INVASIVE ECOLOGY OF THE AMERICAN BULLFROG (*RANA CATESBEIANA*) IN HAWAI`I

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ABSTRACT

The American Bullfrog, *Rana catesbeiana*, is considered one of the most successful and damaging invasive amphibians, having spread by various means from the eastern US, currently established in Europe, Asia, South America and Oceania. Through various ecological interactions including competition, predation, and possible transmission of pathogens, this amphibian is extremely damaging to the aquatic ecosystems it invades. Numerous studies have documented devastating ecological impacts of bullfrogs in non-native ranges that include the western US, but very little information on this topic is available in the Hawaiian Islands, despite having been established for over 120 years. This project investigated aspects of bullfrog invasion ecology in Hawai`i, including morphological divergence from mainland populations in terms of total body size, head width, and leg length, characters which substantially affect bullfrog interactions with native Hawai`ian ecosystems. Hawai`i bullfrog specimens differed significantly from most mainland groups for all morphological traits, exhibiting larger bodies, narrower heads, and shorter legs than observed in the native range. I also characterized bullfrog diet at two Oahu coastal wetlands, Kualoa Ranch and James Campbell National Wildlife Refuge. Finally, I assessed genetic diversity, inferred source populations, and evaluated the occurrence of the genetic invasion paradox in Hawai`i bullfrog populations. This information will inform prioritization of management action and utilization of personnel and resources in an effective manner. Invasive ecology on islands like Oahu can also serve as a useful model for bullfrog-invaded ecosystems worldwide.

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Introduction

As a result of their extreme geographic isolation, the Hawai`ian islands are characterized by ecosystems that are simultaneously unique, exceptionally biodiverse, and highly sensitive to degradation. Species radiations tend to be found in the lineages that have colonized remote volcanic island chains such as the Hawai`ian islands, resulting in high rates of endemism (Gillespie et al., 1994; Freed et al., 1987). For instance, species endemism in Hawai`ian flowering plants is estimated to be greater than 90%, with approximately 850 endemic species (Craddock, 2000). Fossil evidence indicates Hawai`i historically supported over 100 endemic bird species, with approximately 92% endemism (Olson & James, 1982; Pyle & Pyle, 2017). Endemism in Hawai`ian terrestrial snail species is approximately 99%, with more than 800 described endemic species (Cowie et al., 1999), while insect endemism is approximately 98% with 5,366 described endemic species (Nishida, 2002).

Anthropogenic activities have extirpated an increasing proportion of these native taxa. An estimated 9% of Hawai`ian angiosperm flora are extinct and over 50% are at risk, due to habitat loss and pollinator declines (Sakai et al., 2002). Over 60% of endemic Hawai`ian birds are extinct as a result of anthropogenic activity, including the introduction of non-native species (Boyer, 2008; Olson & James, 1982). According to Regnier et al. (2015), as many as 95% of the endemic land snail species may already be extinct, due primarily to the impacts of invasive predators. The conservation status of Hawai`ian insects and arachnids is largely unknown due to the challenges of monitoring small cryptic species. Nevertheless, entomologists have implicated invasive plants, ungulates, rodents, ants, and parasitoid wasps in the decline of native insect populations (Rubinoff, 2007; Medeiros et al. 2013). Natural history studies in Hawai`i emphatically identify introduced species as a persistent threat to biodiversity.

In addition to exceptional species radiations, the geographic isolation of the Hawai`ian islands has also drastically limited colonization by freshwater fauna, resulting in the presence of relatively few native freshwater species. Many of these species derive from marine taxa and are therefore highly endemic and characterized by unique life history patterns (Brasher et al., 2006). Hawai`ian freshwater fish include the endemic Oopu Akupa (Sandwich Island sleeper*) Eleotris sandwicensis,* which occur close to stream mouths, as well as three endemic species of goby: the Oopu Alamoo (bicolored stream goby, *Lentipes concolor*), Oopu Nopili (*Sicyopterus stimpsoni*), and Oopu Naniha (Hawai`ian blackbar goby, *Stenogobius Hawai`iensis*) (Brasher et al., 2006). These goby species possess morphological adaptations that allow them to climb waterfalls and live at high elevations. Hawai`ian freshwater stream systems also contain two native crustaceans, the Opae Kalaole (mountain shrimp, *Atyoida bisulcate*) and the Opae Oehaa (*Macrobrachium grandimanus*) (Brasher et al., 2006). Two species of gastropods also occur in Hawai`i freshwater systems, Hihiwai or *Neritina granosa*, and Newcomb's snail or *Erinna newcombi* (Brasher et al., 2006).

Today, these freshwater ecosystems face numerous challenges, primarily in the form of habitat alterations as well as aquatic predators and competitors introduced within the past century (Martin & Tsang; Walter et al., 2012; Christensen et al., 2021). The destructive anthropogenic changes to these ecosystems include reduction and diversion of freshwater flow patterns, stream bed structure alteration and channelization, pollution by effluent release, and eutrophication from urban and agricultural runoff (Ramirez et al., 2012). In addition, large invasive predatory species present an under-recognized and under-studied threat to freshwater ecosystems in Hawai`i. Invasive aquatic species range from relatively small crayfish and cichlids to the sizable South American peacock bass (*Cichla kelberi*), Asian snakehead fish (*Channa striata*), Chinese softshelled turtle *Palea spp*. (Dong et al., 2016; Marchetti & Engstrom 2016) and American bullfrog (*Rana catesbeiana*). Many streams in the Hawai`ian islands have also been colonized, or are utilized for reproduction, by numerous other predatory non-native species, including red-eared sliders (*Trachemys scripta elegans*), smallmouth bass (*Micropterus salmoides*), armored catfish (*Hypostomus spp.*), rainbow trout (*Oncorhynchus mykiss*), various cichlids including multiple species of tilapia, western mosquitofish (*Gambusia affinis*), Tahitian prawn (*Macrobrachium lar*), Asiatic freshwater clam (*Corbicula fluminea*), several species of crayfish, and cane toads (*Bufo marinus*) (Yamamoto & Tagawa 2000). Because the freshwater ecosystems of Hawai`i historically lacked any large predators, stream fauna have evolved in the absence of predatory pressure (Cox & Lima, 2006; Kraus et al., 2011). This phenomenon, termed "evolutionary prey naiveté," is proposed as one of the primary causes of extinctions attributable to invasive predators (e.g. Mathis et al., 1993; Kovalenko et al., 2010).

While invasive plants, mammals and insects have received the majority of scientific attention due to their significant economic impacts, ecological effects attributable to invasive herpetofauna are poorly understood (Reed & Kraus, 2010). As many as 26 herpetofauna species have become established on the archipelago over the past century, including 10 primarily aquatic predatory species (Van Kleeck & Holland, 2018). To date, the Jackson's chameleon has been studied most thoroughly, in terms of its movement behavior, predation on rare Hawai`ian taxa, and adaptations to novel environmental gradients in the Hawai`ian islands (Holland et al., 2010; Kraus et al., 2012a; Kraus et al., 2012b; Chiaverano & Holland, 2014; Wright et al., 2014; Van Kleeck & Holland, 2015; Van Kleeck et al., 2018). *Palea steindachneri* and *Pelodiscus sinensis*, two softshell turtles at high conservation risk in their native ranges, have been evaluated as assurance colonies (colonies that may serve to repopulate the native range in case of extirpation) in Hawai`i, although their ecological impacts on native species remain unresolved (Dong et al., 2016). Invasive coqui and greenhouse frogs have been hypothesized to reduce native arthropods or compete with endemic birds (Beard & Pitt, 2005; Kraus et al. 1999; Olson et al., 2011; Olson et al., 2012); however, both frogs appear to predate exclusively on non-native species in Hawai`i, having established populations only in urbanized lowlands where few native species occur. In contrast, both Jackson's chameleons and Japanese wrinkled frogs have invaded inland forests and exploit native insects and gastropods (Van Kleeck & Holland, 2018). The impacts of non-native herpetofauna may be moderated by the species' habitat preferences, prey availability, and adaptations to novel environments.

The American bullfrog, *Rana catesbeiana*, is among the most successful invasive amphibians, having spread from their native range in the eastern U.S. and become established in 40 countries on four continents (Funk et al., 2011) - Europe, Asia, South America and Oceania (Lever, 2003). Through ecological interactions including competition, predation, and transmission of pathogens (Daszak et al., 2004) this amphibian can be highly damaging to the aquatic ecosystems it invades. Biological features that enhance the ability of this species to spread, compete and become established include generalist predatory behavior, exceptional reproductive capacity (producing approximately 80,000 eggs per year in the tropics), relatively large body size, and high mobility (Moyle, 1973; Adams et al., 2007; Kaefer et al., 2007). Bullfrogs have been implicated in devastating ecological degradation in their non-native ranges, (Lawler et al., 1999), but little information about this species has been documented in the Hawai`ian islands despite this species having been established for over 120 years. Insights into their distribution, population connectivity, adaptations, and trophic interactions will enable us to assess the threat bullfrogs pose to Hawai`ian ecosystems.

Research on bullfrog foraging ecology in Hawai`i may have conservation implications for native species with distributions that overlap with those of *R. catesbeiana*. Four endangered Hawai`ian waterbirds establish nests in coastal wetlands where bullfrogs occur: the Hawai`ian Duck (*Anas wyvilliana*, Koloa Maoli), Hawai`ian Stilt (*Himantopus mexicanus knudseni*, Ae‘o), Hawai`ian Coot (*Fulica alai*, ‘Alae Kea), and Hawai`ian Gallinule (*Gallinula galeata sandvicensis*, `Alae `Ula). A U.S. Fish and Wildlife Service investigation of Hawai`ian Stilt hatchling success conducted by Eijzenga (2004) documented impacts of bullfrogs at the James Campbell National Wildlife Refuge on Oahu in 2003 and 2004. During this study, 49 newly hatched Hawai`ian Stilts were radio-tagged and monitored until fledging; of these individuals, there were 13 confirmed deaths, 10 (77%) of which were due to bullfrog predation. The resulting technical report (Eijzenga, 2004) suggested that bullfrog predation on stilts may account for hatchling mortality at a frequency that impedes their population recovery. To evaluate the threat of invasive species beyond low elevation streams and wetlands, Martin and Tsang (2019) assessed the ability of 14 non-native aquatic taxa to bypass waterfalls in Hawai`i. Although waterfalls are considered natural barriers to upstream dispersal of aquatic invasives, *Rana catesbeiana* was identified as one of only two species able to bypass these obstacles and colonize upstream reaches (Martin & Tsang, 2019). Bullfrogs have potential to interact with many Hawai`ian endemic aquatic and wetland fauna, even in relatively pristine high-elevation habitat.

As a result of adaptations to novel conditions, the habitat preferences, dispersal behaviors, and trophic interactions of a species in one environment do not necessarily predict its ecological impacts in other environments (Van Kleeck & Holland, 2018). The scale of impact caused by invasive bullfrogs in Hawai`i may be modulated by their morphological and behavioral divergence from populations in their native range. In some cases, invasive herpetofauna have adapted to novel island environments based on the unique in situ selective forces – often the very conditions that have led to the explosive adaptive radiations of endemic lineages (Van Kleeck et al., 2015; Amorim et al., 2017). These conditions include steep environmental gradients in humidity, rainfall, and temperature across restricted geographic scales, as well as habitat diversity and resource and niche availability (Barajas-Barbosa et al., 2021). Adaptive traits, which result from genetic mutations or phenotypic plasticity, can increase the success of an invasive species and shape how it interacts with the native ecosystem. For example, average body length in an invasive population may increase due to factors such as temperature regulation, greater ecosystem productivity, or predator release, thereby increasing the size of exploitable prey (Cloyed & Eason, 2017). In their non-native range bullfrogs exhibit behavioral adaptations, extending annual spawning activity based on photoperiod and temperature (Medeiros et al., 2016) and decreasing metamorphosis duration from two years to four months in wetlands with short hydroperiods (Cook et al., 2013). Morphological microevolution is documented in other invasive amphibians, including the cane toad (Hudson et al., 2016), but the extent and pace at which bullfrogs adapt phenotypically to non-native environments is unresolved.

Elucidation of the distributional range, phenotypic adaptations, and diet of bullfrogs in Hawai`i is needed to understand their ecosystem impacts and evaluate the urgency with which control actions must be taken. To obtain a thorough understanding of bullfrog invasion ecology in Hawai`i, I will address three elements of invasion ecology: shifting morphology, genetics, and diet. 1) I will test whether bullfrogs exhibit morphological adaptations unique to the Hawai`ian islands, including snout-urostyle length, head width, and limb length, relative to native and non-native populations in the contiguous US. 2) A phylogeographic analysis of US bullfrog populations will indicate whether any morphometric differences result from plasticity or genetic variation. Molecular analysis will quantify genetic diversity, identify gene flow between Hawai`ian populations and suggest historical propagule pressure. 3) Lastly, bullfrog gut contents will be analyzed to document short-term diet in the collected specimens. This foundational information is critical to the prioritization of management actions and effective allocation of personnel and resources.

Background

Distribution, Behavior, and Habitat Use

Known Bullfrog Distribution

Bullfrogs have been present in Hawai`i since 1879 (Bryan, 1932; Yamamoto & Tagawa, 2000), when they were introduced for commercial pest control and human consumption. Early records indicate that bullfrogs became established in Hawai`i after 72 frogs were shipped to Hilo, Hawai`i from Contra Costa County, California to establish a farm (Jennings & Hayes, 1985). To our knowledge, the geographic range and distribution of bullfrog populations in Hawai`i have not been systematically studied or documented. Personal communications with biologists from the Department of Land and Natural Resources (DLNR) and the US Fish and Wildlife Service (USFSW), the Oahu Army Natural Resources Program (OANRP) and local university researchers indicate that field surveys of bullfrog distribution have not taken place since this species became established in Hawai`i.

For a growing number of species, geospatial data can be acquired from online databases such as the VertNet project (VertNet.org) and iNaturalist (iNaturalist.org). VertNet records indicate bullfrogs are present on the islands of Kauai, Oahu, Maui, and Hawai`i (VertNet.org, 2019). iNaturalist citizen sightings confirm this distribution (iNaturalist.org, 2019). These open source records suggest that in Hawai`i bullfrogs tend to occur in low elevation wetlands adjacent to human development, although this distribution likely reflects areas of higher search effort. An environmental impact statement prepared by the Bishop Museum's Hawai`i Biological Survey for a timber harvest in the South Hilo District of Hawai`i island observed bullfrogs at elevations up to 880 meters (Englund et al., 2002), while on Oahu they have been observed in the Ko`olau range at elevations up to 600 meters (Brenden Holland, personal communications).

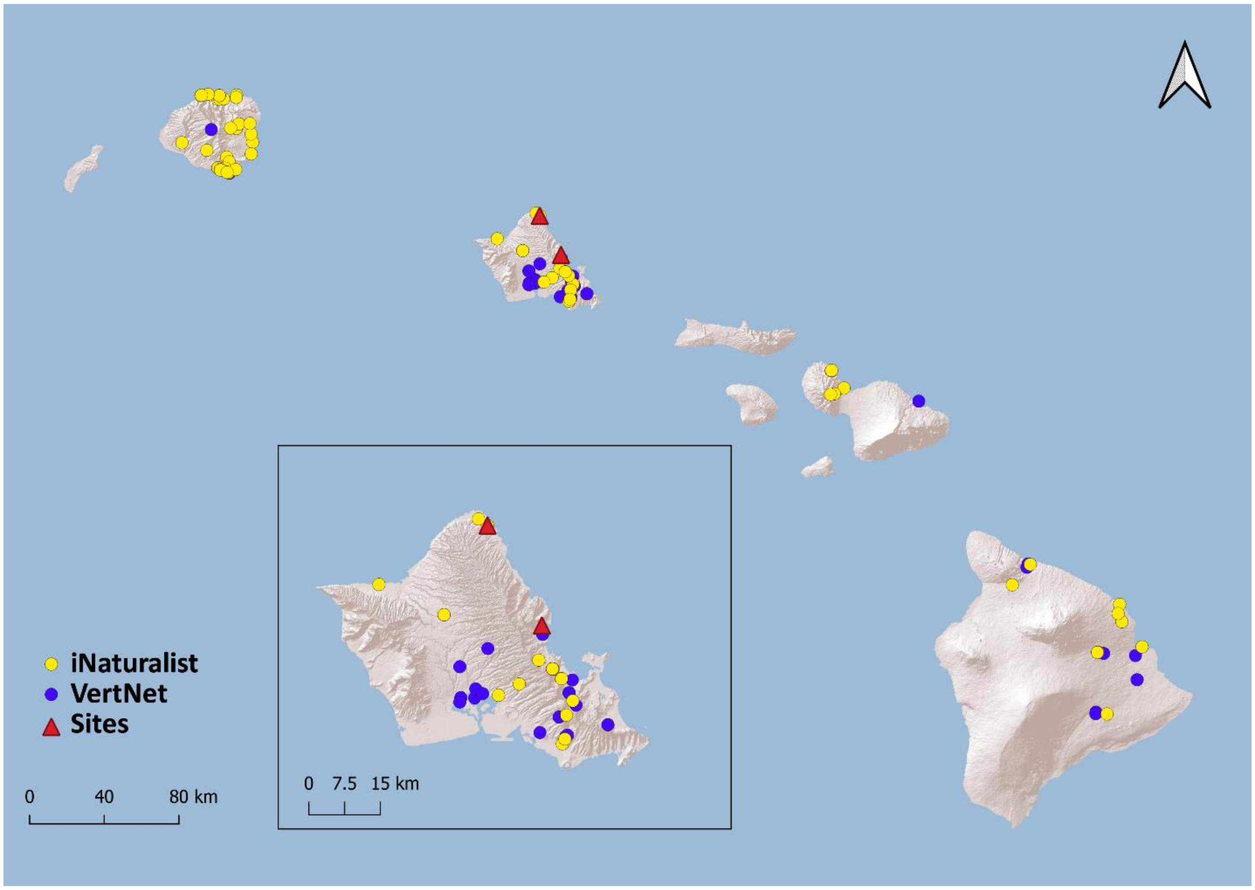

Figure 1. Geospatial data from VertNet (represented with blue points) and iNaturalist (represented with yellow points) records of *R. catesbeiana* observations. Red triangles represent study sites from which *R. catesbeiana* during the course of this thesis project. Oahu is shown enlarged in the inset.

Reproductive Behavior

Permanent water sources were historically considered critical for the establishment of bullfrog populations; in the native range, adult females lay eggs in permanent water bodies during the breeding season and tadpoles require two years to metamorphose (Ficetola et al., 2010). An invasive population in Oregon, however, has evolved to metamorphose in as little as four months (Cook et al., 2013). A single bullfrog can lay between 1,000 and 25,000 eggs in a single clutch and population densities in breeding ponds can reach nearly 2,000 adults per acre (Adams & Pearl, 2007). Medeiros et al. (2016) examined bullfrog reproduction in a subtropical ecosystem outside of their native range, documenting the frequency of mating vocalizations and searching for spawning over the course of a year in order to assess the relationship between bullfrog reproductive activity and multiple environmental predictors. Not only where photoperiod and temperature found to be the most statistically significant predictors of reproductive activity, but bullfrogs became torpid for much shorter timespans in subtropical ecosystems than in their temperate native range (Medeiros et al. 2016). This finding suggests that bullfrogs have longer reproductive periods in tropical locations like Hawai`i, potentially remaining highly active throughout the year particularly at low elevation sites.

Water Sources and Habitat Utilization

The life history of *R. catesbeiana* determines the distribution and dispersal of this species. Like most amphibians, this obligate aquatic species modifies its utilization of various water and terrestrial resources during the different phases of its life cycle. In accordance with the dramatic ontogenetic changes associated with anuran metamorphosis, bullfrogs spend a portion of their lives on land and in water. *R. catesbeiana* individuals hatch from aquatic eggs as aquatic tadpoles that breath via gills before metamorphosing to the semi-aquatic tetrapod adult stage, breathing air using lungs and highly porous skin.

Gahl et al. (2009) examined bullfrog occupancy patterns and movement between breeding ponds and ephemeral water sources. This study used mark-resighting and radio telemetry techniques over two breeding periods per wetland sampling area. Bullfrogs appear to preferentially occupy ponds with depth of 20 centimeters or greater and disperse when the water levels drop below this depth. However, ephemeral ponds provide food subsidy and refugia that allow for greater dispersal distances, and provide important non-breeding habitat for juvenile and subadult frogs. Ficetola et al. (2010) used habitat suitability models to determine the relationship between historical land use patterns and bullfrog distribution using MAXENT, a machine learning program. This study found a strong association between bullfrog presence and agricultural lowlands, which were characterized by complex networks of ditches, reservoirs, and other permanent water sources. Environmental conditions that correlated with bullfrog dispersal and establishment include summer and winter precipitation, permanent waterbodies, high maximum temperatures, and proximity to anthropogenically modified habitat (Ficetola et al., 2007).

Dispersal and Movement Patterns

Amphibian populations experience regular patterns of local extinction and repopulation based on hydrological regimes (Peterson et al., 2013). The ability of this species to disperse and move between water sources is therefore necessary for the establishment of stable metapopulations. Bullfrogs have been documented migrating distances of up to 1.6 kilometers, and greater mobility has been observed in female and subadult bullfrogs due to the territoriality of adult male frogs (Gahl et al., 2009). Gahl et al. (2009) revealed that adult males regularly traverse between breeding ponds (long-term water sources) and non-breeding ephemeral, seasonal pools. This suggests a broad impact on wetland ecosystems, in that adult bullfrogs are not restricted to long-term water sources and regularly predate on species in ephemeral water bodies. Hawai`i waterbirds have also been known to exploit ephemeral wetlands (Engilis & Pratt, 1993) and therefore may be vulnerable to chick predation and diet overlap with bullfrogs at these sites (USFWS biologist Kauaoa Frailoa, personal communication, 2020). Youngquist and Boone (2014) examined how bullfrog movement changed based on habitat edge permeability in agricultural landscapes by releasing individuals at the interface of two habitats and tracking frog movement with fluorescent pigment powder. Bullfrogs tended to move along edges of agricultural fields due to their structural simplicity, suggesting that agricultural landscapes and habitat fragmentation facilitate bullfrog dispersal (Younguist & Boone, 2014).

Plasticity and Microevolution

Bullfrogs may exhibit measurable adaptive morphological differences among Hawai`ian populations as well as between the Hawai`ian specimens and those collected in their native range and invasive continental range. Morphological divergence has been documented in recently isolated populations in association with a number of biotic factors. Van Kleeck et al. (2015) recently examined the relationship between exploited prey characteristics and head-size in invasive Jackson's chameleons, generalist predators much like the bullfrog, in the Hawai`ian islands. Van Kleeck et al. (2015) identified a significant positive correlation between exploited prey hardness and chameleon head-size, as consumption of harder prey requires larger, stronger jaws. There was also a correlation between head size and precipitation; harder prey are generally more resistant to desiccation and can thrive in arid habitats. Morphological differentiation occurred in these isolated island populations over only 50 years. A similar study examined head size of geckos on islands created by the development of a hydroelectric dam and subsequent valley flooding (Amorim et al., 2017). These recently isolated gecko populations rapidly diverged from their mainland counterparts over approximately 15 years, increasing in head size as their dietary niche breadth increased due to the local extinction of their dominant competitor species (Amorim et al., 2017).

As noted by Van Kleeck et al. (2015), this morphological divergence in recently isolated populations may be the result of phenotypic plasticity or rapid microevolution. Phenotypic plasticity is the phenomenon by which genetically identical individuals differ phenotypically for particular traits, in different environments, based on differential gene expression (Gomez-Mestre & Jovani, 2013). Plasticity can be an important ecological and evolutionary factor in the successful establishment, range expansion, and adaptive capacity of an invasive species in novel habitats (Winwood-Smith et al., 2015). This may be especially true when there is limited genetic diversity in a colonizing population that has experienced some degree of a population bottleneck during the process of introduction and release (Gomez-Mestre & Jovani, 2013).

Several studies suggest the potential for morphological plasticity to contribute to bullfrog invasion success. Research by Relyea and Werner (2000) documented morphological plasticity in bullfrog tadpoles in response to strong predation pressure and found that the presence of predatory dragonfly larvae triggered bullfrog eggs to develop into significantly shorter, wider bodied tadpoles. This response was hypothesized to be associated with increased foraging efficiency, thus decreasing the overall time and effort spent foraging. Anurans including Ranids have also been found to exhibit morphological, developmental, and behavioral plastic responses to environmental conditions during larval development, varying in relative limb and head size, duration to metamorphosis, and size at metamorphosis, among other traits (Tejedo et al., 2010).

Plasticity in traits may allow invasive *R. catesbeiana* populations to adapt successfully to novel environments despite relatively low genetic diversity in founding individuals. Bullfrogs in Hawai`i may exhibit plasticity in traits that allow for successful dispersal, predation, and competitive dominance.

Potential Bullfrog Impacts on Native Species

Impact on Water Birds

A technical report authored by J. Eijzenga for the Oahu National Wildlife Refuge Complex in 2004 provides the only comprehensive research on bullfrog impacts in Hawai`i. This unpublished report documents the causes of stilt chick mortality during two different fledging seasons. The fledging period lasts two weeks and constitutes the length of time during which hatchlings are most vulnerable to bullfrogs. Using radio telemetry, Eijzenga (2004) tagged a total of 49 one-day-old chicks and monitored their survival until fledging. Bullfrogs accounted for half of the known mortalities one year and 100% of known mortalities the next year. Eijzenga (2004) also included eight tagged stilts in Waiawa and Honouliuli (sites which were not inhabited by bullfrogs in 2004), of which only one chick did not survive to adulthood. While the results of this study suggest that bullfrogs are a substantial cause of stilt mortality, the geographic range was limited to a single wetland refuge. It is also difficult to attribute the fledging success in Waiawa and Honouliuli to the absence of bullfrogs, given the small sample size. Despite the limitations of this study, its findings reveal a serious and previously unidentified threat to waterbird hatchlings; a situation that clearly warrants further investigation. Prior to this study, there had been only one documented observation of a bullfrog predating on a Hawai`ian waterbird, specifically a moorhen chick on Kauai (Viernes, 1995).

Though Eijzenga (2004) only studied Hawai`ian Stilts, land managers believe bullfrogs are a threat to several other endemic waterbird species that utilize similar habitats (Underwood & Jackson, 2016). Three other species in particular occupy low elevation wetland habitats where bullfrogs generally reach the highest densities. These birds are the Hawai`ian Coot, the Hawai`ian Duck, and the Hawai`ian Gallinule, all of which are listed as endangered. Because the ranges of these species (including the stilt) overlap extensively, they all may face similar environmental stresses and therefore stand to benefit from the same management actions (Reed et al., 2011). In recent years, these species have experienced modest population increases on Oahu and Kauai, islands with greater areas of protected wetlands (Reed et al., 2011). Although no surveys have documented the extent or any details of bullfrog dispersal behavior in Hawai`i, citizen-science observations have verified their presence on all the main islands (iNaturalist.org). Bullfrog presence may compound issues like habitat degradation on the islands with less wetland protection.

Impact on Invertebrates

Previous analyses of bullfrog gastrointestinal contents suggest invertebrates comprise the majority of their diet, although they are highly opportunistic. While beetles, isopods, and crayfish are often the numerically dominant prey (Jancowski & Orchard, 2013), visual diet analysis is likely biased towards hard prey that require longer digestion times. Several authors have suggested that bullfrogs may be immune to natural predator defenses, consuming wasps, rough-skinned newts, scorpions, rattlesnakes, and stickleback fish (Jancowski & Orchard, 2013). A long-term study from Vancouver, Canada examined the stomach contents of 5,075 bullfrogs from 60 sites, and found the remains of 15 different classes of organisms, including frogs, newts, turtles, passerine birds, voles, and shrews (Jancowski & Orchard, 2013). Of the identifiable prey remains, 84.1% were insects, 13% were arachnids, and 4.1% were snails (Jancowski & Orchard, 2013). Odonata, or dragonflies and damselflies, were found to be an important food source for all bullfrog age classes in the springtime.

The reliance of *R. catesbeiana* on invertebrates, especially odonates and gastropods, suggests this invasive may predate on Hawai`i's highly endemic and endangered species. An environmental impact statement produced by the Bishop Museum's Hawai`i Biological Survey group noted an instance of bullfrog predation on adult Hawai`ian damselfly (*Megalagrion blackburni*) (Englund et al., 2002). A diet study on the Japanese wrinkled frog (*Glandirana rugosa*), another nonnative generalist amphibian with established populations in Hawai`i, was published in 2018 (Van Kleek & Holland, 2018). The diet of these frogs may be comparable to that of bullfrogs due to their similar foraging strategies and niche spaces, although the wrinkled frog is substantially smaller. In this study by Van Kleeck & Holland (2018), snails comprised about 5% of the prey items (by count and volume) consumed by the wrinkled frog. The rate of snail consumption is particularly concerning due to the high extinction rate of Hawai`ian land snails, estimated at 95% by Regnier et al. (2015). Bullfrogs, while common in low elevation wetlands, are evidently capable of dispersing to higher elevation habitats that overlap with some of the rarest endangered endemic snail ranges. A population has been observed at mid-elevation in the heart of pristine native forest in Poamoho Pond, where a number of endangered insects and tree snails are known to occur (Brenden Holland, personal comm.). It is highly likely that bullfrogs have multiple negative impacts on Hawai`i's fragile ecosystems.

Table 1. Bullfrog diet studies from native and non-native locations (Jancowski & Orchard, 2013).

Location	Invasive alien status	Sample size	Number of sites	Reference
Argentina: Buenos Aires	Non-native	35	3	Barrasso et al. 2009
Argentina: Calingasta	Non-native	169	1	Quiroga et al. 2015
Brazil: Minas Gerais	Non-native	113	1	Silva et al. 2009
Canada: British Columbia	Non-native	13	1	Irwin 1994
Canada: British Columbia	Non-native	150	4	Govindarajulu et al. 2006
China: Daishan Island	Non-native	121	1	Wu et al. 2005
China: Yunnan Province	Non-native	214	2	Liu et al. 2015
Cuba: Artemisa	Non-native	18	1	Garcia-Padron et al. 2021
Germany: Baden Wuerttemberg	Non-native	44	1	Laufer 2004
Japan: Hokkaido	Non-native	469	1	Sarashina & Yoshida 2021
Japan: Kyoto	Non-native	128	1	Hirai 2004
Japan: Okinawajima	Non-native	89	1	Nakamura & Tominga, 2021
Korea: Gyeonggi	Non-native	139	1	Park et al. 2018
Mexico: Baja California	Non-native	64	1	Ortiz-Serrato et al. 2013
Uruguay: Cerro Largo	Non-native	126	1	Laufer et al. 2021
USA: California	Non-native	5	1	Jennings & Cook 1998
USA: California	Non-native	30	1	Carpenter et al. 2002
USA: California	Non-native	107	2	Hothem et al. 2009
USA: Hawai`i	Non-native	44	2	Cutia et al. 2022
USA: Michigan	Native	166	2	Werner et al. 1995
USA: Missouri	Native	455	1	Korschgen & Moyle 1955
USA: Missouri	Native	4	1	Beringer & Johnson 1995
USA: Nebraska	Non-native	1	1	Bomberger Brown & Brown 2009
USA: Nevada	Non-native	28	2	Cross & Gerstenberger 2002
USA: New Mexico	Non-native	138	1	Stuart & Painter 1993
USA: New Mexico	Non-native	85	1	Krupa 2002
USA: Ohio	Native	158	1	Bruggers 1973
USA: Ohio	Native	1	1	Spetz & Spence 2009
USA: Oklahoma	Native	52	1	McCoy 1967
USA: Wyoming	Non-native	112	3	Flynn et al. 2017
Venezuela	Non-native	338	1	Diaz De Pascual & Guerrero 2008
Total for all locations		3616	43	

Diet Analysis through Visual identification

Visual identification of prey species in the consumer’s stomach, gut, and feces can characterize diet over the short duration between consumption and complete digestion, an interval with a wide range, from approximately 6 to 48 hours (Nielsen et al., 2018). Of the dietary approaches described by Nielsen et al. (2018), only visual techniques were able to characterize the life stages of ingested prey items. The results may be biased toward larger prey or those with indigestible body parts; in a diet study of predatory fish, juvenile and larval fish prey rapidly became unidentifiable visually, in only 30 to 60 minutes after ingestion, due to their soft tissues (Carreon-Martinez et al., 2011). Depending on the expertise of the observer and the extent of digestion, a large proportion of the gastrointestinal contents may be unidentifiable or identified at a low taxonomic resolution. However, visual analysis is often selected due to the low cost and simplicity of the process, and has been successfully utilized in many bullfrog diet studies, including by Jancowski and Orchard (2013) in Canada, Garcia-Padron et al. (2021) in Cuba, Liu et al. (2015) in China, and Flynn et al. (2017) in Wyoming, USA, among others.

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Chapter 2

Head Size Variation and Diet in American Bullfrog Populations in Hawai`i

Introduction

The American Bullfrog, *Rana catesbeiana*, has the largest native range of any North American amphibian, spanning from the Atlantic coast westward to the Rocky Mountains, and is the largest frog in North America, reaching 185 mm in snout-vent length (SVL) (Ortiz-Serrato et al., 2014). Widely considered one of the most successful invasive amphibians (Kraus, 2009; Lowe et al., 2000), bullfrogs have spread from their native range to become established in 40 countries on four continents - Europe, Asia, South America and Oceania (Funk et al., 2011; Lever, 2003). Known to predate on invertebrates, bats, birds, reptiles, amphibians, fish, and small mammals, bullfrogs have been implicated in devastating ecological degradation in their non-native ranges, including the extinction or extirpation of native species (Kraus, 2009; Lawler et al., 1999; Cloyed & Eason, 2017; Werner et al., 1995; Hirai, 2004). Because introduced bullfrogs may substantially alter aquatic ecosystems as their distribution expands, it is critical to understand the traits and mechanisms associated with bullfrog invasion success.

The broad ecological niche of bullfrogs in their native range likely contributes to their successful establishment in invaded habitats. Current research suggests that generalist amphibians with large native ranges have higher probability of successful invasion, having adapted to a wide range of climatic, geographic, and habitat features (Vasquez, 2006). *R. catesbeiana* exhibits relatively high morphological variability which facilitates establishment in habitats that differ considerably from the conditions in which the species evolved, a trait observed in other invasive anurans (Cloyed & Eason, 2017; Escoriza et al., 2014). Lopez et al. (2017) found that invasive bullfrog populations in Mexico occupy climatically distinct environmental niches from their native range, indicating high niche flexibility. Similarly, adaptations to exploit broad trophic resources in the native range are hypothesized to increase the invasion success of introduced species (Leivas et al., 2012; Vasquez, 2006). Invasive species can undergo environmental and dietary niche shifts in novel habitats via the mechanisms of microevolution, phenotypic plasticity, and release from predators and competitors (Davies et al., 2018). The high morphological and physiological variability found in bullfrogs may indicate a greater propensity for these processes via either genetic variation or plasticity-mediated trait variation (Ledon-Rettig et al., 2008).

Head shape and jaw width are morphological traits likely to influence the trophic niche space occupied by an invasive anuran (Cloyed & Eason, 2017). In contrast to most vertebrates, anuran jaws tend to be weak and ancillary to predation; instead, non-larval anurans rely on derived tongue projection mechanisms to capture prey (Schwenk, 2000). The gape width of anuran predators generally constrains the size of prey they can consume (Schwenk, 2000). For gape-limited anuran species, individuals with smaller gapes more commonly consume smaller prey, while individuals with wider gapes generally capture larger prey (Cloyed & Eason, 2017; Ortiz-Serrato et al., 2014). Examining gape width in five anurans, Cloyed and Eason (2017) found support for the niche variation hypothesis, which postulates that populations with broad ecological niches exhibit high phenotypic variability relative to populations with more constrained niches. High phenotypic variation and resource partitioning among bullfrogs of different gape widths decrease intraspecific competition and likely increase ecological flexibility and probability of establishment success (Cloyed & Eason, 2017).

The mechanism underlying the high phenotypic variability in bullfrog gape widths may be environmentally-induced plasticity or genetic variation (Van Kleeck et al., 2015). Genetic differentiation or adaptive radiation can occur when individuals are introduced to novel environments, experiencing both release from native selective pressures and exposure to new abiotic and biotic pressures. When ongoing genetic exchange occurs between populations, morphological differentiation between groups may be attributable to a plastic response in gene expression to environmental variables. In some cases, trait plasticity appears to evolve rapidly following colonization of a novel environment in response to changing and broadening niche availability (Svanback & Schluter, 2012). Herpetofauna, in particular, appear to exhibit morphological plasticity which contributes to localized variation and broadened norm of reaction in non-native habitats compared to other vertebrate taxa (Aguilar-Kirigin & Naya, 2013; Joahnsson, 2007; Levis & Pfennig, 2018; Losos et al., 2000; Shine et al., 2012).

Island invasion ecology provides opportunities to investigate the patterns and mechanisms underlying niche expansion and phenotypic adaptations. Geographically isolated islands, in particular, may prompt accelerated genetic and morphological variation in colonizing species (Amorim et al., 2017; Gross, 2006). Oceanic archipelagos like Hawai`i can be highly dynamic and environmentally heterogenous, with extreme elevational and thermal gradients, orographic precipitation regimes, and rain shadow effects (Barajas-Barbosa et al., 2021). This heterogeneity in climate, topography, and soil type shapes biogeographical processes, imposes clinal selective pressures and distribution constraints, and can generate rapid divergence in colonizing species (Barajas-Barbosa et al., 2021). For example, Amorim et al. (2017) observed rapid morphological divergence in lizards on newly created islands. Over only 15 years, these fragmented island populations experienced release from competitors and developed larger head sizes relative to nearby mainland lizards, accompanied by increased mean prey size and dietary niche breadth (Amorim et al., 2017). Similarly, morphological differentiation has occurred in Hawai`ian populations of Jackson’s chameleons (*Chameleo jacksonii xantholophus*), which were introduced to the archipelago in 1972 (Van Kleeck et al., 2015). Through dietary analysis and assessment of three head size variables, Van Kleeck et al. (2015) identified significant variation in head size and prey hardness among and within island populations, coinciding with precipitation in each location. Invertebrate prey cuticle hardness, which moderates desiccation risk in arid regions, has likely exerted selective pressure for enhanced bite force in geographically isolated chameleon populations in Hawai`i, resulting in rapid phenotypic divergence (Van Kleeck et al., 2015).

The establishment of *R. catesbeiana* in Hawai`i provides an opportunity to assess localized morphological adaptation to novel environmental conditions on short timescales. This species was introduced to the island of Hawai`i in 1879 to control Japanese beetles and as a food source (Bryan, 1932; Yamamoto & Tagawa, 2000), and have since spread to all of the main Hawai`ian islands (Yamamoto & Tagawa, 2000). Hawai`i's steep environmental gradients and geographic isolation from the bullfrog's native range may promote adaptive phenotypic variation both among Hawai`ian populations and between Hawai`ian and mainland populations. Following a similar approach to Van Kleeck et al. (2015), an investigation into the gape width, diet composition, and genetic diversity of Hawai`i bullfrog populations may help to elucidate the adaptive morphology and underlying mechanisms that contribute to the notorious invasion success of this species. To pursue this objective, I collected specimens from two coastal wetlands where bullfrogs occur in relatively high densities and overlap in habitat with endangered endemic Hawai`ian waterbird nesting sites. At each site, stomach contents were extracted from bullfrog individuals for analysis. I also analyze North American patterns in bullfrog morphology derived from hand-captured specimens as well as preserved museum specimens to identify adaptive traits that may have resulted from selective pressures present in Hawai`i. I hypothesize that the average head width of American bullfrog populations in Oahu differ from each other and from mainland populations.

Methods

Field Sampling

Two Hawaii sites were selected for collection of specimens based on bullfrog abundance and accessibility. The Kualoa Ranch (KU; 21.5061, -157.8540) site is a privately-owned restored taro patch located near Waikane, along Highway 83 on Oahu's Northshore. This permanent waterbody has an established bullfrog population, as well as at least two breeding pairs of endangered Hawai`ian Stilts (Genevieve Hao, personal communication, 2020). The site was selected in consultation with Kualoa Ranch land managers to enhance and assist their restoration program. The second site, James Campbell National Wildlife Refuge (JC; 21.6847,-157.9566) is managed by U.S. Fish and Wildlife Service (USFWS). These wetlands provide habitat for endangered waterbirds including the Hawai`ian Stilt (*Himantopus mexicanus knudseni*, Ae'o), Hawai`ian Duck (*Anas wyvilliana,* Koloa Maoli), Hawai`ian Coot (*Fulica alai,* 'Alae Kea), Gallinule (*Gallinula galeata sandvicensis,* `Alae `Ula). Because of the restricted access at James Campbell NWR, specimens were gaffed and frozen at -20ºC by USFWS biologists over the winter season of 2020 - 2021. At Kualoa Ranch, I surveyed along the shoreline at night from July 2020 to January 2021, using flashlights to detect adult frog eyeshine. Specimens were collected using a dipnet and humanely euthanized via benzocaine gel (Orajel), followed by pithing or a BB gunshot to the brain in accordance with the IACUC protocol. Euthanized specimens were immediately placed on ice and then transferred to a -20ºC freezer to prevent degradation of stomach contents.

Diet Analysis

Bullfrog stomach contents were identified visually to assess prey. The alimentary canal, from the lower esophagus to the pyloric sphincter, was dissected and all contents were removed for analysis. Vegetation and soft, highly degraded and unidentifiable prey matter were not included in the analysis. Visual identification of prey species was used to characterize bullfrog diet over the short duration between consumption and complete digestion, a somewhat imprecise interval from approximately 6 to 48 hours from ingestion (Nielsen et al., 2018). I quantified prey items by frequency of occurrence in bullfrog stomachs, rather than by bulk volume or weight. Although bulk quantification methods are commonly used to determine the dietary importance of prey groups, the degraded nature of stomach contents and the varying rates and stages of digestion drastically limit the accuracy of separating, identifying, and quantifying individual components (Baker et al., 2013).

Morphometric Data Collection

In addition to my own specimen collection, I conducted morphological measurements on all available *R. catesbeiana* specimens housed in the collection at the Los Angeles Natural History Museum in Los Angeles, and the Bernice Pauahi Bishop Museum in Honolulu, HI, and morphological data were obtained from University of Toronto PhD candidate Hollis Dahn. Each individual was assigned an identifier number with which its measurements were associated. Measurements of snout-vent length (SVL) and jaw width (JW, measured between tympana) were collected for each individual using Vernier millimeter calipers.

In total, morphological measurements were obtained for 294 specimens. Individuals were grouped by biologically relevant geography, in this case major North American drainage basins as defined by United States Geological Survey (cec.org, 2022). These groups are the Atlantic-

Seaboard (n=37), Atlantic-Saint Lawrence River (n=108), Colorado River (n=20), Gulf of Mexico (n=17), Hawai`i (n=91), and the mainland Pacific coastline (n=21). Drainage basin boundaries and specimen collection localities are depicted in Figure 2.

Table 2. Number of specimens from which morphological measurements were obtained by drainage basin. Table includes the name of the person who performed measurements and the source of the specimens, including Royal Ontario Museum (ROM), Natural History Museum of Los Angeles (NHMLA), and Bishop Museum of Honolulu (BM). “Wild-caught” indicates specimens were collected in the wild by the measurer.

Group	*n*	*Measured by:*	*Source*
Atlantic-Seaboard	27	Hollis Dahn	Wild-caught
	5	Hollis Dahn	ROM
	5	Heidi Cutia	NHMLA
Atlantic-Saint Lawrence River	108	Hollis Dahn	ROM
Colorado River	20	Heidi Cutia	NHMLA
Gulf of Mexico	17	Heidi Cutia	NHMLA
Hawai`i	47	Heidi Cutia	BM
	44	Heidi Cutia	Wild-caught
Pacific-Seaboard	21	Heidi Cutia	NHMLA

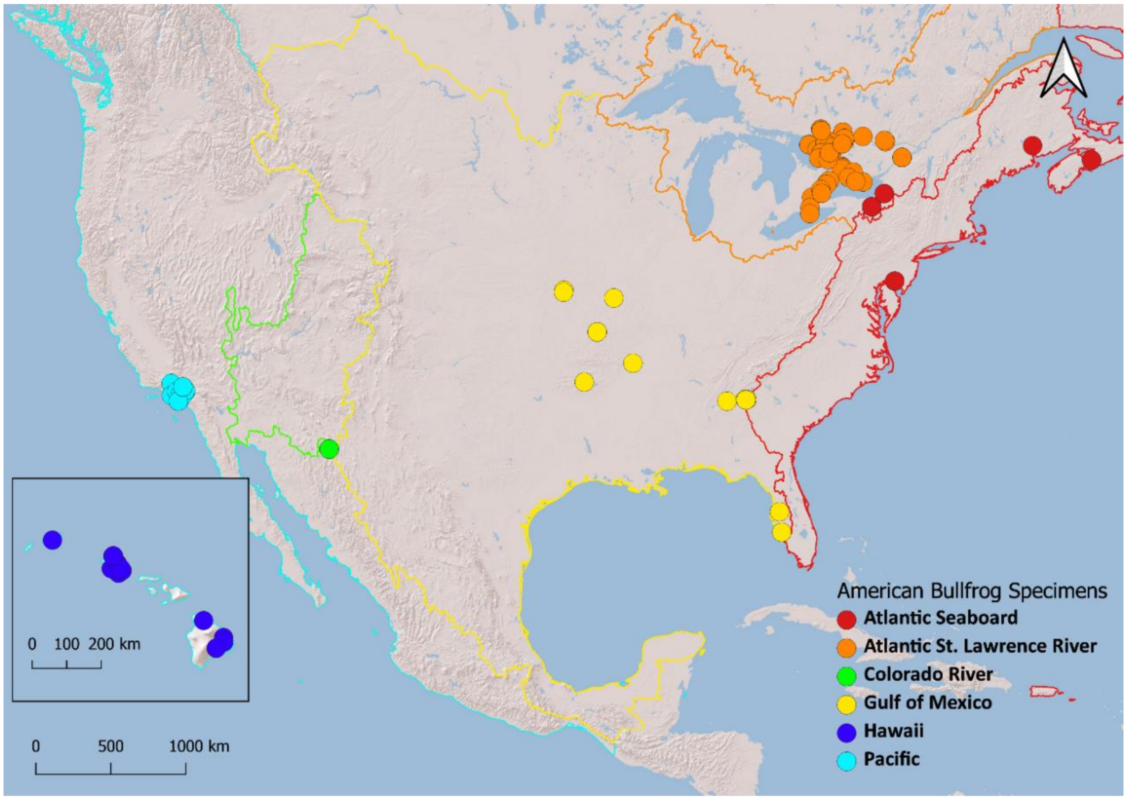

Figure 2. Locations from which *R. catesbeiana* measurements were obtained.

Statistical Analyses

To evaluate differences in average jaw width between groups, I first removed body size bias using the ratio method, wherein jaw width measurements were divided by SVL to obtain a unitless ratio (Glazier, 2022). While the Lleonart and Thorpe equation is often used to remove body size effects from morphometric data, I found jaw width growth to be highly isometric with increasing SVL, eliminating the necessity for allometric body size correction (Lleonart et al., 2000; Chan & Grismer, 2021). After ratio-correcting the tibiofibula measurements, I performed Levene's test for homogeneity of variance and determined that variances were unequal among groups (F value=6.16, df=5, p=0.00002). I performed a Welch's ANOVA on ratio-corrected jaw width between geographic groups and tested the residuals for normality. Based on the skew (-0.07), kurtosis (2.39), and quantile comparison plot, the residuals were normally distributed and the ratio-corrected jaw widths were treated as normal. A MANOVA was used to compare ratio-corrected jaw widths among geographic groups, with sex as a fixed factor. All statistical analyses were performed in R (R Core Team, 2020) and RStudio (RStudio Team, 2019), and significance was assessed at the $\alpha= 0.05$ level.

Results

Patterns of Gape Width in North American Bullfrogs

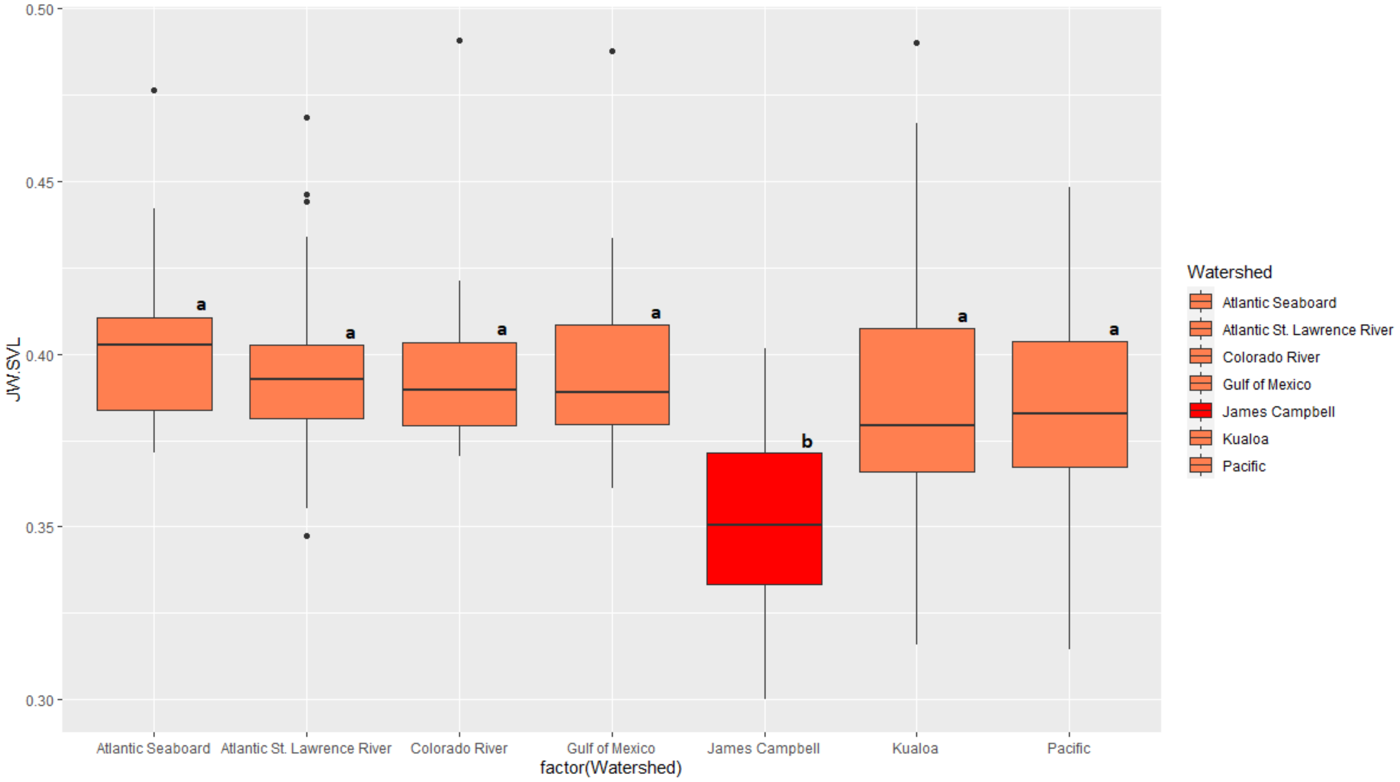

Figure 3. Boxplot of ratio-corrected jaw width by drainage basin. Letters shared between groups indicate no statistical difference between those groups at a significance level of 0.05. Colors correspond to letter designations.

Table 3. Numerical summary of average jaw widths and average ratio-corrected jaw widths by drainage basin. Table includes standard deviation (sd) and number of samples (n). Two Hawaii sites from which the majority of samples were collected are included; sample sizes for other Hawaii sites were small and therefore not included in statistical analyses.

Group	***Mean (mm)***	***Mean sd (mm)***	***Size-corrected mean***	***Size-corrected mean sd***	***n***
Atlantic Seaboard	33.19	11.46	0.40	0.020	37
Atlantic St. Lawrence River	32.73	11.47	0.39	0.019	108
Colorado River	49.30	5.40	0.40	0.027	20
Gulf of Mexico	30.06	11.97	0.40	0.032	17
Hawai`i	38.79	15.29	0.37	0.038	84
James Campbell NWR	42.80	15.62	0.35	0.027	40
Kualoa Ranch	38.83	12.21	0.38	0.037	28
Pacific	32.95	16.56	0.39	0.034	21

The MANOVA indicated a significant difference between size-corrected jaw widths among geographic locations at the $\alpha=0.05$ level (F=15.10, df = 5, $p=4.32e^{-13}$), significant differences between sexes (F=5.79, df=2, p=0.003), and a significant sex:watershed interaction (F= 4.19, df=7, p= 0.0002). A post-hoc ANOVA identified significant differences in relative jaw width among geographic groups (F= 12.79, df = 5, $p=3.29e^{-11}$). Figure 3 presents boxplots of jaw width values among groups.

Table 4. Post-hoc Tukey test comparing ratio-corrected jaw width means among geographic groups. Bolded values are significant at $\alpha=0.05$.

Comparison	***Adj p***
Atlantic St. Lawrence River - Atlantic Seaboard	0.681665
Colorado River - Atlantic Seaboard	0.990916
Gulf of Mexico - Atlantic Seaboard	0.999232
James Campbell - Atlantic Seaboard	**0.000000**
Kualoa - Atlantic Seaboard	0.317664
Pacific - Atlantic Seaboard	0.290384
Colorado River - Atlantic St. Lawrence River	0.999540
Gulf of Mexico - Atlantic St. Lawrence River	0.995132
James Campbell - Atlantic St. Lawrence River	**0.000000**
Kualoa - Atlantic St. Lawrence River	0.921544
Pacific - Atlantic St. Lawrence River	0.869467
Gulf of Mexico - Colorado River	0.999995
James Campbell - Colorado River	**0.000000**
Kualoa - Colorado River	0.906504
Pacific - Colorado River	0.854667
James Campbell - Gulf of Mexico	**0.000000**
Kualoa - Gulf of Mexico	0.841512
Pacific - Gulf of Mexico	0.780620
Kualoa - James Campbell	**0.000002**
Pacific - James Campbell	**0.000055**
Pacific - Kualoa	0.999991

Table 5. Diet composition of *R. catesbeiana* from Kualoa Ranch (KU, n=18) and James Campbell National Wildlife Refuge (JC, n=22) on Oahu, from July 2020 to January 2021. Table includes number of identified prey (NP), numerical percentage not including empty stomachs (%N), month of collection grouped by dry (July to October) and wet (November to January) months, and the number of prey from each order found in bullfrogs of each sex and size class. Total numbers of bullfrogs associated with each season, sex, and size class are listed in the final row.

			Month		*Sex*		*Size Class (mm)*			*Location*	
Prey item	**NP**	**%N**	**Jul - Oct**	**Nov - Jan**	**M**	**F**	**≤86**	**87-127**	**≥128**	**JC**	**KU**
Coleoptera	11	26.2	4	7	3	3	0	4	2	1	5
Decapoda	8	19	0	8	3	8	2	9	0	2	9
Stylommatophora	6	14.2	1	5	1	0	0	0	1	1	0
Lepidoptera	4	9.5	3	1	3	1	1	2	2	1	3
Araneae	4	9.5	0	4	0	2	0	1	1	1	1
Orthoptera	3	7.1	0	3	0	1	1	0	0	0	1
Hymenoptera	2	4.8	0	2	1	0	0	1	0	0	1
Diptera	1	2.4	0	1	2	1	0	2	1	2	1
Odonata	1	2.4	0	1	1	7	0	1	7	8	0
Ephemeroptera	1	2.4	0	1	2	2	0	1	3	3	1
Blattodea	1	2.4	1	0	1	0	0	1	0	0	1
Empty stomach	11	-	1	10	4	7	1	3	7	10	1
***R. catesbeiana* total**	-	-	14	30	15	29	5	17	18	22	18

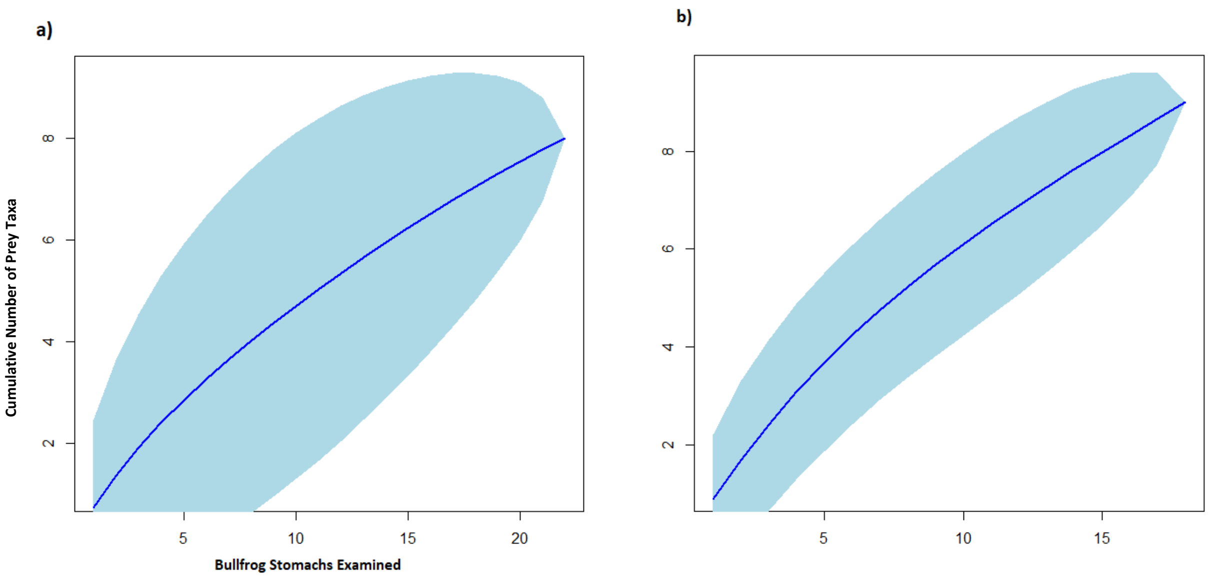

Figure 4. Species accumulation curves for a) James Campbell NWR and b) Kualoa Ranch. Horizontal axis represents number of bullfrog stomachs examined and the vertical axis represents the cumulative number of prey orders observed. The shaded areas indicate 95 percent confidence intervals. Using Chao1 correction for to account for rare species, 24 prey taxa are estimated as the asymptote for Kualoa Ranch, while 11 taxa are estimated for James Campbell NWR.

Stomach contents were obtained from 40 *R. catesbeiana* individuals (51 – 162 mm SVL) on Oahu, including 18 from Kualoa Ranch and 22 from James Campbell National Wildlife Reserve, between July 2020 and January 2021. Of these specimens, a total of 11 frogs had empty stomachs, including four males and seven females, and were excluded from analysis. Four additional female specimens contained only highly degraded and unidentifiable stomach contents. From the remaining stomachs, 42 prey items were identified to at least the order level, spanning 11 orders. The resulting data are likely biased toward larger prey or those with hard, indigestible body parts; in a diet study of predatory fish, juvenile and larval fish prey rapidly became unidentifiable visually, in only 30 to 60 minutes after ingestion, due to their soft tissues (Carreon-Martinez et al., 2011). Vertebrate bones, teeth, and claws, arthropod chitin and keratin, and mollusk calcium carbonate shell material are also generally more identifiable relative to soft tissues.

Coleoptera occurred with the greatest frequency (26.2%), followed by Decapoda (19.0%). For males, Stylommatophora, Coleoptera, and Lepidoptera were the numerically dominant prey items, while females most frequently consumed Coleoptera and Decapoda (specifically Red Swamp Crayfish, *Procambarus clarkia*). Size classes were selected following the classifications used by Ortiz-Serrato et al. (2014). Bullfrogs from the smallest size class (≤86 mm SVL) were only observed to have consumed Odonata (dragonflies and damselflies), Coleoptera (beetles), and Lepidoptera (moths and butterflies), while young adults (87 mm to 127 mm) predated upon nearly every order except Odonata and Diptera (flies), most frequently consuming Coleoptera and Stylommatophora (pulmonated gastropods). Individuals of the largest size class (≥128 mm SVL) predated upon crayfish more frequently than other prey items, and also had the greatest frequency of empty stomachs of all size classes. Rarefaction curves (Figure

4) suggest more sampling is needed at both sites to identify all common prey items, as horizontal asymptotes were not reached.

Discussion

Results of this study suggest that the Hawai`i bullfrog population at James Campbell NWR has significantly shorter body size-corrected jaw widths than any of the mainland populations examined, with the exception of the geographically nearest sampling region, the Pacific group, with an average jaw width-to-SVL ratio of 0.35 compared to the mainland's cumulative average of 0.394. While the Kualoa Ranch bullfrog population did not exhibit significant differences in jaw width from mainland groups, the size-corrected average jaw width was smaller than all mainland groups at 0.38. Despite the morphological divergence of Hawai`i populations from the mainland, the mean absolute jaw width for Hawai`i is the second highest of all groups at 38.8 mm (15.29 s.d.), reflecting the significantly larger body size found in Hawai`i populations compared to the Atlantic-Seaboard, Atlantic-Saint Lawrence River, Gulf of Mexico, and Colorado River populations (see Chapter 3). This phenotypic differentiation in Hawai`i populations may be a result of exposure to certain environmental conditions during larval development or selective pressures unique to these island ecosystems.

Intraspecific variation in relative head width is associated with both the individual and population food niche breadth and trophic ecology. Larger prey may provide an energetic advantage that diminishes at larger body sizes as metabolic requirements increase (Amorim et al., 2017). Increasing head size relative to body size allows for the consumption of larger available prey, without significantly increasing handling time or metabolic energy requirements, a process which has been observed on relatively short timescales in recently isolated populations of both geckos and chameleons (Amorim et al., 2017; Van Kleeck et al., 2015). In some anuran genera, relative head width also positively correlates with more generalist diets, with narrow heads indicating specialization (Toft, 1995). The greater relative head width in mainland bullfrog populations may indicate predominantly larger prey or more generalist feeding behavior; conversely, the small relative gape width and large body size observed in Hawai`i bullfrog populations may reflect specialization toward abundant small prey and a lack of selective pressure to consume larger prey. However, the large body size and absolute gape width in these populations suggest that access to large prey is not diminished by this morphological divergence, at least for the larger size classes of bullfrogs. It is important to note that despite the narrower heads observed in frogs from James Campbell NWR, the largest prey type identified (crayfish) was consumed with high frequency at this location.

Patterns in total body size may be influenced by a number of other factors, including temperature, precipitation, and length of growing season (refer to Chapter 3 for further background on geographic patterns and drivers in bullfrog body size); however, factors that increase the overall growth rate in *R. catesbeiana* may not affect growth rate of all skeletal components proportionately (Blouin & Brown, 2000). The environmental conditions that promote large body size in Hawai`i bullfrog populations therefore may not increase the head width at the same rate. Because the absolute head width observed in Hawai`i is large relative to the other populations in this study, average-sized bullfrogs in this region likely consume prey of similar or larger size than most mainland populations.

The small relative gape width observed in Hawai`i bullfrogs may result from plastic responses during larval development to environmental conditions. Four key ecological factors during larval development have been observed to drastically influence post-metamorphic anuran morphology: temperature, type and quantity of available trophic resources, pond desiccation and presence of predators (Tejedo et al., 2010). Tejedo et al. (2010) conducted a meta-analysis to elucidate relationships between environmental variables and plasticity in anuran head shape. Unlike other genera included in this study, post-metamorphic *Rana* individuals appeared to exhibit narrower heads relative to body length when exposed to food scarcity during their larval stage. This pattern may also occur in *R. catesbeiana*, providing a possible explanation for the head size patterns I observed; Hawai`i bullfrog larvae experience caloric limitation via either greater competition or fewer available resources. Release from predators and parasites often allows introduced species to reach greater population densities than in their native habitat, which could generate greater intraspecific competition than experienced in their native range (Govindarajulu et al., 2006). However, environmental stresses including food scarcity and competition are generally associated with smaller body size and reduced body condition at metamorphosis, which correlate closely with mature post-metamorphic body size (Crespi & Warne, 2013). Bullfrogs in Hawai`i display larger body sizes than most mainland populations (see Chapter 3), potentially challenging resource scarcity as an explanation for smaller gape widths in these populations.

In addition, Tejedo et al. (2010) noted that higher temperatures during larval development also resulted in a reduction in head width across all of the species included in this comparative analysis. Previous research suggests that increased temperature results in more rapid anuran larval development and smaller body size at metamorphosis, again leading to smaller average sizes in adulthood (Atkinson, 1994, 1996; Richter-Boix et al., 2006; Brady & Griffiths, 2000). However, a common garden study of temperature-induced plasticity in closely clustered populations of the frog *Pelophylax lessonae* found strong differentiation in plastic responses among populations, with diverging responses to temperature treatments among populations

(Orizaola & Laurila, 2009). Orizaola & Laurila (2009) posited that local environmental conditions including temperature, desiccation, and predation risk can select for adaptive microgeographic variation in trait plasticity. *R. catesbeiana* may exhibit a plastic response to increased temperature that differs from previous *Rana* studies or even between populations.

In this study, Hawai`i bullfrog populations present the greatest variance in relative gape width of all geographic groups. Cloyed and Eason (2017) assessed anuran gape widths in relation to the Niche Variation Hypothesis (NVH), which postulates that populations with wider ecological niches will exhibit greater phenotypic variability than populations with narrower niches at both the interspecific and intraspecific level. Intraspecific niche variation benefits populations by decreasing competition, where varied phenotypes among individuals allow for the exploitation of different prey resources, with the tradeoff that each individual niche breadth may be limited. Cloyed and Eason (2017) observed that diet variation among individuals of a species and variation in relative gape width were correlated with the niche breadth of that species. *R. catesbeiana* exhibited the greatest coefficient of variation for relative gape width compared to the other four anurans in the study (Cloyed & Eason, 2017). The high variation in gape width of Hawai`ian bullfrog populations may suggest that this group exploits a greater niche breadth than mainland populations and may limit intraspecific competition via the differentiation of individual diets.

In previous *R. catesbeiana* diet studies a number of patterns in prey preference have emerged. Insects are often the numerically dominant available prey, especially beetles (Coleoptera) (Korschgen & Moyle, 1955; Bruggers, 1973; Werner et al., 1995; Hirai, 2004; Barrasso et al., 2009; Hothem et al., 2009), although other arthropods including isopods and crayfish are also commonly exploited (Bruggers, 1973; Krupa, 2002; Ortiz-Serrato et al., 2014).

In Missouri farm ponds, native bullfrogs consume crayfish, Odonates, Lepidopterans, and ground beetles with the greatest frequency (Korschgen & Moyle, 1955), while in California, Coleoptera, Hemiptera, and Araneae are the dominant prey (Hothem et al., 2009). The prey items identified in Hawai`i bullfrog specimens spanned 11 taxonomic orders, the most frequent being Coleoptera, followed by Decapoda and Stylommatophora. Prey identified to the species level included the rosy wolfsnail (*Euglandina rosea***)**, giant African land snail (*Lissachatina fulica*), cane spider (*Heteropoda venatoria*), and Red Swamp Crayfish (*Procambarus clarkia).*

Although the occurrence frequencies of prey taxa observed at each Oahu site are distributed similarly to other locations, the reduced head width of bullfrogs in Hawai`i may shift the prey types consumed by each size class compared to other populations. For example, in a Baja California population, crayfish were the second most important prey to bullfrogs of the two smaller size classes (≤86 and 87 – 128 mm SVL) (Ortiz-Serrato et al., 2014) and juveniles (averaging 95 mm SVL) in a northern California population consumed birds, mammals, and fish (Hothem et al., 2009). These findings contrast with the diet of the smallest size class in Hawai`i, which included only small insects. Korschgen and Moyle (1955) observed that bullfrog densities increased in ponds after crayfish introduction, suggesting that crustaceans represent an important food source. A systematic review of bullfrog trophic ecology found strong evidence that this species often narrows its diet in invaded ranges, and noted that crayfish were often over-represented in stomach contents relative to their abundance in the community (Bissatini & Vignoli, 2017). Bissatini & Vignoli (2017) suggested that crayfish mitigate the detrimental effects of bullfrog predation in invaded locations. However, if only the larger size classes of Hawai`i consume crayfish, then smaller size classes of bullfrogs may rely on smaller prey.

Although bullfrogs in other regions frequently consume chordates (Jancowski & Orchard, 2013; Flynn et al., 2017; Barrasso et al., 2009; Wu et al., 2005), none were identified in the course of this study. However, because visual analysis can only capture dietary information on relatively short timescales, co-occurring chordates, including fish, turtles, birds, juvenile rodents, and other invasive anurans, may still contribute to bullfrog diet in Hawai`i. Bullfrogs have been observed to predate on the endangered Hawai`ian stilt (*Himantopus mexicanus knudseni*, Ae‘o) (Eijzenga, 2004) and likely opportunistically consume other wetland-nesting waterbirds. Due to restricted access at James Campbell NWR during waterbird nesting season, bullfrog specimens were not collected during the nesting or fledging period, and therefore waterbird hatchlings were not available prey during the sampling period. An environmental impact statement produced by the Bishop Museum's Hawai`i Biological Survey group also noted an instance of bullfrog predation on an endemic adult Hawai`ian damselfly (*Megalagrion blackburni*) (Englund et al., 2002). Bullfrogs at higher-elevation streams have a greater probability of interacting with native Hawai`ian invertebrates.

Stable isotope analysis and dietary DNA analysis may provide greater insight into the trophic interactions of *R. catesbeiana* in Hawai`i. Data on small, soft-bodied, or infrequently consumed prey are likely not accurately encapsulated by visual stomach contents analysis. The reduction in head width observed in Hawai`ian bullfrog populations may influence their food resource exploitation in more pristine, higher elevation streams. Although the presence of invasive crayfish can mitigate the damaging ecological effects of invasive bullfrogs in other regions (Bissatini & Vignoli, 2017), juvenile and young adult frogs in Hawai`i may have limited ability to consume large, hard-shelled freshwater crustaceans. Insects are considered relatively poor sources of calcium (Latney & Clayton, 2014), so this population may exhibit increased consumption of gastropods to fulfill nutritive calcium requirements, a dietary shift that would have implications for Hawai`i's endemic and endangered snails, including the aquatic Newcomb's snail (*Erinna newcombi*). Further research on this topic is essential to understand how *R. catesbeiana* impacts Hawai`i's vulnerable aquatic ecosystems and the morphological changes that alter this species' ecology in novel habitats.

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Chapter 3

Body Size and Limb Length Variation in Invasive Bullfrog Populations

Introduction

Several biogeographical rules have been proposed to describe intraspecific and interspecific patterns in body size based on climate, latitude, and population insularity. These macroevolutionary principles may have predictive value as climate change and human activity shift species distributions and alter regional environmental conditions, but the generality and underlying causal mechanisms of these principals remain contentious (Teplitsky & Millien, 2013; McCoy, 2012; Millien et al., 2006; Sheridan & Bickford, 2011; Pincheira-Donoso et al., 2008; Rollinson & Rowe, 2018). Because body size can correlate with a number of ecological, physiological, and natural history factors, including local habitat features such as food availability and temperature, rate of metabolism, population density, and life history including the duration of juvenile development, the evaluation of these large-scale biogeographic patterns is complicated (Olalla-Tarraga & Rodrigo, 2007; Lomolino et al., 2006; Vinarski, 2014). In particular, a species' method of thermoregulation, or lack thereof, may influence how biogeographical laws relating to climate and temperature can be applied. Species that thermoregulate endothermically, like mammals, or behaviorally, like frogs, exhibit different clinal patterns than thermoconforming species such as salamanders (Olalla-Tarraga & Rodrigo, 2007). Thus, the existence and robustness of geographical body size patterns in ectothermic taxa remains controversial (Olalla-Tarraga & Rodrigo, 2007; Vinarski, 2014). Investigating these trends as they relate to ectotherms may provide valuable insight into the effects of rapid anthropogenically-driven shifts in global species distribution (Boivin et al., 2016; Blackburn et al., 2018).

Among the most frequently discussed geographical patterns in body size are Bergmann's rule, the converse Bergmann's rule, Allen's rule, and Foster's rule (Bergmann, 1847; Allen, 1877; Mousseau, 1997; Foster, 1963). Bergmann's rule states that organisms of a species in cooler climates will be larger than their interspecific or conspecific counterparts in warm climates (Bergmann, 1847). This pattern, originally developed for endotherms, is based on the organism's surface-area-to-volume ratio. In cold climates, the organism's relative surface area is reduced to limit heat loss to the environment. Similar to Bergmann's rule, Allen's rule hypothesizes that organisms in colder climates will have shorter limbs than their counterparts in warmer climates to conserve body heat (Allen, 1877). The converse Bergmann's rule suggests that body size decreases with increasing latitude due to shorter growing seasons, which limit the time for foraging, development, and growth (Blanckenhorn & Demont, 2004). Some research supports thermoregulating ectotherms, such as anurans that display heat conserving behavior, exhibiting body size gradients along climatic clines (Olalla-Tarraga & Rodrigo, 2007; Ashton, 2002; Blanckenhorn & Demont, 2004), though this trend is not consistent (Hu et al., 2011; Lui et al., 2018). The Island rule, also known as Foster's rule, describes a pattern in which insular populations of small organisms trend toward gigantism and large organisms trend toward dwarfism in relation to their mainland counterparts (Foster, 1963). Potential mechanisms for this phenomenon include reduced predation and competition and a shift in resource availability.

Hawai`i, as an isolated archipelago with a relatively warm tropical climate, presents an interesting challenge in the application of these biogeographical rules. Whereas Foster's rule and the inverse Bergmann's rule may predict an increase in body size relative to mainland populations, Bergmann's Rule may suggest a decrease in body size relative to conspecifics at higher latitudes of North America. In particular, recently introduced species in Hawai`i pose an opportunity to assess how morphological changes, as predicted by biogeographical rules, occur on a short timescale through either plastic or genetic mechanisms. Established non-native populations can be compared to their origin populations from which they have been isolated to understand adaptive responses to novel climates. Hawai`i's sensitive and highly endemic ecosystems face an emerging threat in the growing number of invasive species now established on the island chain (Juvik & Juvik, 1984; Chiaverano & Holland, 2014; Shell & Rehan, 2019; Joe & Daehler, 2008; Messing et al., 2007; Plentovich et al., 2021). In a global assessment, the Hawai`ian islands host among the greatest richness of established non-native species (Dawson et al., 2017). Exploring climatic patterns in invasive ectotherm morphology may contribute to our understanding of their local impacts and global colonization ability.

Herpetofaunal body size trends likely have important implications for Hawai`i's ecosystems, which have been colonized by at least 26 non-native reptiles and amphibians. Body size influences how an individual interacts with the ecosystems via competition, predation, and habitat utilization (Wang et al., 2007). Hirai (2002) studied ontogenetic shifts in the diet of the pond frog (*Pelophylax nigromaculata),* an East Asian ranid that may have established populations on Oahu. As pond frogs increase in size, they exploit larger prey taxa and forego less energetically profitable smaller taxa (Hirai, 2002). Pond Sliders (*Trachemys scripta*) collected from Kawai Nui Marsh on Oahu also exhibit significant dietary shifts with growth, with the largest size class consuming a greater proportion of animal matter relative to plant matter compared to smaller size class individuals (Works & Olson, 2018). Articulating biogeographic body size trends of non-native Hawai`ian herpetofauna can allow us to understand their habitat utilization and resource exploitation in their current distributions and to predict how these species will adapt to novel environments.

The American Bullfrog (*Rana catesbeiana*) is a notoriously destructive invasive amphibian, whose adaptive morphology is of particular significance to Hawai`ian aquatic ecosystems. Like most anurans, bullfrogs are gape-limited predators (Vignoli et al., 2017). An increased average body size may result in predation on larger and potentially higher trophic level prey; as their body size increases, the percentage of insects in their diet appears to decrease (Carpenter & Morrison, 1973). Here, I assess North American patterns in bullfrog body size and leg length, comparing average snout-vent and tibiofibula length among Hawai`ian populations and both native and non-native mainland populations. I evaluate correlations between body size and climatic variables to assess support for Bergmann's and Allen's rule in this *Rana* species. With these data, I hope to expand the scientific understanding of biogeographic rules, as they relate to a highly invasive amphibian species and ectotherms in general.

Methods

Field and Museum Data Collection

Two sites were selected for collection of specimens based on bullfrog abundance and accessibility. The Kualoa Ranch (KU; 21.5061, -157.8540; n = 22) site is a privately-owned restored taro patch located near Waikane, along Highway 83. This site has an elevation of approximately 10 feet above sea level and ranges between an annual temperature minimum of approximately 19.5 ºC and an annual temperature maximum of approximately 28 ºC (Giambelluca et al., 2014). Total monthly rainfall ranges from approximately 90 mm in summer months to 220 mm in winter months. This permanent waterbody has an established bullfrog population, as well as at least two breeding pairs of endangered Hawai`ian Stilts (Genevieve Hao, personal communication, 2020). The site was selected in consultation with Kualoa Ranch land managers to enhance and assist their restoration program. The second site, James Campbell National Wildlife Refuge (JC; 21.6847,-157.9566; n = 22) lies along Oahu's north shore and is managed by U.S. Fish and Wildlife Service (USFWS). This site has an elevation of approximately 5 feet above sea level and ranges between an annual temperature minimum of approximately 15 ºC and an annual temperature maximum of approximately 20.5 ºC (Giambelluca et al., 2014). Total monthly rainfall ranges from approximately 45 mm in summer months to 135 mm in winter months. These wetlands provide habitat for endangered waterbirds including the Hawai`ian Stilt (*Himantopus mexicanus knudseni,* Ae‘o), Hawai`ian Duck (*Anas wyvilliana,* Koloa Maoli), Hawai`ian Coot (*Fulica alai,* ‘Alae Kea), Gallinule (*Gallinula galeata sandvicensis,* `Alae `Ula). Because of the restricted access at James Campbell NWR, specimens were gaffed and frozen at -20ºC by USFWS biologists over the winter season of 2020 - 2021. At Kualoa Ranch, I surveyed along the shoreline at night from July 2020 to January 2021, using flashlights to detect adult frog eyeshine. Specimens were collected using a dipnet and humanely euthanized via benzocaine gel (Orajel), followed by pithing or a BB gunshot to the brain in accordance with the IACUC protocol. Euthanized specimens were immediately placed on ice and then transferred to a -20ºC freezer to prevent degradation of stomach contents.

In addition to my own specimen collection, I conducted morphological measurements on all available *R. catesbeiana* specimens at the Los Angeles Natural History Museum and Bernice Pauahi Bishop Museum and obtained further morphological data from University of Toronto PhD candidate Hollis Dahn.

Morphological Analyses

Each individual was assigned an identifier number with which its measurements were associated. Measurements of snout-vent length (SVL) and tibiofibula length (TB) were collected for each individual using Vernier millimeter calipers, with a resolution of 0.02 mm. In total, morphological measurements were obtained for 294 specimens (Table 6 and Appendix Table 13).

Individuals were grouped by biologically relevant geography, in this case major North American drainage basins as defined by United States Geological Survey (cec.org, 2022). These groups are Atlantic-Seaboard (AS, n=37), Atlantic-Saint Lawrence River (ASLR, n=108), Colorado River (CR, n=20), Gulf of Mexico (GOM, n=17), and Pacific (PAC, n=21). Hawaii specimens were primarily from two sites, James Campbell NWR (n = 40) and Kualoa Ranch (n = 28) including any specimens found within 5 miles of each site. Other Hawaii sites were not included in MANOVA and ANOVA tests due to insufficient sample size. I tested for significant differences in SVL between groups with a MANOVA test, with sex as a fixed factor, followed by an ANOVA tested for SVL differences between geographic groups and a post-hoc Tukey test.

To evaluate differences in average tibiofibula length between groups, I first removed body size effects using the ratio method. Tibiofibula measurements were divided by SVL measurements to obtain a unitless ratio. While the Lleonart and Thorpe equation is often used to remove body size effects from morphological data, I found tibiofibula growth to be highly isometric with increasing SVL, eliminating the necessity for allometric body size correction (Lleonart et al., 2000). After ratio-correcting tibiofibula measurements and testing for normality and homoscedasticity, I performed a MANOVA test to evaluate differences in average TB between geographic groups with sex as a fixed factor, followed by an ANOVA of TB differences between geographic groups and a post-hoc Tukey test.

Environmental Correlations

Latitudinal patterns in body size are often hypothesized to be driven by temperature, precipitation, or productivity-related factors. The following datasets were selected as potential explanatory variables for variation in SVL: latitude; longitude; yearly maximum, mean, and minimum normalized difference vegetation index (NDVI) (via NASA, 2020); maximum, average, and minimum monthly average air temperature per year in degrees Celsius (UDel Air Temperature dataset, 2017); and maximum, average, and minimum monthly average precipitation per year in millimeters (UDel Precipitation dataset, 2017). I reduced the number of explanatory variables by first using Pearson correlations to assess their relationships with SVL and then selecting the variables with the greatest significance for further analysis. Due to multicollinearity between the environmental variables (see Appendix, Table 14), a principal components analysis (PCA) was conducted and multiple regressions were performed with single principal components (PCs) and with combinations of two and three PCs. Akaike Information Criterion evaluated these models to identify the regression with the greatest explanatory power and least overfitting for SVL.

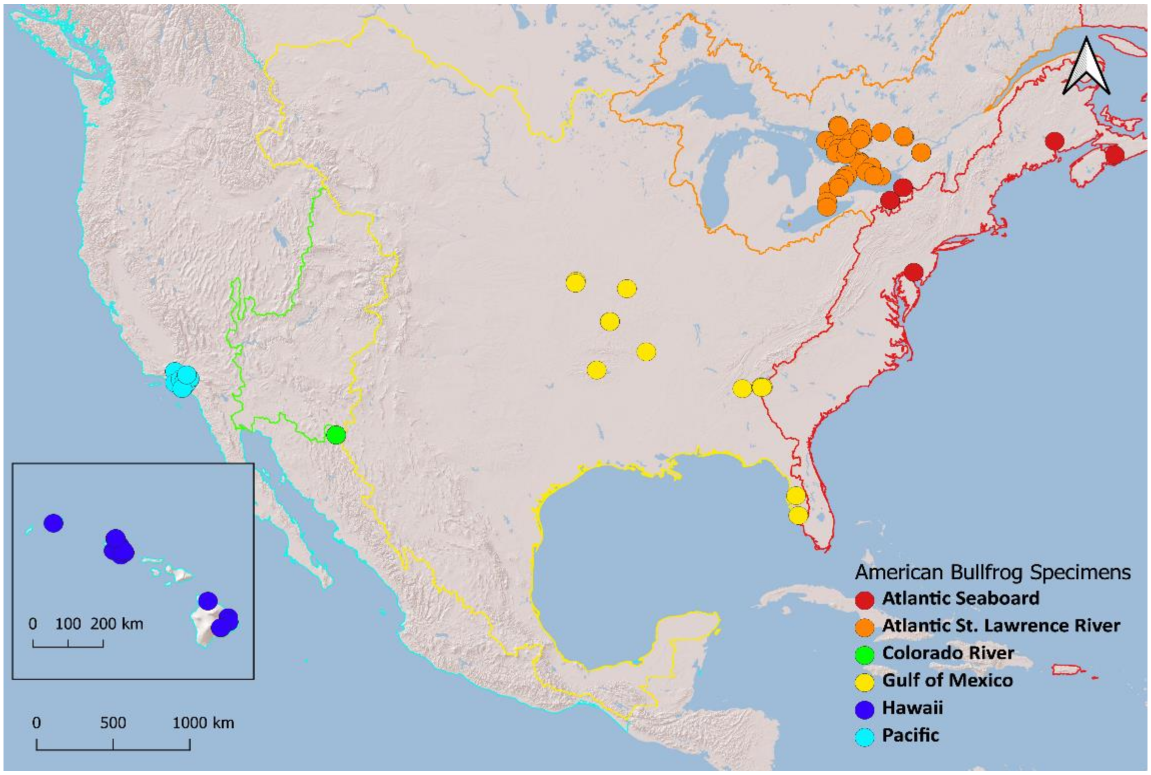

Figure 5. Map of North American drainage basins and locations from which *R. catesbeiana* morphological measurements were obtained.

Results

Snout-Vent Length

No outliers in SVL were identified via boxplot or Grubbs test (p=1). Normality of each group was checked by the Shapiro-Wilk method, and the results suggest apparent violation of the assumption for the Atlantic Saint Lawrence River and James Campbell populations (W=0.96, p=0.021 and W=0.76, p=1.76e^{-6} respectively). Levene's test of homogeneity also indicated unequal variances occur (F=3.03, df=6, p=0.007). However, the Box and Cox maximum likelihood method to select a transformation suggested that no transformation was needed (λ = 1.84135, df= 1, p = 0.174). A Welch's ANOVA was conducted and the residuals were normally distributed, based on Q-Q plot, histogram, skew, and kurtosis. The parametric MANOVA test was therefore performed and identified statistically significant differences between sexes (F=29.69, df=2, p=2.46e^{-12}) and watersheds (F=13.78, df=6, p=1.39e^{-13}), as well as a significant sex:watershed interaction (F=5.41, df=9, p=8.40e^{-7}). A follow-up ANOVA confirmed significant differences in SVL between geographic groups (F=14.34, df=6, p=3.42e^{-14}), and a Tukey test evaluated pairwise differences between groups.

Table 5. Numerical summary of snout-vent length (mm) by drainage basin. This table includes sample size (n), mean, standard deviation (SD), the interquartile range (IQR), and the 0th, 25th, 50th, 75th, and 100th percentile. The two Oahu sites from which most specimens originated (James Campbell National Wildlife Refuge [JC] and Kualoa Ranch [KU]) are included.

	n	**Mean**	**SD**	**IQR**	**0%**	**25%**	**50%**	**75%**	**100%**
Atlantic Seaboard	37	82.92	28.52	45.79	40.59	58.21	83.55	104	131.69
Atlantic St. Lawrence River	108	83.22	29.42	46.72	33.92	56.18	86.95	102.90	150.63
Colorado River	20	125.10	15.89	19.25	95	115.75	126.5	135	152
Gulf of Mexico	17	76.00	30.49	53	30	55	73	108	116
Hawai`i	90	108.80	40.86	68.75	28	75	121.5	143.75	164
Oahu - JC	46	123.11	40.52	29.5	28	119.5	140.5	149	164
Oahu - KU	27	101.48	29.19	44	38	80.5	112	124.5	141
Pacific	21	83.67	37.37	61	35	55	73	116	146

Average SVL of bullfrogs from the Colorado River drainage was found to be significantly different from the Atlantic Seaboard, Atlantic - Saint Lawrence River, Pacific, and Gulf of Mexico. Average SVL of bullfrogs from Kualoa Ranch in Hawaii were significantly different from the Atlantic - Saint Lawrence River drainage, while average SVL of bullfrogs from James Campbell NWR differed from the Atlantic Seaboard, Atlantic – Saint Lawrence River, Gulf of Mexico, and Pacific specimens.

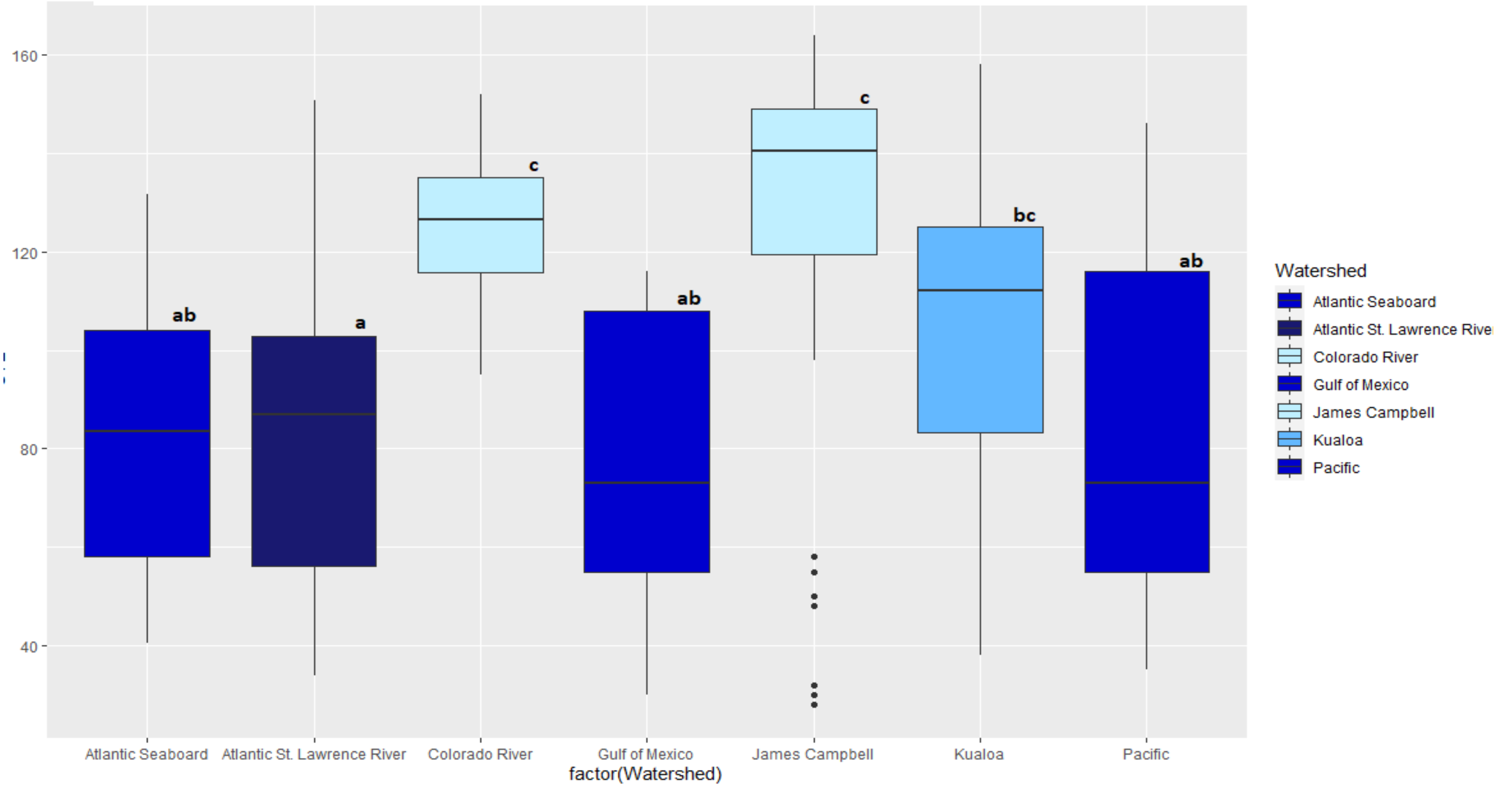

Figure 6. Boxplot of SVL (mm) by drainage basin. Letters shared between groups indicate no statistical difference between those groups at a significance level of 0.05. Colors correspond to letter designations.

Table 6. Tukey test, comparison of SVL by group. Bolded values are significant at α=0.05.

Comparison	P.adj
Atlantic St. Lawrence River - Atlantic Seaboard	1.0000000
Colorado River - Atlantic Seaboard	**0.0000494**
Gulf of Mexico - Atlantic Seaboard	0.9892444
James Campbell - Atlantic Seaboard	**0.0000005**
Kualoa - Atlantic Seaboard	0.1287430
Pacific - Atlantic Seaboard	1.0000000
Colorado River - Atlantic St. Lawrence River	**0.0000023**
Gulf of Mexico - Atlantic St. Lawrence River	0.9756121
James Campbell - Atlantic St. Lawrence River	**0.0000000**
Kualoa - Atlantic St. Lawrence River	**0.0420955**
Pacific - Atlantic St. Lawrence River	1.0000000
Gulf of Mexico - Colorado River	**0.0000774**
James Campbell - Colorado River	0.9999856
Kualoa - Colorado River	0.2293943
Pacific - Colorado River	**0.0006998**
James Campbell - Gulf of Mexico	**0.0000060**
Kualoa - Gulf of Mexico	0.0722282
Pacific - Gulf of Mexico	0.9895640
Kualoa - James Campbell	0.1320017
Pacific - James Campbell	**0.0000678**
Pacific - Kualoa	0.3108263

Table 7. Pearson's correlation coefficients, p-value, and degrees of freedom between SVL and 11 environmental variables. Bolded values are statistically significant at α=0.5.

	Pearson's r	p-value	df
Latitude	-0.324	**$2.297e^{-8}$**	282
Longitude	-0.321	**$3.011e^{-8}$**	282
Yearly NDVI maximum	-0.081	0.174	282
Yearly NDVI mean	-0.350	**$1.281e^{-9}$**	282
Yearly NDVI minimum	-0.228	**$1.031e^{-4}$**	282
Maximum monthly average air temperature	0.330	**$1.179e^{-8}$**	282
Mean monthly average air temperature	0.340	**$4.083e^{-9}$**	282
Minimum monthly average air temperature	0.332	**$1.034e^{-8}$**	282
Maximum monthly average precipitation	-0.076	0.2002	282
Mean monthly average precipitation	-0.167	**$4.842e^{-3}$**	282
Minimum monthly average precipitation	-0.194	**$9.852e^{-4}$**	282

Principal components analysis (PCA) was run across the four environmental variables selected for analysis (latitude [$r(282) = -0.322$, $p = 2.686e^{-8}$]; mean monthly average air temperature [r(282) = 0.338, p= $3.627e^{-9}$]; minimum average monthly precipitation [r(282) = -0.188, p=0.0013], and yearly mean NDVI [r(282) = -0.350, p-value = 8.349e-10). Environmental variables with loadings greater than 0.5 or less than –0.5 were identified as providing a strong contribution to the principal component. The first component accounted for 76 percent of the variance, while PC1 and 2 cumulatively accounted for 96 percent of the variance, and PC1, 2, and 3 accounted for 99 percent of the variance in total. Regressions were performed for each PC individually, for all combinations of PCs 1, 2, and 3, and for all three variables together, with SVL as the response variable. All 7 PC regressions, as well as separate regressions for each environmental variable, were assessed using the Akaike Information Criterion (AIC). The model with the lowest AIC score included only PC1 as a predictor variable.

Component loadings:	**PC1**	**PC2**	**PC3**
Latitude	**0.555088**	0.19982	0.255351
Yearly mean NDVI (2020)	**0.554834**	0.106032	**-0.80954**
Mean monthly average air temperature (2017)	**-0.55519**	-0.1699	**-0.52556**
Minimum average monthly precipitation (2017)	0.275322	**-0.95915**	0.056798

The fitted regression model was: SVL = 94.02 + (-7.276)PC1. The overall regression was statistically significant (adjusted $R^2 = 0.1207$, F(1, 282) = 39.86, p = $1.06e^{-9}$), and both the intercept (p < $2e^{-16}$) and PC1 were significant (p = $1.06e^{-9}$). The AIC score for this model was 2812.97.

Tibiofibula Length

I first divided each tibiofibula length measurement by its associated SVL measurement to remove the effect of body size. After ratio-correcting tibiofibula data, I tested for normality and outliers.

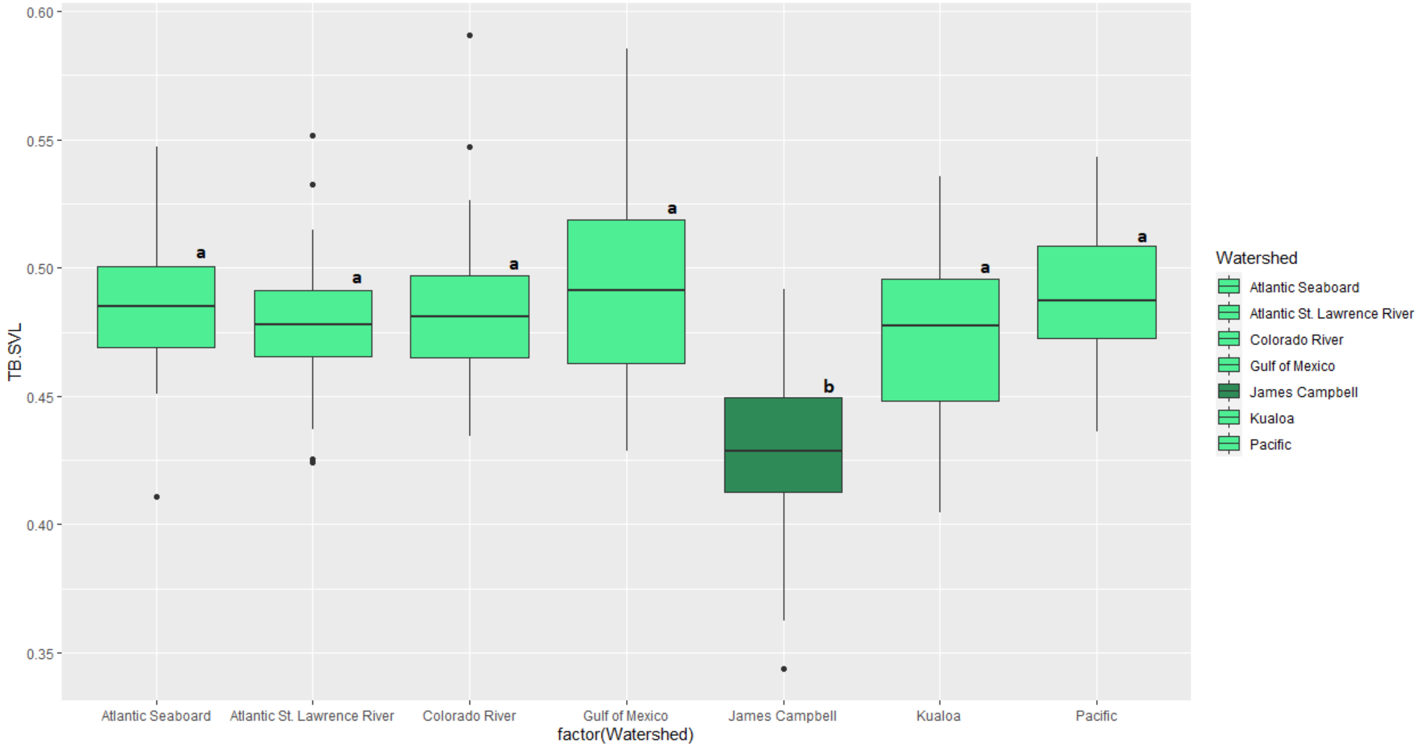

Figure 7. Boxplot of ratio-correct TB by drainage basin. Letters shared between groups indicate no statistical difference between those groups at a significance level of 0.05. Colors correspond to letter designations.

Table 8. Numerical summary of body size-corrected tibiofibula length. This table includes sample size (n), mean, standard deviation (SD), the interquartile range (IQR), and the 0th, 25th, 50th, 75th, and 100th percentile. The two Oahu sites from which most specimens originated (James Campbell National Wildlife Refuge [JC] and Kualoa Ranch [KU]) are included.

	n	**Mean**	**SD**	**IQR**	**0%**	**25%**	**50%**	**75%**	**100%**
Atlantic Seaboard	37	0.487	0.026	0.031	0.411	0.469	0.485	0.501	0.547
Atlantic St. Lawrence River	108	0.478	0.020	0.026	0.424	0.466	0.478	0.491	0.552
Colorado River	20	0.487	0.036	0.032	0.434	0.465	0.481	0.497	0.591
Gulf of Mexico	17	0.491	0.040	0.056	0.429	0.463	0.491	0.519	0.585
Hawai`i	90	0.449	0.043	0.053	0.344	0.423	0.446	0.476	0.594
Oahu - JC	46	0.427	0.031	0.037	0.344	0.413	0.429	0.450	0.492
Oahu - KU	28	0.477	0.037	0.047	0.405	0.448	0.478	0.496	0.536
Pacific	21	0.489	0.029	0.036	0.436	0.472	0.487	0.509	0.543

I assessed the normality of corrected TB data by geographic group using the Shapiro-Wilk test, as well as by assessing Q-Q plots and histograms, and determined the data were normal. The data were found to have unequal variances using Bartlett's test of homogeneity of variances (Bartlett's K^2=56.901, holm-adjusted p=$3.212e^{-11}$). I performed a Welch's ANOVA test (not assuming equal variances) to identify differences between average corrected TB among drainage basins and tested the residuals for normality. The residuals of the Welch's ANOVA were normally distributed based on the histogram, skew, kurtosis, and Q-Q plot. A MANOVA was therefore used to assess the effects of sex and geographic group on TB measurements. Both sex (F=3.68, df=2, p=0.027) and geographic groups (F=26.75, df=6, p=$<2e^{-16}$) had a significant effect on TB length. A post-hoc Tukey test was used to identify differences in size-corrected tibiofibulas between geographic groups. James Campbell NWR bullfrog TB measurements differed significantly from all other geographic groups, as demonstrated in Table 9.

Table 9. Tukey test p-values indicating significantly different average ratio-corrected TB by drainage basin. Bolded values are significant at α=0.5.

Group comparison	P adj.
Atlantic St. Lawrence River-Atlantic Seaboard	0.698946
Colorado River-Atlantic Seaboard	1.000000
Gulf of Mexico-Atlantic Seaboard	0.997483
James Campbell-Atlantic Seaboard	**0.000000**
Kualoa-Atlantic Seaboard	0.853958
Pacific-Atlantic Seaboard	0.999887
Colorado River-Atlantic St. Lawrence River	0.843087
Gulf of Mexico-Atlantic St. Lawrence River	0.553489
James Campbell-Atlantic St. Lawrence River	**0.000000**
Kualoa-Atlantic St. Lawrence River	1.000000
Pacific-Atlantic St. Lawrence River	0.656166
Gulf of Mexico-Colorado River	0.999396
James Campbell-Colorado River	**0.000000**
Kualoa-Colorado River	0.901408
Pacific-Colorado River	0.999989
James Campbell-Gulf of Mexico	**0.000000**
Kualoa-Gulf of Mexico	0.681383
Pacific-Gulf of Mexico	0.999986
Kualoa-James Campbell	**0.000000**
Pacific-James Campbell	**0.000000**
Pacific-Kualoa	0.779563

Discussion

The relatively large body size found in James Campbell NWR and Colorado River watershed populations of *R. catesbeiana,* as well as Kualoa Ranch to a lesser extent, may be the result of the population age structure, an increased growth rate associated with environmental factors, heterochrony in relation to metamorphosis, or anthropogenic selection resulting from the intentional introduction of larger individuals to be used as a food source. Biotic selective pressures in these watersheds, including the presence or absence of bullfrog predators and competitors, as well as available prey species, may drive the morphological changes observed in these drainage basins. The mechanism underlying differences in body size is outside the scope of the current study; however, documented biogeographic patterns and environmental correlations from the literature may provide insight into this phenomenon.

The principal components regression (PCR) identified a significant negative correlation between SVL and the first principal component, which had strong positive loadings for latitude and yearly mean NDVI, and a negative loading for mean monthly average air temperature. Both the PCR and Pearson's correlations indicate that *R. catesbeiana* body size correlates positively with mean monthly average air temperature and negatively with latitude and NDVI. Pearson's correlations indicate a decrease in body size with higher maximum, mean, and minimum monthly average precipitation, although precipitation did not contribute greatly to the significant principal component in the regression. These patterns have a range of possible explanations. A longer frost-free growing season at warmer temperatures and lower latitudes may also allow this amphibian to acquire more resources than at higher latitudes. Invertebrates, the dominant prey consumed by bullfrogs, are generally more abundant in warmer conditions at lower latitudes, so bullfrogs may be larger in Hawai`i and Arizona due to greater food resources (Well, 2007). Larger body size in warmer, more arid locations may allow for adaptive water retention, thereby reducing desiccation risk and increasing fitness (Olalla-Tárraga et al., 2009). Fewer predators in these invaded locations may increase bullfrog longevity and thereby reach greater average size.

The *R. catesbeiana* body size patterns observed in this study contrast with the Bergmann's cline observed in some other anuran studies. In a meta-study, Ashton (2002) found a significant positive interspecific association between body size and latitude or elevation in amphibians, although there was no significant relationship between body size and environmental temperature. A number of explanations were proposed: smaller size may lower maintenance costs in warm climates; large body size may be related to fasting endurance during overwintering periods in cooler, seasonal environments; and ecological variables such as predators, competitors, and food resources may also account for body size trends. Olalla-Tárraga and Rodriguez (2007) tested for interspecific geographical patterns in the body size of 265 North American and European amphibian species and observed a size gradient for anurans consistent with Bergmann's rule, with larger body size in northern regions. These authors also analyzed several explanatory variables, including mean annual temperature, annual potential evapotranspiration, primary productivity, growing season length, water availability. Annual potential evapotranspiration and length of growing season explained the variance in body size, with larger anurans in low-energy areas. Their analysis supports the heat balance hypothesis, that larger body size is advantageous in cold environments by reducing the surface area to volume ratio and allowing for greater heat conservation. Olalla-Tárraga et al. (2009) later examined interspecific body size patterns in Brazil and found the largest species in warm, water deficit areas near the equator while significantly smaller species occurred in wet southern regions, suggesting that the dominant driver of anuran body size may shift from heat conservation in the Holarctic to water retention near the equator.

While the community assemblage approach utilized by Ashton (2002), Olalla-Tárraga and Rodriguez (2007), and Ollala-Tárraga et al. (2009) have identified significant interspecific body size patterns, intraspecific variation may result from species-specific physiological, ecological, and social contexts (Boaratti & De Silva, 2015). For example, Boaratti and De Silva (2015) note that frogs of different species exhibit different dehydration rates under the same experimental conditions, suggesting that the significance of precipitation, relative to other environmental variables influencing body size, may differ between species. Lui et al. (2018) examined latitudinal body size patterns of *Fejervarya limnocharis* in China, and obtained similar results to the present study – size correlated positively with temperature and negatively with latitude, but had no significant relationship with precipitation. In China, Xuan et al. (2009) found that invasive bullfrog populations exhibit slower growth rates at cold, high altitudes, resulting in smaller average body size even after controlling for skeletochronologically-determined age; this study supports the finding that bullfrogs exhibit an inverse Bergmann's cline.

Large body size also appears to correlate with likelihood of a species to be intentionally introduced to a new ecosystem. Blackburn and Cassey (2007) identified non-random patterns in the invasive avifauna of Florida and found significantly greater body mass in introduced species relative to a random sample of birds, even when controlling for family. A meta-analysis by Blackburn et al. (2009) confirmed that large body mass increases the likelihood of establishment success in birds at the species level. Tingley et al. (2010) examined traits of introduced and non-introduced amphibians to identify predictors for introduction event probability, and found body size to be highly significant. These authors note that organisms with large body size are advantageous for biocontrol purposes because they tend to consume greater biomass. Large body size is also preferable when introducing organisms as a food source. Intentionally introduced amphibians are also usually subject to care in captivity, unlike unintentionally introduced species, potentially increasing the probability of successful establishment in the new environment (Kraus, 2009).

The data obtained by this study suggest that Hawai`i bullfrog populations have significantly shorter tibiofibulas relative to total body length than all mainland populations, both native and non-native. Anuran leg length has been found to correlate with a number of environmental factors. A metastudy of environmental conditions and plasticity in anuran hindlimb length (Tejedo et al., 2010) found that hindlimb length correlated positively with resource availability. Post-metamorphic *Rana* species also appear to exhibit shorter hindlimbs after larval exposure to pond desiccation and predation risks. Pond desiccation does not appear to pose a risk to the Hawai`i populations from which I collected specimens; however, predator presence is a potential explanation for the short tibiofibulas observed in these populations as damselfly nymphs and waterbirds may predate on bullfrog tadpoles. Reduced resources including food and habitat may influence leg length in these populations as well, although further study of the aquatic ecology in these wetlands is needed.

Intraspecific relative hindlimb length has also been observed to correlate negatively with time to metamorphosis in frogs (Emerson, 1986). The variables associated with development time include population density, hydroperiod, length of frost-free period (Emerson, 1986; Cook et al., 2013; Collins, 1979). In their native range, bullfrogs exhibit a latitudinal gradient in the duration of the larval stage, spanning 2 to 3 years in Nova Scotia and Quebec, to less than a year in the southern United States (Govindarajulu et al., 2006). However, some invasive populations have displayed larval development that contradicts this trend. Bullfrog tadpoles from non-native ephemeral habitats in southern Oregon have been documented to metamorphose in four months (Cook et al., 2013). Cook et al. (2013) performed a common garden experiment to evaluate plasticity in the duration of the larval stage and suggest this trait is likely genetic, rather than plastic. Hawai`i *R. catesbeiana* populations may experience novel pressures, such as increased population density and longer hydroperiods relative to mainland populations, that select for longer larval stages and result in shorter tibiofibulas.

Reduced tibiofibula length may have physiological consequences for bullfrogs in Hawai`i. Tejedo et al. (2010) note that an increase in anuran relative hindlimb length under conditions of high resource availability resulted in a substantial increase in size-adjusted maximum jumping distance (for example, an 8.8 percent increase in relative hindlimb length for *Pelodytes punctatus* individuals resulted in an increased maximum jump distance of 34 percent). In this study, the average relative tibiofibula length of Hawai`i bullfrog populations was 6.9 percent shorter than the overall mainland population average, suggesting that Hawai`i bullfrogs may have significantly reduced jumping distance. This trait may be associated with less effective predator evasion and shifts in dispersal behavior or ability.

Our study revealed significant morphological differentiation between Hawai`ian and mainland populations of *R. catesbeiana*, with larger average size and shorter relative tibiofibula length. These results have implications for Hawai`i's aquatic ecosystems, suggesting potential for bullfrogs to predate on larger and potentially higher trophic level prey than observed in native populations. The data also suggest that bullfrogs across the native and non-native North American range exhibit an inverse Bergmann's cline, a pattern which may be particularly significant in tropical locations. The manner in which Hawai`i bullfrogs' reduced leg length correlates with or influences dispersal behavior and predator avoidance is a topic for future research. Research on the duration of the bullfrog larval stage in tropical regions may contribute to our understanding of their population dynamics and invasion success.

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Chapter 4

Comparative Phylogeography of American Bullfrog Populations (*Rana catesbiana*), from Native and Invasive Ranges in North America and Hawai`i

Introduction

Invasive species are widely recognized as drivers of large-scale ecological change, impacting native species population levels, critical habitats, community structure, and entire ecosystems by both direct and indirect means (Sakai et al., 2001). In addition to ecological costs, overall global financial burdens continue to mount and accelerate, with recent estimates of US$1.288 trillion (2017 US dollars) over the past few decades (1970–2017), an annual mean cost of US$26.8 billion, and a staggering estimated annual mean cost of US$162.7 billion in 2017 (Diagne et al., 2021). Population genetic approaches, including application of molecular markers for analysis of the genetic diversity, phylogeographic structure, microevolution and phenotypic plasticity, have potential to improve our understanding and management of biological invasions (Sakai et al., 2001; Ficetola et al., 2008; Estoup & Guillemaud, 2010; Dufresnes et al., 2019). The occurrence of multiple introductions tends to correlate with invasion success by increasing propagule pressure and genetic variation, depending on the number of individuals translocated and assuming source populations are genetically diverse (Sakai et al., 2001). Despite the fact that biological invasions typically result in significant genetic bottlenecks, and founding populations are generally relatively restricted in size, and therefore susceptible to strong homogenizing forces of genetic drift causing loss or fixation of alleles, a substantial percentage of these taxa become established and expand their ranges. Those introduced species are successful at establishing and expanding their populations in novel environments despite small founding populations and strong bottleneck effects. Genetic analyses have identified a fairly frequent phenomenon, now known as the invasion paradox, where introduced species that have undergone severe genetic bottlenecks, rapidly respond and adapt to novel selective pressures despite their depleted genetic diversity (Allendorf & Lundquist, 2003; Estoup et al., 2016). Data regarding invasion history, geographic source, comparative genetic variation of native to invaded range, rate and extent of secondary spread, and introduction pathways can provide important biological information relevant to control, prevention and eradication strategies.

As a result of their extreme geographic isolation, the Hawai`ian islands host unique, exceptionally biodiverse ecosystems that are highly sensitive to degradation. Species radiations tend to be found in the lineages that have colonized remote volcanic island chains such as the Hawai`ian islands, resulting in high rates of endemism (Cowie & Holland, 2008; Gillespie et al., 1994; Freed et al., 1987; Liebherr, 2007; Kier et al., 2009). Many endemic Hawai`ian lineages have been devasted by anthropogenic habitat disturbance and disruption, and now experience extraordinarily high extinction rates (Boyer, 2008; Cowie, 2001; Regnier et al., 2015). Over 60% of endemic Hawai`ian birds (Boyer, 2008) and as many as 95% of the endemic land snail species may already be extinct (Regnier et al. 2015), due to anthropogenic activity including the introduction of invasive predators (Hadfield et al., 1993; Holland et al., 2010; Chiaverano et al., 2014). The conservation status of Hawai`ian insects and arachnids is largely unknown due to the challenges relating to monitoring small cryptic species. Nevertheless, invasive plants, ungulates, rodents, ants, and parasitoid wasps are implicated in the decline of native insect populations (Rubinoff, 2007; Medeiros et al. 2013).

While invasive plants, mammals and insects have received the majority of scientific attention due to their significant economic impacts, ecological effects attributable to invasive herpetofauna are poorly understood (Reed & Kraus, 2010). As many as 26 herpetofauna have become established on the archipelago over the past century, including 10 primarily aquatic predatory species (Van Kleeck & Holland, 2018). The few studies on the herpetofauna in Hawai`i suggest potential for substantial ecological impacts. A recent observation of a native

Hawai`ian tree snail in the stomach of a Jackson's chameleon marked the first documentation of an introduced reptile impacting native taxa (Holland et al., 2010; Chiaverano et al., 2014). Japanese wrinkled frogs have invaded relatively pristine inland forests and exploit native insects and gastropods (Van Kleeck & Holland, 2018). A more thorough ecological understanding of introduced reptiles and amphibians in Hawai`i will inform management effort and strategies. Population genetic analysis of these species can reveal the geographic sources of Hawai`i populations, indicate genetic diversity, and provide insight into their capacity for adaptation to novel habitats.

American bullfrogs (*Rana catesbeiana*), native to eastern North America, have become established worldwide with populations in 40 countries on four continents (Funk et al., 2011). Bullfrogs have been implicated in significant alteration to ecosystems in their non-native range, outcompeting and predating on native aquatic species (Lawler et al., 1999; Pearl et al., 2004; Adams & Pearl, 2007). The invasion history of *R. catesbeiana* varies widely among regions, with numerous intentional introductions in some locations and small numbers of accidental introductions in others (Ficetola et al., 2006; Jennings & Hayes, 1985; Lawrence et al., 2005). For example, European bullfrog populations were introduced from the native range and exhibit relatively low haplotype diversity, with simulations suggesting that these populations were derived from only six breeding females (Ficetola et al., 2006). Chinese bullfrog populations have similarly low haplotype diversity, but likely originated from non-native populations in Cuba (Bai et al., 2012). A recent global analysis of bullfrog colonization history suggests that high inbreeding and bottleneck effects do not diminish this species' ability to establish populations outside their native range (Jorgewich-Cohen et al., 2020, preprint).

R. catesbeiana was introduced to the Hawai`i archipelago as a food source and to control Japanese beetles in 1879 (Bryan, 1932; Jennings & Hayes, 1985; Yamamoto & Tagawa, 2000), when 72 frogs were shipped to Hilo, Hawai`i from Contra Costa County, California to establish a farm. This species now occurs on all of the main Hawai`ian islands (Yamamoto & Tagawa, 2000). Environmental conditions that correlate with bullfrog dispersal and establishment include summer and winter precipitation, permanent waterbodies, high maximum temperatures, and proximity to anthropogenically modified habitat (Ficetola et al., 2007); consequently, Hawai`i's watersheds are highly susceptible to bullfrog colonization. Able to disperse up to 1.5 kilometers in a single season (Ingram & Raney, 1943), bullfrogs pose a threat to Hawai`i's aquatic ecosystems from the anthropogenically altered coastal wetlands to the relatively pristine mid-elevation streams and waterbodies (Martin & Tsang, 2019). Elucidating the invasion history and current genetic makeup of Hawai`ian bullfrog populations can reveal paths of introduction, suggest population connectivity, and provide insight into the plastic or genetic adaptations that enable this species to spread so successfully.

Here, I investigate 1) geographic source of introductions, 2) the degree to which the Hawai`i population has undergone a genetic bottleneck relative to population level genetic partitioning within its native range, 3) levels of molecular divergence between invasive Hawai`i populations and those in their native range, and 4) evidence of the invasion paradox, where other invasive herpetofauna have been shown to exhibit genetic bottlenecks and yet still become established and flourish in novel habitats. I focused DNA sequencing efforts on mtDNA, since it tends to vary in tractable and moderately rapid fashion, and I selected the gene target that has the highest representation in NCBI Genbank (Cytb). After DNA sequences were obtained, edited and aligned, phylogenetic analyses and descriptive statistics were generated, including mean molecular divergence values within and between sampling localities, Tajima's test for neutrality using MEGAX (Kumar et al., 2018), and a maximum parsimony haplotype network using PopART (Leigh & Bryant, 2015).

Methods

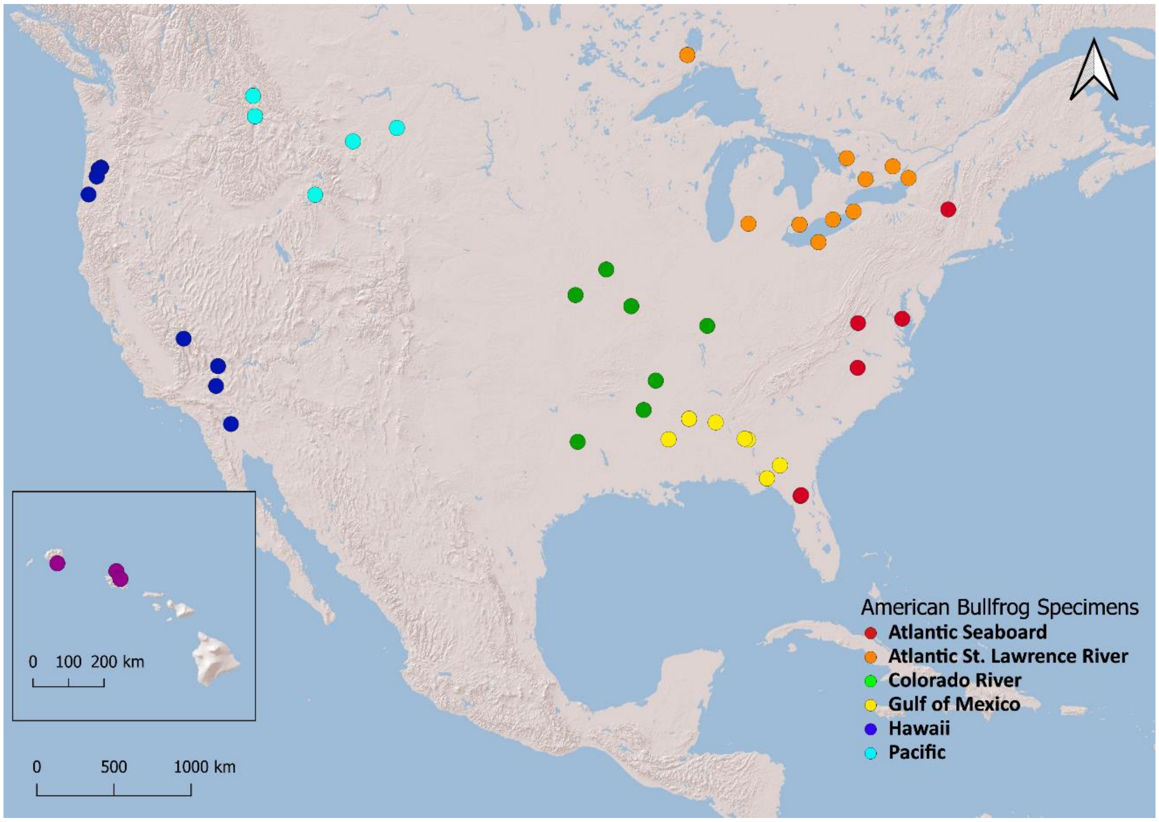


Figure 8. Locations from which genetic samples of *R. catesbeiana* were obtained via specimen collection, from the GenBank database, and from tissue samples provided by the Los Angeles natural History Museum.

Bullfrogs were collected from each site using a dipnet. I surveyed along the shoreline and collected any bullfrogs that were observed. I collected tissue samples for DNA analysis from 22 frogs per Hawai`i sampling site for phylogeographic analysis. The specimens were humanely euthanized via benzocaine gel (Orajel), followed by pithing or a BB gunshot in accordance with the IACUC protocol. Frogs were stored whole at -20ºC to prevent decomposition. Each individual was assigned an identification number with which its morphological measurements and tissue samples were associated. DNA samples were collected via toe clips and immediately processed.

DNA was extracted from the tissue samples using a Macherey-Nagel NucleoSpin Tissue DNA extraction kit. The mitochondrial cytochrome b (cytb) gene was selected for amplification. Primers L14850 (forward) and H15410 (reverse) were used to amplify a 584 basepair segment of the cytb gene (Goebel et al., 1999; Tanaka et al., 1996). This targeted segment overlaps with the 408-bp segment utilized by Austin et al. (2004), Ficetola et al. (2008); Funk et al. (2011); Jorgewich-Cohen et al. (2020, preprint) and Bai et al. (2012).

Conquest PCR Master Mix Optimizing Pack (Lamda Biotech, Catalog#: D911-Mix1234) reagents were used for all polymerase chain reactions (PCR). PCR reagents consisted of 20 µL total volume per sample, with approximately 2 ng template DNA, 10 µL Taq DNA polymerase Mastermix, 50 µM of each primer, and distilled sterile H_2O to volume. Thermocycling conditions included initial hot-start denaturation at 95°C for 2 min; 35 cycles of 95°C for 30 s, 52°C for 45 s, and 72°C for 45 s; and final extension at 72°C for 10 min, using a MyCycler^{TM} Thermal Cycler (Bio-Rad, Hercules, CA, USA). Successful PCR products were purified using Macherey-Nagel NucleoSpin$^{®}$ Gel and PCR Clean-up columns (Takara Bio, Inc. USA, 1290 Terra Bella Ave., Mountain View, CA 94043, USA; Reference #740609.50). Purified PCR products were submitted to the University of Hawai`i Advanced Studies in Genomics, Proteomics, and Bioinformatics (ASGPB) Laboratory for Sanger dideoxy chain termination DNA sequencing.

Hawai`i *R. catesbeiana* cytb sequences were aligned with sequences from both the native and non-native ranges throughout North America, analysis revealed 24 distinct haplotypes. North American sequences were obtained from GenBank (National Center for Biotechnology

Information [NCBI], 2022), as well as from Dr. Chris Funk (Colorado State University, personal communication). A total of 112 nucleotide sequences, including 24 from Hawai`i, were analyzed in this study. Locations, GenBank accession numbers, and original authors are listed in the Appendix, Table 15. The sequences obtained from these samples were manually aligned and analyzed using MEGA X software (Kumar et al., 2018) and PopART software for haplotype network reconstruction (Leigh & Bryant, 2015). Sequences were grouped by biologically relevant geography, in this case North American drainage basins including Hawai`i (n = 24), Columbia River (n = 6), Mississippi River system (n = 10), Saint Lawrence River (n = 13), Gulf of Mexico (n = 10), Atlantic continental coast (n = 6), and Pacific continental coast (n = 41). One outgroup sequence from the congeneric *Rana clamitans* was included in analyses. I used 1000 bootstrap replicates to assess statistical support for the maximum likelihood phylogeny.

Results

Hawai`i *R. catesbeiana* sequences showed highest molecular divergence (md) values relative to the Mississippi River basin (md = 0.01456), as well as the Columbia River watershed to a lesser extent (m.d. = 0.01007), and displayed the greatest similarity to the Atlantic (m.d. = 0.00596) and Saint Lawrence River (m.d. = 0.00635) populations. The maximum likelihood phylogeny placed one Kauai sequence in a monophyletic clade with both sequences from Kansas in the Mississippi River basin and a Montana sequence in the Columbia River basin. However, most Hawai`i sequences were grouped monophyletically with sequences from New York, Ohio, Ontario, Maryland, Florida, Montana, and North Carolina, validating the lower molecular divergence values between Hawai`i and both Atlantic and Saint Lawrence River basins. Despite records suggesting that bullfrogs were introduced to the Hawai`ian islands from northern California (Jennings & Hayes, 1985), the Hawai`ian populations appear to share greater genetic similarity with those from the northeastern North American native range. Sequences from California and Oregon differed most from the groups within the native range and are most similar to sequences from the Columbia River basin and Hawai`i.

Table 10. Estimates of Evolutionary Divergence over Sequence Pairs between Clades (as identified in Figure 9). The number of base substitutions per site from averaging over all sequence pairs between groups are shown. Standard error estimates are shown above the diagonal. Analyses were conducted using the Kimura 2-parameter model (Kimura, 1980). This analysis involved 110 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 326 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

	Clade 1	**Clade 2**	**Clade 3**	**Clade 4**	**Clade 5**	**Clade 6**
Clade 1		0.00400	0.00326	0.00445	0.00798	0.00707
Clade 2	0.00648		0.00315	0.00439	0.00773	0.00682
Clade 3	0.00413	0.00387		0.00308	0.00700	0.00593
Clade 4	0.00802	0.00776	0.00386		0.00697	0.00596
Clade 5	0.02095	0.02068	0.01654	0.01727		0.00308
Clade 6	0.01702	0.01674	0.01268	0.01350	0.00452	

Watershed	**K2P**	**SE**
Pacific	0.0072	0.0031
Atlantic	0.0068	0.0028
Gulf of Mexico	0.0111	0.0033
Saint Lawrence River	0.008	0.0027
Mississippi River system	0.0101	0.0037
Hawai`i	0.0048	0.0019
Columbia River	0.0116	0.0042

Table 11. Estimates of Average Evolutionary Divergence over Sequence Pairs within Groups. The number of base substitutions per site from averaging over all sequence pairs within each group are shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using The Kimura 2-parameter model (Kimura, 1980). This analysis involved 112 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). A total of 326 basepairs were in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Table 12. Results from Tajima's Neutrality Test (Tajima, 1989). This analysis involved 112 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 326 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). *Abbreviations*: m = number of sequences, n = total number of sites (n = 326), S = Number of segregating sites, $p_s = S/n$, $\Theta = p_s/a_1$ (where a_1 is a correction factor), π = nucleotide diversity, and D is the Tajima test statistic. Bold text indicates significance of Tajima's D value at α = 0.05.

	M	***S***	p_s	Θ	π	***D***
Hawai`i	22	6	0.018405	0.004929	0.004657	-0.1668
Kualoa Ranch	15	0	0	0.000000	0	n/c
James Campbell	7	5	0.015337	0.006260	0.008764	**1.98152**
Columbia River	6	8	0.024540	0.010747	0.011452	0.386471
Mississippi River	10	9	0.027607	0.009759	0.010020	0.117398
St Lawrence River	13	10	0.030675	0.009885	0.007865	-0.81619
Gulf of Mexico	10	11	0.033742	0.011927	0.010975	-0.35765
Atlantic	6	6	0.018405	0.008061	0.006748	-0.931688
Pacific	41	6	0.018405	0.004302	0.007085	1.717383
All	112	72	0.220859	0.04174	0.012717	-2.23163

Sequences from Hawai`i exhibited the lowest nucleotide diversity (π = 0.004657) of all groups, with one densely populated site (Kualoa Ranch) characterized by a single haplotype observed in 15 specimens. The Columbia River and Pacific sequences, which were also collected within nonnative regions, have moderate nucleotide diversity relative to other groups, likely due to greater population connectivity and a greater number of introductions relative to Hawai`i. A negative Tajima's D was identified in Hawai`i overall, indicating population expansion following a genetic bottleneck; sequences from USFWS James Campbell National Wildlife Reserve , however, were associated with a positive Tajima's D, which might be explained by ongoing control efforts on the bullfrog population at this site

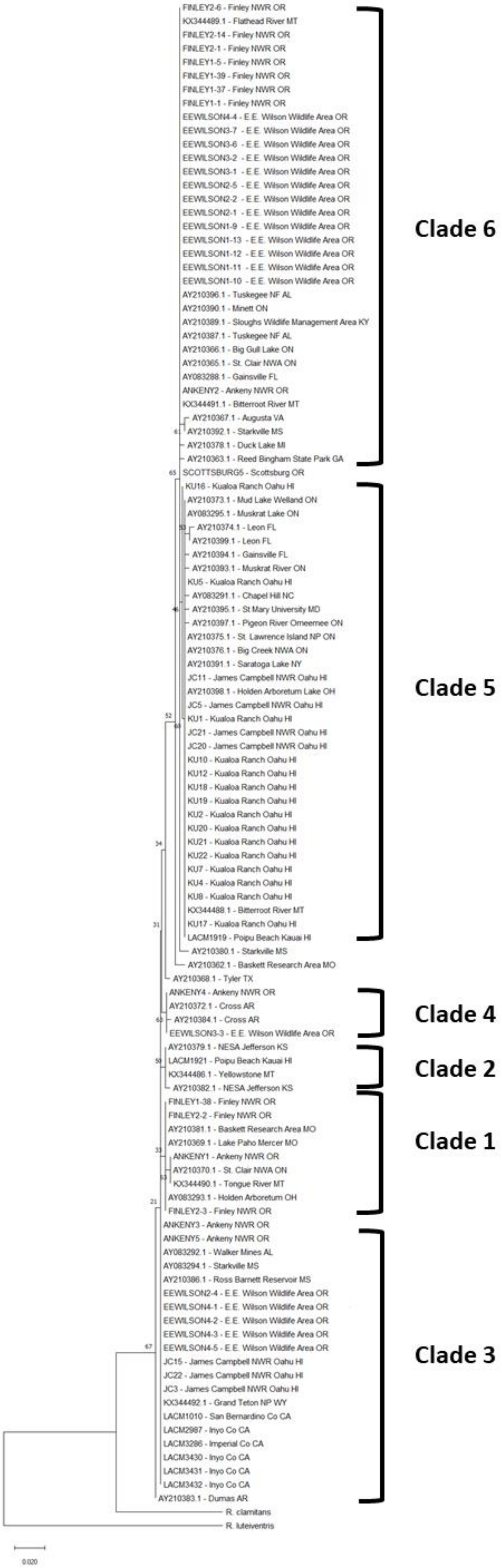


Figure 9. Maximum Likelihood analysis of R. catesbeiana from North America using cytochrome b. The evolutionary history was inferred using the Maximum Likelihood method and Kimura 2-parameter model with discrete gamma distribution(Kimura, 1980). This substitution model was identified as having the best fit using the Bayesian Information Criterion value. The tree with the highest log likelihood (-896.74) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.2787)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 112 nucleotide sequences. There were a total of 326 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). Appendix Figure 12 presents a maximum likelihood tree with compressed branches.

I inferred the source populations of Hawai`i *R. catesbeiana* using the geographical distribution of haplotypes in mainland ranges, both native and non-native, and through phylogeographic relationships determined via a maximum likelihood phylogenetic tree. Three unique haplotypes were observed in Hawai`i, with two identified on Oahu (Haplotypes 8 and 16 in Figure 10) and one found only on Kauai (Haplotype 7). Haplotype 7 appears to have originated from the central and western regions of the species’ native range, whereas Haplotype

8 occurs in the southern native range and along the Pacific coast. Haplotype 16 was found in the northernmost regions of the native range, as well as in the Columbia River basin, but was not present in sequences from the Pacific coast. This haplotype is the most frequent in Hawai`i and accounted for 18 of the 24 sequences sampled on Oahu and Kauai.

Phylogeographic analysis and tree reconstruction resulted in generally low resolution relationships due to limitations in sequence and haplotype polymorphism. The maximum likelihood phylogeny identified one Kauai specimen as recently diverged from specimens in Kansas and Montana, while the other Kauai sequence and most Oahu sequences were positioned in a clade with Ontario, Maryland, Florida, Kentucky, North Carolina, New York, Ohio, Montana, and Virginia. Other Oahu sequences in this tree were placed in a poorly resolved group with sequences from California, Wyoming, Oregon, Alabama, and Mississippi. These clades align with the geographic haplotype distribution shown in Figure 11.

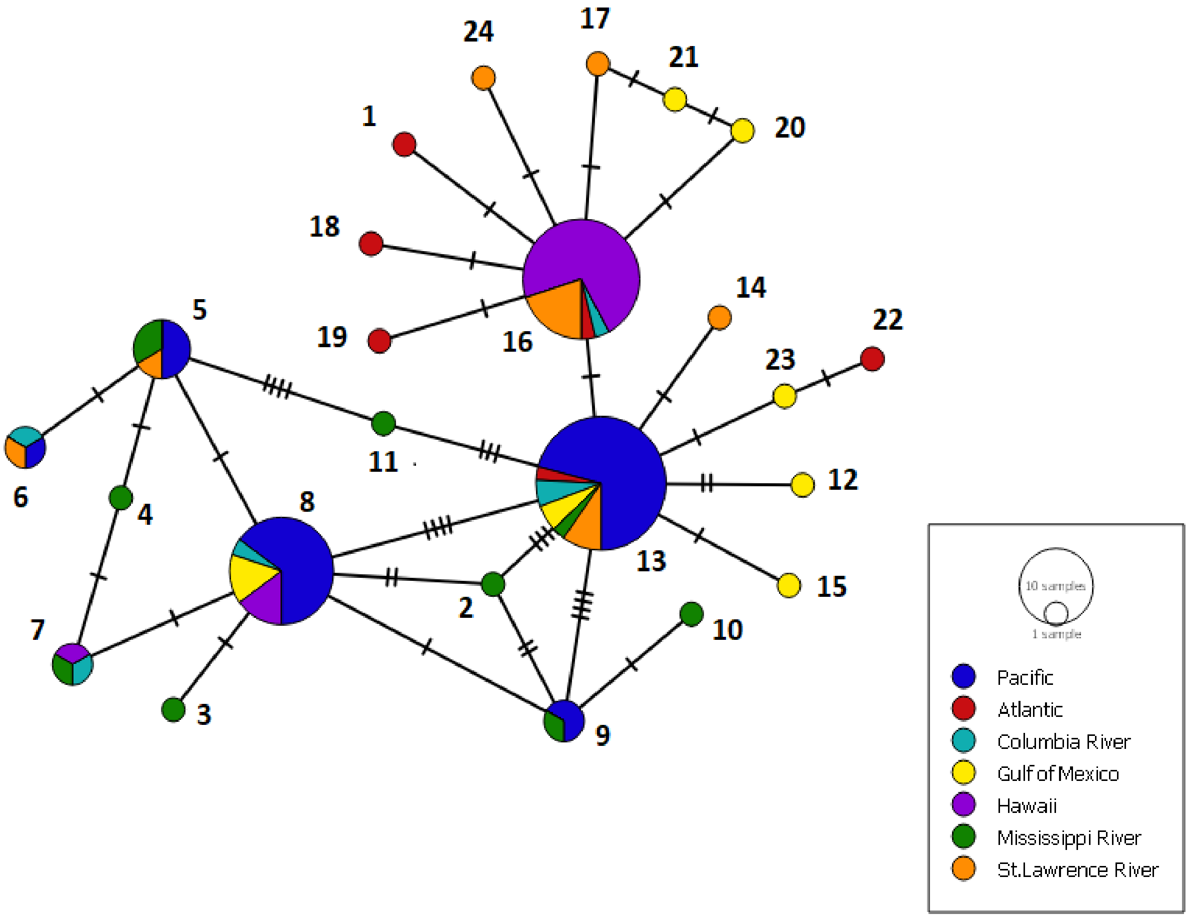
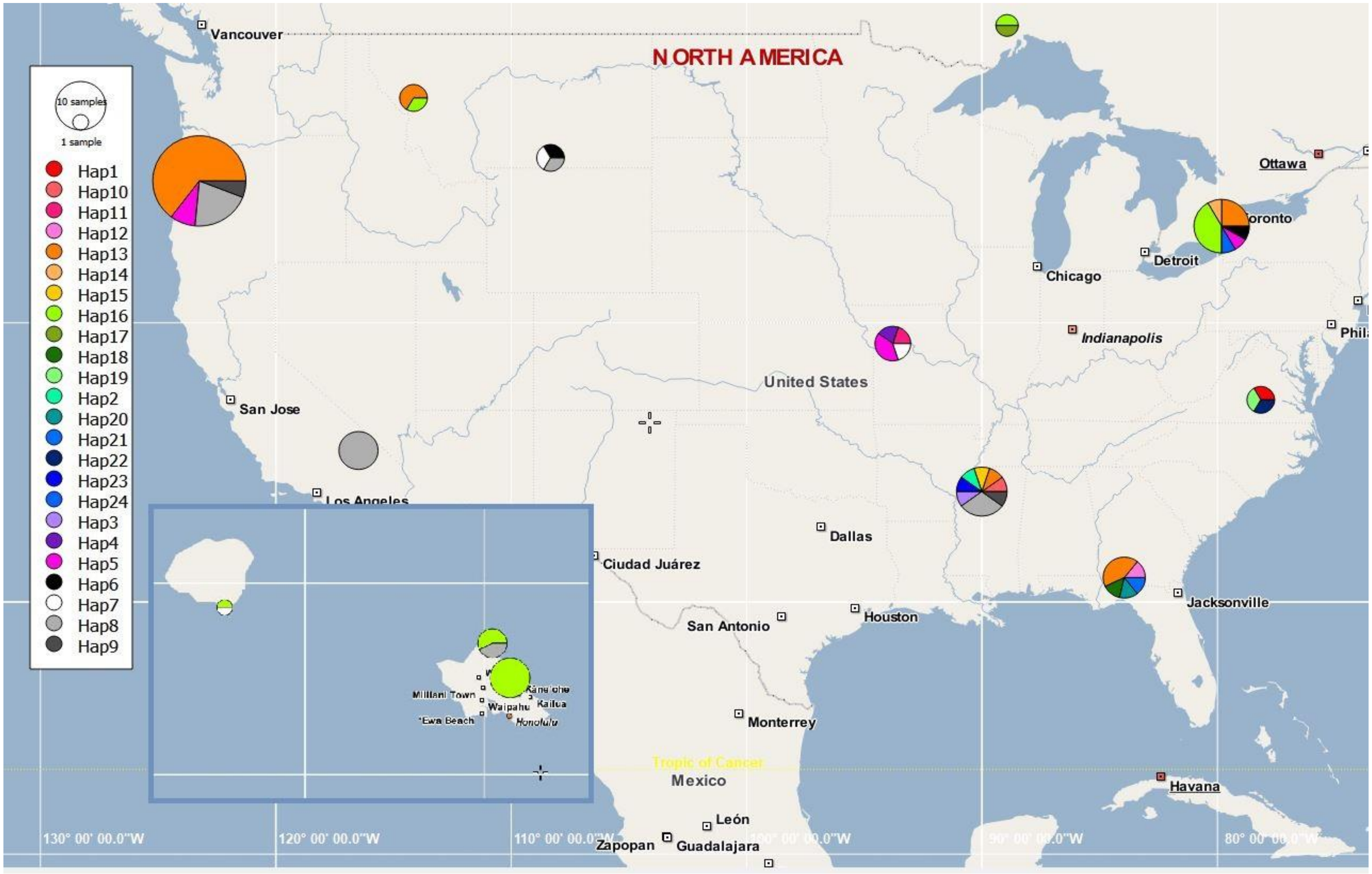


Figure 10. Ninety-five percent statistical parsimony haplotype network for Cytb from 108 North American *R. catesbeiana* specimens, produced with PopART version 1.7 for Windows (Leigh & Bryant, 2015). Circles represent haplotypes and colors represent the drainage basin from which genetic samples were taken, as noted in the legend. Circle sizes represent the number of individuals sharing each haplotype. Haplotypes are numbered consistently with Figure 11 below. Dash marks along the branches represent the number of mutations between haplotypes.

Figure 11. Geographic distribution of haplotypes observed in bullfrog populations sampled throughout the native and non-native North American range, produced using PopART (Leigh & Bryant, 2018). Circle size is proportional to the number of individuals sampled in each region, and samples are clustered using the k-means algorithm. Sequence data were obtained from GenBank, from Austin et al. (2003; 2004), Kamath et al. (2016), and Funk et al. (2011) in addition to sequence data generated for this study



Discussion

Biological invasions are among the most daunting threats to global biodiversity and pose urgent global conservation challenges (Pimentel et al., 2005). The initial introduction process often determines the success or failure of an invasion, presenting barriers to establishment such as genetic bottlenecks and novel environmental conditions to which the species has not adapted. However, evidence suggests the American Bullfrog experiences considerable invasion success despite extraordinarily low propagule pressure in some cases (Ficetola et al., 2008). Elucidation of invasion history and genetic diversity can improve scientific understanding of highly successful invasive species like the American bullfrog. Phylogeographic analysis of *R. catesbeiana* throughout the native and non-native North American range using mitochondrial cytochrome b offers insight into the sources and genetic characteristics of Hawai`i populations, and provides genetic context for the morphological variation described in Chapters 2 and 3.

Three unique haplotypes were identified in Hawai`i bullfrog populations, which, in the context of North American haplotype distributions, phylogeographic trees, and historical documentation, suggest multiple, rather than a single invasion pathway. Two sampling locations, James Campbell NWR, Oahu, and Poipu Beach, Kauai, revealed two distinct haplotypes, while only a single haplotype was identified in specimens from Kualoa Ranch, Oahu. I also sequenced six tissue samples from three counties in California (Inyo, San Bernardino, and Imperial counties) and identified only a single haplotype (Haplotype 8). Jennings and Hayes (1985) reviewed records documenting the establishment of bullfrogs in California and found that this species was likely introduced to Contra Costa county, California, from Maryland and Florida. Frogs from farms in this California location were later shipped to Hilo, Hawai`i. Haplotype 8 is present on Oahu and may have been introduced to Hawai`i from California during this early translocation event. This haplotype was not observed in specimens from the Atlantic coast and more likely originated from the southern native range, as illustrated by the monophyletic clades grouping California and Oahu with Mississippi, Alabama, and Arkansas (Figure.9). Haplotype 16, found in populations from both Hawai`ian islands sampled, is widely distributed in mainland populations from Ontario, Ohio, Florida, Montana, Maryland, North Carolina, and New York. This haplotype may be present in northern California (not sampled), reflecting the invasion pathway described in historical records, but more sequence data are needed in order to confirm this potential pathway. Haplotype 7, identified from Kauai, suggests another introduction event from populations in either Kansas in the native range or Wyoming in the invaded range, the only mainland regions where this haplotype was observed.

Haplotype diversity in Hawai`i was limited compared to haplotype diversity of populations sampled in mainland drainage basins, which span greater geographic areas and have potential for genetic connectivity. Despite the relatively low genetic diversity observed in Hawai`i, specimens from Oahu displayed the greatest variation in leg length and head width relative to body size compared to mainland regions with much greater genetic diversity, and have significantly diverged morphologically from mainland populations (see Chapter 2 and 3). Tajima’s D test statistic was negative for Hawai`i, suggesting population expansion following a genetic bottleneck, presumably the founder event that occurred in this species during its transport to the Hawai`ian archipelago. The high morphological variation and low genetic diversity may indicate that bullfrogs in Hawai`i exhibit trait plasticity in response to novel environmental conditions. Adaptive phenotypic plasticity can assist colonizing species in overcoming limited genetic diversity, shedding light on the genetic paradox of invasion described by Estoup et al. (2016). Recent studies have identified loci with environmentally sensitive alleles and regulatory loci that rapidly modulate gene expression in response to environmental factors, as well as epigenetic processes such as DNA methylation that may generate plasticity in adaptive traits (Estoup et al., 2016; Des Marais et al., 2013).

An invasion pathway from multiple non-native geographic regions including California and Wyoming suggests that *R. catesbeiana* that eventually arrived and became established in Hawai`i have historically undergone sequential bottleneck events, having established populations from small founder populations outside of their native North American distribution, and again upon introduction of a limited number of individuals to Hawai`i (Estoup et al., 2011). Jorgewich-Cohen et al. (2020) identified similar events in their genetic analysis of the global invasion history of bullfrogs, noting that Japan was the source of *R. catesbeiana* to all Asian countries besides China, and Brazilian bullfrogs may have arrived from China. Although the drastically reduced genetic diversity caused by small founding populations should theoretically induce deleterious effects, such as reduced fitness due to inbreeding depression and a limited ability to adapt to novel environmental conditions, Ficetola et al. (2008) found that European bullfrog populations may have originated from as few as six breeding females.

Genetic diversity and the potential for adaptive plasticity in an invasive population can impact control strategies in invaded locations. For example, artificially draining managed wetlands has been suggested as a control method for *R. catesbeiana*, as tadpoles in the native range require a year or two to metamorphose and are thus reliant on permanent waterbodies (Cook et al., 2013). However, non-native populations in Oregon have experienced microevolutionary adaptation, where they are able to undergo metamorphosis after only four months. Here wetland draining strategies may artificially, rapidly select for individuals with accelerated development (Cook et al., 2013). Likewise, populations of *R. catesbiana* that have become established in tropical environments with year-round warm condition, such as those in Hawai`ian aquatic ecosystems, experience drastically different conditions that those of the native North American range where cold winters help to slow and regulate the metamorphic process. In Hawai`i American bullfrogs may be released from this restrictive environmental control system. In this case, selective release from seasonal environmental thermal fluctuation, may allow accelerated reproduction, ontogeny, and exponential population growth. The ability of hearty generalist predators such as the American bullfrog to adapt to a broad spectrum of environmental conditions, despite extraordinarily low genetic diversity, warrants additional research in order to develop novel, effective management strategies and should provide strong incentives to prevent further introductions.

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Appendix

Table 13. Morphological data summaries for Hawaii *R. catesbeiana* specimens by collection site. Table includes tibiofibula (TB), jaw width (JW), snout-vent length (SVL), ratio-corrected TB and JW values, and the number of individuals of each sex (NA indicates sexually dimorphic traits were ambiguous).

			Sex							
Watershed	***Site***	***Island***	**M**	**F**	**NA**	***TB***	***JW***	***SVL***	***TB/ SVL***	***JW/ SVL***
Hawaii	Hilo	Hawaii	0	1	0	41.0	33.0	69.0	0.594	0.478
Hawaii	Honolulu	Oahu	0	1	0	61.0	48.0	125.0	0.488	0.384
Hawaii	James Campbell NWR	Oahu	15	27	0	56.5	46.4	132	0.430	0.354
Hawaii	Kahuku	Oahu	0	0	4	11.8	10.0	30.0	0.393	0.334
Hawaii	Kaneohe	Oahu	2	2	2	36.7	31.0	94.4	0.429	0.359
Hawaii	Kauai	Kauai	1	0	0	54.0	45.0	113.0	0.478	0.398
Hawaii	Kawainui Marsh	Oahu	0	1	0	70.0	58.0	158.0	0.443	0.367
Hawaii	Kualoa Ranch	Oahu	7	15	0	56.4	43.3	112.9	0.501	0.385
Hawaii	Manoa Valley	Oahu	1	0	0	59.0	48.0	136.0	0.434	0.353
Hawaii	Nuuanu	Oahu	0	2	0	36.5	27.0	74.0	0.490	0.364
Hawaii	Puna	Hawaii	2	0	0	53.0	42.5	109.0	0.486	0.390
Hawaii	Volcanoes NP	Hawaii	0	0	2	19.5	14.5	44.5	0.438	0.325
Hawaii	Waimanalo	Oahu	1	0	2	28.0	23.0	66.3	0.427	0.321
Hawaii	Waipahu	Oahu	0	0	2	15.0	12.5	35.0	0.424	0.356
Hawaii	Waipio Valley	Hawaii	0	1	0	30.0	23.0	64.0	0.469	0.359

Table 14. Pearson correlations between environmental variables used in Chapter 3. R values are below the diagonal and p values are above the diagonal. P values significant at the α=0.05 level are bold.

Pearson correlations:	***Latitude***	***Longitude***	***NDVI yearly max***	***NDVI yearly mean***	***NDVI yearly min***	***Air temp max monthly mean***	***Air temp mean monthly mean***	***Air temp min monthly mean***	***Precip max monthly mean***	***Precip mean monthly mean***	***Precip min monthly mean***
Latitude	-	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
Longitude	0.8415	-	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
NDVI yearly max	0.2907	0.3695	-	**<.0001**	0.1503	**<.0001**	**<.0001**	**0.0003**	**<.0001**	**<.0001**	**<.0001**
NDVI yearly mean	0.7419	0.8067	0.6775	-	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
NDVI yearly min	0.278	0.3078	0.1037	0.5128	-	**<.0001**	**0.0003**	**0.0020**	**0.0009**	**<.0001**	**<.0001**
Air temp max monthly mean	-0.9681	-0.8711	-0.291	-0.7687	-0.3005	-	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
Air temp mean monthly mean	-0.9733	-0.8182	-0.2994	-0.7377	-0.2561	0.99	-	**<.0001**	**<.0001**	**<.0001**	**<.0001**
Air temp min monthly mean	-0.9451	-0.7754	-0.2551	-0.664	-0.2204	0.9687	0.9854	-	**<.0001**	**<.0001**	**<.0001**
Precip max monthly mean	0.5837	0.7924	0.6684	0.7963	0.2374	-0.5692	-0.5363	-0.4701	-	**<.0001**	**<.0001**
Precip mean monthly mean	0.6628	0.8767	0.6162	0.8712	0.3000	-0.6752	-0.6267	-0.5501	0.9711	-	**<.0001**
Precip min monthly mean	0.6743	0.9135	0.529	0.8697	0.3415	-0.7072	-0.639	-0.5573	0.9092	0.9768	-

Table 15. Summary of Genbank sequences used in phylogenetic analyses, including author and publication date, locality as identified by authors in publication, and Genbank accession number.

Locality	*GenBank Accession Number*	*Authors*
Leon, FL	AY210399.1	Austin et al. (2004)
Holden Arboretum, Lake, OH	AY210398.1	Austin et al. (2004)
Pigeon River, Omemee, ON	AY210397.1	Austin et al. (2004)
Tuskasgee National Forest, Macon, AL	AY210396.1	Austin et al. (2004)
St. Mary's University Campus, MD	AY210395.1	Austin et al. (2004)
Gainsville, Alachua, FL	AY210394.1	Austin et al. (2004)
Muskrat River, ON	AY210393.1	Austin et al. (2004)
Starkville, Oktibbeha, MS	AY210392.1	Austin et al. (2004)
Saratoga Lake, Saratoga, NY	AY210391.1	Austin et al. (2004)
Minett, ON	AY210390.1	Austin et al. (2004)
Sauerherber Unit, Sloughs Wildlife Management Area, Henderson, KY	AY210389.1	Austin et al. (2004)
Tsinia Wildlife Viewing Area, Tuskasgee National Forest, Macon, AL	AY210387.1	Austin et al. (2004)
Ross Barnett Reservoir, Lake Harbor Road, Rankin, MS	AY210386.1	Austin et al. (2004)
L'Anguille, CR 221 and CR 220, Cross, AR	AY210384.1	Austin et al. (2004)
Dumas, Route 165 between Oakwood Bayou and Canal #43, Desha, AR	AY210383.1	Austin et al. (2004)
NESA, Jefferson, KS	AY210382.1	Austin et al. (2004)
E. Shelton Lane, Baskett Research Area, Boone MO	AY210381.1	Austin et al. (2004)
Starkville, Oktibbeha, MS	AY210380.1	Austin et al. (2004)
NESA, Jefferson, KS	AY210379.1	Austin et al. (2004)
Duck Lake, Allegan, MI	AY210378.1	Austin et al. (2004)
Big Creek Natl. Wildlife Area, Port Rowan, ON	AY210376.1	Austin et al. (2004)
St. Lawrence Isl. Natl. Park, Grenadier Island, ON	AY210375.1	Austin et al. (2004)
Leon , FL	AY210374.1	Austin et al. (2004)
Mud Lake, Welland, ON	AY210373.1	Austin et al. (2004)
L'Anguille, CR 221 and CR 220, Cross, AR	AY210372.1	Austin et al. (2004)
Dumas, Route 165 between Oakwood Bayou and Canal #43, Desha, AR	AY210371.1	Austin et al. (2004)
St. Clair Natl. Wildlife Area, ON	AY210370.1	Austin et al. (2004)
Lake Paho, Mercer, MO	AY210369.1	Austin et al. (2004)
Tyler, TX	AY210368.1	Austin et al. (2004)
Augusta, VA	AY210367.1	Austin et al. (2004)
Bug Gull Lake, Clarendon and Miller Township, ON	AY210366.1	Austin et al. (2004)
St. Clair Natl. Wildlife Area, ON	AY210365.1	Austin et al. (2004)
Reed Bingham State Park, Colquitt, GA	AY210363.1	Austin et al. (2004)
E. Shelton Lane, Baskett Research Area, Boone MO	AY210362.1	Austin et al. (2004)
Muskrat Lk., Pembroke, ON	AY083295.1	Austin et al. (2003)
Starkville, MS	AY083294.1	Austin et al. (2003)
Holden Arboretum, OH	AY083293.1	Austin et al. (2003)
Walker Mines, AL	AY083292.1	Austin et al. (2003)
Chapel Hill, NC	AY083291.1	Austin et al. (2003)
Gainesville, FL	AY083288.1	Austin et al. (2003)
Grand Teton NP, WY	KX344492.1	Kamath et al. (2016)
Bitterroot River, MT	KX344491.1	Kamath et al. (2016)
Tongue River, MT	KX344490.1	Kamath et al. (2016)
Flathead River, MT	KX344489.1	Kamath et al. (2016)
Bitterroot River, MT	KX344488.1	Kamath et al. (2016)
Yellowstone, MT	KX344486.1	Kamath et al. (2016)
Ankeny National Wildlife Refuge, OR	ANKENY1	Funk et al. (2011)
Ankeny National Wildlife Refuge, OR	ANKENY2	Funk et al. (2011)
Ankeny National Wildlife Refuge, OR	ANKENY3	Funk et al. (2011)

Ankeny National Wildlife Refuge, OR	ANKENY4	Funk et al. (2011)
Ankeny National Wildlife Refuge, OR	ANKENY5	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON1-9	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON1-10	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON1-11	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON1-12	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON2-5	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON3-2	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON3-3	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON4-5	Funk et al. (2011)
William L Finley National Wildlife Refuge, OR	FINLEY1-1	Funk et al. (2011)
William L Finley National Wildlife Refuge, OR	FINLEY1-37	Funk et al. (2011)
William L Finley National Wildlife Refuge, OR	FINLEY1-39	Funk et al. (2011)
William L Finley National Wildlife Refuge, OR	FINLEY2-1	Funk et al. (2011)
Scottsburg, OR	SCOTTSBURG5	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON1-13	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON2-1	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON2-2	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON2-4	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON3-1	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON3-6	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON3-7	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON4-1	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON4-2	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON4-3	Funk et al. (2011)
E.E. Wilson Wildlife Area, OR	EEWILSON4-4	Funk et al. (2011)
William L Finley National Wildlife Refuge, OR	FINLEY1-5	Funk et al. (2011)
William L Finley National Wildlife Refuge, OR	FINLEY1-38	Funk et al. (2011)
William L Finley National Wildlife Refuge, OR	FINLEY2-2	Funk et al. (2011)
William L Finley National Wildlife Refuge, OR	FINLEY2-3	Funk et al. (2011)
William L Finley National Wildlife Refuge, OR	FINLEY2-6	Funk et al. (2011)
William L Finley National Wildlife Refuge, OR	FINLEY2-14	Funk et al. (2011)
Waiohai Marriott, Poipu Beach, Kauai, HI	LACM1919	Cutia et al. (2022)
Waiohai Marriott, Poipu Beach, Kauai, HI	LACM1921	Cutia et al. (2022)
.Afton Canyon, Mojave River, San Bernadino-Inyo Co., CA	LACM1010	Cutia et al. (2022)
Southwestern White Mountain foothills , Warm Springs, Inyo Co., CA	LACM2987	Cutia et al. (2022)
O Drain, off State Hwy 111, Imperial Co., CA	LACM3286	Cutia et al. (2022)
Old State Highway 127, Shoshone, Inyo Co., CA	LACM3430	Cutia et al. (2022)
Old State Highway 127, Shoshone, Inyo Co., CA	LACM3431	Cutia et al. (2022)
Shoshone Hot Spring, Shoshone, Inyo Co., CA	LACM3432	Cutia et al. (2022)
James Campbell National Wildlife Refuge, Oahu, HI	JC11	Cutia et al. (2022)
James Campbell National Wildlife Refuge, Oahu, HI	JC15	Cutia et al. (2022)
James Campbell National Wildlife Refuge, Oahu, HI	JC20	Cutia et al. (2022)
James Campbell National Wildlife Refuge, Oahu, HI	JC21	Cutia et al. (2022)
James Campbell National Wildlife Refuge, Oahu, HI	JC22	Cutia et al. (2022)
James Campbell National Wildlife Refuge, Oahu, HI	JC3	Cutia et al. (2022)
James Campbell National Wildlife Refuge, Oahu, HI	JC5	Cutia et al. (2022)
Kualoa Ranch, Oahu, HI	KU1	Cutia et al. (2022)
Kualoa Ranch, Oahu, HI	KU10	Cutia et al. (2022)
Kualoa Ranch, Oahu, HI	KU12	Cutia et al. (2022)
Kualoa Ranch, Oahu, HI	KU18	Cutia et al. (2022)
Kualoa Ranch, Oahu, HI	KU19	Cutia et al. (2022)
Kualoa Ranch, Oahu, HI	KU2	Cutia et al. (2022)
Kualoa Ranch, Oahu, HI	KU20	Cutia et al. (2022)

Kualoa Ranch, Oahu, HI	KU21	Cutia et al. (2022)
Kualoa Ranch, Oahu, HI	KU22	Cutia et al. (2022)
Kualoa Ranch, Oahu, HI	KU4	Cutia et al. (2022)
Kualoa Ranch, Oahu, HI	KU5	Cutia et al. (2022)
Kualoa Ranch, Oahu, HI	KU7	Cutia et al. (2022)
Kualoa Ranch, Oahu, HI	KU8	Cutia et al. (2022)

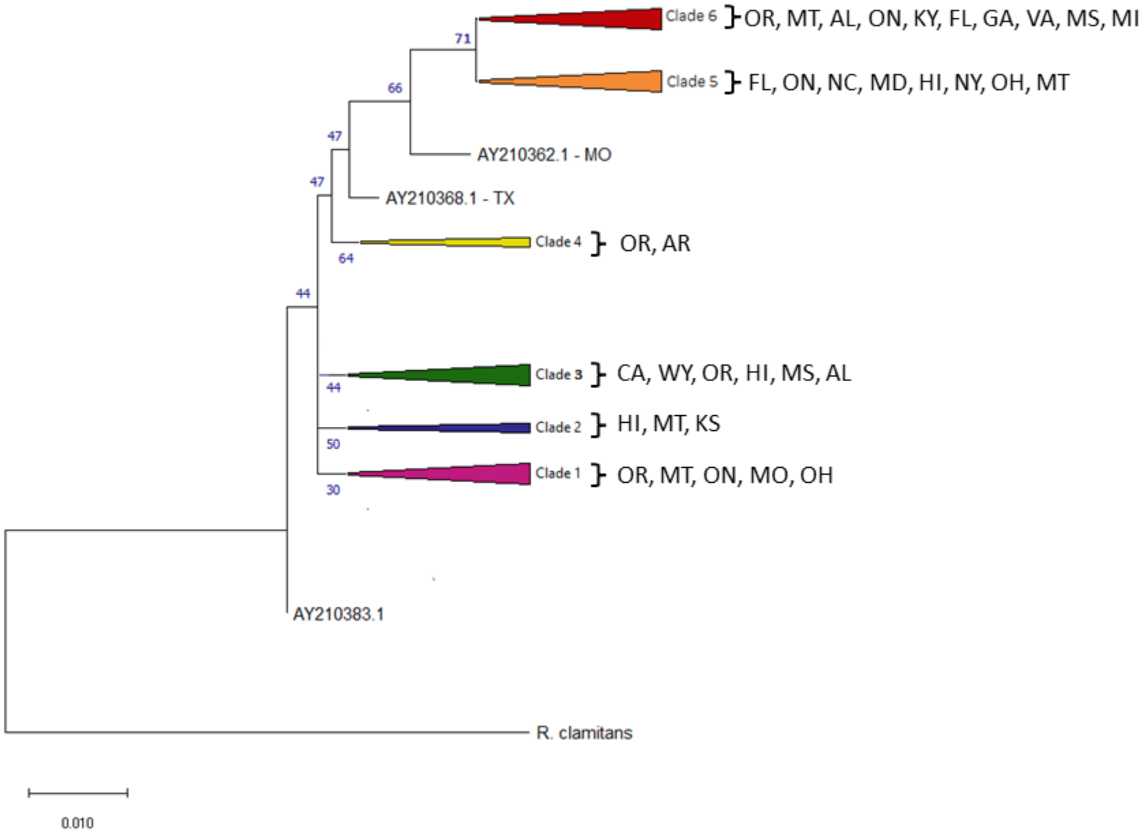

Figure 12. Maximum Likelihood analysis of R. catesbeiana from North America using cytochrome b. The evolutionary history was inferred using the Maximum Likelihood method and Kimura 2-parameter model with discrete gamma distribution(Kimura, 1980). This substitution model was identified as having the best fit using the Bayesian Information Criterion value. The tree with the highest log likelihood (-896.74) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.2787)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 112 nucleotide sequences. There were a total of 326 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).