Introduction

Recent evolutionary radiations on island chains such as the Hawaiian Islands can provide insight into evolutionary processes, such as genetic drift and adaptation (Wallace 1880, Grant and Grant 1994, Losos and Ricklefs 2009). For limited mobility species, colonization processes hold important evolutionary stories not just among islands, but within islands as well (Holland and Hadfield 2002, Parent 2012). One such radiation produced at least 91 species of Hawaiian tree snails in the endemic subfamily Achatinellinae, on at least five of the six main Hawaiian Islands: O‘ahu, Maui, Lana‘i, Moloka‘i, and Hawai‘i (Pilsbry and Cooke 1912–1914, Holland and Hadfield 2007). As simultaneous hermaphrodites with the ability to self-fertilize, colonization events among islands may have occurred via the accidental transfer of a single individual by birds (Pilsbry and Cooke 1912–1914), or via land bridges that connected Maui, Molokai, and Lanai at various points in geologic history (Price and Elliot-Fisk 2004). Early naturalists attributed speciation solely to genetic drift, noting that this subfamily was “still a youthful group in the full flower of their evolution” (Pilsbry and Cooke 1912–1914). However, as these species evolved over dramatic precipitation and temperature gradients, natural selection and adaptation may have been quite rapid as species expanded to fill unexploited niches along environmental gradients, early in this subfamily’s history. As such, species in the subfamily Achatinellinae provide an excellent system for examining both neutral and adaptive processes of evolution.

Habitat loss, predation by introduced species, and over-harvesting by collectors led to the extinction of more than 50 species in the subfamily Achatinellinae, and resulted in the declaration of all remaining species in the genus *Achatinella* as Endangered (Hadfield and Mountain 1980; U.S. Fish and Wildlife Service 1981; Hadfield 1986). Of these, *Achatinella mustelina* (Mighels 1845) is the most abundant and locally widespread, with at least 2000 individuals remaining in the wild.

A study of *A. mustelina* based on a single barcoding gene, cytochrome oxidase I (COI), synonymized many of the subspecies that had been characterized based on shell morphology, and identified six evolutionarily significant units (ESUs) whose distribution generally correlated with geographic features such as ridgelines (Holland and Hadfield 2002). In the last twenty years the field of genetics has transitioned from this type of single or multi-gene study to genomic methods (Stapley et al. 2010), but many researchers working on non-model organisms have been left out of this revolution (Garvin et al. 2010). Reduced-representation sequencing has made genomic approaches more affordable for those working on non-model organisms (Helyar et al. 2011, Toonen et al. 2013). This total information approach includes thousands of sites from across the genome, and may generate better-resolved phylogenies (Rokas et al. 2003), improving the management of endangered species that previously lacked this high-resolution information (Harrison and Kidner 2011).

In this study we had several goals. First, we examined whether the relationships uncovered utilizing a single barcoding gene, cytochrome oxidase I (Holland and Hadfield 2002), were consistent with relationships identified using whole mitochondrial genome comparisons. Next, we asked whether mitochondrial relationships were consistent with those that were found utilizing a genome-wide approach in which thousands of variable sites (single-nucleotide polymorphisms, or SNPs) were examined across the genome (Toonen et al. 2013). We asked whether these relationships among populations of *A. mustelina* were consistent with population-level or species-level relationships, by constructing mitochondrial and SNPs-based phylogenies that included species in all four genera within the subfamily Achatinellinae (*Achatinella, Newcombia, Partulina, Perdicella*), as well as from two genera within the family, but outside of the subfamily Achatinellinae (*Auriculella*, *Tornatellides*).

Methods

Field Sites, Sample Collection, and Preparation. The current range of *Achatinella mustelina* extends about 25 kilometers north to south in the Waianae Mountain Range along elevational clines of 450–

1200 m (Holland and Hadfield 2007). These elevational clines correlate with rainfall and temperature, with a rainshadow effect between the windward and leeward sides of the mountain range.

Sample collection and DNA extraction. Between October 2014 and June 2016 small tissue samples were collected in a nonlethal manner from 4–50 individuals per population and individually preserved in 100% ethanol until DNA extraction (Thacker and Hadfield 2000). DNA was individually extracted from tissue samples using a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. Extracted DNA was quantified using the Biotium AccuClear Ultra High Sensitivity dsDNA quantitation kit with 7 standards. Equal quantities of DNA from each individual within a population were pooled to a total of 1 µg. From these pools, libraries were prepared for genome scanning using the ezRAD protocol (Toonen et al. 2013) version 2.0 (Knapp et al. 2016). Samples were digested with the frequent cutter restriction enzyme DpnII from New England Biolabs®. They were then prepared for sequencing on the Illumina® MiSeq using the Kapa Biosystems Hyper Prep kit following the manufacturers guidelines with the exception of the size selection, which was modified to select for DNA fragments between 350–700 bp. All samples were amplified after size selection for the recommended cycles to generate 1 µg of adapter-ligated DNA. Once complete, all libraries were run on a bioanalyzer and with qPCR to validate and quantify them to ensure equal pooling on the MiSeq flow cell. Quality control checks and sequencing were performed by the Hawaii Institute of Marine Biology Genetics Core Facility.

After cleaning and pairing forward and reverse reads we obtained a total of 301,350,630 sequences from 22 populations of *A. mustelina*, as well as between one and six populations of 24 other species from five genera (Table 1).

Table 1. Populations and species sequenced in this project (*Achatinella mustelina*) and in a concurrent project funded through the Hawaii Division of Forestry and Wildlife (DOFAW; all other species).

Subfamily	Genus	Species	Code	ESU	Population
Achatinellinae	*Achatinella*	*apexfulva*	AAP1		
Achatinellinae	*Achatinella*	*bulimoides*	ABU1		
Achatinellinae	*Achatinella*	*concavospira*	ACO1		
Achatinellinae	*Achatinella*	*decipiens*	ADE1		
Achatinellinae	*Achatinella*	*fulgens*	AFUL1		
Achatinellinae	*Achatinella*	*fulgens*	AFUL2		
Achatinellinae	*Achatinella*	*fuscobasis*	AFUS1		
Achatinellinae	*Achatinella*	*lila*	ALI2		
Achatinellinae	*Achatinella*	*lila*	ALI1		
Achatinellinae	*Achatinella*	*lila*	ALI3		
Achatinellinae	*Achatinella*	*mustelina*	AMU1	ESUA	Kahanahaiki
Achatinellinae	*Achatinella*	*mustelina*	AMU2	ESUA	Pahole
Achatinellinae	*Achatinella*	*mustelina*	AMU3	ESUB	Koiahi
Achatinellinae	*Achatinella*	*mustelina*	AMU4	ESUB	Ohikilolo
Achatinellinae	*Achatinella*	*mustelina*	AMU5	ESUB	Culvert 39
Achatinellinae	*Achatinella*	*mustelina*	AMU6	ESUB	Culvert 56/57
Achatinellinae	*Achatinella*	*mustelina*	AMU7	ESUC	Skeet Pass
Achatinellinae	*Achatinella*	*mustelina*	AMU8	ESUC	Haleauau
Achatinellinae	*Achatinella*	*mustelina*	AMU9	ESUD	SBW-R
Achatinellinae	*Achatinella*	*mustelina*	AMU10	ESUD	Makaha
Achatinellinae	*Achatinella*	*mustelina*	AMU11	ESUD	Puu Hapapa
Achatinellinae	*Achatinella*	*mustelina*	AMU12	ESUD	Puu Kalena
Achatinellinae	*Achatinella*	*mustelina*	AMU13	ESUD	Puu Kumakalii
Achatinellinae	*Achatinella*	*mustelina*	AMU14	ESUE	Ekahanui
Achatinellinae	*Achatinella*	*mustelina*	AMU15	ESUF	Palikea
Achatinellinae	*Achatinella*	*mustelina*	AMU16	ESUE	H1-H4 Huliwai
Achatinellinae	*Achatinella*	*mustelina*	AMU17	ESUE	NH1-NH4
Achatinellinae	*Achatinella*	*mustelina*	AMU18	ESUD	K1-K6
Achatinellinae	*Achatinella*	*mustelina*	AMU19	ESUD	S1-S6
Achatinellinae	*Achatinella*	*mustelina*	AMU20	ESUB	Kaawa 1-13
Achatinellinae	*Achatinella*	*mustelina*	AMU21	ESUD	MAK-G 1-15
Achatinellinae	*Achatinella*	*mustelina*	AMU22	ESUD	MAK-F 1-7
Achatinellinae	*Achatinella*	*sowerbyana*	ASO1		
Achatinellinae	*Achatinella*	*sowerbyana*	ASO2		
Achatinellinae	*Achatinella*	*sowerbyana*	ASO3		
Achatinellinae	*Achatinella*	*sowerbyana*	ASO4		
Achatinellinae	*Achatinella*	*sowerbyana*	ASO5		
Achatinellinae	*Achatinella*	*sowerbyana*	ASO6		
Achatinellinae	*Achatinella*	*sp. Oahu*	AUN1		
Achatinellinae	*Newcombia*	*cumingi*	NCU1		
Achatinellinae	*Partulina*	*mighelsiana*	PMI1		

Subfamily	Genus	Species	Code
Achatinellinae	*Partulina*	*perdix*	PPE1
Achatinellinae	*Partulina*	*perdix*	PPE2
Achatinellinae	*Partulina*	*physa*	PPH1
Achatinellinae	*Partulina*	*proxima*	PPR1
Achatinellinae	*Partulina*	*proxima*	PPR2
Achatinellinae	*Partulina*	*proxima*	PPR3
Achatinellinae	*Partulina*	*redfieldii*	PRE1
Achatinellinae	*Partulina*	*redfieldii*	PRE2
Achatinellinae	*Partulina*	*redfieldii*	PRE3
Achatinellinae	*Partulina*	*redfieldii*	PRE4
Achatinellinae	*Partulina*	*semicarinata*	PSE1
Achatinellinae	*Partulina*	*terebra*	PTER1
Achatinellinae	*Partulina*	*tesselata*	PTE1
Achatinellinae	*Partulina*	*variabilis*	PVA1
Achatinellinae	*Perdicella*	*helena*	PHE1
Achatinellinae	*Perdicella*	*helena*	PHE2
Achatinellinae	*Perdicella*	*sp.* Maui	PER1
Auricullelinae	*Auriculella*	*sp.*	AUR1
Auricullelinae	*Auriculella*	*sp.*	ACR1
Tornatellidinae	*Tornatellides*	*iridescens*	TIR1

Mitochondrial Genomes. Utilizing the data from 15 populations of *A. mustelina*, we assembled the complete mitochondrial genome of *A. mustelina* (GenBank accession number KU525108). Reads (69,178,116 sequences) were initially mapped to the reference mitogenome of *Albinuria coerulea* (Hatzoglou et al. 1995). The alignment of mapped sequences was inspected, and a consensus sequence was generated. This consensus sequence was used as a reference for the next iteration, in which all ~69 million sequences from a given population were mapped against the consensus sequence achieved in the previous round of alignment. This process was repeated until the complete mitochondrial genome was obtained. In total, 30,695 reads mapped to the complete mitochondrial genome, with coverage ranging from 10x to 4409x per site (256 ± 50). Annotation of mitochondrial elements was carried out with DOGMA (Wyman et al. 2004) and MITOS (Bernt et al 2013).

Once the *Achatinella mustelina* mitogenome was obtained, reads for populations of other species were mapped to the reference mitogenome of *Achatinella mustelina* (Price et al. 2016).

Through an iterative process, whole and partial mitogenomes of all populations were constructed. In total, 969–7474 reads per population mapped to the complete mitochondrial genome, with coverage ranging from 1X to 1030X per site (46.3 ± 73.6). Annotation of mitochondrial elements was carried out with DOGMA (Wyman et al. 2004) and MITOS (Bernt et al 2013). Multiple sequence alignments were performed with MUSCLE v3.8.31 (Edgar 2004) under default parameters, visual inspection of the alignment found no regions that appeared to be poorly aligned. Maximum likelihood trees were generated with RAxML v. 8.1.16 (Stamatkis 2014) with the GTRGAMMA model and optimization of rate parameters and bootstrap support values based on 500 replicates.

Genome-wide Analyses. Initial trials were conducted with the programs pyRAD or ipyRAD, however these large ezRAD libraries (~6 million reads up to 300bp long) were slow to process due to the high number of loci (a single library took up to a month to process on a high-end work station). The dDocent pipeline v. 2.2.19 was used to process raw reads with several steps modified in order to quickly process a large number of libraries (n=59), and to account for pooled populations. *De-novo* assembly was first performed on members of the *Achatinella* genus (n=39) in order to construct a reference sequence for reference mapping against the total dataset. The *de-novo* assembly options were: Clustering_Similarity% = 0.85, Mapping_Reads? = Yes; Mapping_Match_Value = 1; Mapping_MisMatch_Value = 4; Mapping_GapOpen_Penalty = 6. All libraries were mapped to the *Achatinella* reference using mapping parameters as above. The program Freebayes v1.0.2-29 was used to call variants from the merged bam file produced by the dDocent pipeline, with stringent filters, ignoring multi-nucleotide polymorphisms and complex events, under the pooled continuous model with a minimum coverage of 5 reads (i.e. -0 -E 3 -z .1 -X -u -n 4 -K --min-coverage 5 --min-repeat-entropy 1 -V). The resulting vcf file was examined in R (R Development Core Team 2011), using the heatmap.bp function in the package vcfR (Knaus and Grunwald 2017) in order to evaluate coverage across libraries and loci (Figure S1), which was fairly even with the exception of the outgroups and few libraries with

very low coverage that were dropped from further analysis using VCFtools (Danacek et al. 2011). VCFtools which was also used to determine depth and heterozygosity information of the libraries. For phylogenetic analysis, the SNPhylo (Lee et al. 2014) was used in order to generate a fasta formatted file containing variable positions. The number of sites was higher than allowed by the automated pipeline, and subsets of the data were analyzed under a broad array of program settings; however these trees generally resulted in low support values and odd placements of taxa (results not shown) therefore, trees were generated with RAxML v. 8.1.16 with the GTRGAMMA model and optimization of rate parameters and bootstrap support values based on 500 replicates.

Results

Mitochondrial genomes. The *Achatinella mustelina* mitogenome is similar to those of other Pulmonates (White et al. 2011), with 13 protein-coding genes, two rRNA genes, and 22 tRNA genes. The total length is 16,323 bp, slightly larger than other Pulmonates (White et al. 2011). The base composition of the genome is: A (34.7%), T (42.6%), C (12.7%), and G (10.0%). This is the first mitochondrial genome sequenced within the Achatinelloidea superfamily (Price et al. 2016).

When whole and partial mitochondrial genomes were compared across populations within *A. mustelina*, for the most part, the same patterns were observed as in previous studies using only one mitochondrial gene (Fig. 1ab). However, some of the populations near previously identified ESU boundaries grouped in slightly different ways. For example, samples from several populations thought to be ESU D clustered with the samples from Ekahanui (ESU E). When analyses of mitochondrial genomes included all species, the differentiation among populations in ABC and those in DEF appeared to be consistent with species-level differences among other species (Fig. 1a). Overall, populations grouped into five or six clusters, consistent with ESUs ABCDEF. Populations in ESUs ABC grouped together, and populations in ESUs DEF grouped together, with strong support values (Fig. 1b).

Total information approach. When thousands of sites from across the genome were used to examine relationships, patterns generally followed ESU patterns, with a few exceptions. The Makaha population ("AMU10") grouped with ESU B populations, rather than ESU D populations (Figure 2). When all 59 samples were analyzed using the total information approach, patterns were similar over all, but there were low support values on multiple branches within *A. mustelina* (Figure 3).

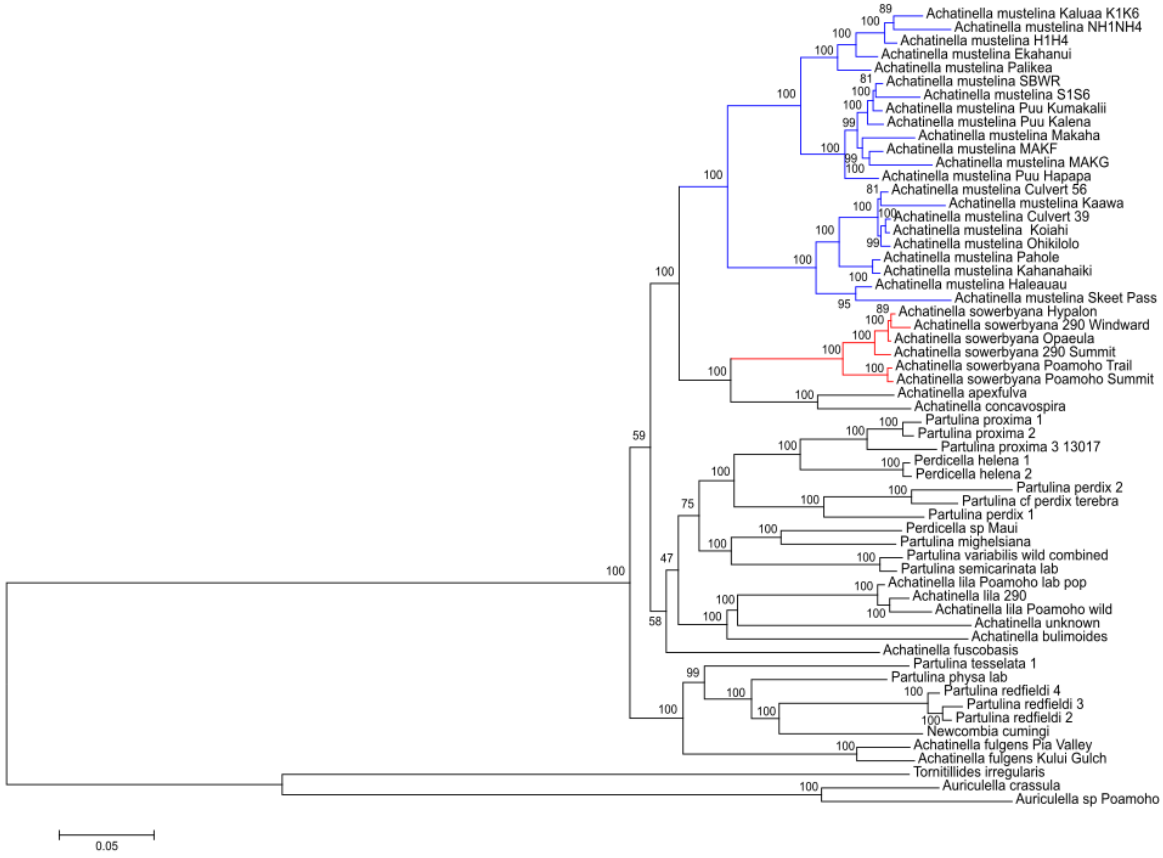


Figure 1a. Mitochondrial tree with all populations and species sequenced, including 22 populations of *Achatinella mustelina* and 37 populations representing 24 additional species.

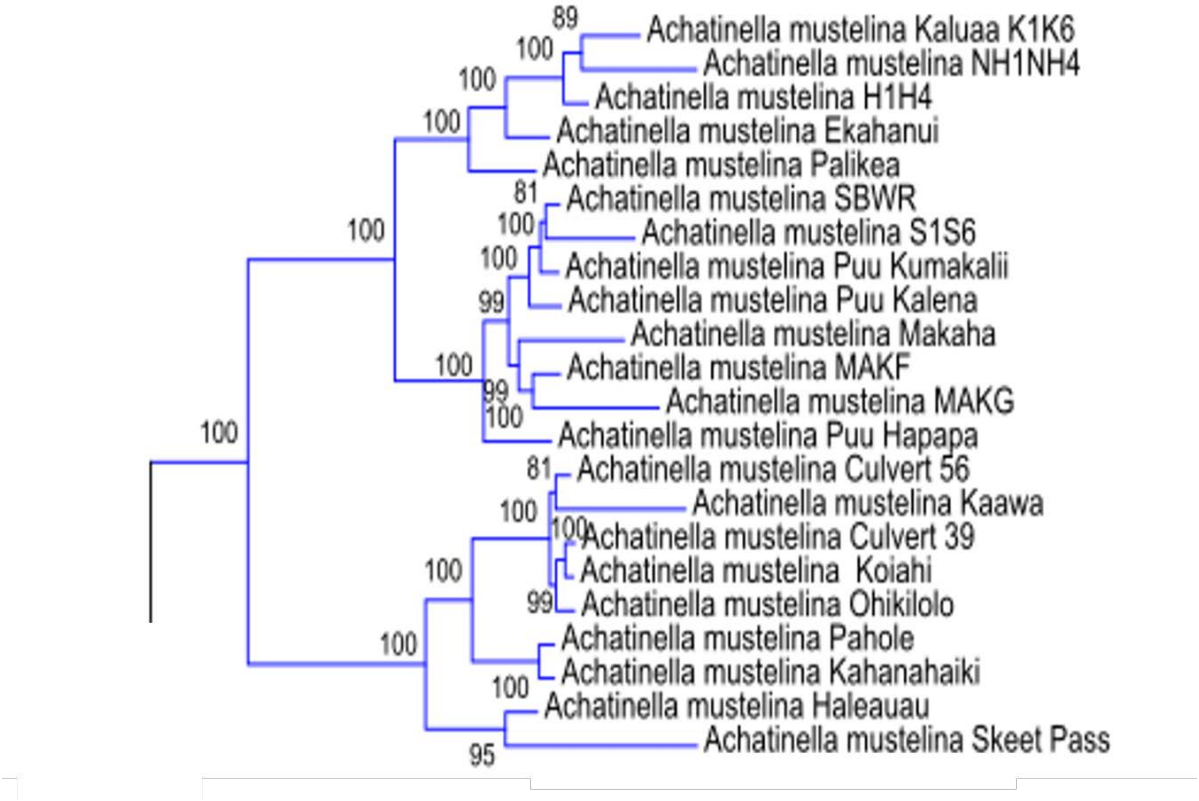


Figure 1b. Only populations of *Achatinella mustelina*, from figure 1a, for viewing convenience.

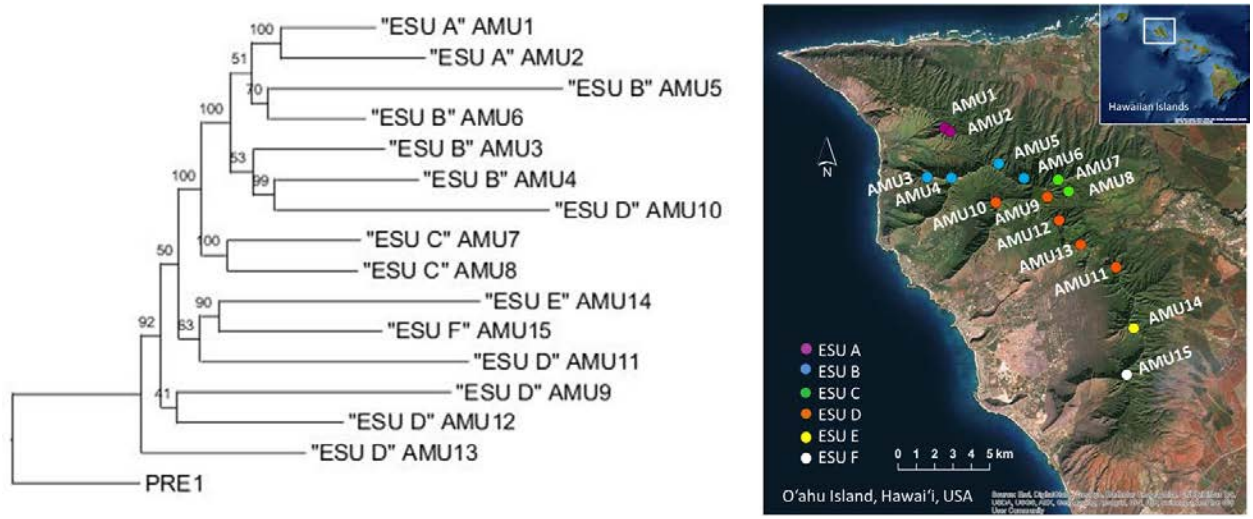
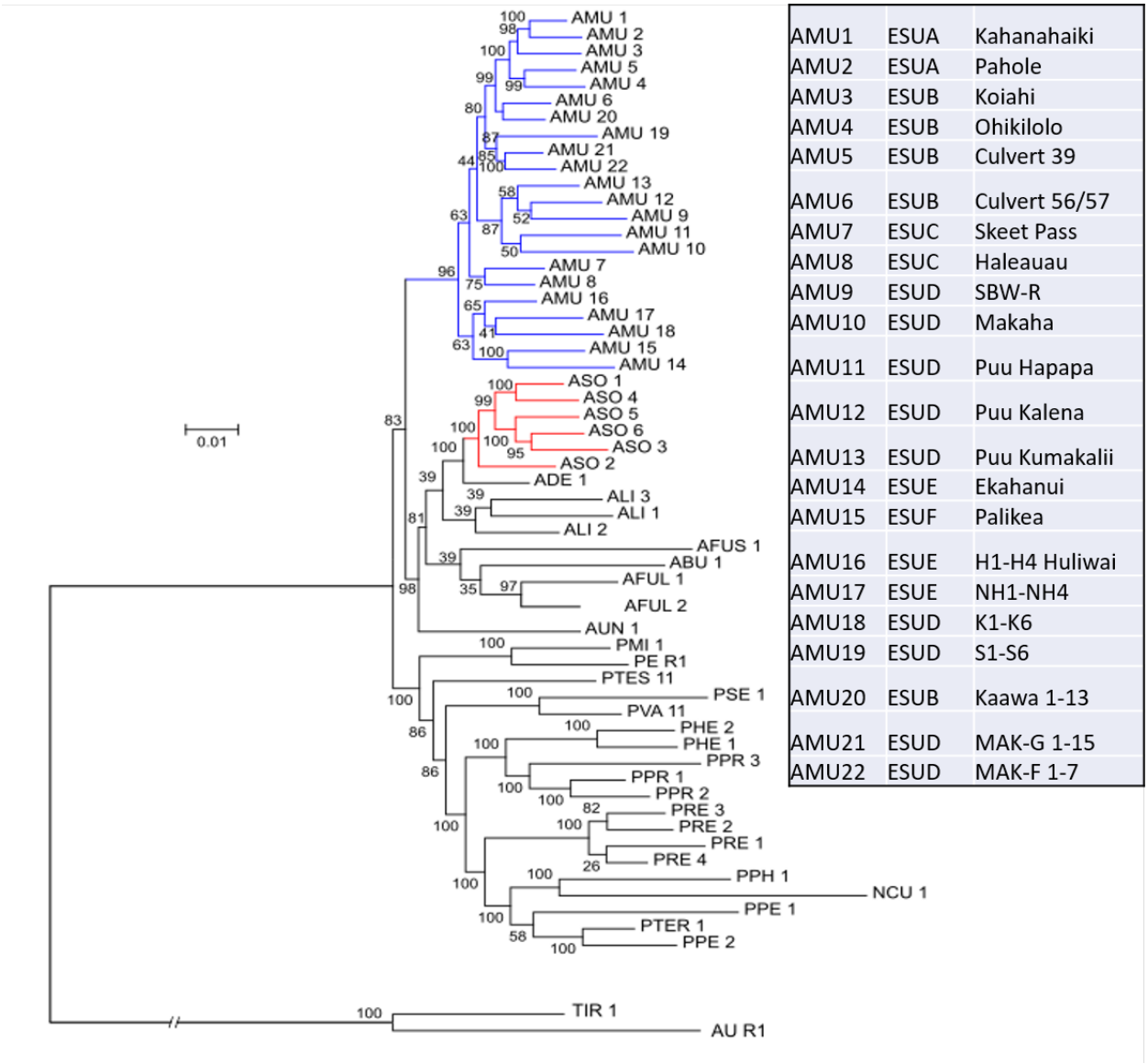


Figure 2. Phylogenetic tree generated using a total information approach using the program iPyrad, with geographic locations shown for each population.



AMU1	ESUA	Kahanahaiki
AMU2	ESUA	Pahole
AMU3	ESUB	Koiahi
AMU4	ESUB	Ohikilolo
AMU5	ESUB	Culvert 39
AMU6	ESUB	Culvert 56/57
AMU7	ESUC	Skeet Pass
AMU8	ESUC	Haleauau
AMU9	ESUD	SBW-R
AMU10	ESUD	Makaha
AMU11	ESUD	Puu Hapapa
AMU12	ESUD	Puu Kalena
AMU13	ESUD	Puu Kumakalii
AMU14	ESUE	Ekahanui
AMU15	ESUF	Palikea
AMU16	ESUE	H1-H4 Huliwai
AMU17	ESUE	NH1-NH4
AMU18	ESUD	K1-K6
AMU19	ESUD	S1-S6
AMU20	ESUB	Kaawa 1-13
AMU21	ESUD	MAK-G 1-15
AMU22	ESUD	MAK-F 1-7

Figure 3. All populations and species analyzed using a total information approach (both nuclear and mitochondrial variable sites).

Discussion

Populations within *A. mustelina* are now managed to maintain the genetic distinctiveness of the ESUs, by only "mixing" snails within, but not among, ESUs. Management efforts for the remaining populations include four *in situ* predator-free enclosures (two in ESU A, one in ESU D, and one in ESU F), and rat removal in large populations outside of predator-free enclosures, along with other habitat

management efforts. There is general agreement that predator-free enclosures are the only way to protect tree snails from all three invasive predators, since there is, as yet, no effective method for removing the predatory snail *Euglandina rosea* or Jackson's chameleons, which have both devastated native mollusks and other invertebrates in habitats where they are present. However, enclosures are expensive to build and require accessible land with a minimal incline, which is scarce in the high elevations of the Waianae Mountain Range. ESUs B, C, and E do not yet have enclosures due to these constraints, and many populations within these ESUs are declining due to high rates of predation from *E. rosea* and Jackson's Chameleons. However, current policy, based on the existing understanding of genetic structure in this species, prevents the movement of vulnerable populations into existing enclosures that contain tree snails belonging to a different ESU.

Our methods have captured 50-90% of mitochondrial genomes for each population examined. Whole mitochondrial genomes have been compared across the range of Achatinella mustelina, and for all species sequenced as part of this study. These results suggest the same management approach as COI alone (Holland and Hadfield's 2002 paper), with no change to the current management approach of 5 or 6 discrete ESUs, with populations grouping along the Waianae ridgelines.

However, when nuclear evidence was considered (a scan/survey of thousands of sites across the entire genome), we observed a more nuanced picture. For example, Makaha (ESU D) always groups with Koiahi and Ohikilolo (ESU B). Puu Hapapa (ESU D) groups with Ekahanui (ESU E) about 50% of the time. On the other hand, some populations are very much the same for both nuclear and mitochondrial markers. Populations in ESU C (Haleauau and Skeet Pass) always group together, separate from the others. The populations on the three ridges that meet on top of Mt. Kaala (from ESUs B, C, D) separate out from one another with both mitochondrial and whole-genome approaches.

Another result consistent across both mitochondrial and genome-wide approaches is that the differences among some ESUs are similar to species-level differences across the subfamily. Evaluations

of morphology and further examination of genetic data are needed before any major conclusions may be drawn, but these results are highly suggestive that major differences exist among two groups if ESUs (ABC, DEF), and outcrossing depression could result if geographically distant populations, particularly from different ESUs, are combined. The location of the divide between the two groups (ABC, DEF) is roughly consistent with a faultline near the top of Mt. Kaala, which correlates with historical, but not current, geological features that may have formed geographic barriers to gene flow in the past (Figure 4). However, we lack modern geographic features to explain the lack of gene flow between ABC and DEF.

Balancing concerns regarding predators, inbreeding, and climate change. Given concerns regarding high predation on tree snail populations, and our limited ability to remove two out of three predators, protection of declining tree snail populations remains a priority. Over the past few years a number of other concerns have been raised, including the potential for inbreeding depression in small, isolated populations, as well as impacts of climate change, such as an increasing number of drought events leading to high juvenile mortality. When translocating snails into enclosures or into areas with rat-trapping grids to protect them from predation, potential impacts of inbreeding or outbreeding depression, as well as potential impacts from climate change, must be considered.

Overall, there are four conditions under which translocations are currently being considered. In the majority of situations, translocation is being considered because of drastic population declines caused by high predation by rats, Jackson chameleons, or *E. rosea*. Translocation may also be important when genetic rescue is needed due to low heterozygosity and inbreeding depression. In this case, diversity may be increased by combining populations or simply translocating a few individuals into an enclosure. Third, in the case of assisted evolution, we may wish to combine populations to add genetic diversity that increases the likelihood of critical populations adapting to climate change. Finally, we may

wish to move a critical population that is not predicted to survive climate change in its current location, to a location where it is more likely to survive climate change, a process called assisted colonization.

Unsurprisingly, total DNA evidence suggests that snail populations that are closer together geographically are more closely related genetically, and snail populations that are farther apart are less related. Pulling snails from nearby populations (< 1 km) into enclosures should be enough to combat inbreeding. Outbreeding depression may be a concern if tree snails from more distant populations are combined. Phylogenetic trees generated in this study may be used as general guidelines, particularly for branches with high bootstrap values (>70), but consultation is strongly encouraged in cases where snails will be moved > 1 km. In light of climate change, we still recommend moving snails to wetter, cooler locations, and never to locations that are warmer or drier than source locations. Also based on projections of shifts in suitable climate under likely climate change scenarios (A. Vorsino, in prep), we recommend moving snails in ESUs D, E, and F north (toward Mt. Kaala), but not south.

For populations in the southern Waianae Mountains, in particular (ESUs DEF), that are adapted to hotter, drier, conditions, populations must be carefully monitored for response to droughts and high-temperature conditions. In consultation with the Snail Extinction Prevention Program and USFWS, OANRP may wish to consider trials in which tree snails from ESUs E and F are crossed under lab conditions, to determine whether outbreeding depression is a concern. These trials should be undertaken before the population size of ESU E declines further.

Moving forward, actions should be taken and populations prioritized based on whether the loss of the population would likely mean the loss of an entire ESU, whether the population has unique genetic characteristics that contribute to ESU or species-level diversity, and whether the population is predicted to survive through the end of the century under hotter, drier conditions.

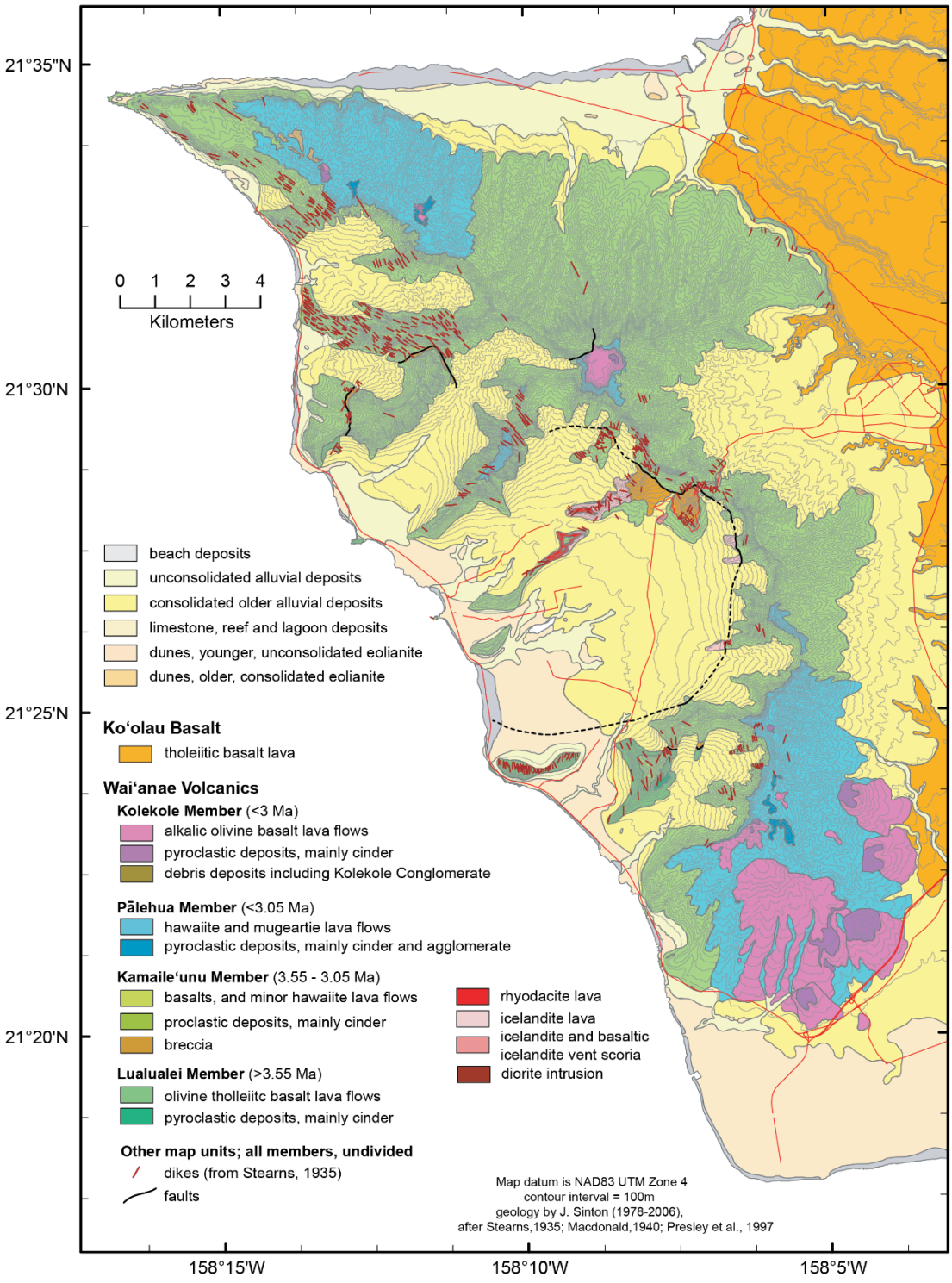


Figure 4. Geology of the Waianae Mountains (from Presley et al. 1997, updated by J. Sinton 2016).

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