuina Trail	21.32935N, 157.82326W	18-Mar-08
51	Maui, Haleakala National Park	20.7721N, 156.23566W	23-Mar-08
52	Oahu, Nahuina Trail	21.32935N, 157.82326W	18-Mar-08
53	Maui, Haleakala National Park	20.7721N, 156.23566W	23-Mar-08
54	Oahu, Pu'u Ualaka'a State Park	21.31527N, 157.82045W	18-Mar-08
55	Oahu, Nahuina Trail	21.32935N, 157.82326W	18-Mar-08
56	Maui, Haleakala National Park	20.7721N, 156.23566W	23-Mar-08
57	Maui, Haleakala National Park	20.7721N, 156.23566W	23-Mar-08
58	Molokai, Kamakou Preserve	21.1184N, 156.9027W	21-Jul-08
59	Molokai, Kamakou Preserve	21.11818N, 156.90814W	22-Jul-08
60	Molokai, Molokai Forest Preserve	21.13067N, 156.92191W	20-Jul-08
61	Molokai, Kapu Ranch	Unknown	23-Jul-08
62	Molokai, Kamakou Preserve	21 07.130"N, 156 56.126"W	24-Jul-08
63	Maui, Haleakala National Park	20.7557N, 156.2227W	30-Mar-08
64	Unknown	Unknown	Unknown
65	Maui, Haleakala National Park	20.7596N, 156.2307W	30-Mar-08
66	Molokai, Molokai Forest Preserve	21.13300N, 156.93242W	20-Jul-08
67	Molokai, Kamakou Preserve	21 06.922"N, 156 56.122"W	24-Jul-08
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69	Hawaii, Hawaii Volcanoes N. Park	19.49215N, 155.38599W	9-Aug-08
70	Hawaii, Honomolino Preserve	19.20613N, 155.82213W	8-Aug-08
71	Hawaii, Honomolino Preserve	19.21520N, 155.77654W	8-Aug-08
72	Hawaii, Kahilipali Preserve	19.10392N, 155.62238W	8-Aug-08
73	Hawaii, Mauna Kea Forest Reserve	19.80861N, 155.39743W	4-Aug-08
74	Hawaii, Mauna Kea Forest Reserve	19.80861N, 155.39743W	4-Aug-08
75	Hawaii, Kohala Mt. Rd.	20.04793N, 155.73683W	5-Aug-08
76	Hawaii, Makaula-Ooma Mauka Tract	19.72203N, 155.94734W	3-Aug-08
77	Hawaii, Kona Region	19.70770N, 155.92415W	3-Aug-08
E1	Kauai	Unknown	Unknown
E2	Kauai	Unknown	Unknown
E3	Hawaii	Unknown	Unknown
E4	Maui Nui	Unknown	Unknown
E5	Oahu	Unknown	Unknown
E6	Maui Nui	Unknown	Unknown
E7	Hawaii	Unknown	Unknown
E8	Hawaii	Unknown	Unknown

Table 1 (continued)

E9	Kauai	Unknown	Unknown
E10	Oahu	Unknown	Unknown
E11	Kauai	Unknown	Unknown
E12	Kauai	Unknown	Unknown
E13	Hawaii	Unknown	Unknown
E14	Maui Nui	Unknown	Unknown
E15	Maui Nui	Unknown	Unknown
E16	Kauai	Unknown	Unknown
E17	Kauai	Unknown	Unknown
E18	Oahu	Unknown	Unknown
E19	Kauai	Unknown	Unknown
E20	Kauai	Unknown	Unknown
E21	Kauai	Unknown	Unknown
E22	Kauai	Unknown	Unknown
E23	Kauai	Unknown	Unknown
E24	Hawaii	Unknown	Unknown
E25	Oahu	Unknown	Unknown
E26	Hawaii	Unknown	Unknown
E27	Maui Nui	Unknown	Unknown
E28	Oahu	Unknown	Unknown
E29	Hawaii	Unknown	Unknown
E30	Maui Nui	Unknown	Unknown
E31	Hawaii	Unknown	Unknown
E32	Maui Nui	Unknown	Unknown
E33	Maui Nui	Unknown	Unknown
E34	Maui Nui	Unknown	Unknown

Table 2: A list of primers used to compile the data set in this study. Primer names are listed along with the gene that they amplify in a PCR reaction, and are followed by the unique primer base sequence and the original citation.

Gene	Primer	Sequence (5' to 3')	Reference
COI	L6625	CCGGATCCTTYTGRTTYTTYGGNCAYCC	(Hafner et al., 1994)
	H7005	CCGGATCCACNACRTARTANGTRTCRTG	(Hafner et al., 1994)
12S	12Sai	AAACTAGGATTAGATACCCTATTAT	(Simon et al., 1994)
	12Sbi	AAGAGCGACGGGCGATGTGT	(Simon et al., 1994)
EF1α	EF1-For3	GGNGACAAYGTTGGYTTCAACG	(Danforth & Ji, 1998)
	Cho10	ACRGCVACKGTYTGHCKCATGTC	(Danforth & Ji, 1998)

Table 3: Sequence divergences as Kimura-2-parameter values as percentages. These were calculated in MEGA 6. Specimens are grouped together by island and compared against one another, as well as with islands, to check how informative the data is in this project.

	All Taxa	**Hawaii**	**Maui Nui**	**Molokai**	**Oahu**	**Kauai**
Within Group Mean Divergence	13%	10.39%	12.70%	10.46%	12.28%	13.39%
Within Group Max Divergence	23.7%	19.85%	21%	17%	19%	20%
Between Group Mean Divergence		12%	12.9%	12%	13.1%	14.6%

Figure 2: Phylogenetic Tree. This tree is composed of a Bali-Phy topology with Bali-Phy posterior probability / MrBayes posterior probability / Garli bootstrap support values. ‘Groups’ A, B and C are designated for the ease of discussion. This ‘groups’ become monophyletic in later analyses. All nodes used for time calibrations for BEAST dated analyses are highlighted in yellow circles.

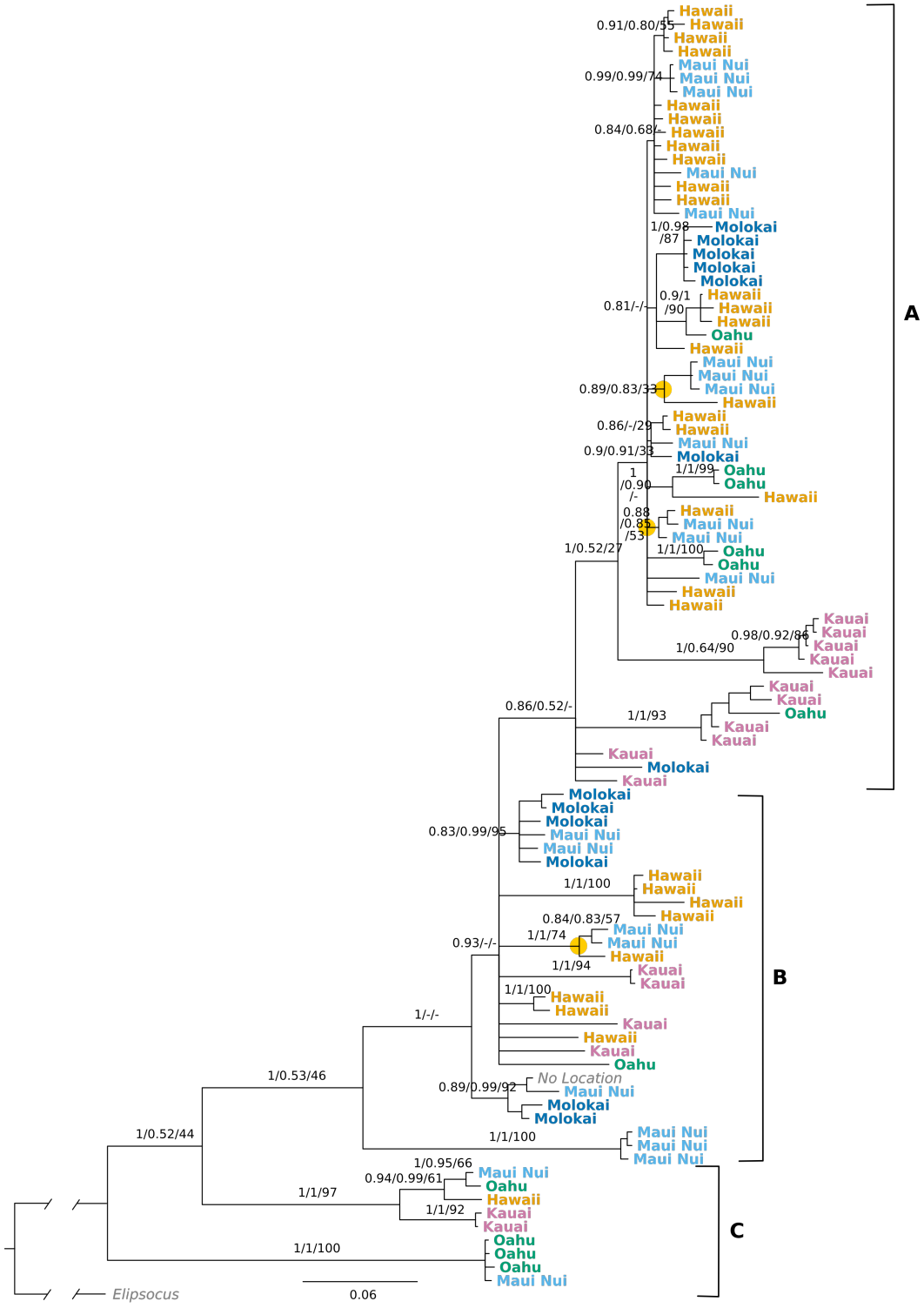


Figure 3: BEAST dated phylogenetic tree. This phylogeny was generated in BEAST set at 30 million generations, sampling every 1000 generations. Calibrated nodes were set at the date of the uplift of the island of Hawaii at 0.43 mya with a lognormal distribution and a standard deviation of 0.01. The color of the branches indicates the level of support, with blue being the highest and red being the lowest support. 'Groups' A, B and C match with those of Figure 2.

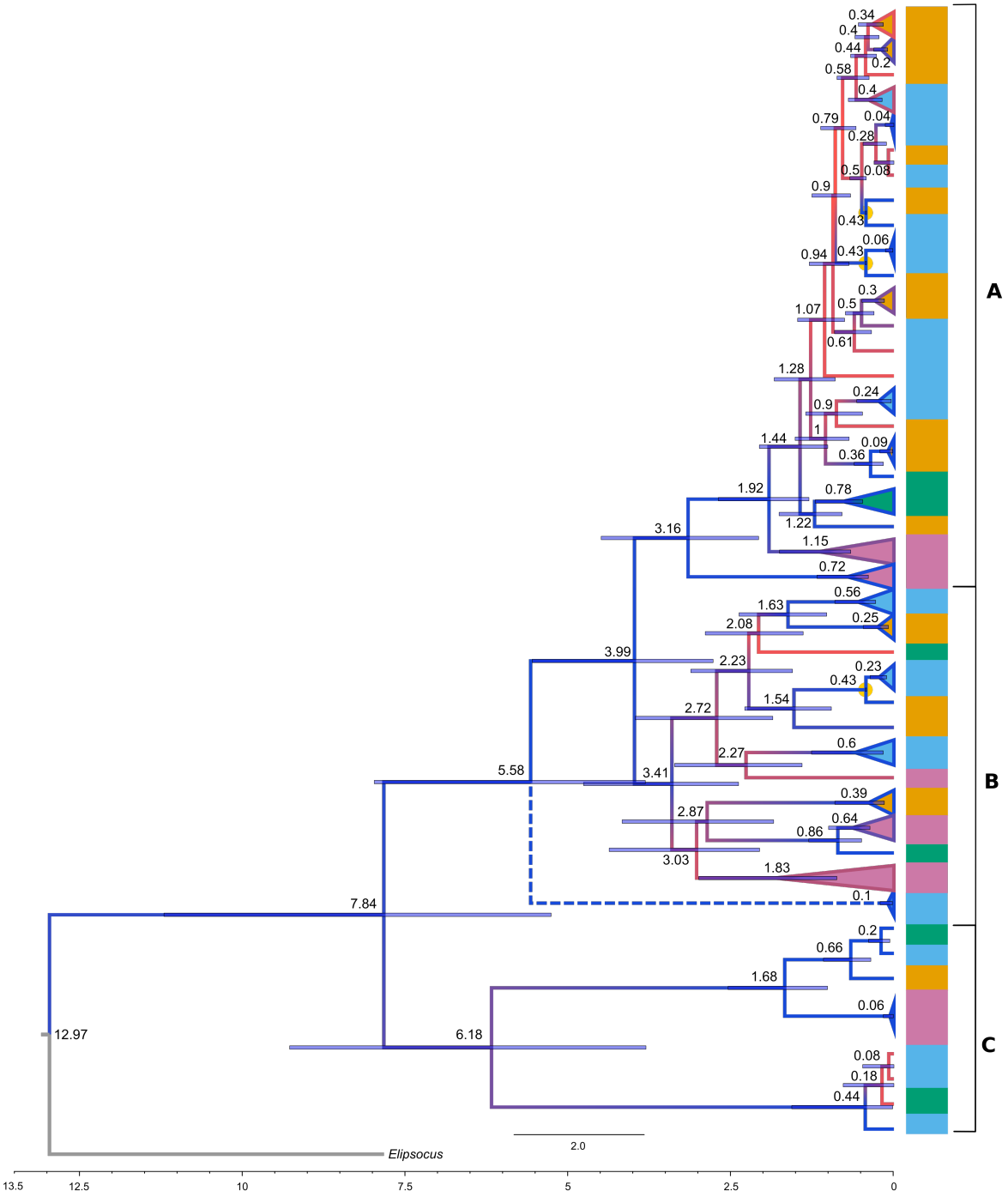
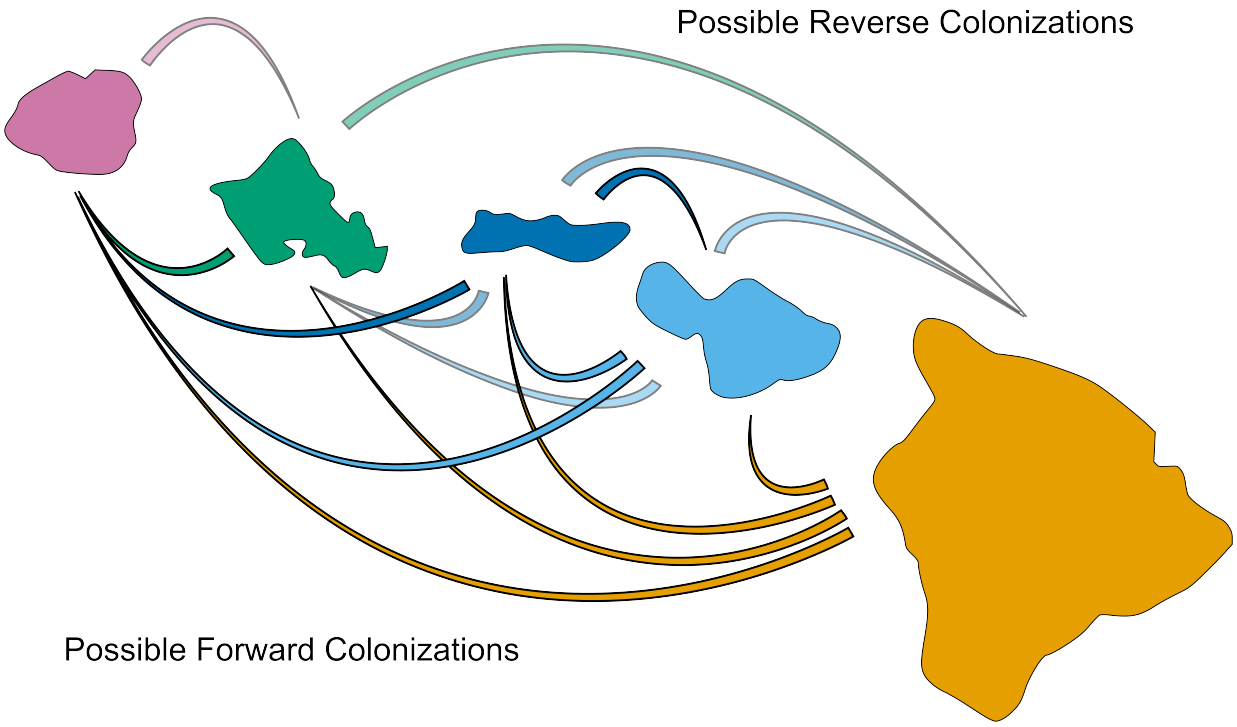


Figure 4: Possible Colonization Routes of *Kilauella*. These routes are potential pathways of movement between the islands in the chain, as inferred from the phylogeny in Figure 2. More transparent routes represent a route with a lower probability / lower frequency in the phylogeny.



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