SYSTEMATICS, EVOLUTION, AND BIOGEOGRAPHY AMONG DRACAENOID GENERA: DRACAENA, PLEOMELE, AND SANSAVIERIA (ASPARAGACEAE)

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By

Pei-Luen Lu

DISSERTATION COMMITTEE:

Clifford W. Morden, Chairperson
Stacy Jørgensen
Sterling C. Keeley
Will C. McClatchey
Thomas A. Ranker

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ABSTRACT

This study presents the molecular evolutionary relationships among the ambiguous three plant genera Dracaena Vand. ex L., Pleomele Salisb., and Sansevieria Thunb. (dracaenoids) first described in the 18th century. The taxonomy of dracaenoids has many ambiguities and until present there have been no molecular systematics study addressing this issue. This dissertation provides the first complete molecular phylogenetic study to include a thorough representative collection of dracaenoids and discusses their systematics and biogeography on a global scale. At a more local scale, population genetics of two endemic Hawaiian Pleomele species (one endangered and one common) were done for expanding our biological knowledge of these species and assessing conservation needs based on results of random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) data. The relationships of dracaenoids with combined chloroplast DNA sequences were reconstructed. The combined dataset was analyzed using parsimony, Bayesian, and maximum likelihood. The combined dataset was analyzed using parsimony, maximum likelihood, and Bayesian analysis. Results show that 1) the monophyly of dracaenoids is confirmed; 2) the Hawaiian Pleomele species are the sister group to the remainder of the dracaenoid phylogeny; 3) the species Dracaena and Pleomele are intermixed; 4) Sansevieria is monophyletic, but is nested within Dracaena; 5) the Central American species D. americana and D. cubensis are the basal group to the remainder of the dracaenoid species. The common ancestor of dracaenoids is from Asia rather than Africa where the center of diversity among dracaenoids is located. Based on these results, a new combination recognizing the Hawaiian species of Pleomele as a distinct genus, Chrysodracon, and combining the three remaining genera all into Dracaena is proposed.
Chapter 3 examines the evolutionary history and biogeography of the endemic tree genus *Chrysodracon* (formerly the Hawaiian species of *Pleomele*). The results provide clear evidence of the monophyletic relationship of these species, their biogeographical patterns among the main Hawaiian Islands, and provide evidence for a new Hawaiian *Chrysodracon* species using molecular data. Evidence suggests that there was an initial colonization of the islands to Kauai or Maui, or both concurrently, followed by dispersal and speciation in the other islands.
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ABSTRACT

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Chapter 1. Introduction, Literature Review, Hypotheses, and Methodology
INTRODUCTION

*Dracaena* Vand. ex L., *Sansevieria* Thunb., and *Pleomele* Salisb. are genera with a significant presence in flora’s of the tropic and subtropic regions worldwide. Species in these genera represent an important example of evolution of Asparagaceae for their rapid adaptation to a diversity of habitats. Moreover, they are widely used in horticulture and medicine. However, there are several issues related to the three genera that are in need of resolution. For example, the systematic classification of species within these genera are replete with misidentifications, the classification scheme has not been reexamined since the early 20th century, and no molecular systematic approach has been used to address species and genera relationships (Bureki et al., 2009). Most treatments of the genera are limited to regional taxonomies (Wagner et al., 1990; Bos, 1998; Staples and Herbst, 2005; Mwachala and Mbugua; 2007), and little research into the broader biogeography and evolution of species has been pursued. Of greater urgency recently is that many of the species are increasingly becoming rare (both worldwide and in Hawaii) and conservation efforts are being made to preserve dwindling populations even though species relationships among them have not been clearly resolved. Therefore, the purpose of this study is to clarify the classification of species among *Dracaena*, *Sansevieria*, and *Pleomele*. In so doing, a better understand of their systematics, evolution, and biogeography will be possible which will in turn allow land managers to better understand the conservation status of the species. In addition, these results will help shed light on the ethnobotanical use of these species and their potential use in horticulture and medicine.
LITERATURE REVIEW

SECTION I: Family and relationship

_Dracaena, Pleomele, and Sansevieria_ had been placed in the family Liliaceae (Brown, 1914 and 1915). Among the characteristics that support this include a superior ovary, leaves that are not twisted at base, bulbs present, and fleshy fruits (Brown, 1914 and 1915; Bos, 1980; Waterhouse, 1987). _Dracaena_ species are woody, unlike the typically herbaceous Liliaceae. Others have classified _Dracaena, Pleomele, and Sansevieria_ in the family Agavaceae based on the features of flowers with six stamens, paniculate inflorescences, and plants with rosettes of fleshy fibrous leaves (Hutchinson, 1973; Huang, 1993; Staples and Herbst, 2005). The ovary in species of these genera is superior, unlike other Agavaceae.

Salisbury (1866) defined the family Dracaenaceae that included the genera _Dracaena, Sansevieria, Pleomele, and Liriope_ (Salisbury, 1866). Since then, the family has been variously classified with the number of genera expanded to six (Mabberley, 1997) or to as few as one (Bos, 1998) or two (Watson and Dallwitz, 1992). Nakai (1936) later classified the three primary genera of Dracaenaceae (_Dracaena, Sansevieria, and Pleomele_) into the family Sansevieriaceae, although this was subsequently viewed as a synonym.

With the completion of detailed phylogenetic analyses resulting in clarification of ordinal and family relationships (APGII, 2003; APGIII, 2009), the family alignment among dracaenoid genera was reevaluated. _Dracaena, Pleomele, and Sansevieria_ were classified within the family Ruscaceae (APGII, 2003; Judd et al. 2007). A close relationship among these genera was demonstrated among the genera included in this family. This was determined by molecular analysis of 18S rDNA, _rbcL, atpB_, and _matK_, and with morphological characters.
including the presence of resin canals in leaves and bark (APGII, 2003; Hilu et al. 2003; Judd, 2003; Judd et al. 2007). Key characters for inclusion of these genera within the Ruscaceae included a superior ovary, berry fruit, and the primary photosynthetic organ being leaves rather than stems (Judd et al. 2007). However, subsequent analyses with additional representatives of related genera resulted in an unclear relationship among taxa and questionable family alignments (APGIII, 2009). Ultimately, genera were submerged into the large and complex family Asparagaceae, subfamily Nolinoideae (Chase et al., 2009).

The most recent treatment of the dracaenoid genera was completed by Brown (1914) where he separated the three genera based leaf differences. Leaves of Dracaena are thick and rigid; leaves of Pleomele are thin, flat (never fleshy), and usually with an evident and prominent abaxial mid-rib; leaves of Sansevieria are cylindrical or laterally compressed, flattened leaves sometimes with an inconspicuous mid-rib. Flowers of Sansevieria and Dracaena are macro-morphologically similar, but different from Pleomele. Leaves of Pleomele and Dracaena are macro-morphologically similar, but different from Sansevieria. The following sections will review the taxonomic history and other attributes of each genus in detail.

SECTION II: Dracaena

Dracaena was first described in 1762 by Vandelli and validly published later by Linnaeus (1767) with the type species of D. draco L. Dracaena can be categorized into two groups: arborescent and arbustial (Mabberley, 2008), the arbustial species being the popular horticultural plants. Several morphological features characterize Dracaena (Brown, 1914; Bos,
1998; Staple and Herbst, 2005). The leaves are alternate, distichously or spirally arranged, sword-shaped to oblong, often thickened and usually amplexicaulous with a sheathing base. Guard cells, unlike those in Nolinoideae, do not contain oil. Aerial stems often have obvious leaf scars. The inflorescence is terminal on each branch and flowers are often grouped together on articulated pedicels. Perianths have a short tube and are divided into six segments. The ovary is superior, and cylindrical to bottle-shaped with three locules, and one ovule per cell. *Dracaena* has six styles, septal nectaries are present, and the stigma is capitate with three lobes. Fruits are fleshy berries. The pollen grains are sulcate (Erdtman, 1952). The chromosome number of *Dracaena draco* is $2n=38$ (Bowden, 1945).

Reproductive traits associated with the ecology of *Dracaena* species suggest night pollination. *Dracaena* are nocturnal flowering species with a strong fragrance during the late evening when nectar is produced to attract night-active pollinators (Bos, 1998). This adaptation is variable. Habitats can vary from semi-desert to rainforest, near seashores, grasslands and cliff areas, and from sunny exposed areas to forest understory (Bos, 1998). Some *Dracaena* species are thermophile (Marrero et al., 1998; Adolt and Pavlis, 2004).

**Subsection I: Historical Biogeography of Dracaena**

*Dracaena* comprises between 60 to 80 species worldwide, mainly in the tropics and subtropics with the exception of South America (Mabberley, 2008; Bos et al., 1992; Bos, 1998; Staples and Herbst, 2005; Judd et al., 2007). Africa is regarded as the center of diversity for *Dracaena* with Southeast Asia regarded as a secondary region also high in diversity (Bos, 1984 and 1998; Marrero et al., 1998; Mwachala and Mbugua, 2007). Most species are native to Africa with some species distributed in Madagascar, Asia, Socotra, Mediterranean regions,
Central America, Cuba, Micronesia, Northern Australia, and Pacific Islands (Gwyne, 1966; Bos, 1998; Marrero et al., 1998; Staples and Herbst, 2005). The distribution of paleogeography was Holarctic, Paleotropical, Neotropical, and the cape region of South Africa (Watson and Dallwitz, 1992).

Species similar to modern dracaenoids are found in the fossil records. Two extinct *Dracaena* species from the Neogene (23 ± 0.05 million years ago) have been identified based on the analysis of pollens in these fossils (Van Campo and Sivak, 1976). *Dracaena saportae* is recorded from Bohemia, Czech Republic, and *Dracaena guinetii* is recorded in Tunis, Tunisia Republic (Van Campo and Sivak, 1976; Bonde, 2005). Six *Dracaena* fossil species recorded from the Eocene to Neogene (55 to 23 ± 0.05 million years ago) have been identified based on the analysis of pollens and ensiform leaf structure (Van Campo and Sivak, 1976). They are *Dracaenites brongniartii, Dracaenites sapultus, Dracaenites narbonensis, Dracaenites resurgens, Dracaenites minor, and Dracaenites pusillus* recorded in the vicinity of the Tethys Sea (Saporta, 1862, 1865, 1873ab, 1888, 1889).

**Subsection II: Medicinal Use of *Dracaena***

Some species of *Dracaena* are highly valued for their medicinal properties (Lee, 1975; Chun, 1994; Edward et al., 2001). One of the common names for *Dracaena* is “dragon’s blood” (Milburn, 1984). The name originated from the deep red liquid that exudes from injured bark. This liquid has been shown to have some ability to cure injuries (Lee, 1975; Edward et al., 2001; Langenheim, 2003). The resin can be dried to produce many cultural herbal medicines, and has been used in Chinese, Asian, Arab, ancient Greek, Roman, Indian, and African cultures (Lee, 1975; Milburn, 1984; Miller and Morris, 1988; Chun, 1994; Edward et al., 2001;
Langenheim, 2003). *Dracaena draco*, *D. cinnabari*, *D. ombet*, *D. cambodiana*, and *D. cochinchinensis* were species reported to have used for the medicinal function of “Dragon’s blood” (Miller and Morris, 1988; Wu and Raven et al., 1994; Edward et al., 2001; Langenheim, 2003; Gonzalez et al., 2004). Although comprehensive information on the chemical composition of the dragon’s blood resin is available (Himmerlreich et al., 1995; Edward et al., 2001; Vesela et al., 2002; Langenheim, 2003; Gonzalez et al., 2004), too few studies of the relationship to its medicinal function, specific compounds, and systematics have been completed.

In traditional Chinese medicine, dragon’s blood has been called Chi-lin-chieh (麒麟竭) or Hsueh-chieh (血竭) (Lee, 1975). Lee (1975) indicated that in 659 A.D., *Tong Ben Cao* (Su, 2005), the first official medical book written and published by the government (Tong Dynasty, from 618 A.D. to 917 A.D.) in traditional Chinese history, recorded Chi-lin-chieh as an “essential” Chinese medicinal plant (Lee, 1975 and 2004). Since the Tong Dynasty, Chinese have used Chi-lin-chieh to cure wounds, internal injuries, hemorrhages, and surgical trauma (Lee, 1975 and 2004). The medicinal material was the resin from chopping and then burning the plant stems (Lee, 1975 and 2004). Afterward, the red gum was packed into large dark masses, and the medicinal material was transported to traditional Chinese medicinal shops (Lee, 1975 and 2004). There, it was usually made into a tasteless powder that would be soluble when mixed with spirits or wine (Lee, 1975 and 2004).

**Subsection III: Horticultural Use of Dracaena**

Several species of *Dracaena* are valued horticultural plants (Bos, 1980, 1984, 1992, and 1998; Staples and Herbst, 2005). Because of interesting patterns of leaf variegation, many
Dracaena species are popular, especially in America and Europe. People decorate their homes with Dracaena, particularly in temperate zones (Bos, 1998; Staples and Herbst, 2005). In horticulture, Dracaena species are very important with at least 40 Dracaena species being utilized in decoration (Staples and Herbst, 2005; John Griffis, personal communication). Many horticulturists aim to produce new Dracaena cultivars by cross breeding. Because of this, it is often difficult to find correct scientific names for the Dracaena horticultural plants from markets as many Dracaena horticultural species are reproduced asexually and often rarely produce flowers and fruits where identifications could be made. Not only does this confuse scientists, but it also baffles the growers who want to grow new species of Dracaena. Moreover, due to uncertain positions of classifications for Dracaena, breeding a new horticultural species of Dracaena takes longer time. Most importantly, in application of horticulture, if one horticulturist knows the precise classification, he/she can breed it in proper seasons and methods, thereby shortening the breeding time from 5 years to 1 year. Therefore, establishing the systematics of Dracaena will help to improve identification and breeding efforts in horticulture.

SECTION III: Pleomele

Pleomele was first named and its type species identified as Pleomele fragrans by Salisbury (1796). The genus consists of 40 to 50 species worldwide though some estimates suggest that there may be as many as 100 species in the Old World tropics (Wagner et al., 1990; 2003). Because of lacking phylogenetic evidence, the monophyletic status of Pleomele is not affirmed even though the genus is regarded as a monophyletic based on several morphological
treatments (Bos, 1980 and 1998; St. John, 1985; Wagner et al., 1990; Staples and Herbst, 2005). To date, the center of diversity of Pleomele is unknown, and little biological information is available.

There are six species of Pleomele presently recognized as being endemic the Hawaii Islands (Wagner et al., 1990; Wagner and Herbst, 2003). St. John (1985, 1987) recognized 10 Pleomele species in Hawaii and described their morphological features. However, Wagner et al. (1990, 2003) reclassified the genus and recognized only six species in the Hawaiian flora. Pleomele aurea (H. Mann) N. E. Brown is found on Kauai. Pleomele auwahiensis St. John is present on Maui and Molokai. Pleomele fernaldii St. John is restricted to Lanai. Pleomele forbesii Degener and Pleomele halaapepe St. John are on Oahu. Pleomele hawaiiensis Degener is only in Hawaii (Big Island). Jankaski (2008) did not consider Pleomele to be a distinct genus from Dracaena but recognized the Hawaiian species as a distinct new subgenus. He showed that these species did not fit the description of Pleomele based as characterized by Brown (1914), and identified these species as Dracaena subgenus Chrysodracon. Most species of Hawaiian Pleomele were discovered sibequent to Brown (1914), and thus he was unable to examine these species. Taxonomists placed the Hawaiian species into Pleomele as this was considered the most appropriate classification (St. John, 1985).

Subsection I: Questionable Relationship of Pleomele and Dracaena

Pleomele has had a long and intimate relationship with Dracaena. The type species, Pleomele fragrans, had been previously described as Dracaena fragrans (Ker Gawler, 1808). Brown (1914) separated Pleomele from Dracaena based on floral differences. Dracaena has a very short perianth tube and staminal filaments thickened near the middle. In contrast, the
perianth tube of *Pleomele* has at least one-third connate tepal length (Brown, 1914; Wagner et al., 1990). However, in recent studies, *Pleomele* was addressed as the synonym of *Dracaena* based on similar morphological characteristics (Stevens, 2001; Staple and Herbst, 2005; Mabberley, 2008). Taxonomic ambiguity regarding the uncertain relationship of the two genera, *Dracaena* and *Pleomele*, is in need of resolution.

Subsection II: Medicinal Use of *Pleomele*

Hawaiians call some *Pleomele* species *hala pepe* or *le‘ie*. The Hawaiians used it both culturally and medicinally. It was used as one of five different species at Hula altars to honor Laka, the deity of hula along with *maile*, *`ie`ie*, *`ôhia lehua*, and *palapalai*. In native Hawaiian herbal medicine, *hala pepe* was used to cure fever by pounding it into fresh liquid (Pukui and Elbert, 1986; Chun, 1994). After Hawaiians ground fruits and bark of *hala pepe*, it was then burned until the red liquid it became colloid but not become coke. Patients would drink the mixture in the morning and in the evening to cure symptom (Chun, 1994).

Subsection III: Species Rarity and Hawaiian *Pleomele* Species

The Hawaiian Archipelago includes various endemic species that are the result of speciation subsequent to isolation from source populations (Carlquist, 1970 and 1980; Ziegler, 2002). Although many lineages of plants are species rich in Hawaii, few of them have been studied in detail. The mesic and dry forests of the Hawaiian Islands have been reduced due to habitat loss by human development and the introduction and spread of invasive plants and animals (Gagne and Cuddihy, 1999). More than 90% of Hawaiian dry forests are already lost (DLNR, 1992). Genetic deterioration may result from decreasing population size. Many studies
have shown that small populations tend to have lower genetic variation, especially among rare plant species (Ellstrand and Elam, 1993; Fischer and Matthies, 1998; Morden and Loeffler, 1999; Vergeer et al., 2003; Oliver et al., 2005; Will et al., 2007). Therefore, conservation of genes, species, populations, and ecosystems are essential to maintain biodiversity and population dynamics.

According to International Union for the Conservation of Nature and Natural Resources (IUCN) red list of threatened species (2007), *Pleomele hawaiiensis*, *Pleomele forbesii*, and *Pleomele fernaldii* are endangered species. The US Fish and Wildlife Service (USFWS) listed *Pleomele hawaiiensis* as an endangered species in 1996, and *Pleomele forbesii* and *Pleomele fernaldii* became candidates for endangered species status in 1997 and 2001, respectively. *Pleomele hawaiiensis* exists only in 6-8 populations totaling approximately 300-400 individuals. *Pleomele forbesii* currently totals approximately 500 individuals from 12 populations. *Pleomele fernaldii* is found in three populations totaling fewer than 300 individuals in rare dry forests on the leeward side of Lanai. To date, nothing is known about genetic variation of these species. Notably, the total individuals of *Pleomele fernaldii* are only 200 individuals, but the USFWS only lists it as a candidate, not an endangered species. This species may possibly be extinct in few years if no preservation actions are taken.

**SECTION IV: Sansevieria**

Thunberg (1794) first described *Sansevieria* and its lectotype of *Sansevieria hyacinthoides* (L.) Druce later described (McVaugh, 1974). Common name of some
Sansevieria species include “snake plant” or “Bowstring hemp,” and the famous horticultural plant “mother-in-law’s tongue” (S. trifasciata) (Staple and Herbst, 2005). The genus name has been variously spelled by different names more than one century (Sanverinia, and Sanseveria), but the spelling Sansevieria was conserved by Harms in 1904 (Harms, 1904; Marais, 1973; McVaugh, 1974). Plants are usually xerophytic perennials that are often rhizomatous, and their habit is herbaceous (Staple and Herbst, 2005). The leaves are alternate with entire margins, and parallel venation, usually stiff and often succulent (Brown, 1915). Flowers are crowded in dense racemes or panicles, bisexual, and hypogynous. Pedicels often have disc-like articulations. Perianths are in two series of three each and petaloid. Stamens are in two series of three and exserted. Ovaries are superior and three-locular. There is one ovule per locule and the placentation is axillary. Fruits are berries with up to 3 seeds per fruit. (Brown, 1915; Bos, 1998; Mwachala and Mbugua, 2007). Sansevieria trifasciata is pollinated nocturnally; plants flower with a strong fragrance during the late evening and produce nectar on the inflorescence to attract night-active pollinators (personal observation).

Subsection I: Historical Biogeography of Sansevieria

Sansevieria comprises about 61 species worldwide, mainly in the tropics and subtropics of the Old World (Mabberley, 2008; Marais, 1973; Bos, 1998; Staples and Herbst, 2005). Some species have medicinal and horticultural value (Neuwinger, 1996; Khalumba and Mbugua; Bos, 1998; Staples and Herbst, 2005). Africa is the center of diversity for Sansevieria (Mabberley, 2008; Brown, 1915; Morgenstern, 1979). Although most species are native to Africa, the distribution extends North into the Arabian Peninsula, and east to Southeast Asia (Morgenstern, 1979; Carlquist and Schneider, 2007). Species generally grow in dry areas (Brown, 1915). The
ecology and reproduction mechanism of Sansevieria are similar to those of Dracaena (Bos, 1998).

Subsection II: Questionable Relationship of Sansevieria and Dracaena

It has been proposed several times that Sansevieria be united with Dracaena (Baker, 1875; Brown, 1914; Bos, 1984). Recent classifications have treated Sansevieria as a synonym of Dracaena based on their overlapping morphological characteristics (Bos, 1998; Mabberley, 2008). However, Sansevieria is conserved as a genus by several botanists (McVaugh, 1974; Newton, 2002; Jankaski, 2003). Recent molecular work has focused mainly on deciphering the family level phylogenetics. These studies have generally only sampled one or two species of each genus (and did not include type species) and showed they are closely related, but did not resolve their status (APGII, 2003; APGIII, 2009; Kim et al., 2010). Taxonomic ambiguity regarding the uncertain placement between Dracaena and Sansevieria should be resolved by molecular phylogenetics with more complete inclusion of taxa in the future.

Subsection III: Ethnobotany of Sansevieria

Sansevieria aethiopica, S. bagamoyensis, S. ehremberggi, S. liberica, S. trifasciata, S. senegambica and S. roxburghiana are species known for their leaf juices that have ethnomedicinal function. Ailments they are believed to cure include fever, pain, bleeding, and inflammation by many cultures throughout Africa and in India (Neuwinger, 1996; Binojkumar, 2002; Haldar et al., 2010). The medicinal function is similar to the extant reports as Pleomele and Dracaena.

Fibers in the leaves of some species are known to be a valuable economic source in
many African indigenous cultures (Neuwinger, 1996; Khalumba and Mbugua, 2005). Since 1887, when the Royal Botanical Gardens, Kew established the biggest cultivated collection of Sansevieria species in the world, more people and governmental organizations have been interested in the development of fibers from Sansevieria (Brown, 1915). This trend was also extended to the America’s and this practice continued up until World War II and in the Cold War (Dodge, 1983; Pate, et al., 1960; Koller and Thomas, 1988; Khalumba and Mbugua, 2005).

**HYPOTHESES**

The present study was undertaken to explore the phylogenetic relationships within and among species of Dracaena, Pleomele, and Sansevieria. Specifically, these include addressing phylogenetic relations among genera and among species, their biogeography, evolution, population genetics of endemic Pleomele species, and the potential medicinal usages based on these phylogenetic relationship.

**Objective 1:** to understand the relationships of Dracaena, Sansevieria, and Pleomele using molecular phylogenetic evidence.

**Hypothesis 1:** Dracaena, Pleomele, and Sansevieria are each monophyletic.

Recent molecular systematics at the family level of Agavaceae and Ruscaceae (Bogler and Simpson, 1995, 1996; Hilu et al., 2003; Kim et al., 2010) support Dracaena as a monophyletic group with other genera under those families. Brown (1914) and St. John (1985) distinguished Pleomele from Dracaena based on morphological characters. Sansevieria can be
separated from *Dracaena* and *Pleomele* based on their morphological characteristics (Brown, 1914, 1915; Newton, 2002).

**Objective 2:** to elucidate the biogeography of *Dracaena, Pleomele,* and *Sansevieria* throughout their distributions around the world based on molecular studies. Such a model of dracaenoid biogeography may also contribute to my third and fourth objective.

**Hypothesis 2: The common ancestor for the dracaenoids is from an African *Dracaena* species.**

The center of biodiversity for *Dracaena* and *Sansevieria* is located in Africa. The *Dracaena* fossil record is located in the Europe and North Africa and in the vicinity of the old Tethys Ocean (Van Campo and Sivak, 1976; Bonde, 2005; Mabberley, 2008). Thus, it is expected that modern dracaenoids would be descended from species originating in this vicinity.

**Objective 3:** to understand the evolution and the biogeography of *Pleomele* in the Hawaiian Archipelago.

**Hypothesis 3: The ancestor of Hawaiian *Pleomele* originated in Asia**

The majority of Hawaiian species are descended from colonists arriving from Asia or the Indo-Pacific region (Wagner et al., 1990). Species of Pleomele are known in these regions as well as on islands of the south Pacific, and it is conjectured that the Hawaiian species are related to these taxa (Wagner et al., 1990).
Hypothesis 4: Hawaiian *Pleomele* followed a general pattern of west-to-east (Kaua‘i to Hawai‘i) migration down the Hawaiian Island chain.

With only one exception, species of Hawaiian *Pleomele* are island specific. It is suspected that initial colonization was to the oldest of the Hawaiian Islands (Kauai if not a now submerged high island among the Northwest Hawaiian Islands) followed by a stepping stone colonization down the island chain as suitable habitat became available (Wagner and Funk, 1995; Ziegler, 2002).

Objective 4: to investigate the genetic structure within and among populations of *P*. *hawaiiensis*, a rare and endangered species, and compare this variation to the more common and non-endangered species *P. auwahiensis*. This will be done to determine the relationship between population size and the level of diversity by using RAPD markers. Understanding the population genetic structure of Hawaiian *Pleomele* species may be desirable to provide insight needed to establish proper conservation strategies to preserve the biodiversity of an island ecosystem.

Hypothesis 5: Genetic variation is low within and among populations of the endangered *Pleomele hawaiiensis*.

Many studies have shown that endangered species have lost genetic diversity (Ellstrand and Elam, 1993; Fischer and Matthies, 1998; Vergeer et al., 2003; Olver et al., 2005; Will et al., 2007). The endangered species *P. hawaiiensis* on Hawaii Island has fewer than 300 wild individuals in several populations along the entire length of the Kona coast. Inbreeding with concomitant loss of alleles is expected have resulted in very low diversity within populations.
To address the stated hypotheses, phylogenetic will be used to assess monophyly of the three genera, including the analysis of relationship of those medicinal species, to elucidate relationships among the Hawaiian *Pleomele* taxa, including an assessment of possible new species separated from *P. auwahiensis* and to investigate historical biogeography of the three genera.

RAPD and ISSR analyses were employed to examine population genetic diversity and differentiation, and to assess gene flow among the populations for *P. hawaiiensis* and *P. auwahiensis*, respectively. The comparison between the two endemic Hawaiian species will then be discussed. Results from the analyses of *P. auwahiensis* were also used to address questions of speciation among its populations; the results from *P. hawaiiensis* will be useful for proper conservation management of this species.

**MATERIALS AND METHODS**

*Taxon sampling for phylogenetics*

A total of 32 *Dracaena* species, 34 *Sansevieria* species, and 31 *Pleomele* species representing the global distribution of these genera were examined in this study. Six undescribed *Dracaena* species native to Thailand discovered by Paul Wilkin (Kew Herbarium, London, UK) were included. Sources of DNA for sequencing included freshly collected leaves, herbarium specimens and the DNA Bank at Royal Botanic Gardens, Kew, United Kingdom. Table 1 includes specimen source, voucher information, locality, and collection number. All novel sequences generated for this study will be deposited in GenBank.
selection of five outgroup taxa from the Asparagaceae (Nolinoideae) was based on the results of previous studies and were shown to be closely allied to dracaenoid genera (Bogler and Simpson 1996; Rudall et al., Kim et al., 2010). Four of the outgroup species (Comospermum yedoensis, Disporopsis pernyi, Liriope muscari, Speirantha gardenii) are distributed in East Asia and one is from Africa (Eriospermum flagelliforma).

**DNA extraction and amplification**

Total genomic DNA was extracted from 1.0 g of fresh or 0.2 g of silica gel-dried leaves using the CTAB method (Doyle and Doyle, 1987) with modification (Morden et al., 1996), or using the Qiagen DNeasy plant mini kits (Qiagen Corporation, Valencia, CA, USA) following manufacturer specifications. DNA samples were accessioned into the Hawaiian plant DNA library (Morden et al. 1996).

Data for this study includes four chloroplast intergenic spacer regions: trnL-trnF, ndhF-rpl32, trnQ-rps16, and rpl32-trnL. The polymerase chain reaction (PCR) was performed using Eppendorf (Westbury, New York, USA) Mastercycler gradient or MJ Research Thermal PCR machine (GMI, Inc. Ramsey, Minnesota, USA). The PCR reaction was performed in a 25 µl reaction mixture containing 5 µl 5X Green or colorless GoTaq Flexi PCR buffer, 1.5 µl 25 mM MgCl2, 0.5 µl 10 mM dNTPs, 0.5 µl 10 mM primers, 0.2 µl GoTaq polymerase (5 U/ul) (Promega, Madison, WI, USA), and 14.5 µl ddH2O. The rpl32-trnL region was amplified using standard primers pairs rpl32 (CAGTTCCAAAAAACGTACTTC) and trnL (CTGCTTCCTAAGAGCAGGT) (Shaw et al., 2007). The trnQ-rps16 region was amplified using standard primers pairs trnQ (GCGTGCCAYGMATATT) and rps16 (GTTGCTTTYTACCACATCGTTT) (Shaw et al., 2007). The rpl32-trnL and trnQ-rps16 with
the following PCR conditions: 5 min at 80°C, followed by 30 cycles at 95°C for 1 min, 50°C for 1 min (a ramp 0.3°C/sec to 65°C), 65°C for 4 min, plus a final extension of 5 min at 65°C. The *ndhF-rpl32* region was amplified by the primer pairs Forward (GCATATTGATAKGTATGTTCCA) and Reverse (ATMGAAGTRCGTTTYTTTTGG) (Scarcelli et al., 2011). Two parameters were used to amplify this region. The first one with the following PCR conditions: 3 min at 94°C, followed by 35 cycles at 94°C for 30 sec, 42°C for 30 sec, 72°C for 1 min, plus a final extension of 10 min at 72°C. The second one with the following PCR conditions: 5 min at 80°C, followed by 35 cycles at 94°C for 1 min, 42°C for 1 min (an increasing 3°C/sec), 65°C for 4 min, plus a final extension of 10 min at 65°C. The *trnL-F* region was amplified by the forward primer (e; GGTTCAAGTCCCTATCCC) and the reverse primer (f; ATTTGAACGTGATACACGAG) of Taberlet et al. (1991) with the following PCR conditions: 80°C for 5 min followed by 29 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 4 min, plus a final extension of 5 min at 72°C (Shaw et al. 2005). The first spacer region of the internal transcribed spacer (ITS1) amplification was design here for dracaenoid specificity: *Chrysodracon*-forward (CTTACGTKGTTCTTCATCGATGC) and *Chrysodracon*-reverse (GGAAGGATCATTGTCGTGAC) with the following PCR conditions: 95°C for 2 min, 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, final extension of 72°C for 7 min. Non-dracaenoid species were amplified using standard primers found in the literature (Bogler and Simpson 1996) based on soybean 18S rDNA, position 1762-1787 (AAGTCGTAACAAGGTTTCCGTAGGTG) and on rice 26S rDNA, position 52-67 (TTTCTTTTCTCCGTGCT) with the following PCR conditions: 94°C for 2 min, 35 cycles of 94°C for 30 sec, 50°C for 2 min, 72°C for 2 min, plus a final extension of 72°C for 10 min. All amplifications were verified on 1.5% agarose gel.
DNA amplification by polymerase chain reaction (PCR), and template purifications were performed with Go Taq PCR Core Kit (Promega, Madison, WI, USA) and all PCR products were prepared for sequencing using ExoSAP-IT (USB Corporation, Cleveland, OH, USA) or similar treatment with shrimp alkaline phosphatase and exonuclease I following the USB ExoSAP-IT PCR product cleanup protocol of incubation for 37°C for 15 min followed by 80°C for 15 mins. Samples were bidirectionally sequenced at the University of Hawaii’s ASGPB Sequencing Facility (http://cgpbr.hawaii.edu/) using BigDye Terminator chemistry (Applied Biosystems, Foster City, California, USA) and visualized on an ABI 3730XL capillary-based DNA sequencer (Applied Biosystems).

**Sequence Alignment and Phylogenetics analysis**

Contiguous sequences were constructed and edited for all ITS1, trnL-trnF, ndhF-rpl32, trnQ-rps16, and rpl32-trnL sequences using MEGA 5 (Tamura et al., 2011). All sequences were aligned initially in ClustalW (ver. 2.1; Larkin et al., 2007) and Muscle (Edgar 2004) and then manually adjusted in MEGA 5 following the guidelines of Kelchner (2000) to minimize indels. Sequences from all four cpDNA regions were combined into one dataset and indels were excluded from subsequent phylogenetic analyses. ModelTest version 3.7 (Posada and Crandall, 1998) was used to determine the best model of DNA substitution. The best-fit models as determined in evolutionary models were selected by Akaike information criterion (AIC; Akaike, 1974) for each partition and the combined dataset, evaluating all models against defaults of the program. The GTR+G model (a general time reversible model with a gamma-shaped distribution of rates across sites) was chosen for the four regions combined data matrix and the nuclear region combined with chloroplast regions as the best-fitting among the 24
models compared and was used to construct the ML and Bayesian trees. The models for separate and combined data partitions are as follows: ITS1: Tamura 3-parameter (T-92); \textit{ndhF-trnL}: T92; \textit{TrnF-trnL}: T92; \textit{trnL-rpl32}: T92+G (T92: Tamura 3-parameter with a discrete Gamma distribution); \textit{trnQ-rps16}: T92+G. Sequences were concatenated and aligned according to codon positions in a NEXUS file and phylip file prior to conversion by Mesquite 2.7.4 (Maddison and Maddison, 2009) into the appropriate file format necessary for each tree search application. The data were separated into two major partitions, nuclear (ITS1) and chloroplast (\textit{trnF-trnL}, \textit{trnQ-rps16}, \textit{ndhF-rpl32}, \textit{rpl32-trnL}). The data partitions were compared using the incongruence length difference (ILD) test (Farris et al., 1994), implemented as the partition homogeneity test in PAUP* version 4.0b10 (Swofford, 2002). For each test, 1000 replicates were performed using heuristic searches, tree-bisection-reconnection (TBR) branch swapping with random addition for three replicates, nchuck=2, and chuckscore=1.

Maximum parsimony (MP), maximum likelihood (ML) and Bayesian methods were used to estimate phylogenies for all data sets (nuclear, chloroplast and combined). Maximum parsimony search were conducted using PAUP* 4.0b10 (Swofford, 2002). In all MP analyses, heuristic searches were done using a starting tree built from stepwise addition with 1000 random addition replicates and TBR branch swapping. Bootstrap analyses based on 1000 replicates with 10 random additions per replicate were used to assess confidence in clades.

Maximum likelihood (ML) analyses as an optimality criterion (Felsenstein 1981) was conducted for each of the three datasets using RAxML v7.2.7 (Stamakis, 2006; Stamakis et al., 2008). RAxML uses the GTR model with six categories of rate variation, and use of a separate support for individual branches was assessed using the nonparametric bootstrap
(Felsenstein, 1985) via the rapid bootstrap procedure of Stamatakis et al. (2008) with bootstrapping stopped automatically using a frequency based criterion (Pattengale et al., 2010). Maximum likelihood bootstrap proportions (MLBS) >70% were considered strong support (Hills and Bull, 1993).

MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2005) was used to conduct Bayesian analyses under a time-free model. Bayesian analyses were run using four incrementally heated chains for 1,000,000 generations and a sampling frequency of 100 generations. Tree samples and parameter estimates from the first 25% of all trees (2500) were designated as the burn-in period and discarded. Posterior probability (PP) support values >0.95 were considered strong support for individual clades (Erixon et al., 2003; Huelsenbeck and Rannala, 2004).

Plant collection for population genetics

Leaf tissues were randomly collected from extant populations of each species in Hawai‘i (P. hawaiiensis) and Maui (P. auwahiensis). Each population for both species has voucher specimens deposited in Bishop Museum (Table 1). The endangered species permit for P. hawaiiensis is No. P-159. Permits are attached in the Appendix 2. Total genomic DNA was extracted from fresh leaf tissue using a slightly modified version of the CTAB extraction protocol (Doyle and Doyle, 1987) or from silica dried samples using the Qiagen DNeasy Plant Mini kit according to the manufacturer’s instructions (Qiagen Corporation, Valencia, CA, USA). DNA sequences will be submitted to GenBank.
Amplification of random amplified polymorphic DNA (RAPD) and intersimple sequence repeats (ISSR)

Approximately 25ng of DNA was amplified via the polymerase chain reaction performed in a MJ Research Thermal PCR machine (GMI, Inc. Ramsey, Minnesota, USA) in 15µl volume reactions. Conditions for RAPD reactions were 0.2 µM random 10-mer oligonucleotide primers, 0.2 mM each of dNTP, 1× Taq polymerase PCR buffer, 1.5 mM MgCl₂, 0.01 g/ml concentration 1% Bovine Serum Albumins (BSA) in the total reaction volume, and 1 unit of Taq polymerase (Promega, Madison, WI, USA). RAPD PCR conditions were for one cycle at 94°C for 3min, 35°C for 30s, and 72°C for 2 min, followed by 43 cycles at 94°C for 45s, 35°C for 30s, and 72°C for 2 min, and a final cycle at 94°C for 45 s, 35°C for 30 s, and 72°C for 6 min. Conditions for ISSR reactions were 0.4 µM primer, 0.2 mM each dNTP, 1× Taq polymerase PCR buffer, 2.5 mM MgCl₂, 5% 0.01 g/ml concentration BSA in the total reaction volume, and 1 unit of Taq Polymerase (Promega, Madison, WI, USA). ISSR PCR conditions were 94°C for 90 s, followed by 34 cycles of 94°C for 40 s, 45°C for 45 s, and 72°C for 90 s, followed by 94°C for 45 s, and 45°C for 45 s, ending with 5 min at 72°C after cycling was completed.

Amplification products were mixed with loading dye (20 mm EDTA, 10% glycerol, 1% sarcosyl with bromophenol blue and xylene cyanol) and separated in 1.5% agarose gels in 0.5 × TBE (tris-borate-EDTA) buffer with 125 ng ethidium bromide per liter. Sizes of the amplification products were estimated by comparison to a Promega 100 bp ladder (Promega, Madison, WI, USA). RAPD primers (Operon Technology, CA, USA; kits OPA-OPI) and ISSR primers (University of British Columbia Primer Kit #9) were screened for amplification of Chrysodracon DNA, and selected primers were then used for amplification of all individuals.
Molecular markers were identified by the primer used to generate them and the approximate size of the band as estimated from the 100 bp ladder.

The reproducibility of amplification was tested for each primer prior to data collection. GelAnalyzer 1D image analysis software (Dr. Istvan Lazar, www.gelanalyzer.com) was initially used to estimate the number of base pairs represented by each amplified fragment and manually adjusted based on eye observation. Loci were scored as diallelic (1 = band present, 0 = band absent). Gels were scored independently by me and another researcher to produce unbiased and unambiguous analysis of RAPD and ISSR amplifications.

Analysis of RAPD and ISSR

Assumptions regarding of RAPD marker analysis were described by Lynch & Milligan (1994), which also apply to ISSR analysis. RAPD and ISSR markers were determined to be polymorphic if estimated allele frequency was less than 95%. In practice, a population marker was considered polymorphic when amplification was present in one or more individuals of the population or if a null (no amplification) occurred in one or more individuals. Absence of a marker within a population, although present in others, was assumed to indicate that individual to be a null/null homozygote rather than there having been a loss of the locus. Expected heterozygosity was calculated for each population ($H_3$) and species ($H_T$) for each locus as follows: $H = 1 - (p^2 + q^2)$ where $p$ is the frequency of the amplified allele and $q$ is the frequency of the null allele. Allele frequencies were estimated from the number of null/null homozygotes present in the population (Hartl and Clark, 2007). Lynch and Milligan (1994) point out that only markers present with an observed frequency of less than $1 - (3/N)$ (where $N$ represents the sample size) are used to reduce a potential bias when analyzing dominant markers. Principal
coordinate analysis (PCO) was employed using MVSP 3.0 (Multi-Variate Statistical Package; Kovach Computing Services 1986–2011) for the analysis of genetic relationships within and among populations with the Gower general similarity coefficient (Gower, 1966 and 1971), and matrixes of genetic similarity analysis within and among populations for each species with Gower similarity coefficient analysis (Gower, 1966 and 1971). Population grouped similarity coefficients from the triangular data matrix in order to calculate an average similarity value within and among populations. Summary statistics of average similarity measures (means, standard errors, and t-tests) were calculated using Excel (Microsoft Office 2007). Distribution of genetic variation within and among populations was estimated using Shannon’s information index \( I \) (Lewontin, 1972). Shannon’s information index was calculated as:

\[
H_o = - \sum p_i \log_2 p_i
\]

where \( p_i \) is the frequency of a given RAPD or ISSR phenotype within a population or species group. Analysis of Molecular Variance (AMOVA) is a method of estimating population differentiation directly from molecular data and testing hypotheses such as population differentiation. The genetic structure was further investigated using AMOVA (Excoffier et al. 1992) as implemented in GenAlex 6.1 (Peakall et al., 2006) to estimate variance components and to test the significance of partitioning of RAPD and ISSR variations within and among populations. The AMOVA approach computes \( \Phi_{ST} \), a statistic analogous to \( F_{ST} \), which estimates the level of genetic differentiation between populations. As in \( F_{ST} \), values of \( \Phi_{ST} \) range from 0 (complete genetic homogeneity) to 1 (complete genetic separation).


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Chapter 2. Phylogenetic Relationships among *Dracaena, Pleomele, and Sansevieria*  

*(Asparagaceae: Nolinoideae)*
ABSTRACT

This study presents a global evolutionary pattern to resolve the ambiguous relationships of the three dracaenoid plant genera *Dracaena*, *Pleomele*, and *Sansevieria* using molecular systematics. The classification of the three genera has been disputed due to different taxonomic interpretation and the difficulty in morphological characterization of the species and genera. Molecular systematics was essential for resolving the problem of taxonomical classification of dracaenoid genera. In this study, phylogenetic analysis of 97 species representing all three genera was explored using four combined chloroplast intergenic spacer DNA regions (*trnL-trnF*, *ndhF-rpl32*, *trnQ-rps16*, and *rpl32-trnL*). The combined dataset was analyzed using parsimony, maximum likelihood, and Bayesian analysis. Results show that 1) the monophyly of dracaenoids is confirmed; 2) the Hawaiian *Pleomele* species are the sister group to the remainder of the dracaenoid phylogeny; 3) the species *Dracaena* and *Pleomele* are intermixed; 4) *Sansevieria* is monophyletic, but is nested within *Dracaena*; 5) the Central American species *D. americana* and *D. cubensis* are the basal group to the remainder of the dracaenoid species. The Hawaiian *Pleomele* are morphologically and genetically distinct from the remaining dracaenoids and are recognized as the distinguished genus *Chrysodracon*. Species of *Sansevieria* and *Pleomele* combined with and recognized as species of *Dracaena*. These findings contribute towards the understanding of the evolutionary history and relationships among dracaenoid species.

Key-words: Asparagaceae, biogeography, chloroplast DNA, *Chrysodracon, Dracaena, phylogeny, Pleomele, Sansevieria.*
INTRODUCTION

Taxonomic issues surrounding the relations among the three globally important genera *Dracaena* Vand., *Pleomele* Salisb., and *Sansevieria* Thunb. (Asparagaceae subfamily Nolinoideae) have been ongoing since the 18th century. The three plant genera, collectively referred to as the dracaenoids, represent an unusual evolutionary history within tropical and subtropical Asia, Africa, and Central America and in the remote Hawaiian Islands, (Rudall et al., 2000; APGII, 2003; APGIII, 2009). In addition, they have valued application in horticulture, medicine, the fiber industry, and for worshiping in ceremonies of different countries and cultures (Lee, 1975; Koller and Thomas, 1988; Chun, 1994; Neuwinger, 1996; Bos, 1998; Binojkumar, 2002; Langenheim, 2003; Staples and Herbst, 2005; Haldar et al., 2010). The three genera have been variously classified in the families Liliaceae (Brown 1914, 1915), Agavaceae (Hutchinson, 1973; Staples and Herbst, 2005), Dracaenaceae (Salisbury, 1866, Watson and Dallwitz, 1992, Bos, 1998), Ruscaceae (APGII, 2003), and now the Asparagaceae (APG III, 2009). Dracaenoids (Rudall et al., 2000) share the derived characteristics of having soft berries that lack phytomelanin in the seed testa.

*Dracaena* comprises 60-80 species worldwide, mainly in the tropics and the subtropics with the exception of South America where there are only two extant species recorded (Mabberley, 2008; Bos, et al., 1992; Bos, 1998; Staples and Herbst, 2005; Judd et al., 2007). One of the common names recognized for *Dracaena* is “dragon tree” (Milburn, 1984), and several *Dracaena* species are the source of “dragon’s blood” medicine derived from the trees’ sap (Lee, 1975; Edward et al., 2001; Langenheim, 2003). Because of their adaptation to harsh conditions and the interesting patterns of leaf variegation, several *Dracaena* species are also
popular horticultural plants, particularly in temperate zones (Bos, 1998; Staples and Herbst, 2005). Africa is thought to be the region of center of diversity for *Dracaena* (Bos, 1984; Bos, 1998; Marrero et al., 1998; Mwachala and Mbugua, 2007). Species are mainly native to Africa with some distributions into Madagascar, Asia, Socotra, the Mediterranean regions, Central America, Micronesia, northern Australia, and the Pacific islands (Gwyne, 1966; Wu et al., 1994; Bos, 1998; Marrero et al., 1998; Staples and Herbst, 2005). Great morphological variation has been shown in *Dracaena* with species generally categorized into two groups: arborescent and arbustial dracaenas (Marrero, 2000). Arborescent dracaenas are usually restricted to semi-desert regions surrounding the Red Sea, whereas the arbustial dracaenas form the remainder of the species distribution (Bos, 1998).

*Pleomele* consists of 40-50 species worldwide, mainly in the tropics and the subtropics with the exception of America (Brown 1914; Wagner et al., 1990). There are six endemic *Pleomele* species in the Hawaiian Islands (Wagner et al., 1990). *Pleomele* is regarded as a monophyletic lineage based on morphological characteristics (Brown, 1914; St. John, 1985; Wagner et al., 1990). The center of diversity of *Pleomele* should be in tropical Asia (Brown, 1914). To date, little biological information is available. Hawaiians utilize *Pleomele* species for cold medicine and ceremonial functions; the sap was not used for external purposes as dragon’s blood.

*Sansevieria* comprises about 60 species worldwide, mainly in dry or arid areas of the Old World tropics and subtropics (Brown, 1915; Marais, 1973; Bos, 1998; Staples and Herbst, 2005; Mabberley, 2008). Some species have medicinal and horticultural value (Neuwinger, 1996; Khalumba and Mbugua; Kubitski, 1998; Staples and Herbst, 2005). Common English names for these species are snake plant or bowstring-hemp along with the widely cultivated
horticultural plant “mother-in-law’s tongue” (*S. trifasciata*) (Staples and Herbst, 2005). Africa is the center of diversity for *Sansevieria* (Mabberley, 2008; Brown, 1915; Morgenstern, 1979) with some species distributed in the Arabian Peninsula, South Asia, and Southeast Asia (Brown, 1915; Morgenstern, 1979; Carlquist and Schneider, 2007). Plants are usually xerophytic perennials that are often rhizomatous, and they can be herbs, shrubs or trees (Staple and Herbst, 2005).

*Pleomele* has an ambiguous relationship to the genus *Dracaena*. Brown (1914) distinguished *Pleomele* from *Dracaena* on the basis of differences in floral morphology. *Dracaena* has a very short perianth tube with the tepals divided to the base, and thickened staminal filaments. In contrast, the perianth tube of *Pleomele* is slender and longer with tepals fused at least two-third their length, and with filiform staminal filaments. However, other authors have considered these characteristics to be variable and treated *Pleomele*, including the type species *P. fragrans*, as a synonym of *Dracaena* (Ker Gawler, 1808; Stevens, 2001; Staples and Herbst, 2005; Mabberley, 2008; Jankaski, 2008). Recently, Jankaski (2008) combined the two genera and recognized them as subgenera (subg. *Dracaena* and subg. *Pleomele*). He also recognized a third new subgenus for the Hawaiian species, subg. *Chrysodracon*, because those taxa do not fit subg. *Dracaena* or subg. *Pleomele* having longer flowers with broader floral tubes and yellow (rather than white, green or purple) tepals (Jankaski, 2008).

Like *Pleomele*, *Sansevieria* has also been variously treated in its relationship to *Dracaena* (Baker, 1875; Brown, 1914; Bos, 1984). Several authors have placed *Sansevieria* in synonymy with *Dracaena* based on their similar floral characteristics (Bos, 1998; Mabberley, 2008). However, *Sansevieria* is conserved as a genus by several botanists (Harms, 1904; Marais, 1973; McVaugh, 1974; Newton, 2002; Jankaski, 2003) because of their usually shorter stature and
herbaceous nature, thick leathery leaves, and presence of a creeping rhizome.

Several issues exist in the classification of the three dracaenoid genera. The systematic relationships among them are unclear and outdated, and no examination of their biogeography or evolution has been made. Recent molecular phylogenetic studies have focused only on the large-scale relationships at the family level among Agavaceae, Dracaenaceae, Ruscaceae, and Asparagaceae (Begler and Simpson, 1995 and 1996; Rudall et al., 2000; Kim et al., 2010). These studies only sampled one to two species of each genus (and did not include type species) and showed they are closely related, but did not further resolve their relationships. No previous molecular study on a global scale has tested the relationships among dracaenoid genera and examined their evolutionary history and biogeography. The present study employed molecular systematic analyses to assess the circumscription of these genera, the evolution of dracaenoids, and their biogeographic affinities.

MATERIALS AND METHODS

Taxon sampling for phylogenetics

A total of 32 Dracaena species, 34 Sansevieria species, and 31 Pleomele species representing the global distribution of these genera were examined in this study (Table 2.1). Six undescribed Dracaena species native to Thailand discovered by Paul Wilkin (Kew Herbarium, London, UK) were included. Sources of DNA for sequencing included freshly collected leaves, herbarium specimens and the DNA Bank at Royal Botanic Gardens, Kew, United Kingdom. Table 1 includes specimen source, voucher information, locality, and collection number. All novel sequences generated for this study will be deposited in GenBank.
The selection of five outgroup taxa from the Asparagaceae (Nolinoideae) was based on the results of previous studies and were shown to be closely allied to dracaenoid genera (Bogler and Simpson, 1996; Rudall et al., Kim et al., 2010). Four of the outgroup species (Comospermum yedoensis, Disporopsis pernyi, Liriope muscari, Speirantha gardenii) are distributed in East Asia and one is from Africa (Eriospermum flagelliforma).
Table 2.1: List of taxa used in this study, voucher information, and GenBank accession numbers for the four regions studied. The following abbreviations are used for herbaria and location: BISH = Bishop Museum, USA; PTBG= Herbarium, National Tropical Botanical Garden, USA; NY = New York Botanical Garden, USA; NCU= Herbarium, University of North Carolina, USA; WBG=Waimea Botanical Garden, Hawaii, USA; UNC: Universities of North Carolina, at Chapel Hill, USA; WAG: Wageningen University, Wageningen, Netherlands; TAN: Parc de Tsimbazaza, Antananarivo, Madagascar; CBG: Curepipe Botanical Garden, Curepipe, Mauritius; K=Royal Botanic Gardens at Kew, UK; SING=Singapore Botanic Gardens, Singapore; BZ=Herbarium Bogoriense, Bogor, Java, Indonesia; HITBC=Xishuangbanna Tropical Botanical Garden, Academia Sinica, Xishuangbanna, Yunnan, China; HAJB=Jardín Botánico Nacional, Habana, Cuba; HAW: University of Hawaii at Manoa, USA
<table>
<thead>
<tr>
<th>Taxa</th>
<th>Voucher specimen</th>
<th>Location</th>
<th>TrnQ-rps16</th>
<th>TrnL-trnF</th>
<th>TrnL-rpl32</th>
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<td><strong>Pleomele phylloides</strong></td>
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<td>Nouvelle Decouverte, Mauritius. East Africa to SE. Asia</td>
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<td><strong>Pleomele umbraculifera</strong></td>
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<td><strong>Sansevieria aubrytiana</strong></td>
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<td><strong>Sansevieria ballyi</strong></td>
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<td>Cult. source, RBG Kew, UK. SE. Kenya to Tanzania</td>
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<td>PL52519</td>
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<td>PL52520</td>
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<td>Q1525623</td>
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<td>Q1525624</td>
<td>PL52523</td>
<td>rp25725</td>
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<td>PL52524</td>
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<td>PL52528</td>
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<td><strong>Sansevieria ehrenbergii</strong></td>
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<td>Q1525630</td>
<td>PL52529</td>
<td>rp25731</td>
<td>n1525832</td>
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<td><strong>Sansevieria gracilis</strong></td>
<td>RBGKew, LivColl.1965-2001</td>
<td>Cult. source, RBG Kew, UK. Native from Ethiopia to S. Africa</td>
<td>Q1525632</td>
<td>PL52531</td>
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<td><strong>Sansevieria grandis</strong></td>
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<td>Cult. source, RBG Kew, UK. Native from Kenya to S. Africa</td>
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<td>PL52532</td>
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<td><strong>Sansevieria humbertiana</strong></td>
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<td>Taita Distr., Kenya. Native from E. Africa to S. Africa</td>
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<td>PL52533</td>
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<td>Q1525635</td>
<td>PL52534</td>
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<td><strong>Sansevieria kirkii</strong></td>
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<td>PL52535</td>
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<td><strong>Sansevieria metallica</strong></td>
<td>RBGKew, LivColl.1974-1487</td>
<td>Réunion (France, DOM-ROM). Native from Malawi to Reunion</td>
<td>Q1525638</td>
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<td><strong>Sansevieria nilotica</strong></td>
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<td>Rubondo Is., Tanzania. Native from C. Africa to Tanzania</td>
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<td><strong>Sansevieria parva</strong></td>
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<td><strong>Sansevieria patens</strong></td>
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<td>Cult. source, RBG Kew. UK. Native to Kenya</td>
<td>Q1525641</td>
<td>PL52540</td>
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<td>Cult. source, RBG Kew. UK. Native to Southern Africa</td>
<td>Q1525642</td>
<td>PL52541</td>
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<td>Cult. source, RBG Kew. UK. Native from Kenya to Tanzania</td>
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<td>PL52542</td>
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<td>PL52543</td>
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<td>PL52544</td>
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<td>n1525847</td>
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<td>Wierenga et al. 5823, WAG</td>
<td>Cameroon. Native from W. to C. Africa</td>
<td>Q1525646</td>
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<td>Neghell-Filta, Ethiopia. Native from Ethiopia to S. Africa</td>
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<td>Cult. source, RBG Kew, UK</td>
<td>Q1525648</td>
<td>PL52547</td>
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<td><strong>Sansevieria suffruticosa</strong></td>
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<td>Korro Moro to Ngobit Rd, Kenya. Native from Kenya to S. Africa</td>
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<td>PL52548</td>
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<td>Cult. source, WBG, USA. Native from Mayotte to Mozambique</td>
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<td>PL52549</td>
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<td>Lau 2852, BISH</td>
<td>Cult. source, WBG, USA. Native from Nigeria to WC. Africa</td>
<td>Q1525651</td>
<td>PL52550</td>
<td>rp25752</td>
<td>n1525853</td>
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<td><strong>Sansevieria volksii</strong></td>
<td>RBG Kew, LivColl.1975-302</td>
<td>Serengeti Plain, Kenya. Native from Zaire to Somalia</td>
<td>Q1525652</td>
<td>PL52551</td>
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<td><strong>Speirantha gardenii</strong></td>
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<td>Cult. source, RBG Kew, UK. Native to South China</td>
<td>Q1525653</td>
<td>PL52552</td>
<td>rp25754</td>
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<td>Chase 833, K</td>
<td>Cult. source, RBG Kew, UK. Native to South Japan</td>
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<td>PL52553</td>
<td>rp25755</td>
<td>n1525856</td>
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<td><strong>Disporopsis perryi</strong></td>
<td>Chase 493, K</td>
<td>Nanjing, China (Native to South China and Taiwan)</td>
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<td>PL52554</td>
<td>rp25756</td>
<td>n1525857</td>
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<td><strong>Lilope muscaria</strong></td>
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<td>Cult. source, UNC USA. Native to East Asia</td>
<td>Q1525656</td>
<td>PL52555</td>
<td>rp25757</td>
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<td><strong>Enospermum flagelliforme</strong></td>
<td>Chase 2051, K</td>
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<td>Q1525657</td>
<td>PL52556</td>
<td>rp25758</td>
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DNA extraction and amplification

Total genomic DNA was extracted from 1.0 g of fresh or 0.2 g of silica gel-dried leaves using the CTAB method (Doyle and Doyle, 1987) with modification (Morden et al., 1996), or using the Qiagen DNeasy plant mini kits (Qiagen Corporation, Valencia, CA, USA) following manufacturer specifications. DNA samples were accessioned into the Hawaiian plant DNA library (Morden et al. 1996).

Data for this study includes four chloroplast intergenic spacer regions: trnL-trnF, ndhF-rpl32, trnQ-rps16, and rpl32-trnL. The polymerase chain reaction (PCR) was performed using Eppendorf (Westbury, New York, USA) Mastercycler gradient or MJ Research Thermal PCR machine (GMI, Inc. Ramsey, Minnesota, USA). The PCR reaction was performed in a 25 µl reaction mixture containing 5 µl 5X Green or colorless GoTaq Flexi PCR buffer, 1.5 µl 25 mM MgCl2, 0.5 µl 10 mM dNTPs, 0.5 µl 10 mM primers, 0.2 µl GoTaq polymerase (5 U/ul) (Promega, Madison, WI, USA), and 14.5 µl ddH2O. The rpl32-trnL region was amplified using standard primers pairs rpl32 (CAGTTCCA AAAAAACGTACTTC) and trnL (CTGCTTCCAAGAGCAGCGT) (Shaw et al., 2007). The trnQ-rps16 region was amplified using standard primers pairs trnQ (GCGTGGCCAYGMATATT) and rps16 (GTTGCTTTYTACCACATCGTTT) (Shaw et al., 2007). The rpl32-trnL and trnQ-rps16 with the following PCR conditions: 5 min at 80°C, followed by 30 cycles at 95°C for 1 min, 50°C for 1 min (a ramp 0.3°C/sec to 65°C), 65°C for 4 min, plus a final extension of 5 min at 65°C. The ndhF-rpl32 region was amplified by the primer pairs Forward (GCATATTGATAKGTATGTTCCA) and Reverse (ATMGAAGTRCGTTTYTTTGG)
(Scarcelli et al., 2011). Two parameters were used to amplify this region. The first one with the following PCR conditions: 3 min at 94°C, followed by 35 cycles at 94°C for 30 sec, 42°C for 3 sec, 72°C for 1 min, plus a final extension of 10 min at 72°C. The second one with the following PCR conditions: 5 min at 80°C, followed by 35 cycles at 94°C for 1 min, 42°C for 1 min (an increasing 3°C/sec), 65°C for 4 min, plus a final extension of 10 min at 65°C. The \textit{trnL-F} region was amplified by the forward primer (e; GGTTCAAGTCCCTATCCC) and the reverse primer (f; ATTTGAACTGTGACACGAG) of Taberlet \textit{et al.} (1991) with the following PCR conditions: 80°C for 5 min followed by 29 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 4 min, plus a final extension of 5 min at 72°C (Shaw \textit{et al.} 2005). All amplifications were verified on 1.5% agarose gel.

DNA amplification by polymerase chain reaction (PCR), and template purifications were performed with Go Taq PCR Core Kit (Promega, Madison, WI, USA) and all PCR products were prepared for sequencing using ExoSAP-IT (USB Corporation, Cleveland, OH, USA) or similar treatment with shrimp alkaline phosphatase and exonuclease I following the USB ExoSAP-IT PCR product cleanup protocol of incubation for 37°C for 15 min followed by 80°C for 15 mins. Samples were bidirectionally sequenced at the University of Hawaii’s ASGPB Sequencing Facility (http://cgpbr.hawaii.edu/) using BigDye Terminator chemistry (Applied Biosystems, Foster City, California, USA) and visualized on an ABI 3730XL capillary-based DNA sequencer (Applied Biosystems).
Contiguous sequences were constructed and edited for all \textit{trnL-trnF}, \textit{ndhF-rpl32}, \textit{trnQ-rps16}, and \textit{rpl32-trnL} sequences using MEGA 5 (Tamura et al., 2011). All sequences were aligned initially in ClustalW (ver. 2.1; Larkin et al., 2007) and Muscle (Edgar, 2004) and then manually adjusted in MEGA 5 following the guidelines of Kelchner (2000) to minimize indels. Sequences from all four cpDNA regions were combined into one dataset and indels were excluded from subsequent phylogenetic analyses. ModelTest version 3.7 (Posada and Crandall, 1998) was used to determine the best model of DNA substitution. The best-fit models as determined in evolutionary models were selected by Akaike information criterion (AIC; Akaike, 1974) for each partition and the combined dataset, evaluating all models against defaults of the program. The GTR+G model (a general time reversible model with a gamma-shaped distribution of rates across sites) was chosen for the four regions combined data matrix and the nuclear region combined with chloroplast regions as the best-fitting among the 24 models compared and was used to construct the ML and Bayesian trees. The models for separate and combined data partitions are as follows: \textit{ndhF-trnL}: T92; \textit{TrnF-trnL}: T92; \textit{trnL-rpl32}: T92+G (T92: Tamura 3-parameter with a discrete Gamma distribution); \textit{trnQ-rps16}: T92+G. Sequences were concatenated and aligned according to codon positions in a NEXUS file and phylip file prior to conversion by Mesquite 2.7.4 (Maddison and Maddison, 2009) into the appropriate file format necessary for each tree search application. The data were separated into two major partitions, nuclear (ITS1) and chloroplast (\textit{trnF-trnL}, \textit{trnQ-rps16}, \textit{ndhF-rpl32}, \textit{rpl32-trnL}).

Maximum parsimony (MP), maximum likelihood (ML) and Bayesian methods were used to estimate phylogenies for all data sets (nuclear, chloroplast and combined). Maximum
parsimony search were conducted using PAUP* 4.0b10 (Swofford, 2002). In all MP analyses, heuristic searches were done using a starting tree built from stepwise addition with 1000 random addition replicates and TBR branch swapping. Bootstrap analyses based on 1000 replicates with 10 random additions per replicate were used to assess confidence in clades.

Maximum likelihood (ML) analyses as an optimality criterion (Felsenstein, 1981) was conducted for each of the three datasets using RAxML v7.2.7 (Stamakis, 2006; Stamakis et al., 2008). RAxML uses the GTR model with six categories of rate variation, and use of a separate support for individual branches was assessed using the nonparametric bootstrap (Felsenstein, 1985) via the rapid bootstrap procedure of Stamatakis et al. (2008) with bootstrapping stopped automatically using a frequency based criterion (Pattengale et al., 2010). Maximum likelihood bootstrap proportions (MLBS) >70% were considered strong support (Hills and Bull, 1993).

MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2005) was used to conduct Bayesian analyses under a time-free model. Bayesian analyses were run using four incrementally heated chains for 1,000,000 generations and a sampling frequency of 100 generations. Tree samples and parameter estimates from the first 25% of all trees (2500) were designated as the burn-in period and discarded. Posterior probability (PP) support values >0.95 were considered strong support for individual clades (Erixon et al., 2003; Huelsenbeck and Rannala, 2004).

RESULTS

A total of 408 new sequences were generated from the four gene regions and the 102 species examined. GenBank accession numbers for each sequence are available in Table 1. The length
of the combined dataset was 3263 bp. Table 2.2 presents a complete list of gene regions, the length of each region, and combined data matrix statistics. Of the four sampled regions, \textit{trnQ-rps16} is the longest region sampled (1003 bp) and is \textit{trnL-trnF} the shortest (380 bp). \textit{ndhF-rpl32} was the most parsimony informative gene region with 13.0% of the site variable while \textit{rpl32-trnL} was the least parsimony informative with 6.6% variable. Of the combined data, total parsimony informative characters were 10.0%. Overall, the four regions had consistency index (CI) values ranging from 0.51 (\textit{trnL-trnF}) to 0.68 (\textit{rpl32-trnL}) and the combined dataset CI value was 0.67. Retention index (RI) values ranged from 0.53 (\textit{ndhF-rpl32}) to 0.78 (\textit{rpl32-trnL}) and the combined dataset RI was 0.70.

\textit{Parsimony analysis based on combined cpDNA dataset}

Maximum parsimony (MP) analysis was conducted on the combined dataset of all regions. The strict consensus tree is shown with bootstrap percentages (BP) greater than 50% associated with the branches (Fig. 2.1). The dracaenoids were strongly supported (100% BP) as a monophyletic lineage relative to the outgroup genera. However, species of \textit{Dracaena}, \textit{Pleomele}, and \textit{Sansevieria} were polyphyletic. \textit{Sansevieria} was monophyletic and terminal within the dracaenoid clade (100% BP). The monophyly of Hawaiian \textit{Pleomele} was strongly supported (94% BP) and positioned as the sister group to a large clade containing the remainder of ingroup taxa. The two Central American species, \textit{D. americana} and \textit{D. cubensis}, were supported as the most ancestral taxa among the remaining ingroup species (100% BP) followed by \textit{D. cinnabari} from Socotra (100% BP). Species derived following \textit{D. cinnabari} are variously associated with distributions throughout Africa or Asia.
Table 2.2. Statistics for the gene regions analyzed in the Maximum Parsimony (MP) analysis.

PIC= parsimony informative characters; CI= consistence index; RI= retention index.

<table>
<thead>
<tr>
<th>Statistic</th>
<th>trnL-trnF</th>
<th>rpl32-trnL</th>
<th>trnQ-rps16</th>
<th>ndhF-rpl32</th>
<th>combined cpDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aligned length</td>
<td>380</td>
<td>975</td>
<td>1003</td>
<td>905</td>
<td>3263</td>
</tr>
<tr>
<td>Constant sites</td>
<td>332</td>
<td>887</td>
<td>831</td>
<td>723</td>
<td>2931</td>
</tr>
<tr>
<td>Variable sites</td>
<td>36</td>
<td>68</td>
<td>183</td>
<td>125</td>
<td>432</td>
</tr>
<tr>
<td>PIC</td>
<td>0.51</td>
<td>0.68</td>
<td>0.56</td>
<td>0.59</td>
<td>0.67</td>
</tr>
<tr>
<td>CI</td>
<td>0.66</td>
<td>0.78</td>
<td>0.69</td>
<td>0.53</td>
<td>0.70</td>
</tr>
</tbody>
</table>
Figure 2.1. Strict consensus of the maximum parsimony tree resolved the combined dataset.

Bootstrap percentages >50% are above branches. Locations are based on each species original publication, the catalogue of life (2011), and approximation. Each location labels color. Pink: Hawaii Islands, Blue: Central America, Black: Africa, Orange: Macaronesia, Red: East Asia, Purple: Arabia, Grey: Southeast Asia, Green: broad distribution or North Australia, Dark brown: Mauritius.
Maximum likelihood analysis based on combined cpDNA dataset

Maximum likelihood (ML) analysis of the combined dataset produced a topology similar to that of the MP strict-consensus tree (Fig. 2.2). Placement of some species within Dracaena differ from the MP tree although overall relationships are similar. The monophyly of all dracaenoids was strongly supported (100% BP). As previously described, Dracaena, Pleomele and Sansevieria were polyphyletic with Sansevieria in a terminal position within the clade and Dracaena and Pleomele species intermixed. The monophyly of Hawaiian Pleomele were strongly supported (95% BP), as was their sister relationship to all other dracaenoids (95% BP). Sansevieria was also supported as monophyletic lineage (100% BP). There were clades within Sansevieria with strong support (>90% BP) and several small clades within the Dracaena/Pleomele clade with widely varying support (>80% BP).
Figure 2.2. Maximum-likelihood tree of the combined dataset. Bootstrap percentages > 50% are above branches.
Figure 2.3. Posterior probabilities consistent with the strict consensus tree are shown above each branch, but groups with PP lower than 50 are not indicated.
Bayesian analysis based on combined cpDNA dataset

The majority rule consensus tree from the combined Bayesian analysis is shown in Figure 3 with the posterior probability (PP) summarized from the set of recovered post-burn-in trees. The phylogenetic relationships of the three genera were overall similar to what was found in the MP and ML analyses. Dracaenoides were strongly supported as a monophyletic lineage (1.0 PP), but species of Dracaena, Pleomele and Sansevieria were polyphyletic. The African Sansevieria was supported as monophyletic (1.0 PP). The Hawaiian Pleomele were supported as monophyletic (1.0 PP) and as sister to the clade containing all other dracaenoid species (1.0 PP). The relation of D. cubensis, D. americana (Central America) and D. cinnabari (Socotra) as the basal taxa in the remainder of the dracaenoid clade is also strongly supported and consistent with other analyses.

DISCUSSION

Dracaenoid phylogeny

Dracaenoids are a well-supported monophyletic group, consistent with previous molecular phylogenetic analyses at the family level (Bogler and Simpson, 1995 and 1996; Rudall et al. 2000). The combined analysis provides evidence of a primary division within the dracaenoid radiation into three main clusters. The Hawaiian Pleomele was basal within the dracaenoid clade and constitute the sister group to the remainder of the dracaenoid species (Dracaena sensu lato). Support for this relationship was strong in all three analyses. The remainder of the Dracaena and Pleomele species are intermixed in the central region of the tree and do not form identifying
groups related to these genera. *Sansevieria* species cluster together in a strongly supported terminal position within the tree.

**Monophyly of the Hawaiian dracaenoids**

This is the first time that all species of Hawaiian *Pleomele* have been included in a comprehensive phylogenetic analysis. The affinities of Hawaiian *Pleomele* have long been debated (Brown, 1914; Bos, 1984 and 1998; St. John, 1985; Wagner et al., 1990). Results here demonstrate that the six extant Hawaiian *Pleomele* species are strongly supported as monophyletic and support Jankaski’s classification separating them as a distinct taxon from the remainder of *Dracaena* and *Pleomele* (2008). Additional phylogenetic analyses including a nuclear marker will be presented and discussed in Chapter 3.

Although classified as such, the Hawaiian *Pleomele* do not fit well within the morphological circumscription of the genus as described by Salisbury (1796) or Brown (1914). Hawaiian *Pleomele* species are unique in having yellow tubular flowers that are long and wide and in pendent panicles. The non-Hawaiian *Pleomele* species have white or greenish slender tubular flowers and the flowers are much smaller than those of Hawaiian species. Also, Hawaiian *Pleomele* have a diurnal flowering and fragrant cycle, in contrast to the nocturnally fragrant flowering of *Pleomele, Dracaena* and *Sansevieria* (Bos, 1984 and 1998, and 1992; Mwachala, 2005; Jankaski, 2008; personal observation). These traits in the Hawaiian species led Jankaski (2008) to place them in a separate subgenus, *Dracaena* subg. *Chrysodracon*.

Results here indeed support Jankaski’s (2008) separation of the Hawaiian clade as a distinct group although they do not support his assumption that they are derived from *D.*
american or D. cubensis from central America. The species are clearly monophyletic and form the sister group to the remainder of Dracaena sensu lato. Based on these morphological differences and the clear genetic differentiation of the Hawaiian species from the remainder of the dracaenoids, this unique clade should be elevated to the rank of genus as Chrysodracon. The taxonomic revision of the species and genera will be treated in a subsequent publication (Lu and Morden in prep).

Relationships among Dracaena and Pleomele

Excluding the Hawaiian clade, the relationship among species of Pleomele and Dracaena are polyphyletic with no identifiable groupings associated. Therefore, these data do not support Brown’s (1914) or Jankaski (2003) classification. Brown (1914) separated Pleomele from Dracaena based on differences in the division of perianth parts and filament thickness. Jankaski’s (2003) had retained Dracaena and Pleomele as subgenera of Dracaena based on these morphological distinctions. The results here thus suggest that variation in these traits is convergent rather than homologous and, as such, the two should be recombined as a single genus with no subgeneric designation.

The base of the clade leading to the remainder of the dracaenoid species is composed of either individual species or clusters of two or three species. The two Central American species, Dracaena americana and D. cubensis, form a clade that is most basal among the Dracaena sensu lato. Dracaena americana is widely distributed throughout Central America, whereas D. cubensis is restricted to the island of Cuba (Marie-Victorin, 1942). The adjacent lineage is Dracaena cinnabari, an endemic to Socotra Island, Yemen. Nested next to this is the first of the
African species with *D. adami*, *D. angustifolia*, and *D. aletriformis*. *Dracaena angustifolia* is a widely distributed species with populations also in Asia and islands of the Pacific Ocean whereas *D. adami* is found in West Africa and *D. aletriformis* is southern Africa (Bos, 1984; Mwachala, 2005).

The center of diversity among species of *Dracaena* was identified to be in West Africa (Bos, 1984) or East Africa (Mwachala, 2005). Although numerous species from both African regions and Asia were used in this analysis, there were no subclades with obvious clusters of species from any specific region. In contrast, the several subclades present have representative taxa from both Africa and Asia. There does not appear to be any strong correlations between phylogenetic relationships among species and their current biogeographical distribution. This pattern suggests that dispersal between Africa and Asia was common. This supports the suggestion of Bos (1998) that birds are the most likely dispersal agents for dracaenoids, and such a pattern of speciation suggests that this may have occurred on a regular basis between these two continents.

The relation between arborescent and arbustial dracaenoids was examined and results here demonstrate that differences in woody habit are polyphyletic. Molecular systematics do not support Brown’s classification for *Dracaena* to separate arborescent *Dracaena* from arbustial *Dracaena* (Brown 1914). The arbustial dracaenoids are ancestral within the phylogeny, being present also among the Hawaiian species, and arborescence has arisen numerous times independently.
The 31 Sansevieria taxa form a well-supported clade that is nested within Dracaena. Although monophyletic, its position within the phylogeny makes Dracaena sensu lato a paraphyletic assemblage and, as such, Sansevieria must be considered a clade within the broader interpretation of Dracaena. These results support the classifications of Bos (1984 and 1998) that subsume Sansevieria into Dracaena, rather than the classification of Jankaski (2003) that maintains their separation as distinct genera. All Sansevieria species in this analysis are native to Africa; there are four species native to Asia described by Brown (1915) that were unavailable for analysis and should be analyzed to better understand the origins of this group. Biogeographical patterns among species here suggest that the oldest lineages are in east Africa with subsequent dispersal to southern and west Africa.

Sansevieria taxa radiated rapidly with little genetic differentiation among the species. This is best exemplified in the Maximum Likelihood analysis where internode length among the branches is very short. Bayesian analysis showed clear resolution among the species of Sansevieria and with strongly supported clades. However, phylogenetic orders of species relationships among the three analyses are not in concordance. As such, further examination of these taxa should be carried out to include additional genetic regions to resolve these species relationships.

Results here do not support the morphological classification based on gross leaf forms identified by Brown (1915). A flat leaf is the ancestral leaf-type and is present in all other dracaenoids. The unique cylindrical leaf-type Brown (1915) described in his monograph of Sansevieria is not a synapomorphic character. This trait is present in 11 species and has multiple
origins within the molecular phylogeny shown here. These would entail having evolved separately on each occasion or possibly seven separate occasions if an allowance is made for multiple back mutations to a flat leaf form (Fig. 2.4). Compared to the other dracaenoid species, Sansevieria species are better adapted to extreme drought and high solar irradiation conditions (Sreenivasan, 2011). Their thick, leathery leaf combined with their rhizomatous nature make them very efficient at water maintenance. The round leaf forms are even more water efficient in this regard (Sreenivasan, 2011).

_Evolution of Dragon’s Blood_

The name “Dragon’s blood” is applied to the deep red liquid that exudes from injured bark. This liquid has been shown to have medicinal properties (Lee, 1975; Edward et al., 2001; Langenheim, 2003) and its use has been documented in several species including _D. draco_, _D. cinnabari_, _D. ombet_, _D. cambodiana_, and _D. cochinchinensis_ (Miller and Morris, 1988; Wu and Raven et al., 1994; Edward et al., 2001; Langenheim, 2003; Gonzalez et al., 2004). The resin is dried and used to produce many traditional herbal medicines among the Chinese, Southeast Asian, Arab, ancient Greek, Roman, Indian, and African cultures (Lee, 1975; Milburn, 1984; Miller and Morris, 1988; Chun, 1994; Edward et al., 2001; Langenheim, 2003). The main medicinal function is to cure wounds, internal injuries, hemorrhages, and surgical trauma as an external medicine and anti-inflammatory by directly treating on the injured areas (Lee, 1975 and 2004; Langenheim, 2003). It was usually made into a tasteless powder that is soluble in a little spirits or wine (Lee, 1975, 2004; Langenheim, 2003).
Many dracaenoid species produce the secondary metabolite red resin on the leaf base although only the few mentioned above have been used medicinally. It has also been found in several Hawaiian species (personal observation), but no documented use has been made of them. The red resin has not been found in any Sansevieria taxa. These species can survive in extreme environments, such as limestone and lava (St. John, 1985; Marrero et al., 1998; Langenheim, 2003), and often grow on cliffs or steep embankments. The red resin may be related to this group’s specific mechanism for adaptation to xerophytic environments. It has not been identified in species adapted to wet habitats, such as those from southeast Asia and Indonesia (person observation). During the process of macroevolution when species became more adapted to the extreme arid habitats, tree or shrub forms reduced to the almost stemless xeromorphic form present among Sansevieria species and production of red resin lost.

Although no Sansevieria species contain dragon’s blood, several African Sansevieria species have been used for their anti-inflammatory properties. Specific taxa used for their anti-inflammatory properties include S. aethiopica, S. bagamoyensis, S. ehremberggi, S. liberica, S. trifasciata, and S. senegambica (Neuwinger, 1996; Binojkumar, 2002; Haldar et al., 2010). These taxa do not cluster together in the phylogenetic analyses conducted here. This suggests that medicinal properties are inherent within the dracaenoid lineage and all species should be considered candidates for future screening in phytochemical and pharmacological studies.
Figure 2.4. Detailed examination of *Sansevieria* species for leaf form based on Bayesian analysis. Green = cylindrical leaves; black = flat leaves. This morphological description is mostly based on Brown (1915). Posterior probabilities (>0.50) are provided for above branches. Arrow indicates position where mutation to round leaf type evolved affecting multiple species in the radiation.
Results here and previously by Kim et al. (2010) indicate that the dracaenoid’s are descended from Asparagaceae genera native to the temperate and sub-tropical regions of Asia. Previous hypotheses have been that dracaenoids were arose in Africa where their center of diversity is located (Bos, 1984 and 1998; Mwachala, 2005). However, all lineages ancestral to the dracaenoids are tropical and subtropical Asian. Dracaenoids form a sister clade to genera of Ruscaceae sensu stricto (Kim et al., 2010) that are distributed in temperate Asia. A split occurred in these lineages from their ancestors giving rise to Ruscaceae sensu stricto to the west across temperate regions of central Asia and Europe, and dracaenoids remaining in the east.

Although results suggest that the likely origin of the dracaenoids was in East Asia, it is apparent that the ancestral lineages from this region have since gone extinct. The most ancestral lineages of dracaenoids are the Hawaiian or Central American taxa; the Hawaiian dracaenoids are the sister group of the remaining dracaenoid phylogeny, and the most ancestral of the remaining dracaenoids are the two Central American species. This would suggest that the ancestral Asian lineage went extinct after dispersal to the remote Hawaii Archipelago. This surprising result is similar to what was found in the Begoniaceae (Clement et al., 2004). In their well-supported molecular phylogeny, the monotypic species *Hillebrandia sandwicensis*, endemic to Hawaii, is placed at the base of the phylogeny and is ancestral to all other species of *Begonia* (ca. 1400 species) located in Africa, America, and Asia (Clement et al., 2004). Their hypothesis was that *H. sandwicensis* represents a relict species dating back to an early colonization of now subsided high Hawaiian islands 20 MYA and has island hopped down the chain as new islands formed.
Several dispersal events are necessary to explain the present-day distribution of the dracaenoids. Extant taxa occur widely in tropical and subtropical regions of the continents of Asia, Africa, and Central America (Brown, 1914; Bos, 1998; Mwachala, 2005). Dracaenoids also occur on many oceanic islands that are in close proximity to a continental land mass (Bos, 1998), the Hawaiian radiation being the one lone exception due to their isolation. With the origins of dracaenoids being in the south or Southeast Asia region (above), the bird mediated dispersal (Bos, 1998) that gave rise to extant lineages is complex. First, an early separation to the Pacific islands leading to the Hawaiian radiation must have taken place. This suggests that colonization is likely to have occurred to a previous high island that has now subsided, followed by a stepping stone distribution from the older to younger islands as they formed. The oldest high island in the Hawaiian archipelago is Kauai dated to ca. 5 mya (Carson and Clague, 1995), and *Dracaena*-like pollen from the Neogene period (ca. 23 mya) suggests that the dracaenoid lineage is considerably older than this (Van Campo and Sivak, 1976).

Second, a similar long distance dispersal event from the original source region in Asia to Central America occurred. The species in Central America did not radiate in to multiple species as it has in other regions or, if they did, the other species have gone extinct. Third, dispersal occurred from the original source region in Asia to the Arabian Peninsula. This in turn led to further dispersal with a large radiation of species into mainland Africa and subsequently to Madagascar (and Mauritius), Australia and Papua New Guinea, and periodic dispersal back to South and Southeast Asia. Following this third major dispersal event, the original source population went extinct.
Conclusions

New circumscriptions for the dracaenoid species are proposed. The Hawaiian species were the first clade to form and are distinct both genetically and morphologically from the remainder of the dracaenoids. As such, they are to be considered as a distinct genus based on the subgeneric name used for the Hawaiian species as classified by Jankaski (2008). Formal circumscription and species delineations of Chrysodracon (Jankaski) Lu and Morden, *gen. nov.* (Lu and Morden *in prep*) will be made in a separate publication.

The remainder of the dracaenoid lineage forms a single large clade. Species of *Dracaena* and *Pleomele* are intermixed and show now apparent clustering of taxa traditionally ascribed to these genera. Although *Sansevieria* species do form a monophyletic lineage, that lineage is within the larger *Dracaena/Pleomele* clade. As such, this paraphyletic relationship must necessarily be resolved by combining the species traditionally regarded in *Dracaena, Pleomele*, and *Sansevieria* into a single large genus. The generic circumscription of *Dracaena* Vandelli ex Linnaeus (1767) has priority over *Sansevieria* Thunberg (1794) and *Pleomele* Salisbury (1796), and thus *Dracaena* is the conserved name for species in this clade.

Future Directions

The results presented here provide a good framework for more intensive studies to be carried out among dracaenoid species. Questions still exist in regards to the placement and biogeography of several species that are ecologically important or have broad distribution with little known concerning their evolution. *Dracaena angustifolia* has a distribution in tropical
regions throughout the world, yet it is not clear if populations represent different species or what the biogeographical relationship is among them. *Dracaena marginata* from Madagascar is used extensively in horticultural trade, but its status as a species is suspect and warrants closer inspection. *Dracaena reflexa* is a wide-spread species with numerous varieties that have been identified, but their relationships are obscure and in need of closer evaluation.

Further molecular analysis is needed to compare groups within the dracaenoid clade. The present study was done using chloroplast genetic markers, and it would be beneficial to examine relationships with the inclusion of nuclear markers. In particular, little variation was found using four cpDNA gene regions among most of the *Dracaena* and *Sansevieria* taxa. Use of a nuclear marker (such as the intergenic transcribed spacer, ITS) would provide additional variation and would also allow for examination of potential hybridization among the species.

Greater analysis of morphological and anatomical characteristics should be used further to clarify distinctions between dracaenoid taxa. Several morphological features were identified that correlate with the distinction of the Hawaiian species from the remainder of dracaenoid species. However, the relation of the New World species to the Old World species is also questionable, and should be examined more fully. Characteristics of pollen or seed architecture, leaf anatomy, etc. should be closely examined to in conjunction with the molecular phylogeny produced. As morphological traits are examined, these features (if available) should similarly be examined among the fossil specimens of allied dracaenoid taxa previously discovered (*Dracaenites* species; Saporta, 1862, 1865, 1873 a and b, 1888, and 1889) to better understand their relationships and possible timeframe for the evolution of dracaenoids.

Little is known of the pollination biology of dracaenoid species. Several of the Hawaiian species and many other species throughout the world are endangered. Determining reproduction
biology traits ecological parameters for species will be important features in future conservation management and recovery for many of these species.
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Chapter 3: Evolution and Biogeography of Species in the Endemic Hawaiian Genus *Chrysodracon* (Asparagaceae: Nolinoideae)
ABSTRACT

The Hawaii Archipelago is one of the best places in the world to study evolution, particularly island evolution. Dry forests there contain a high degree of endemism. However, few studies on the taxa of the region have been conducted and many of the taxa are facing severe risk of extinction. This study examines the evolutionary history and biogeography of the endemic tree genus *Chrysodracon* (formerly the Hawaiian species of *Pleomele*). Recent phylogenetic analysis has shown *Chrysodracon* to be the sister group to the remainder of the draceanoid species that are distributed worldwide in the tropics. Phylogenetic analysis using a nuclear marker (ITS-1) and four combined chloroplast DNA regions (*trnL-trnF*, *ndhF-rpl32*, *trnQ-rps16*, and *rpl32-trnL*) was explored and the combined dataset was analyzed using parsimony, maximum likelihood and Bayesian analysis. These results provide clear evidence of the monophyletic relationship of these species, their biogeographical patterns among the main Hawaiian Islands, and provide evidence for a new Hawaiian *Chrysodracon* species using molecular data. Evidence suggests that there was an initial colonization of the islands to Kauai or Maui, or both concurrently, followed by dispersal and speciation in the other islands.

INTRODUCTION

The Hawaiian Islands are an excellent location to study evolutionary processes because of their isolation and the resulting high proportion of endemic species (Carlquist, 1970; Stuessy and Ono 1998; Olson, 2004). The biologically diverse dry forests in Hawaii once covered a significant portion of the leeward sides of all islands (Cuddihy and Stone, 1990; Athens et al. 2002; Myers et al., 2000). Prior to human contact, Hawaii’s lowland dry and mesic forests contained diverse array of native plants (Rock, 1913; Ziegler, 2002). Dry and mesic forests are one of the Hawaiian Islands’ most culturally important habitats because native Hawaiians use plants from these forests routinely for medicines, worship, cultural ceremonies, foods, tools, and building materials (Pukui and Elbert, 1986; Chun, 1994). Unfortunately, these forests were, and continue to be, threatened because they were often the first to be settled and their resources the first to be consumed by human development (Carlquist, 1970; Ziegler, 2002). Understanding the evolutionary history of these endemic taxa now is very urgent for their conservation.

The species of Hawaiian Chrysodracon (formerly Pleomele or Dracaena; Asparagaceae: Nolinoideae) are distributed in mesic and dry forests (Pau et al., 2010). Hawaiians call these species hala pepe or le’ie. The Hawaiians used them as one of five commonly used hula altar plants to honor Laka, the deity of hula. In native Hawaiian herbal medicine, hala pepe was used to cure fever (Pukui and Elbert, 1986; Chun, 1994). Several species of the related genus Dracaena in other regions of the world have also been documented in many cultures and ethnic groups with similar medicinal function as the Hawaiian Chrysodracon (Lee, 1975; Milburn, 1984; Miller and Morris, 1988; Chun, 1994; Edward et al., 2001; Langenheim, 2003). A
common characteristic is the production of red resin or liquid from fruits, leaf scars and leaf bases, or plant trunks that has medicinal attributes commonly used elsewhere.

Species of *Chrysodracon* (Asparagaceae: Nolinoideae) were previously in the widespread genus *Pleomele*, but have been found to be a monophyletic assemblage that is morphologically (Jankaski, 2009) and genetically (Chapter 2) distinct. There were six Hawaiian species recognized previously (Wagner et al, 1990) and these had been recently placed in *Dracaena* subgenus *Chrysodracon* because of their unique floral characteristics (Jankaski, 2008). Extensive phylogenetic analysis of all dracaenoid genera demonstrated that the Hawaiian species are the sister group to the remainder of the dracaenoids (Chapter 2), and subsequently subgenus *Chrysodracon* was elevated to the generic level (Lu and Morden *in prep*). (*Pleomele* was found to be polyphyletic within the larger genus *Dracaena* in that analysis and should be submerged into it.)

The species of *Chrysodracon* are morphological diverse on six of the eight main Hawaiian islands (St. John, 1985). *Chrysodracon aurea* (H. Mann) Lu and Morden is mostly found in wet forests of Kauai and occasionally in mesic coastal area of Kauai. *Chrysodracon auwahiensis* (St. John) Lu and Morden is distributed on Maui and Molokai. *Chrysodracon fernaldii* (St. John) Lu and Morden is restricted to Lanai whereas *C. forbesii* (Degener) Lu and Morden and *C. halaapepe* (St. John) Lu and Morden are on Oahu. *Chrysodracon hawaiiensis* (O. Degener & I. Degener) Lu and Morden is found only on Hawaii island (St. John, 1985; Wagner et al., 1990; Jankaski, 2008; Lu and Morden *in prep*).

Relationships among populations of *C. auwahiensis* have been previously questioned. Rock (1913) observed that plants from Auwahi on the southern slopes of Haleakala, East Maui, (ie, *C. auwahiensis*) were morphologically distinct from those in the Makawao Forest Reserve.
(northern East Maui). St. John (1985) pointed out the ecological differences between these locations (wet forests of Makawao compared to the xeric habitat of Auwahi), but was unable to further distinguish between these populations as he had no floral material (only fruit) available. This population had been previously described as a distinct variety, *P. hawaiiensis* var. *mauiensis* (Degener and Degener, 1980) previous to *P. auwahiensis* being described by St. John (1985). However, St. John (1985) combined this with *P. auwahiensis* until further examination could be made (Wagner et al., 1990).

To date, no clear examination of species relationships among the Hawaiian dracaenoids has been made, particularly using molecular data. The aim of this study is to provide molecular phylogenetics analysis using nuclear and chloroplast DNA regions to solve the questions of species relationships within Hawaiian *Chrysodracon*, biogeographical patterns of dispersal of the species, and to examine issues related to the genetic distinctions among populations of species in the Hawaiian Islands.

**MATERIALS AND METHODS**

*Taxon sampling*

All Hawaiian *Chrysodracon* species, including the Makawao, Maui population, were represented in this analysis. Outgroup taxa included three *Dracaena* species from Central America (*D. americana, D. cubensis*) and Africa (*D. draco*), four *Pleomele* species from Asia (*P. umbraculifera, P. angustifolia*) and Africa (*P. fragrans, P. marginata*), and non-dracaenoids *Liriope muscari* and *Asparagus officinalis* (Asparagaceae). Selection of outgroup taxa in the Asparagaceae was based on results of previous studies (Bogler and Simpson 1996; Rudall et al.,
Plant material was either fresh collected from the field or extracted from herbarium specimens; source, voucher information, locality, and collection number for each sample used was recorded (Table 3.1). All novel sequences generated for this study were deposited in GenBank; two ITS sequences from the outgroup species *Liriope muscari* and *Asparagus officinalis* (Asparagaceae) were previously deposited in GenBank (Bogler and Simpson, 1996).

**DNA extraction and amplification**

Total genomic DNA was extracted from 1.0 g of fresh or 0.2 g of silica gel-dried leaves using the CTAB method (Doyle and Doyle, 1987) with modification (Morden et al., 1996), or using the Qiagen DNeasy plant mini kits (Qiagen Corporation, Valencia, CA, USA) following manufacturer specifications. DNA samples were accessioned into the Hawaiian plant DNA library (Morden et al. 1996).

Data for this study includes four chloroplast intergenic spacer regions: *trnL-trnF, ndhF-rpl32, trnQ-rps16*, and *rpl32-trnL*. The polymerase chain reaction (PCR) was performed using Eppendorf (Westbury, New York, USA) Mastercycler gradient or MJ Research Thermal PCR machine (GMI, Inc. Ramsey, Minnesota, USA). The PCR reaction was performed in a 25 µl reaction mixture containing 5 µl 5X Green or colorless GoTaq Flexi PCR buffer, 1.5 µl 25 mM MgCl2, 0.5 µl 10 mM dNTPs, 0.5 µl 10 mM primers, 0.2 µl GoTaq polymerase (5 U/ul) (Promega, Madison, WI, USA), and 14.5 µl ddH2O. The *rpl32-trnL* region was amplified using standard primers pairs *rpl32* (CAGTTCCAAAAAAACGTACTTC) and *trnL* (CTGCTTCCCTAGAGCGAGCGT) (Shaw et al., 2007). The *trnQ-rps16* region was amplified
using standard primers pairs trnQ (GCGTGGCCAYGMATATT) and rps16 (GTTGCTTTYTACCACATCGTTT) (Shaw et al., 2007). The rpl32-trnL and trnQ-rps16 with the following PCR conditions: 5 min at 80°C, followed by 30 cycles at 95°C for 1 min, 50°C for 1 min (a ramp 0.3°C/sec to 65°C), 65°C for 4 min, plus a final extension of 5 min at 65°C. The ndhF-rpl32 region was amplified by the primer pairs Forward (GCATATTGATAKGTATGTTCCA) and Reverse (ATMGAAGTRCGTTTYTTTGG) (Scarcelli et al., 2011). Two parameters were used to amplify this region. The first one with the following PCR conditions: 3 min at 94°C, followed by 35 cycles at 94°C for 30 sec, 42°C for 30 sec, 72°C for 1 min, plus a final extension of 10 min at 72°C. The second one with the following PCR conditions: 5 min at 80°C, followed by 35 cycles at 94°C for 1 min, 42°C for 1 min (an increasing 3°C/sec), 65°C for 4 min, plus a final extension of 10 min at 65°C. The trnL-F region was amplified by the forward primer (e; GGTTCAAGTCCCTCTATCCC) and the reverse primer (f; ATTTGAAGTGACACGAG) of Taberlet et al. (1991) with the following PCR conditions: 80°C for 5 min followed by 29 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 4 min, plus a final extension of 5 min at 72°C (Shaw et al. 2005). The first spacer region of the internal transcribed spacer (ITS1) amplification was design here for dracaenoid specificity: Chrysodracon-forward (CTTACGTKTCTCATTGCATCGATGC) and Chrysodracon-reverse (GGAAGGATCATGACCGTGATGAGC) with the following PCR conditions: 95°C for 2 min, 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, final extension of 72°C for 7 min. Non-dracaenoid species were amplified using standard primers found in the literature (Bogler and Simpson 1996) based on soybean 18S rDNA, position 1762-1787 (AAGTCGTAACAAGGTTTCCGATG) and on rice 26S rDNA, position 52-67 (TTTCTTTTCCTCCGCT) with the following PCR conditions: 94°C for 2 min, 35 cycles of
94°C for 30 sec, 50°C for 2 min, 72°C for 2 min, plus a final extension of 72°C for 10 min. All amplifications were verified on 1.5% agarose gel.

DNA amplification by polymerase chain reaction (PCR), and template purifications were performed with Go Taq PCR Core Kit (Promega, Madison, WI, USA) and all PCR products were prepared for sequencing using ExoSAP-IT (USB Corporation, Cleveland, OH, USA) or similar treatment with shrimp alkaline phosphatase and exonuclease I following the USB ExoSAP-IT PCR product cleanup protocol of incubation for 37°C for 15 min followed by 80°C for 15 mins. Samples were bidirectionally sequenced at the University of Hawaii’s ASGPB Sequencing Facility (http://cgpbr.hawaii.edu/) using BigDye Terminator chemistry (Applied Biosystems, Foster City, California, USA) and visualized on an ABI 3730XL capillary-based DNA sequencer (Applied Biosystems).

Sequence Alignment and Phylogenetics analysis

Contiguous sequences were constructed and edited for all ITS1, trnL-trnF, ndhF-rpl32, trnQ-rps16, and rpl32-trnL sequences using MEGA 5 (Tamura et al., 2011). All sequences were aligned initially in ClustalW (ver. 2.1; Larkin et al., 2007) and Muscle (Edgar, 2004) and then manually adjusted in MEGA 5 following the guidelines of Kelchner (2000) to minimize indels. Sequences from all four cpDNA regions were combined into one dataset and indels were excluded from subsequent phylogenetic analyses. ModelTest version 3.7 (Posada and Crandall, 1998) was used to determine the best model of DNA substitution. The best-fit models as determined in evolutionary models were selected by Akaike information criterion (AIC; Akaike, 1974) for each partition and the combined dataset, evaluating all models against defaults of the
program. The GTR+G model (a general time reversible model with a gamma-shaped distribution of rates across sites) was chosen for the four regions combined data matrix and the nuclear region combined with chloroplast regions as the best-fitting among the 24 models compared and was used to construct the ML and Bayesian trees. The models for separate and combined data partitions are as follows: ITS1: Tamura 3-parameter (T-92); ndhF-trnL: T92; TrnF-trnL: T92; trnL-rpl32: T92+G (T92: Tamura 3-parameter with a discrete Gamma distribution); trnQ-rps16: T92+G. Sequences were concatenated and aligned according to codon positions in a NEXUS file and phylip file prior to conversion by Mesquite 2.7.4 (Maddison and Maddison, 2009) into the appropriate file format necessary for each tree search application. The data were separated into two major partitions, nuclear (ITS1) and chloroplast (trnF-trnL, trnQ-rps16, ndhF-rpl32, rpl32-trnL). The data partitions were compared using the incongruence length difference (ILD) test (Farris et al., 1994), implemented as the partition homogeneity test in PAUP* version 4.0b10 (Swofford, 2002). For each test, 1000 replicates were performed using heuristic searches, tree-bisection-reconnection (TBR) branch swapping with random addition for three replicates, nchuck=2, and chuckscore=1.

Maximum parsimony (MP), maximum likelihood (ML) and Bayesian methods were used to estimate phylogenies for all data sets (nuclear, chloroplast and combined). Maximum parsimony search were conducted using PAUP* 4.0b10 (Swofford, 2002). In all MP analyses, heuristic searches were done using a starting tree built from stepwise addition with 1000 random addition replicates and TBR branch swapping. Bootstrap analyses based on 1000 replicates with 10 random additions per replicate were used to assess confidence in clades.

Maximum likelihood (ML) analyses as an optimality criterion (Felsenstein, 1981) was conducted for each of the three datasets using RAxML v7.2.7 (Stamakis, 2006; Stamakis et al.,
RAxML uses the GTR model with six categories of rate variation, and use of a separate support for individual branches was assessed using the nonparametric bootstrap (Felsenstein, 1985) via the rapid bootstrap procedure of Stamatakis et al. (2008) with bootstrapping stopped automatically using a frequency based criterion (Pattengale et al., 2010). Maximum likelihood bootstrap proportions (MLBS) >70% were considered strong support (Hills and Bull, 1993).

MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2005) was used to conduct Bayesian analyses under a time-free model. Bayesian analyses were run using four incrementally heated chains for 1,000,000 generations and a sampling frequency of 100 generations. Tree samples and parameter estimates from the first 25% of all trees (2500) were designated as the burn-in period and discarded. Posterior probability (PP) support values >0.95 were considered strong support for individual clades (Erixon et al., 2003; Huelsenbeck and Rannala, 2004).

RESULTS

This study generated 60 new sequences. All regions were initially analyzed separately. Table 3.2 presents the dataset matrix statistics for each analysis: ITS1, cpDNA, and combined datasets. The different regions did not vary in the amount of phylogenetic information they contained. The chloroplast regions examined had more parsimony informative characters than nuclear region examined; however, there was 8.4 times greater cpDNA sequence available than nuclear DNA, and per basepair examined, the two regions were similar. Incongruence length difference tests for the combined matrix revealed that there was no significant level of incongruence (P > 0.01) between the nuclear and chloroplast gene dataset.
Table 3.1. List of taxa used in this study, voucher information, and GenBank accession numbers for the four regions studied. The following abbreviations are used for herbaria and location: BISH = Bishop Museum, USA; PTBG= Herbarium, National Tropical Botanical Garden, USA; NY = New York Botanical Garden, USA; NCU= Herbarium, University of North Carolina, USA; WBG=Waimea Botanical Garden, Hawaii, USA; K=Royal Botanic Gardens at Kew, UK; HAJB=Jardín Botánico Nacional, Habana, Cuba; HAW: University of Hawaii at Manoa, USA.

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Voucher specimen</th>
<th>Source Location</th>
<th>GenBank Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ITS1</td>
</tr>
<tr>
<td>Chrysodracon aurea</td>
<td>Takeuchi Nualolo 17 (BISH)</td>
<td>Kauai, Hawaii, USA</td>
<td>A152463</td>
</tr>
<tr>
<td>Chrysodracon auwahiensis</td>
<td>Oppenheimer H50221 (BISH)</td>
<td>Makawao, Maui, USA</td>
<td>A152464</td>
</tr>
<tr>
<td>Chrysodracon auwahiensis</td>
<td>Hobdy 207 (BISH)</td>
<td>Kauai, Hawaii, USA</td>
<td>A152465</td>
</tr>
<tr>
<td>Chrysodracon fernaledii</td>
<td>Marreo 417 (BISH)</td>
<td>Lanai, Hawaii, USA</td>
<td>A152466</td>
</tr>
<tr>
<td>Chrysodracon forbeii</td>
<td>Perlman 6114 (BISH)</td>
<td>Oahu, Hawaii, USA</td>
<td>A152467</td>
</tr>
<tr>
<td>Chrysodracon halaapepe</td>
<td>Takeuchi 2170 (BISH)</td>
<td>Oahu, Hawaii, USA</td>
<td>A152468</td>
</tr>
<tr>
<td>Chrysodracon hawaiiensis</td>
<td>Takeuchi 5130 (BISH)</td>
<td>Puuwaawaa, Hawaii, USA</td>
<td>A152469</td>
</tr>
<tr>
<td>Dracaena americana</td>
<td>Wendt 3209 (NY)</td>
<td>Veracruz, Mexico</td>
<td>A152456</td>
</tr>
<tr>
<td>Dracaena cubensis</td>
<td>W.W.Thomas et al. 14917 (NY, HAJB)</td>
<td>Municipio Baracoa, Cuba</td>
<td>A152457</td>
</tr>
<tr>
<td>Dracaena draco</td>
<td>Orr and LU 110 (HAW)</td>
<td>Canary Island, Spain</td>
<td>A152458</td>
</tr>
<tr>
<td>Pleomele angustifolia</td>
<td>LU0106 (HAW)</td>
<td>Pingtong, Taiwan</td>
<td>A152459</td>
</tr>
<tr>
<td>Pleomele fragrans</td>
<td>LU 0103 (HAW)</td>
<td>Trop. Africa</td>
<td>A152460</td>
</tr>
<tr>
<td>Pleomele marginata</td>
<td>Lorence 9948 (PTBG)</td>
<td>Madagascar</td>
<td>A152461</td>
</tr>
<tr>
<td>Pleomele umbraculifera</td>
<td>LivColl. 835341 (WBG)</td>
<td>Mauritius</td>
<td>A152462</td>
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<tr>
<td>Liriope muscari</td>
<td>Chase 131 (NCU)</td>
<td>Cult. source, USA</td>
<td>U23981</td>
</tr>
<tr>
<td>Asparagus officinalis</td>
<td>GenBank</td>
<td></td>
<td>U24004</td>
</tr>
</tbody>
</table>
Chloroplast dataset analysis

The combined sequences for the four cpDNA regions examined resulted in an aligned sequence length of 2980 bp. All three phylogenetic analyses indicate the monophyly of the dracaenoids. Figures 1-3 demonstrate this strong support with bootstrap values >50% given for maximum parsimony (MP) and maximum likelihood (ML) analyses or posterior probability values >50% for Bayesian analysis. Only the MP and Bayesian analyses show *Chrysodracon* and the remainder of the dracaenoids as being sister groups (Fig. 3.1 and 3.3). The maximum likelihood (ML) analysis shows the Makawao population of *Chrysodracon auwahiensis* is the basal species of all dracaenoids (Fig 3.2). The MP dataset indicates there were 35% parsimony informative characters. There were 101 equally parsimonious trees; this dataset has high CI values (0.52) and high RI values (0.49).
Table 3.2. Statistics for the gene regions analyzed in the Maximum Parsimony (MP) analysis.

PIC= parsimony informative characters; CI= consistence index; RI= retention index.

<table>
<thead>
<tr>
<th>Statistic</th>
<th>ITS1</th>
<th>combined cpDNA</th>
<th>combined ITS and cpDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aligned length</td>
<td>350</td>
<td>2980</td>
<td>3330</td>
</tr>
<tr>
<td>constant sites</td>
<td>104</td>
<td>1050</td>
<td>1154</td>
</tr>
<tr>
<td>Variable sites</td>
<td>128</td>
<td>885</td>
<td>1013</td>
</tr>
<tr>
<td>PIC</td>
<td>122</td>
<td>1045</td>
<td>1167</td>
</tr>
<tr>
<td>CI</td>
<td>0.5831</td>
<td>0.5249</td>
<td>0.5242</td>
</tr>
<tr>
<td>RI</td>
<td>0.6299</td>
<td>0.4941</td>
<td>0.4928</td>
</tr>
</tbody>
</table>
Figure 3.1. Strict consensus of the maximum parsimony tree resolved the cpDNA combined dataset. Bootstrap percentages > 50% are on branches.
Figure 3.2. Maximum-likelihood tree of the cpDNA combined dataset. Bootstrap percentages > 50 % are on branches.
Figure 3.3. Bayesian tree of the cpDNA combined dataset. Posterior probability values > 50% are above branches.
ITS1 analysis

The dataset for ITS1 was 350 bp long and maximum parsimony (MP) analysis indicates it contained 34% parsimony informative characters (Table 3.2). Figure 4 presents the MP analysis of the strict consensus tree of 15 equally parsimonious trees with bootstrap values indicated on each branch (BP lower than 50% are not indicated). This dataset has high CI values (0.58) and high RI values (0.62). *Chrysodracon aurea* is the basal species of the whole phylogeny with a weak support (BP: 69). It shows that the remaining Hawaiian *Chrysodracon* species formed a clade (BP: 100) and is the sister group of the clade of the *Dracaena* and *Pleomele* species monophyletic (BP: 70).

The ML topology is mostly similar to Bayesian tree (Fig. 3.5 and 3.6). The Central American species *D. americana* and *D. cubensis* are shown as the basal group of the dracaenoid phylogeny, monophyletic group (ML: 100% BP; Bayesian 95% PP) and is a sister group with the clade of African and Asian *Dracaena* and *Pleomele* species.

Combined analysis

The ITS1 and cpDNA analyses were largely compatible in their configuration and, as such, the two datasets were united for a combined sequence of 3330 bp. Maximum parsimony (MP) analysis indicates the combined dataset has 35% parsimony informative characters with a CI values of 0.52 and RI values of 0.49 (Table 3.2). Figure 3.7-3.9 present the strict consensus of 1058 equally parsimonious trees in the MP analysis, ML analysis, and Bayesian analysis,
Figure 3.4. Strict consensus of the maximum parsimony tree resolved the ITS1 dataset. Bootstrap percentages > 50% are on branches.
Figure 3.5. Maximum-likelihood tree of the ITS1 dataset. Bootstrap percentages > 50% are on branches.
Figure 3.6. Bayesian tree of the ITS1 dataset. Posterior probability values > 50% are above branches.
Figure 3.7. Strict consensus of the maximum parsimony tree resolved the ITS and cpDNA combined dataset. Bootstrap percentages > 50% are on branches. Locations are based on each species original publication, the catalogue of life (2011), and approximation. Each location labels color. Black: Hawaii Islands, Blue: Central America, Green: Africa, Orange: East Asia, Grey: Asia, Pink: broad distribution or North Australia. The Hawaiian *Chrysodracon* is Clade A. The *Dracaena* and *Pleomele* form Clade B.
Figure 3.8. Maximum-likelihood tree of the ITS and cpDNA combined dataset. Bootstrap percentages > 50% are on branches.
Figure 3.9. Bayesian tree of the ITS and cpDNA combined dataset. The majority rule consensus tree from the combined Bayesian analysis is with the posterior probabilities (PP) summarized from the set of recovered post-burn-in trees. Posterior probability values > 50% are on branches.
respectively. Bootstrap percentages (BP) are shown on each branch (values below 50% are not indicated) for MP and ML trees, and posterior probability percentages greater than 50% are presented in the Bayesian analysis.

Phylogenetic analysis as shown by the three methods is largely compatible. All three show *Chrysodracon* as monophyletic and sister to the remaining dracaenoid genera, consistent with what has been found previously (see Chapter 2). Two clusters among the Chrysodracon taxa are evident. Clade 1 in both MP and Bayesian analysis is composed of *C. aurea* and the Makawao population of *C. auwahiensis*. ML analysis similarly has these taxa clustered together, but the clade also includes the Kanaio population of *C. auwahiensis*. Clade 2 is composed of the remaining *Chrysodracon* species with relationships among them consistent among the three analyses. The only taxon whose position within the phylogeny shifts is the Kanaio population of *C. auwahiensis* where it is positioned basal to the species of clade 1 in ML, or the species in clade 2 for MP and Bayesian analysis.

**DISCUSSION**

*The Hawaiian Chrysodracon phylogeny*

The combined nuclear and cpDNA data analysis demonstrates that the overall relationship of *Chrysodracon* with *Dracaena* and *Pleomele* are similar to those reported in Chapter 2. The monophyly of the Hawaiian *Chrysodracon* is confirmed and is the sister group to the other dracaenoid taxa. Within *Chrysodracon*, there are two largely consistent clusters of species. One is composed of *C. aurea* (from Kauai) and the Makawao population of *C.
auwahiensis (from Maui). These two species are positioned most ancestrally within the Chrysodracon phylogeny. Four other species (C. halaapepe, C. hawaiiensis, C. forbesii, and C. fernaldii) are consistently associated together in the other cluster. The relative positioning of the Kanaio population of C. auwahiensis is questionable among the three analyses, and as such may be a species intermediate between the two clusters.

Morphological and Ecological traits

Chrysodracon species have similar vegetative and reproductive macromorphological characteristics. They are all evergreen woody plants and typically grow in exposed areas (Wagner et al., 1990). Their large yellowish tubular flowers distinguish these species whereas other dracaenoids have much smaller slender tubular flowers of white, greenish or purplish color (Wagner et al., 1990). Further, Chrysodracon species have a diurnal flowering and fragrant cycle, in contrast to the nocturnally fragrant flowering of Pleomele, Dracaena and Sansevieria (Bos, 1984 and 1998; Mwachala, 2005; Jankaski, 2008; personal observation).

Species of Chrysodracon are found under various moisture regimes. Chrysodracon aurea (Kauai) and the Makawao population of C. auwahiensis are present in mesic to wet forests. One adaptation to the wet environments in C. aurea is the growth of aerial roots on their trunk (personal observation), this not having been reported in other species of this radiation (St. John, 1985). Chrysodracon forbesii grows on slopes in mesic forest of the Waianae Mountain, Oahu. The other species are variously associated with xeric habitats. Chrysodracon hawaiiensis grows on slopes with lava substrate on Hawai‘i Island. Chrysodracon auwahiensis grows on slopes in windy, dry forests on Molokai and at Kanaio, Maui. Chrysodracon fernaldii grow on slopes and
ridges in dry forest on Lanai Island. *Chrysodracon halaapepe* is found on dry to mesic slopes in forests of the Koolau Mountains on Oahu. In these drier environments, the root systems are developed more wide than deep (personal observation), which may be an adaptation to the relative young volcanic substrate in which some species are found. Of all these species, only *C. aurea* has healthy populations with active recruitment of juveniles; all other species have seen drastic reduction to their populations in recent years.

*Biogeography of Chrysodracon in the Hawaiian Islands*

Species of *Chrysodracon* do not follow the common progression rule pattern of biogeography as seen by many other lineages in the Hawaiian Islands (Wagner and Funk, 1995). The progression rule indicates that initial colonization occurs on the oldest island, Kaua‘i, followed by dispersal and evolutionary diversification on each new island as it became available for colonization (Wagner and Funk, 1995; Ziegler, 2002). Six dispersal events are necessary to explain the present-day Hawaiian *Chrysodracon* distribution in the Hawai‘i Archipelago (Figure 3.10). Common ancestor taxa dispersed simultaneously into wet or mesic forests of Maui Nui and Kauai, or colonized one island and soon after the other island. On this likely took place within the past 2 million years when Maui Nui was at its maximum size (Price and Clague, 2002). Such a pattern is consistent with the data that colonization of Hawaii was from tropical regions of Southeast Asia (Chapter 2). Dispersal and adaptation to drier regions of southern Maui Nui to form *C. auwahiensis* subsequently occurred. Dispersal from East Maui (Haleakala) to Molokai may have occurred either through long distance dispersal or by vicariance since these islands are known to have been physically linked in the recent past (Price and Clague, 2002) Dispersal from
Maui or Molokai to the Koolau Mountains of Oahu to form *C. halaapepe* occurred in the next phase. There was a brief land bridge connecting Molokai to the southern Koolau Mountains (Price and Clague, 2002) and vicariance may possibly have been a factor here as well.

Dispersal from the Koolau Mountains of Oahu may have occurred in three different patterns. The most parsimonious manner would be with dispersal and colonization directly to the Waianae Mountains of Oahu, Hawaii Island, and Lanai Island in three separate events. Alternatively, the pattern of dispersal may have been from the Koolau Mountains of Oahu to Hawaii Island with back dispersals to Lanai and Waianae Mountains of Oahu. Although the latter is consistent with the phylogenetic analyses, the branch support for these clades is much weaker. As such, the former pattern that postulates only a single large dispersal from Oahu to Hawaii with smaller events from Lanai and the Waianae Mountains is more plausible. A third alternative way is suggested by the ML tree where dispersal from Maui/Molokai to Oahu and Hawaii Islands may have been nearly simultaneous with subsequent dispersal within Oahu and to Lanai occurring later.

Although many (if not most) species radiations in the Hawaiian Islands follow the progression rule for colonization among the islands (Wagner and Funk, 1995), other patterns of colonization are not uncommon. Here, colonizations are suggested to occur from both younger to older islands as well as older to younger islands. Two radiations with strikingly similar patterns occur among plants and insects. Like *Chrysodracon*, the silversword alliance shows back to Oahu (Carr et al., 1989). Similarly, the Hawaiian species of *Drosophila* have a complex dispersal pattern with some species lineages following the progression rule while others skipping islands or colonizing older islands (Carson, 1983). Both of these radiations have many more species than are present in *Chrysodracon* and are consequently more complex, but the general
pattern of longer distance colonization among the islands (ie, skipping islands) and colonization of younger to older islands is the same.

Conclusion

The evolution of Hawaiian species of *Chrysodracon* presents another striking example of adaptive radiation in the Hawaiian flora. Although not as speciose as other radiations, the species here have a complex pattern of radiation across the islands and demonstrate a shift in ecology from wet to dry habitats. Similar habitat shifts have been observed among species of *Bidens* (Ganders, 1989) and *Dubautia* (Carr, 1987) among other taxa. These results indicate that there are promising areas for future research into the ecology, morphology and reproductive biology of these species. Of the six species now recognized, only *C. aurea* has populations that are reproductively viable; populations of the other five species are in decline and, with a few exceptions, little management or restoration is being conducted for them. Efforts must be made in the future to protect these populations so they may be more carefully examined and their unique evolutionary history better understood before species become extinct.
Figure 3.10: The *Chrysodracon* dispersal pattern based on combined analysis. 1, simultaneous (or nearly so) colonization of wet forests on Kauai and Maui. 2, dispersal and adaptation to drier regions of Maui and Molokai. 3, colonization of Koolau Mountains, Oahu with subsequent colonizations to 4) Hawaii Islands, 5) Waianae Mountains of Oahu, and 6) Lanai.
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Chapter 4: Population genetics of the endemic Hawaiian species *Chrysodracon hawaiiensis* and *Chrysodracon auwahiensis*: insights from RAPD and ISSR variation
ABSTRACT

The genus *Chrysodracon* has six endemic species in Hawaii Islands. *Chrysodracon hawaiensis* is endemic to Hawai‘i Island and was described as a distinct species in 1980. It was listed as an endangered species by USFWS in 1996 and placed on the IUCN Red List in 1997. This woody plant species was at one time common in exposed dry forests, but it became very rare due to grazing pressure and human development. The tree species *Chrysodracon auwahiensis*, endemic to Maui and Molokai, still has large adult populations in dry lands of the islands, but unfortunately no regeneration from seed has been reported in those areas for many years. The Hawaiian dry forests contain an increasingly rare assemblage of species due to habitat destruction, invasive species, and exotic pests. The two endemic species were examined using the molecular technique of random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) to determine the genetic structure of the populations and the amount of variation. Both species possess similar genetic structure. No consequences of genetic bottleneck were found in either species. Larger populations of both species contain similar levels of genetic diversity with smaller populations generally as determined by number of polymorphic loci, estimated heterozygosity, and Shannon’s index of genetic diversity. Although population diversity of *C. hawaiensis* is thought to have remained near pre-disturbance levels, population size continues to decline as recruitment is either absent or does not keep pace with senescence of mature plants. Future recovery efforts for both species must focus on avoiding animals grazing their young seedlings if these and other endemic dry forest species. For *C. hawaiensis*, augmenting populations by outplanting is still the most appropriate method. Collecting seeds and
establishing its seed bank, especially for *C. auwahiesnsis*, is essential as it is necessary to maintain its populations after the adult trees age and die.

Key words: *Chrysodracon*, conservation, Hawaiian species, ISSR, *Pleomele*, population genetics, RAPD

INTRODUCTION

The Hawaiian Islands include a high percentage of endemic species and is one of 25 biodiversity hotspots in the world (Carlquist, 1970 and 1980; Myers *et al.* 2000; Ziegler, 2002). Although many genera are species rich in Hawaii and have special evolutionary histories, few of them have been studied in detail (Ziegler, 2002; Juvik et al., 2008; Pau, 2009). Pacific island species such as those from the Hawaiian Islands show a fragile resource distribution as different island populations are scattered broadly and genetic pools are limited due to the recent founding, isolation from source population, and population size being restricted within island environments (Kay, 1994; Sheely and Meagher, 1996; Ziegler, 2002; Juvik et al., 2008). Many endemic Hawaiian plant species have very low genetic diversity and became endangered once populations were disturbed (Morden and Loeffler, 1999; Kwon and Morden, 2002; Morden and Gregoritza, 2005). The dry forests of the Hawaiian Islands have been seriously reduced due to habitat loss from development and the spread of invasive plants and animals (Gagne and Cuddihy, 1999; Juvik et al., 2008). Notably, more than 90% of Hawaiian dry forests are already lost (DLNR, 1992) and 50% of the extant Hawaiian endemic flora is listed as endangered or rare species in the International Union for the Conservation of Nature and Natural Resources (IUCN, 2011) or
by The US Fish and Wildlife Service (2011). Therefore, study and conservation of genetic resources in species, populations, and ecosystems are essential to maintain biodiversity and population dynamics.

The Hawaiian endemic genus *Chrysodracon* (Jankaski) Lu & Morden, with species previously included among the widely distributed tropical genus *Pleomele* Salisbury, has six endemic species in the Hawaii Islands (Wagner et al., 1990). *Chrysodracon hawaiiensis* (Degener and Degener) Lu & Morden was distinguished as a species in 1980 (Degener and Degener, 1980). Unfortunately, populations of this species have declined rapidly in the past few decades and the US Fish and Wildlife Service listed *Chrysodracon hawaiiensis* (as *Pleomele hawaiiensis*) as an endangered species in 1996 (USFWS 2010). The IUCN also placed it on their red list of endangered and threatened species in 1997 (IUCN, 2011). *Chrysodracon hawaiiensis* exists in only 6-8 populations totaling approximately 300-400 individuals in sunny dry forests on the leeward side of Hawai‘i Island (USFWS, 2011). The largest extant wild population is located in Pu‘uwa‘awa‘a (USFWS, 2011). This species has a unique ability to grow well in young lava substrates on steep slopes. To date, nothing is known about genetic structure of this species. Because of its rarity and small population sizes, it may possibly go extinct or become more severely depressed within a few decades without appropriate conservation management.

Presently, *C. hawaiiensis* is the only species of *Chrysodracon* recognized as occurring on Hawai‘i Island (Wagner et al., 1990). However, St. John (1985) had previously recognized three distinct species (as *Pleomele* species) based on morphological differences, *P. hawaiiensis*, *P. kaupulehuensis* St. John, and *P. konaensis* St. John. These three species were distinguished by leaf width and the length of perianth tube. The perianth of *P. hawaiiensis* is 37-40 mm long with a perianth tube longer than 26 mm whereas the perianth of *P. konaensis* is less than 37 mm with
a perianth tube less than 23 mm, and the perianth of *P. kaupulehuensis* is greater than 43 mm long. The leaf width of *P. hawaiiensis* and *P. konaensis* is less than 22 mm compared to the leaf width of *P. kaupulehuensis* being greater than 23 mm (St. John, 1985). As such, it is important to examine their population differentiation and genetic variation to gain better understanding of the relations between them, which in turn will benefit conservation efforts presently underway.

There were two objectives of this study. First is to investigate the genetic structure within and among populations of the endangered species *C. hawaiiensis*. In doing so, comparisons will also be made of the level of diversity in populations of different size. Understanding the population genetic structure of the endemic Hawaiian *Chrysodracon* species will be desirable to provide insight needed for proper conservation strategies to preserve the biodiversity of island ecosystems, reveal the evolutionary stages of those species and tackle problems that those populations are facing. It will also provide appropriate recovery suggestions for collecting the seeds and artificial pollination from those populations that have the maximum genetic variation. To best examine the population structure of an endangered species, it is also necessary to analyze the population structure of a non-endangered congener species for comparison. Therefore, the second objective of this study was to conduct a genetic survey of *C. auwahiensis* to estimate the level and distribution of genetic diversity among populations on Maui. *Chrysodracon auwahiensis* still has thousands of individuals in several populations on Maui. After completing the population genetics study of both *C. hawaiiensis* and *C. auwahiensis*, a comparison between them will provide an understanding of the type of variation that possibly was present in populations of *C. hawaiiensis* prior to habitat degradation and alteration. Knowledge of the population structure and level of variation will assist in formulating management practices for this species.
MATERIALS AND METHODS

Plant collection and DNA extraction

Leaf tissues were randomly collected from plants in four extant populations of *C. hawaiiensis* on Hawai‘i Island and five extant populations of *C. auwahiensis* on Maui Island (Hawaii State endangered species permit No. P-159 for *C. hawaiiensis*; special use permits of natural area reserves system (NARS) for both species were also obtained from the Hawaii Division of Land and Natural Resources (DLNR) and are attached in Appendix 1). The plants frequently grow on rocky and inaccessible cliffs and, in some cases, sampling was limited due to safety concerns. Because these are rare species, a voucher specimen representative of each population was identified in the collections at B. P. Bishop Museum (BISH) and is listed in Table 1 along with estimates of population size, locality, voucher information, and number of individuals collected. Exact locations of collections were determined using a global position system (GPS) receiver (Table 4.1; Fig. 4.1.1 and 4.1.2). Total genomic DNA was extracted from fresh leaf tissue using the CTAB extraction protocol (Doyle and Doyle, 1987) with modification (Morden et al., 1996), or from silica dried samples using the Qiagen DNeasy Plant Mini kit according to the manufacturer’s instructions (Qiagen Corporation, Valencia, CA, USA). Samples were accessioned into the Hawaiian Plant DNA Library (Morden et al., 1996; Randell and Morden, 1999).
Table 4.1. Species classification, locality, estimated population size (N), number of plant sampling (Ns), and voucher information of the two Hawaiian *Chrysodracon* populations for this study. The topographic map of for the populations locations of two species are shown on Figure 1 and 2.

<table>
<thead>
<tr>
<th>Island</th>
<th>Locality</th>
<th>N</th>
<th>Ns</th>
<th>Voucher</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chrysodracon hawaiiensis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hawai‘i</td>
<td>Pu‘uwa‘awa‘a</td>
<td>200</td>
<td>20</td>
<td>Kondo, Y. 44</td>
</tr>
<tr>
<td></td>
<td>Kohala</td>
<td>20</td>
<td>10</td>
<td>C. Christensen 1</td>
</tr>
<tr>
<td></td>
<td>Manuka</td>
<td>50</td>
<td>13</td>
<td>St. John, H. 11343</td>
</tr>
<tr>
<td></td>
<td>Ka‘upulehu</td>
<td>50</td>
<td>10</td>
<td>Jacobi, J.D.251</td>
</tr>
<tr>
<td><strong>Chrysodracon auwahiensis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maui</td>
<td>Kanaio</td>
<td>600</td>
<td>20</td>
<td>Hobdy, R.W.2552</td>
</tr>
<tr>
<td></td>
<td>Auwahi</td>
<td>600</td>
<td>20</td>
<td>St. John, H. 26869</td>
</tr>
<tr>
<td></td>
<td>Makawao</td>
<td>300</td>
<td>20</td>
<td>Oppenheimer, H.L. H50221</td>
</tr>
<tr>
<td></td>
<td>Iao Valley</td>
<td>300</td>
<td>8</td>
<td>Price, J.C. 19</td>
</tr>
<tr>
<td></td>
<td>Kauaula</td>
<td>300</td>
<td>9</td>
<td>Wood, K.R.11943</td>
</tr>
</tbody>
</table>

Estimate population size of *C. hawaiiensis* from Nick Agorastos (Hawai‘i Natural Area Reserves System) and *C. auwahiensis* from Hank Oppenheimer (Maui Nui Plant Extinction Prevention Program) (personal communication).
Figure 4.1.1 Topographic map of the Hawai‘i Island with the localities of the four *Chrysodracon hawaiensis* populations sampled for this study.
Figure 4.1.2. Topographic map of the Maui Island with the localities of the five *Chrysodracon auwahiensis* populations sampled for this study.
Amplification of random amplified polymorphic DNA (RAPD) and intersimple sequence repeats (ISSR)

Approximately 25ng of DNA was amplified via the polymerase chain reaction performed in a MJ Research Thermal PCR machine (GMI, Inc. Ramsey, Minnesota, USA) in 15µl volume reactions. Conditions for RAPD reactions were 0.2 µM random 10-mer oligonucleotide primers, 0.2 mM each of dNTP, 1× Taq polymerase PCR buffer, 1.5 mM MgCl₂, 0.01 g/ml concentration 1% Bovine Serum Albumins (BSA) in the total reaction volume, and 1 unit of Taq polymerase (Promega, Madison, WI, USA). RAPD PCR conditions were for one cycle at 94°C for 3min, 35°C for 30s, and 72°C for 2 min, followed by 43 cycles at 94°C for 45s, 35°C for 30s, and 72°C for 2 min, and a final cycle at 94°C for 45 s, 35°C for 30 s, and 72°C for 6 min. Conditions for ISSR reactions were 0.4 µM primer, 0.2 mM each dNTP, 1× Taq polymerase PCR buffer, 2.5 mM MgCl₂, 5% 0.01 g/ml concentration BSA in the total reaction volume, and 1 unit of Taq Polymerase (Promega, Madison, WI, USA). ISSR PCR conditions were 94°C for 90 s, followed by 34 cycles of 94°C for 40 s, 45°C for 45 s, and 72°C for 90 s, followed by 94°C for 45 s, and 45°C for 45 s, ending with 5 min at 72°C after cycling was completed.

Amplification products were mixed with loading dye (20 mm EDTA, 10% glycerol, 1% sarcosyl with bromophenol blue and xylene cyanol) and separated in 1.5% agarose gels in 0.5 × TBE (tris-borate-EDTA) buffer with 125 ng ethidium bromide per liter. Sizes of the amplification products were estimated by comparison to a Promega 100 bp ladder (Promega, Madison, WI, USA). RAPD primers (Operon Technology, CA, USA; kits OPA-OPI) and ISSR primers (University of British Columbia Primer Kit #9) were screened for amplification of Chrysodracon DNA, and selected primers were then used for amplification of all individuals.
Molecular markers were identified by the primer used to generate them and the approximate size of the band as estimated from the 100 bp ladder.

The reproducibility of amplification was tested for each primer prior to data collection. GelAnalyzer 1D image analysis software (Dr. Istvan Lazar, www.gelanalyzer.com) was initially used to estimate the number of base pairs represented by each amplified fragment and manually adjusted based on eye observation. Loci were scored as diallelic (1 = band present, 0 = band absent). Gel were scored independently by me and another researcher to produce unbiased and unambiguous analysis of RAPD and ISSR amplifications.

**Analysis of RAPD and ISSR**

Assumptions regarding of RAPD marker analysis were described by Lynch & Milligan (1994), which also apply to ISSR analysis. RAPD and ISSR markers were determined to be polymorphic if estimated allele frequency was less than 95%. In practice, a population marker was considered polymorphic when amplification was present in one or more individuals of the population or if a null (no amplification) occurred in one or more individuals. Absence of a marker within a population, although present in others, was assumed to indicate that individual to be a null/null homozygotes rather than there having been a loss of the locus. Expected heterozygosity was calculated for each population ($H_3$) and species ($H_7$) for each locus as follows: $H = 1 - (p^2 + q^2)$ where $p$ is the frequency of the amplified allele and $q$ is the frequency of the null allele. Allele frequencies were estimated from the number of null/null homozygotes present in the population (Hartl and Clark, 2007). Lynch and Milligan (1994) point out that only markers present with an observed frequency of less than $1-(3/N)$ (where N represents the sample
size) are used to reduce a potential bias when analyzing dominant markers. Principal coordinate analysis (PCO) was employed using MVSP 3.0 (Multi-Variate Statistical Package; Kovach Computing Services 1986–2011) for the analysis of genetic relationships within and among populations with the Gower general similarity coefficient (Gower, 1966 and 1971), and matrixes of genetic similarity analysis within and among populations for each species with Gower similarity coefficient analysis (Gower, 1966 and 1971). Population grouped similarity coefficients from the triangular data matrix in order to calculate an average similarity value within and among populations. Summary statistics of average similarity measures (means, standard errors, and t-tests) were calculated using Excel (Microsoft Office 2007). Distribution of genetic variation within and among populations was estimated using Shannon’s information index \( I \) (Lewontin, 1972). Shannon’s information index was calculated as:

\[ H_0 = - \sum p_i \log_2 p_i \]

where \( p_i \) is the frequency of a given RAPD or ISSR phenotype within a population or species group. Analysis of Molecular Variance (AMOVA) is a method of estimating population differentiation directly from molecular data and testing hypotheses such as population differentiation. The genetic structure was further investigated using AMOVA (Excoffier et al., 1992) as implemented in GenAlex 6.1 (Peakall et al., 2006) to estimate variance components and to test the significance of partitioning of RAPD and ISSR variations within and among populations. The AMOVA approach computes \( \Phi_{ST} \), a statistic analogous to \( F_{ST} \), which estimates the level of genetic differentiation between populations. As in \( F_{ST} \), values of \( \Phi_{ST} \) range from 0 (complete genetic homogeneity) to 1 (complete genetic separation).
RESULTS

**RAPD and ISSR Profiles**

Of the 80 RAPD primers screened, 11 produced repeatable amplification in *Chrysodracon hawaiiensis* that were scored for band presence/absence and yielded 180 scored RAPD markers (Table 4.2). Of these, 173 (96%) loci were polymorphic for all 53 individuals. The same 11 RAPD primers were also used to produce repeatable amplification products in *Chrysodracon auwahiensis*, where a total of 198 RAPD markers were scored and 195 (98%) loci were polymorphic for 77 individuals (Table 4.2). For *C. hawaiiensis*, Pu‘uwa‘awa‘a had three diagnostic (population specific) loci, Manuka had two diagnostic loci, and Ka‘upulehu had two diagnostic loci. For *C. auwahiensis*, Kanaio, Auwahi, Iao Valley, and Kauaula had one diagnostic locus each, whereas Makawao had four diagnostic loci.

Levels of RAPD variation in *C. hawaiiensis*, measured by the percentage of polymorphic markers, exhibited slight differences among populations and displayed similar relationship to the number of individuals sampled in each population (Table 4.3). The Pu‘uwa‘awa‘a population was the largest population sampled and was the most variable (68% polymorphic markers) (Table 4.3). Percent polymorphism in this population was mainly equal to that found in the entire species (70%). The smallest estimated population, Kohala, had approximately the same level of polymorphism (53%) as did both of the moderate size populations Manuka (50%) and Ka‘upulehu (54%).
Table 4.2. RAPD primer name, number of total scored amplification products, number of polymorphic bands, percent polymorphism and the range of band size for *Chrysodracon auwahiensis* and *Chrysodracon hawaiiensis* (11 random oligonucleotide primers). % P= percentage of polymorphism bands

<table>
<thead>
<tr>
<th>Primer</th>
<th>Total scored bands</th>
<th>Polymorphic bands</th>
<th>% P</th>
<th>Size(bp)Min-Max</th>
<th>Total scored bands</th>
<th>Polymorphic bands</th>
<th>% P</th>
<th>Size(bp)Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPA-02</td>
<td>14</td>
<td>13</td>
<td>93</td>
<td>300-1600</td>
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<td>260-1500</td>
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<tr>
<td>OPB-07</td>
<td>23</td>
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<td>260-1500</td>
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<td>100</td>
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<tr>
<td>OPB-14</td>
<td>19</td>
<td>19</td>
<td>100</td>
<td>150-1600</td>
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<tr>
<td>OPC-07</td>
<td>10</td>
<td>9</td>
<td>90</td>
<td>200-2000</td>
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</tr>
<tr>
<td>OPD-02</td>
<td>16</td>
<td>14</td>
<td>88</td>
<td>200-1000</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>OPD-05</td>
<td>16</td>
<td>15</td>
<td>94</td>
<td>200-1200</td>
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</tr>
<tr>
<td>OPD-12</td>
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<td>11</td>
<td>100</td>
<td>350-1000</td>
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<tr>
<td>OPD-15</td>
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<td>18</td>
<td>95</td>
<td>300-2000</td>
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</tr>
<tr>
<td>Total</td>
<td>180</td>
<td>173</td>
<td>96</td>
<td>300-2000</td>
<td>198</td>
<td>195</td>
<td>98</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.3. Genetic variability among populations of *C. hawaiiensis* and *C. auwahiensis* based on RAPD analysis. P: percentage of polymorphic markers. H: Estimate mean heterozygosity over all markers. Hp: Estimate mean heterozygosity over polymorphism markers.

<table>
<thead>
<tr>
<th>Population</th>
<th>P</th>
<th>H</th>
<th>Hp</th>
<th>Shannon's Diversity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chrysodracon hawaiiensis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pu'uwa'awa'a</td>
<td>68</td>
<td>0.218</td>
<td>0.322</td>
<td>1.582</td>
</tr>
<tr>
<td>Kohala</td>
<td>53</td>
<td>0.184</td>
<td>0.350</td>
<td>1.588</td>
</tr>
<tr>
<td>Manuka</td>
<td>50</td>
<td>0.152</td>
<td>0.301</td>
<td>1.576</td>
</tr>
<tr>
<td>Ka'upulehu</td>
<td>54</td>
<td>0.168</td>
<td>0.314</td>
<td>1.552</td>
</tr>
<tr>
<td>All individuals</td>
<td>70</td>
<td>0.254</td>
<td>0.363</td>
<td>1.576</td>
</tr>
<tr>
<td><em>Chrysodracon auwahiensis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keanaio</td>
<td>86</td>
<td>0.240</td>
<td>0.302</td>
<td>1.680</td>
</tr>
<tr>
<td>Auwahi</td>
<td>85</td>
<td>0.241</td>
<td>0.302</td>
<td>1.675</td>
</tr>
<tr>
<td>Makawao</td>
<td>80</td>
<td>0.263</td>
<td>0.363</td>
<td>1.693</td>
</tr>
<tr>
<td>Iao Valley</td>
<td>77</td>
<td>0.238</td>
<td>0.309</td>
<td>1.668</td>
</tr>
<tr>
<td>Kauaula</td>
<td>70</td>
<td>0.226</td>
<td>0.317</td>
<td>1.685</td>
</tr>
<tr>
<td>All individuals</td>
<td>90</td>
<td>0.401</td>
<td>0.432</td>
<td>1.696</td>
</tr>
</tbody>
</table>
The amount of RAPD variation found in populations of *C. auwahiensis* was related to the size of the population, similar to what was found in *C. hawaiiensis*. Of these, Kanaio and Auwahi, the two largest estimated populations, have the largest percentage of polymorphic markers (86% and 85%, respectively), nearly equal to the total amount of 90% for the species (Table 4.1 and 4.3). The two West Maui populations, Iao Valley and Kauaula, which have the smallest estimated population size, show a reduced level of polymorphic loci (77% and 70%, representatively) (Table 4.3). Makawao has approximately the same estimated population size as the West Maui, and their percent polymorphism was also similar at 80% (Table 4.3).

Of the 48 ISSR primers screened, three produced repeatable amplification products in *Chrysodracon hawaiiensis* that were scored for band presence/absence and yielded 49 scored ISSR markers (Table 4.4). Of these, 39 (92%) loci for all 53 individuals were polymorphic. Diagnostic markers were present in three of the populations: Kohala had three unique markers, Manuka had one, and Ka‘upulehu had two. Similarly, three ISSR primers were used to produce repeatable amplification products in *Chrysodracon auwahiensis*. These three primers yielded a total of 40 scored ISSR markers, 35 of which (88%) were polymorphic for the 77 individuals (Table 4.4). Diagnostic markers were present in all five *C. auwahiensis* populations; Kanaio, Auwahi, Iao Valley, and Kauaula had one diagnostic marker each, and Makawao had four diagnostic markers.

Levels of ISSR variation in *C. hawaiiensis*, measured by the percentage of polymorphic markers, exhibited a close relation to population size (Table 4.5). The Pu‘uwa‘awa‘a population was the most variable (73% polymorphic markers) (Table 4.5), and the estimated population size was the largest (Table 4.1). Percent polymorphism in this population was mainly equal to that found in the entire species (76%). Ka‘upulehu (57%) had a moderate estimated population as
Table 4.4. ISSR primer sequences and band statistics for the three primers used.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Total scored bands</th>
<th>Polymorphic bands</th>
<th>% P</th>
<th>Size(bp)Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chrysodracon hawaiiensis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5007 ACACACACACACACAC-C</td>
<td>22</td>
<td>21</td>
<td>95</td>
<td>250-1800</td>
</tr>
<tr>
<td>5009 ACACACACACACACAC-T</td>
<td>13</td>
<td>12</td>
<td>92</td>
<td>480-1500</td>
</tr>
<tr>
<td>5028 GAGAGAGAGAGAGAGA-YT</td>
<td>14</td>
<td>12</td>
<td>86</td>
<td>300-1600</td>
</tr>
<tr>
<td>Total</td>
<td>49</td>
<td>45</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td><em>Chrysodracon auwahiensis</em></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5007 ACACACACACACACAC-C</td>
<td>15</td>
<td>13</td>
<td>87</td>
<td>300-1800</td>
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<tr>
<td>5009 ACACACACACACACAC-T</td>
<td>12</td>
<td>9</td>
<td>75</td>
<td>350-1300</td>
</tr>
<tr>
<td>5028 GAGAGAGAGAGAGAGA-YT</td>
<td>13</td>
<td>13</td>
<td>100</td>
<td>300-1800</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>35</td>
<td>88</td>
<td></td>
</tr>
</tbody>
</table>

% P= percentage of polymorphism bands
Manuka and had slightly lower polymorphic loci than Manuka population (65%) (Table 4.5). The smallest estimated population, Kohala, had the lowest (45%) number of polymorphic markers (Table 4.5).

In contrast to *C. hawaiiensis*, the amount of ISSR population variation based on the percentage of polymorphic loci found in *C. auwahiensis* was not as tightly related to the size of the population. Of these, Kanaio and Auwahi, the two largest estimated populations, had the largest percentage of polymorphic markers (73% and 70%, respectively) (Table 4.1 and 4.5). The two West Maui populations, Iao Valley and Kauaula that had the smallest estimated population sizes showed a reduced level of polymorphic loci (60% and 63%, representatively) (Table 4.3). Makawao had approximately the same estimated population size as the West Maui, and their percentages of polymorphism markers were also similar at 65% (Table 4.3).

**Genetic diversity**

The estimated total RAPD mean heterozygosity over the 70 polymorphic markers within *C. hawaiiensis* was 0.363 (Table 4.3). Of the four populations, Kohala plants displayed the highest level of mean estimated heterozygosity (0.350 over polymorphic loci), Pu‘uwa‘awa‘a and Ka‘upulehu plants displayed less variation (0.322 and 0.314, respectively), and the Manuka population was lowest (0.301). Heterozygosity estimates using all markers showed a pattern different from those of the polymorphic markers with much lower variation (Table 4.3). Total estimated mean heterozygosity over all markers in *C. hawaiiensis* was 0.254. Mean estimated heterozygosity was highest for plants at Pu‘uwa‘awa‘a (0.218), whereas heterozygosity among individuals at Manuka was considerably lower at (0.152). The variation value in Kohala population was 0.184, followed by Ka‘upulehu (0.168).
Table 4.5. Genetic variability among populations of *C. hawaiiensis* and *C. auwahiensis* based on ISSR analysis. P: percentage of polymorphic markers. H: Estimate mean heterozygosity over all markers). $H_P$: Estimate mean heterozygosity over polymorphism markers.

<table>
<thead>
<tr>
<th>Population</th>
<th>P</th>
<th>H</th>
<th>$H_P$</th>
<th>Shannon's Diversity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chrysodracon hawaiiensis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pu‘uwa‘awa‘a</td>
<td>73</td>
<td>0.276</td>
<td>0.375</td>
<td>1.440</td>
</tr>
<tr>
<td>Kohala</td>
<td>45</td>
<td>0.187</td>
<td>0.416</td>
<td>1.333</td>
</tr>
<tr>
<td>Manuka</td>
<td>65</td>
<td>0.223</td>
<td>0.340</td>
<td>1.410</td>
</tr>
<tr>
<td>Ka‘upulehu</td>
<td>57</td>
<td>0.210</td>
<td>0.352</td>
<td>1.395</td>
</tr>
<tr>
<td>All individuals</td>
<td>76</td>
<td>0.316</td>
<td>0.418</td>
<td>1.450</td>
</tr>
<tr>
<td><em>Chrysodracon auwahiensis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kanaio</td>
<td>73</td>
<td>0.257</td>
<td>0.355</td>
<td>1.413</td>
</tr>
<tr>
<td>Auwahi</td>
<td>70</td>
<td>0.216</td>
<td>0.309</td>
<td>1.407</td>
</tr>
<tr>
<td>Makawao</td>
<td>65</td>
<td>0.217</td>
<td>0.230</td>
<td>1.394</td>
</tr>
<tr>
<td>Iao Valley</td>
<td>60</td>
<td>0.184</td>
<td>0.226</td>
<td>1.386</td>
</tr>
<tr>
<td>Kauaula</td>
<td>63</td>
<td>0.181</td>
<td>0.271</td>
<td>1.377</td>
</tr>
<tr>
<td>All individuals</td>
<td>88</td>
<td>0.352</td>
<td>0.403</td>
<td>1.397</td>
</tr>
</tbody>
</table>
Total estimated mean heterozygosity for polymorphic RAPD markers in *C. auwahiensis* was 0.432, higher than for *C. hawaiiensis* (Table 4.3). Values of the mean heterozygosity for five populations were all pretty similar. Makawao plants displayed the highest level of mean estimated heterozygosity (0.363 over polymorphic loci). Iao Valley plants displayed lesser variation (0.309), Kauaula population also has less variation (0.317), and Auwahi and Kanaio populations were lowest (0.302). Heterozygosity estimates using all markers showed a pattern with some difference from those of the polymorphic markers and also much lower than the values of overall polymorphic markers. Total estimated mean heterozygosity over all markers in *C. auwahiensis* was 0.401. Mean estimated heterozygosity was highest for plants from Makawao (0.263), followed by Auwahi (0.241) and Kanaio (0.240). The heterozygosity among individuals of Kauaula (0.226) and Iao Valley (0.238) were the lowest.

Shannon’s Diversity Index estimates based on RAPD analysis for each population and all populations combined are presented in Table 3. Estimates of average genetic diversity within *C. hawaiiensis* based on Shannon’s Index within the four populations were all very similar, ranging from 1.588 (Kohala) to 1.552 (Ka’upulehu). The Shannon’s Index among all individuals was 1.576. Estimates of average genetic diversity based on Shannon’s Index displayed similar disparity in the relative amounts of variation with the four populations, and the result is in contrast with the heterozygosity over all markers estimates, but consistent with the heterozygosity over polymorphism markers among the four populations.

As with *C. hawaiiensis*, the average values of Shannon’s Index based on RAPD analysis within *C. auwahiensis* were all very similar, ranging from 1.668 (Iao Valley) to 1.693 (Makawao). Shannon’s Diversity Index for all five populations combined was 1.696. The data were in contrast with the heterozygosity over all markers estimates, but consistent with the
heterozygosity over polymorphism markers among the five populations. However, the genetic diversity within *C. auwahiensis* also showed similar values in different populations and had moderate diversity for all populations revealing a similar pattern as *C. hawaiiensis*.

In ISSR, total species estimate mean heterozygosity over the 80 polymorphic markers within *C. hawaiiensis* is 0.418 (Table 4.5). Of the four populations, Kohala plants (the smallest population) displayed the highest level of mean estimated heterozygosity over polymorphic markers (0.416), Pu‘uwa‘a‘a (0.375) and Ka‘upulehu plants (0.352) showed similar, but lower, variation (0.352), and the Manuka population was lowest (0.340). Heterozygosity estimates using all markers showed a pattern different from those of the polymorphic markers (Table 4.5). Total estimated mean heterozygosity over all markers in *C. hawaiiensis* was 0.316. Mean estimated heterozygosity was highest for plants on Pu‘uwa‘a‘a (0.276) (Table 4.5). The value in Manuka population was 0.223, followed by Ka‘upulehu (0.210), and Kohala was considerably lower (0.187).

The estimated mean ISSR heterozygosity on total species over the 88 polymorphic markers within *C. auwahiensis* is 0.403. Of the five populations, the Kanaio population displayed the highest level of mean estimated heterozygosity (0.355), Auwahi had less variation (0.309), Kauaula (0.271) had even lesser variation, and Makawao (0.230) and Iao Valley populations had the lowest (0.226). Heterozygosity estimates using all markers showed a slightly difference from those of the polymorphic markers and also lower (Table 4.5). Total estimated mean heterozygosity over all markers in *C. auwahiensis* was 0.352, which was higher than *C. hawaiiensis*. Mean estimated heterozygosity was highest for plants on Kanaio (0.257), followed by the less variation in Makawao (0.217), and then in Auwahi (0.216). The lowest values were found in the Kauaula (0.181) and Iao Valley (0.184) populations.
Shannon’s Diversity Index estimates based on ISSR analysis for each population and all populations combined are presented in Table 4.5. There was no apparent difference between the two species. Estimates of average genetic diversity within *C. hawaiiensis* based on Shannon’s Index within the four populations were similar, ranging from 1.265 (Kohala) to 1.301 (Pu‘uwa‘awa‘a). Estimates of average genetic diversity based on Shannon’s Index displayed similar trend in the relative amounts of variation with the four populations as was present with the heterozygosity over all markers estimates, but differed with the heterozygosity over polymorphic markers. The genetic diversity within *C. hawaiiensis* had moderate diversity and similar values for each population.

The average values of Shannon’s Diversity Index based on ISSR analysis within *C. auwahiensis* were similar across populations and ranged from 1.377 (Kauaula) to 1.413 (Kanaio). The mean Shannon’s Diversity Index for all five populations was 1.397. As with *C. hawaiiensis*, the trend in the data is similar to what was present for the heterozygosity over all markers estimates, but differs with the heterozygosity over polymorphic. Finally, the genetic diversity within *C. auwahiensis* also shows similar values in different populations and has moderate diversity for all populations.

The population differentiation among *C. hawaiiensis* populations is 0.442, which indicates a very significant genetic differentiation. The population differentiation among *C. auwahiensis* populations is slightly lower than *C. hawaiiensis*, and the value is 0.347. Both results indicate that all populations within *C. auwahiensis* on Maui have reached a significant genetic differentiation. All of the results were statistically significant (P=0.001).

ISSR genetic structure estimates from AMOVA are shown in Table 4.7. The variation of *C. hawaiiensis* has slightly less variability within populations (48%) rather than among populations.
(52%). In contrast, the result of the total comparison of C. auwahiensis shows that more variability is attributed to individuals within populations (60%) rather than among populations (40%).

**Genetic Structure**

RAPD genetic structure estimates from AMOVA are shown in Table 4.6. The variation of C. hawaiiensis was slightly greater within populations (56%) than among populations (44%). Similarly, for C. auwahiensis more variation was found within populations (65%) rather than among populations (35%).

There was enough genetic structure defining all populations for both species (Table 4.7). On average, 52% of the variation was partitioned among C. hawaiiensis populations and 40% of the variation was partitioned among C. auwahiensis populations. The population differentiation among C. hawaiiensis populations is 0.519, which indicates a large genetic differentiation. All of the results in C. hawaiiensis were statistically significant (P=0.001). Population differentiation among C. auwahiensis populations is slightly lower than C. hawaiiensis, and the value is 0.401. Both results indicate that all of the populations within C. auwahiensis on Maui have a large degree of genetic differentiation. All of the results in C. auwahiensis were statistically significant (P=0.001).

Results from RAPD and ISSR data were similar for genetic diversity and genetic structure in both species. RAPD and ISSR combined dataset for genetic structure estimates from AMOVA were made and shown in Table 4.8. Overall, the combined dataset produces a similar result compared to both RAPD or ISSR data. The variation of C. hawaiiensis is slightly less within
Table 4.6. Analysis of molecular variance (AMOVA) for 53 individuals in four populations of *C. hawaiiensis* and 77 individuals in five populations of *C. auwahiensis* based on RAPD analysis.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>SSD</th>
<th>MSD</th>
<th>Variance component</th>
<th>%Total</th>
<th>P</th>
<th>Φ&lt;sub&gt;ST&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pleomele hawaiiensis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among the four populations</td>
<td>3</td>
<td>827.12</td>
<td>275.71</td>
<td>19.57</td>
<td>44</td>
<td>0.001</td>
<td>0.442</td>
</tr>
<tr>
<td>Within all populations</td>
<td>49</td>
<td>1208.39</td>
<td>26.66</td>
<td>24.66</td>
<td>56</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td><em>Pleomele auwahiensis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among the five populations</td>
<td>4</td>
<td>974.97</td>
<td>243.74</td>
<td>14.54</td>
<td>35</td>
<td>0.001</td>
<td>0.347</td>
</tr>
<tr>
<td>Within all populations</td>
<td>72</td>
<td>1973.21</td>
<td>27.41</td>
<td>27.41</td>
<td>65</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

d.f., degrees of freedom; SSD, sum of squared deviation; MSD, mean squared deviation; % Total, percentage of total variance contributed by each component; P, probability of obtaining a more extreme component by chance alone; and Φ<sub>ST</sub>.
Table 4.7. Analysis of molecular variance (AMOVA) for 53 individuals in four populations of *C. hawaiiensis* and 77 individuals in five populations of *C. auwahiensis* based on ISSR analysis.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>SSD</th>
<th>MSD</th>
<th>Variance component</th>
<th>%Total</th>
<th>P</th>
<th>Φ&lt;sub&gt;ST&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pleomele hawaiiensis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among the four populations</td>
<td>3</td>
<td>199.02</td>
<td>66.36</td>
<td>4.82</td>
<td>52</td>
<td>0.001</td>
<td>0.519</td>
</tr>
<tr>
<td>Within all populations</td>
<td>49</td>
<td>218.73</td>
<td>4.46</td>
<td>4.46</td>
<td>48</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td><em>Pleomele auwahiensis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among the five populations</td>
<td>4</td>
<td>192.74</td>
<td>48.19</td>
<td>2.94</td>
<td>40</td>
<td>0.001</td>
<td>0.401</td>
</tr>
<tr>
<td>Within all populations</td>
<td>72</td>
<td>316.66</td>
<td>4.4</td>
<td>4.4</td>
<td>60</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

d.f., degrees of freedom; SSD, sum of squared deviation; MSD, mean squared deviation; % Total, percentage of total variance contributed by each component; P, probability of obtaining a more extreme component by chance alone; and Φ<sub>ST</sub>.
populations (46%) rather than among populations (54%). The result of the total comparison of C. auwahiensis shows that more variability is attributed to individuals within populations (65%) rather than among populations (35%).

Genetic structure defined all populations for both species (Table 4.8). The population differentiation among C. hawaiiensis populations was 0.536, which indicates a very strong genetic differentiation. The population differentiation among C. auwahiensis populations, 0.355, was slightly lower than C. hawaiiensis. Both results indicate that all of populations within C. auwahiensis in Maui are genetically distinct.

Genetic similarity indices

Genetic similarity within and among populations was calculated using the Gower similarity coefficient analysis (Gower, 1966 and 1971) where the coefficient ranges between 0 and 1 with the former indicative of complete genetic dissociation and the latter genetic identity. Relationships among population and the relative levels of within population variation are clearly reflected in the matrix of genetic similarity analysis in RAPD and ISSR combined dataset (Table 4.9 and 4.10). As expected, individuals were most similar to members of their own population in both species (Table 4.9 and 4.10). For interpopulation comparisons within C. hawaiiensis, plants from Kohala and Pu‘uwa‘a‘a shared the highest similarity (60%); the Ka‘upulehu and Manuka shared the lowest similarity (47%) (Table 4.9). For interpopulation comparisons within C. auwahiensis, plants from Iao Valley and Kauaula shared the highest similarity (67%); the Kanaio and Auwahi also shared the high similarity (66%); Makawao shared the lowest similarity with the other four populations (51-52%) (Table 4.10).
Table 4.8. Analysis of molecular variance (AMOVA) for 53 individuals in four populations of *C. hawaiiensis* and 77 individuals in five populations of *C. auwahiensis* based on combined RAPD and ISSR combined dataset analysis.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>SSD</th>
<th>MSD</th>
<th>Variance component</th>
<th>%Total</th>
<th>P</th>
<th>Φ&lt;sub&gt;ST&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pleomele hawaiiensis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among the four populations</td>
<td>3</td>
<td>1217.99</td>
<td>406</td>
<td>29.47</td>
<td>54</td>
<td>0.001</td>
<td>0.536</td>
</tr>
<tr>
<td>Within all populations</td>
<td>49</td>
<td>1255.56</td>
<td>25.62</td>
<td>25.52</td>
<td>46</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td><em>Pleomele auwahiensis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among the five populations</td>
<td>4</td>
<td>1167.71</td>
<td>291.928</td>
<td>17.478</td>
<td>35</td>
<td>0.001</td>
<td>0.355</td>
</tr>
<tr>
<td>Within all populations</td>
<td>77</td>
<td>2298.88</td>
<td>31.8</td>
<td>31.8</td>
<td>65</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

d.f., degrees of freedom; SSD, sum of squared deviation; MSD, mean squared deviation; % Total, percentage of total variance contributed by each component; P, probability of obtaining a more extreme component by chance alone; and Φ<sub>ST</sub>.
Table 4.9. Matrix of the average of coefficient genetic similarity within and among populations of *C. hawaiiensis* based on combined RAPD and ISSR dataset analysis. Unit: %

<table>
<thead>
<tr>
<th></th>
<th>Pu'uwa'awa'a</th>
<th>Kohala</th>
<th>Manuka</th>
<th>Ka'upulehu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pu'uwa'awa'a</td>
<td>87</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kohala</td>
<td>60</td>
<td>85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Manuka</td>
<td>56</td>
<td>50</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Ka'upulehu</td>
<td>54</td>
<td>54</td>
<td>47</td>
<td>85</td>
</tr>
</tbody>
</table>
Table 4.10. Matrix of the average of coefficient genetic similarity within and among populations of *C. auwahiensis* based on combined RAPD and ISSR dataset analysis. Unit: %

<table>
<thead>
<tr>
<th></th>
<th>Kanaio</th>
<th>Auwahi</th>
<th>Makawao</th>
<th>Iao Valley</th>
<th>Kauaula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanaio</td>
<td>84</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auwahi</td>
<td>66</td>
<td>83</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Makawao</td>
<td>51</td>
<td>51</td>
<td>86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iao Valley</td>
<td>53</td>
<td>55</td>
<td>52</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Kauaula</td>
<td>53</td>
<td>55</td>
<td>51</td>
<td>67</td>
<td>76</td>
</tr>
</tbody>
</table>
Principal coordinate analysis (PCO) with Gower similarity coefficient analysis for each species is shown in Fig. 4.2 and 4.3 (RAPD), Fig 4.4 and 4.5 (ISSR), and Fig. 4.6 and 4.7 (RAPD and ISSR combined dataset). As expected, both methods showed that individuals in both species were most similar to members of their own population. RAPD analysis in *C. hawaiiensis*, those populations were separated from each other by PCO analysis (Fig. 4.2). The first two principal coordinates account for 20.2% and 17.2% of variation, respectively. For *C. auwahiensis*, the Makawao population is the most isolated of the populations on Maui and is the only singly distinguished population in this analysis (Fig. 4.3). The other four populations have the overlapping individuals (Fig. 4.3). Iao valley and Kauaula populations in West Maui are intermixed, and distinct from the other populations; individuals of Kanaio and Auwahi populations overlap, and are also distinct from the other populations. The first two principal coordinates account for 16.3% and 11.3% of variation, respectively (Fig. 4.3).

Populations of *C. hawaiiensis* in the ISSR analysis were not clearly separated from each other except the Ka'upulehu population in the PCO analysis (Fig 4.4). The first two principal coordinates account for 26.3% and 12.9% of variation, respectively (Fig. 4.4). In *C. auwahiensis*, Makawao population is the most distant from the other populations on Maui and is the only distinguished population in Maui (Fig. 4.5). The populations from Southern East Maui (Kanaio and Auwahi) were broadly overlapped and the West Maui populations (Iao Valley and Kauaula) were also overlapped (Fig. 4.5). As in the RAPD analysis, Iao Valley and Kauaula populations from West Maui and Kanaio and Auwahi from Southern East Maui form distinct clusters. The first two principal coordinates account for 24.2% and 17.1% of variation, respectively.
Figure 4.2. Principal coordinate analysis of RAPD data using all scored markers for *Chrysodracon hawaiensis*.
Figure 4.3. Principal coordinate analysis of RAPD data using all scored markers for *Chrysodracon auwahiensis*. 
Figure 4.4. Principal coordinate analysis of ISSR data using all scored markers for *Chrysodracon hawaiensis*.
Figure 4.5. Principal coordinate analysis of ISSR data using all scored markers for *Chrysodracon auwahiensis*.
The result of the combined dataset strengthens the relationships among populations found in the independent RAPD and ISSR analyses. In *C. hawaiiensis*, the populations form four distinct clusters (Fig 4.6). The first two principal coordinates account for 18.8% and 18.1% of variation, respectively. The first axis clearly distinguishes the southern-most population, Manuka, from the three located in the northern half of Hawâ‘i Island, and to a lesser extent the Kohala/Ka‘upulehu and Pu‘uwa‘a‘a populations; the second axis separates the Kohala and Ka‘upulehu populations. In *C. auwahiensis*, three distinction clusters are readily apparent representations Makawao, Iao Valley and Kauaula, and Kanaio and Auwahi (Fig. 4.7). The first principal component axis separates the Makawao (northern East Maui) from Kanaio and Auwahi (Southern East Maui), and the second principal component axis separates the West Maui populations (Iao Valley and Kauaula) from East Maui. The first two principal coordinates account for 17.1% and 11.2% of variation, respectively.

**DISCUSSION**

*Relative genetic variation*

The present study is the first DNA-level examination within and among populations of *Chrysodracon* species, and establishes a baseline by which comparisons with other species (*Chrysodracon* or other dracaenoids) may be made. Genetic diversity within both of the species was moderate compared to other Hawaiian taxa examined, while among population differentiation was very significant. Percent polymorphism at the species level was 92% for all individuals of *C. hawaiiensis*, and this is higher than what has been found in many other taxa
Figure 4.6. Principal coordinate analysis of combined RAPD and ISSR dataset using all scored markers for *Chrysodracon hawaiensis*.
Figure 4.7. Principal coordinate analysis of combined RAPD and ISSR dataset using all scored markers for *Chrysodracon auwahiensis*.
(Dubautia ciliolata: 70% and Dubautia scabra: 59%, Caraway et al. 2001; 51%, Touchardia latifolia: Morden & Loeffler 1999; Alphitonia ponderosa: 47% and Colubrina oppositifolia: 41%, Kwon and Morden, 2002). However, the population level variation was lower and in a moderate range compared to most other Hawaiian species (Caraway et al., 2001; Kwon and Morden, 2002). Notably, the endangered species C. hawaiiensis shows similar genetic dynamics, as did the common species C. auwahiensis. Because these are long-lived plants, both species still maintain considerable genetic variation reflective of what may have existed prior to the start of the species decline.

Levels of genetic variation based on percent polymorphism indicate that C. hawaiiensis (70%) exhibited moderate levels of relative genetic diversity in comparison to C. auwahiensis (90%). Populations within C. hawaiiensis showed lower diversity (ranging from 50% - 68%) than for the species as a whole. Similarly, levels of variation within populations of C. hawaiiensis show a similar trend. Populations within C. auwahiensis also showed lower diversity (ranging from 70% - 86%) than for the species as a whole. Similarly, levels of variation within populations of C. auwahiensis also show a similar trend.

Both species are long-living woody perennial tree plants. Unfortunately, neither of the two species has natural regeneration of young seedling in the field. There were no reported wild fires destroying the forest at least after 1947 (St. John, 1947; observation by PEP conservation job managers in Maui). As such, the extant genetic diversity is likely representative of the diversity present at least 100 years ago for C. auwahiensis, and the same holds true for C. hawaiiensis as well. Most extant plants are old mature trees in populations that have probably experienced minimal impact from genetic drift. The endangered species C. hawaiiensis has the similar
although slightly lower estimated total polymorphism, heterozygosity, and Shannon’s diversity index over all polymorphic markers as compared to the more common *C. auwahiensis*.

**Comparison of RAPD and ISSR**

In general, the genetic diversity measures in both RAPD and ISSR analyses were very similar and results obtained were highly compatible. Overall, the genetic diversity values were lower in RAPD than ISSR analyses and the values for population differentiation were higher in ISSR than RAPD analyses. The four populations of *C. hawaiiensis* showed similar patterns of clustering in the PCO for each method although the populations were more clearly separated in the RAPD analysis whereas in the ISSR analysis, some individuals from Kohala were overlapping with those from the Pu‘uwa‘awa‘a population. This might be interpreted as the RAPD method being more sensitive than the ISSR method regarding genetic diversity although it may be more an artifact of additional genetic markers being measured for RAPD analyses than ISSR. Similar findings regarding genetic diversity, population differentiation and PCO analyses were also found for *C. auwahiensis*. Although subtle differences may be found when examining the various statistics individually, results from the two methods were highly compatible.

**Population size and genetic diversity**

Genetic diversity within populations reflected the estimated population size in species by RAPD and ISSR analyses. Estimated heterozygosity over all loci or over all polymorphic loci, and estimated genetic diversity was higher in the common species *C. auwahiensis* than in the
endangered species *C. hawaiiensis*. These data suggest that *C. hawaiiensis* populations were at one time much larger, and reduction in population sizes have been recent with some loss in genetic variation. In *C. hawaiiensis*, the estimated population size of Pu‘uwa‘awa‘a was largest and has the higher genetic diversity. The other three populations have similar, but markedly lower, genetic diversity. The disparity in the levels of diversity is undoubtedly related to the estimated population sizes. Individuals in those populations are all long-living old mature plants, and no evidence of seedlings or juveniles in wild are recorded (Personal observation; Nick Agorastos, Hawaii NARS staff, personal communication). Both species have frequently produced flowers and seeds, but the no seedlings were found growing during several years of observation likely because of invasive weeds and insects (Personal observation; H. Oppenheimer, Maui PEP, and Nick Agorastos, Hawaii NARS staff, personal communication). Attrition of individuals from populations without subsequent regeneration may have contributed to the levels of variation now seen there.

Trends in population variation for *C. auwahiensis* were as predicted. The southern East Maui populations, Kanaio and Auwahi, are larger and distributed over a wider geographical area compared to the Makawao or West Maui populations, Iao Valley and Kauaula, and also showed the greatest genetic diversity. The Makawao population is distinct from the populations of East Maui or West Maui in genetic similarity analysis, and this population’s habitat is a wet forest rather than the dry forests of the other populations. Overall, data for *C. auwahiensis* indicates that populations encompassing a larger geographical area retain higher, but not greatly so, genetic diversity compared to those encompassing smaller or more isolated areas. The population diversity here being more similar, as opposed to the greater ranges in diversity within *C. hawaiiensis*, is likely a consequence of all populations still being of a large size.
Distribution of variation

The majority of variation in *Chrysodracon hawaiiensis* was found within rather than among populations, but among rather than within for *C. auwahiensis* populations as estimated by both Shannon’s index and AMOVA analysis in RAPD and ISSR analyses. It has been shown that long-lived plants, especially trees, such as these typically harbor a greater percentage of their variation within populations (Hamrick and Loveless, 1989; Hamrick and Godt, 1990). The study here supports these conclusions for *C. hawaiiensis*, but not *C. auwahiensis*. The numbers of diagnostic alleles unique to each population are possibly signs of differentiation among the populations following selection or genetic drift from the ancestral genetic environment. On the other hand, these alleles may be the representative of new mutations (such as deletions or insertions) that have appeared within populations following their initial dispersal after speciation events.

Population differentiation

Isolation, created by geographical distance and subsequent fragmentation, has provided the initial means for divergence in both species. Significant population differentiation occurs in the four populations within *C. hawaiiensis*. The South Hawai‘i Manuka population has the most distinct differentiation from the other Northern Hawai‘i populations. For *C. auwahiensis*, genetic similarity analysis and PCO analysis in RAPD, ISSR, and combined dataset shows the West Maui populations distinct from the Northern East Maui and Southern East Maui populations. There are noteworthy signs of genetic distinction between the East Maui, Makawao, and West
Maui populations. Because of limited gene flow in both species from habitat alteration between the populations, this differentiation is likely to continue.

Reproductive biology in these species has not been examined up to date, but anecdotal evidence in the course of conservation work suggests that pollen and seed movement among populations is related to birds’ dispersal (Jankaski, 2008). Neither species has had seedlings reported since the last century mainly due to introduced animals eating the leaves and young shoots, and the numbers of introduced animals drastically increasing in the forests in recent years (USFWS 2011). Chrysodracon species have large bell shape yellowish flowers with dark berries, and has been hypothesized to share an association with birds for pollination and seed dispersal (Jankaski, 2008). Although several potential factors may be important in limiting gene flow at this site (i.e., pollinating and seed dispersing extinct birds), the separation of these populations is likely due to habitat destruction and invasive species. There are several lines of evidence to support the continued separation of populations: 1) *C. hawaiiensis* individuals share low genetic similarity; 2) the gene flow among populations is apparently restricted; and 3) localized inbreeding may be occurring reducing the amount variation within populations. Dry forests are typically associated with leeward coast regions of all islands. Chrysodracon species typically survive on steep hillsides or lava substrates with well-drained soils. Thus, seed dispersal and gene flow within island populations may have been considerably greater prior to Polynesian inhabitation and the large-scale destruction of low elevation forests (Athens, 1997), extinction of bird species that followed, invasive weeds competition, and animal and slugs grazing pressure (USFWS, 2011).
Conclusions and Conservation implications

Results of this study demonstrate several important factors regarding the genetic diversity and structure within these species. Patterns of genetic diversity and genetic differentiation within and among populations are similar for both species examined. However, the level of variation found in *C. hawaiiensis*, an endangered species with smaller and more isolated populations, is consistently lower than that found in *C. auwahiensis*, a non-endangered species with much more extensive populations. Populations of *C. hawaiiensis* have been in decline for at least 50 years (Nick Agorastos, Hawaii NARS staff, personal communication), yet a level of genetic diversity nearly equal to that of a non-endangered congener occurring in similar habitats suggests that the affects of inbreeding within populations have not yet had a significantly deleterious impact on their vigor. Genetic diversity at the species level remains very high as levels of polymorphism is above 90% and nearly equal to those species known to have the highest level of genetic diversity yet measured among Hawaiian species (Morden and Gregoritzza, 2005). This is likely a reflection of the species habit (long-lived trees) and habitat (dry forests) that promote slow growth in individuals. Since little to no recruitment of plants within populations has been observed, it is probable that the genetic diversity observed is from individuals that have survived in these environments since before the populations went into decline and that loss of variation is because of population attrition rather than loss of alleles through inbreeding. There are approximately 20 individuals of *C. hawaiiensis* in scattered locations at Hawaii Volcano National Park (HAVO) that were not examined here. Any future study of these species should include the HAVO population.

Long-term survival of *C. hawaiiensis* will not be possible by simply maintaining current population numbers without active conservation management. The impacts from animal grazing
pressure have played a pivotal role in the erosion of plant diversity of Hawaiian dry forests. For *C. auwahiensis*, the additional pressure on the populations by invasive weeds competing with seedlings and invasive slugs that eat seedlings are further threats (H. Oppenheimer, Maui PEP, personal communication). The consequences have been no seedling recruitment in these populations. When it comes to conservation work, the proposed suggestions are collecting seeds from different population of each species because it will increase the genetic variation and benefit to endangered species recovery. Based on the polymorphism data, *C. auwahiensis* in Maui still maintain enough genetic variation (70%-86%) for each population.

Several conservation measures are recommended to protect both species. First, and most importantly, any threats to the plants at the early stages of their development must be removed. This can only be accomplished by building predator proof fences that can exclude introduced herbivorous animals, particularly goats, from those areas. Some snail baits have recently been approved for use in conservation areas, and strategies for their use should be developed to implement this control where snails and slugs are a factor. Second, mature plants readily flower and fruit, and efforts should focus on establishing *ex situ* seed bank for both species. Care should be made while collecting to target widely spaced plants to capture the maximum genetic diversity possible (Cabin et al., 1998). Third, growing plants *ex situ* and outplanting back into the populations when they have attained a size sufficient to withstand existing threats (i.e., slugs and goats) would help maintain the populations integrity until other measures have been implemented that will allow natural recruitment. Fourth, because individual population variation of *C. hawaiiensis* is in decline yet total species variation is high, limited mixing of population progeny is recommended to maintain higher levels of genetic diversity that has been shown to be beneficial for the long-term survival in a wide variety of species (Frankham, et al., 2002). The
loss of genetic variation has been shown to have harmful effect on fitness of individuals of populations (Ledig et al. 1997; Hartl and Clark, 2007). Possible problems associated with outbreeding depression that could occur from mixing different population progeny are minimal, if present at all, and are far less than potential future problems associated with inbreeding depression. Performing hand-pollination crosses among plants from different populations and growing the individuals from such crosses with the purpose to outplant them might also attain this.
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PERMIT FOR THREATENED AND ENDANGERED PLANT SPECIES
Department of Land and Natural Resources
Division of Forestry and Wildlife
1151 Punchbowl Street, Room 325
Honolulu, Hawaii 96813
(808) 587-0165, Fax (808) 587-0160

Permit No. P-159
Date of Issue: February 1, 2011
Expiration Date: January 31, 2012

The Board of Land and Natural Resources hereby grants permission under the authority of Hawaii Administrative Rules §13-104, §13-107, and §13-124, Hawaii Revised Statutes §103D and §195D and all other applicable laws, to the person(s) listed below.

Persons in violation of the terms and conditions of this permit and/or related or appropriate laws may be subject to criminal and/or administrative penalty under Hawaii Administrative Rules §13-104-3 and §13-107-8, Hawaii Revised Statutes §195D-9, §171-6.4 and §171-31.6, or as otherwise provided by law.

Pei-Luen Lu
University of Hawaii-Manoa
3190 Maile Way, Room 101
Honolulu, HI 96822
Phone: 990-8186
Fax: 956-3923
Email: peiluen@hawaii.edu

To take and possess, for the purpose of research, the following plant life:

Pleomele hawaiensis (Endangered)

One leaf per plant may be collected, and only if the plants are in healthy condition. If the population is large, only a subset of up to 40 plants will be sampled. Leaf samples will be used for molecular genetic analysis of the DNA polymorphism in populations of Pleomele hawaiensis, aiming to determine the level of their genetic diversity. Individuals will be collected in Puuwaawaa. Leaves must be collected from the ground or by using a long pole clipper or self-
standing ladder. Climbing of trees or leaning heavy ladders against the trees is prohibited. If individuals of *Pleomele hawaiensis* are located on the Hawaii Experimental Tropical Forest property, the permit holder(s) are required to obtain a permit from HETF. Collection of leaves is permitted in the Manuka NAR only if the permit holder(s) are accompanied by a NAR’s staff and with a NAR’s permit.

Subject to the following conditions:

I. GENERAL CONDITIONS

A. This permit authorizes the permit holder(s) to conduct described activities at location(s) noted, on State Forest Reserves, or lands that are under the control of the Division of Forestry and Wildlife (DOFAW), Department of Land and Natural Resources (DLNR).

B. Activities conducted in DOFAW’s Natural Area Reserves System (NARS) require a Special Use Permit. Activities conducted on other lands under the jurisdiction of DOFAW/DLNR, will require access permits.

C. The permit holder(s) must obtain approval from other landowners on lands where activities are planned, including other divisions of the DLNR, private landowners, tenants, and County, State, and Federal agencies prior to conducting activities on lands under their jurisdiction.

D. This permit is not transferable or assignable. A signed copy must be carried by permit holder(s) while engaging in activities authorized by this permit. Each permit holder is individually responsible and accountable for his or her actions under this permit.

E. This permit does not authorize activities with any other plant species except those stated. Permission to collect additional plant material must be obtained from district DOFAW offices.

F. Appropriate DOFAW district office must be notified in advance of proposed fieldwork, for access, to coordinate collections, plant propagation needs, district requests, and approval of additional field personnel other than the listed associates for state reintroduction projects and/or their island cooperators.

G. Primary repositories are cooperating rare plant nurseries for live storage. A young micropropagation laboratory (for tissue culture) and seed storage facilities (for seed storage) are secondary depositories for these propagules.

H. This permit does not in any way make the Board of Land and Natural Resources of the State of Hawaii liable for any claims of personal injury or property damage to the permit holder(s) or his or her party which may occur while engaged in activities permitted under this permit; further, the permit holder(s) agrees to hold the State harmless against any claims of personal injury, death or property damage resulting from the activities of the permit holder(s).

I. This permit shall become valid upon completion of the following:

1. All persons who are actively involved in activities authorized by this permit have read this permit in its entirety and acknowledge understanding & agreement to abide by its conditions by signing this permit.
2. The signed permit is returned to DOFAW. Upon approval by the DOFAW Administration, a copy of the signed permit will be returned to the principal investigator.

J. The permit holder(s) will provide copies of all publications/reports of any study resulting from the activities of this permit to DOFAW. The permit holder(s) will also provide or make available for inspection any raw data that is obtained under this permit when requested by the Division.

K. Any person violating any of the conditions stipulated under this permit will be subject to the penalty provision provided by law. Further, any infractions of this permit may be cause for revocation of this permit and/or denial of future permit requests.

L. This permit is issued for one year. This permit can be renewed at the end of this period. Please submit plans for the coming year and the need for permit renewal or extension before expiration of present permit.

II. SPECIAL CONDITIONS
A. The purpose of this permit is collection and possession of a rare Hawaiian plant for a research project.

B. Permit holder(s) is strictly prohibited from collecting whole plants unless under specific DOFAW request.

C. In advance of entry, permit holder(s) will notify District DOFAW of communication contacts in the field (cell phone/radio) and notify authorities of specific collection dates and times.

D. The permit holder(s) will adhere to methods that are in accordance with established procedures as published by the Hawaii Rare Plant Restoration Group (HRPRG) for collection of Threatened and Endangered species. Completion of HRPRG Rare Plant Monitoring Forms is required for all collections.

E. New/rare species or species thought to be extinct may be collected under this permit provided the above conditions are followed. For new populations or new species, completion of the HRPRG Rare Plant Monitoring forms is required.

F. Yearly reports with collection information are required and will be in electronic form. If a new population is discovered, GPS information will be supplied when possible.

The undersigned have read, understood, and hereby agree to abide by the conditions as stated above.

Principal Investigators:

[Signature]

Pei-Luen Lu, Principal Investigator

(Date)
State of Hawaii
Department of Land and Natural Resources
Division of Forestry and Wildlife
1151 Punchbowl Street Room 325
Honolulu, Hawaii 96813

License No.  P-159
Date of Issue: February 1, 2011
Expiration Date: January 31, 2012

Associates:

Clifford W. Morden

Mitsuko Yorkston

APPROVED: [Signature]
PAUL CONRY, Administrator,
Hawaii Division of Forestry and Wildlife

Date 2/9/11
SPECIAL USE PERMIT
NATURAL AREA RESERVES SYSTEM
DEPARTMENT OF LAND AND NATURAL RESOURCES
1151 PUNCHBowl STREET, HONOLULU, HI 96813

EFFECTIVE: November 1, 2010 – October 31, 2011
RESERVES: Kanaio, Manuka

The Board of Land and Natural Resources or its authorized representative, with the approval of the Natural Area Reserves System Commission, hereby issues this Special Use Permit in accordance with Section 195-5, Hawaii Revised Statutes; and Title 13-209-5, Department Administrative Rules, to

Ms. Pei-Luen Lu, Ph.D. Candidate
Department of Botany, 3190 Maile Way
University of Hawai‘i Manoa
Honolulu, HI, 96822
Phone: (808) 206-5322 Fax: 956-3923
E-mail: peiluen@hawaii.edu

Dr. Cliff Morden, Major Professor, UH
Dr. Arthur C. Medeiros, USGS
Mr. Chuck Birkland
Mr. Hank Oppenheimer, PEPP

to collect leaf material from halapepe, Pleomele auwahiensis and Pleomele hawaiiensis (Agavaceae, lily family) for genetic analysis. To fully understand the population dynamics all populations must be assessed, so this work will be occurring on other lands as well. A single leaf will be collected form each study plant. Information gained from this research will allow managers to make informed decisions for the best strategies to help maintain their genetic vigor or assist in their recovery. No genetic studies have been done on these species. This research proposal was developed with the explicit purpose to aid the management of these species by understanding the evolution, relationships, and levels of variation present within populations of these species.

STANDARD CONDITIONS:

1. Besides conditions stipulated here, the permit holder will adhere to project specifications given in the permit application.

2. Disturbance of vegetation and wildlife will be avoided as much as possible. Do not leave trails. Activities may not impede public access.

3. Reports: (a) A field report will be submitted within 5 weeks of the project’s completion. (b) Results of the project, as published or unpublished reports, also will be submitted. (c) The reports will identify the Natural Area Reserve as a project site and acknowledge the special-use permit approved by the NARS Commission.

4. Precautions will be taken to prevent introduction of plants or animals not naturally present in the area. Should an infestation develop, Permit Holder is responsible for eradication by methods to be specified by NARS—whether it occurs during or after the permit period, and even though it may be only indirectly attributable to the project activities.
5. This permit is not transferable.

6. This permit does not exempt the permit holder from complying with any other applicable rule or statute.

7. The State of Hawaii shall be released and held harmless from any and all liability for injuries or death, or damage or loss of property however occurring during any activity related to this permit. Provision of Chapters 183, 185, and 195, Hawai‘i Revised Statutes, 1985, as amended, and any other laws applicable thereto, and all rules and regulations of the Department of Land and Natural Resources shall be strictly observed. Infractions or misconducts will constitute grounds for revocation of this permit and criminal prosecution. Any person whose permit has been revoked shall not be eligible to apply for another permit until the expiration of two years from the date of revocation.

8. The Permit Holder is responsible for explaining permit terms to participants and ensuring their compliance at all times; including notifying appropriate DOFAW staff, prior to field entry, for gate keys or other logistical needs.

9. A permit copy will accompany participants in the field at all times.

10. The proposed activities to be conducted in the Natural Area Reserves fall under the Division of Forestry and Wildlife’s exemption list of June 12, 2008, including but not limited to Exemption Class 5: Basic data collection, research, experimental management, and resource evaluation activities which do not result in a serious or major disturbance to an environmental resource (5-2). Division analysis of the proposed resource management actions concluded it will provide a positive environmental benefit and will be done in a manner to have no negative impact on the conditions that define the area. Furthermore, the cumulative impact of these actions over the duration of the permit (1 year) will not have a significant adverse impact and will have minimal or no significant effect on the environment and are exempt from the need to prepare an environmental assessment.

SPECIAL CONDITIONS:

11. One leaf from healthy individuals of each species will be collected for genetic analysis.

12. Leaves will be stored in self-sealing plastic storage bags and returned to the laboratory for processing.

13. No collection of voucher specimens will be made; however photo documentation of plants will be made, which are also acceptable as vouchers along with GPS data (this eliminates the need to over-collect rare species while also providing GPS points for mapping and management purposes. Information will also be provided to Bishop Museum in lieu of voucher specimens, since they are the official repository for the State and hold the data base for the Hawai‘i Biological Survey.

14. Collection of leaves is largely non-destructive to the plants (only one per plant). If a population is large, only a subset of up to 40 plants will be sampled.

15. This permit authorizes Permit Holder and the above named experts to conduct this field work. Collections will be made with field biologists knowledgeable of the area such that minimal impact will be made to the resources of each area, as well as individual plants.

16. Plants that appear stressed will not be collected; GPS locations and noting weather conditions may also inform on stressed plants (particularly in the case of extreme drought or insect attack). If native
or non-native insects are observed, they may be noted and collected for determination back in the lab.

17. Permits are issued on an annual basis; if a renewal is required please submit a report detailing progress to date and an application form detailing future plans.

[Signatures]

LAURA H. THELEN, Chairperson
Board of Land and Natural Resources

PERLBERG I. I.
Permit Holder

2010 Oct 20
CHAPTER 5: SYNTHESIS
Phylogenetic and population genetic analyses were undertaken to examine the evolution and systematics of the relic medicinal plant genera *Dracaena*, *Sansevieria*, and *Pleomele* (collectively referred to as “dracaenoid’s”) both globally and among the Hawaiian Islands. The study revealed both anticipated as well as unanticipated results. The original hypotheses are reassessed in light of these findings.

**HYPOTHESES**

**Hypothesis 1: Dracaena, Pleomele, and Sansevieria are each monophyletic.**

*Rejected.* Results presented in Chapter 2 that *Dracaena*, *Pleomele*, and *Sansevieria* are collectively monophyletic. However, the genera *Dracaena* and *Pleomele* are polyphyletic. Only the species of *Sansevieria* form a monophyletic lineage, but these species form a distinct group clustered within the context of the larger and variable genus *Dracaena*. Such a relationship for *Dracaena* is paraphyletic. Thus, a new taxonomic revision must be considered to merge the species of *Sansevieria* into *Dracaena*. The Hawaiian species previously described as *Pleomele* did form a monophyletic group separate from, and in a sister relationship to, the remaining dracaenoid species. These species had previously been described as a distinct subgenus of *Dracaena* by Jankaski (2008), *D*. subgenus *Chrysodracon*. It is recommended that this group be recognized at the generic level as *Chrysodracon*.

**Hypothesis 2: The common ancestor for the dracaenoid’s is from an African Dracaena species.**

*Rejected.* Results of the biogeography analysis in Chapter 2 were not as expected. Although the
center of diversity among dracaenoid’s is in Africa, this lineage within the Asparagaceae family evolved in regions of Asia or Southeast Asia. The first lineage to branch off from the ancestral type was the Hawaiian clade, Chrysodracon, followed by a colonization to the Central American region, and lastly with a radiation westward from Asia to Africa. Evidence also suggests that there were multiple colonizations to and from Africa and Asia.

**Hypothesis 3:** The ancestor of Hawaiian *Chrysodracon* (previously *Pleomele*) originated in Asia.

*Accepted.* Results from Chapter 2 demonstrate a strong link between the species of *Chrysodracon* endemic to the Hawaiian Islands and the outgroup species from Asia.

**Hypothesis 4:** Hawaiian *Chrysodracon* (previously *Pleomele*) followed a general pattern of west-to-east (Kaua‘i to Hawai‘i) migration down the Hawaiian Island chain.

*Rejected.* Chapter 3 examined in greater detail the species relationships and biogeographic patterns of *Chrysodracon* species using the phylogenetic analysis of combined nuclear (ITS) and chloroplast DNA gene sequences. These analyses demonstrated that species did not follow a progression rule model of colonization from older to younger islands. Instead, the dispersal events indicate a complex pattern of colonization among the islands.

**Hypothesis 5:** Genetic variation is low within and among populations of the endangered *Chrysodracon hawaiiensis* (previously *Pleomele hawaiiensis*).

*Partially Accepted.* Chapter 4 examined the genetic variation within two species of Chrysodracon, the rare *C. hawaiiensis* endemic to Hawaii Island and the more common *C.*
auwahiensis endemic to Maui and Molokai Islands. Genetic variation in C. hawaiiensis is low within population, but high among populations. Overall diversity in C. hawaiiensis is lower than that of C. auwahiensis, but is still high relative to many other Hawaiian endemic species previously examined. Many studies have shown that rare species or species with smaller populations have lost genetic diversity (Ellstrand and Elam, 1993; Fischer and Matthies, 1998; Vergeer et al., 2003; Olver et al., 2005; Will et al., 2007). This is the case also with C. hawaiiensis although there is sufficient overall diversity remaining that proper management of these populations could recoup genetic variation lost from isolated populations.

CONCLUSIONS

A more comprehensive understanding of the evolutionary processes that occurred in dracaenoids (Dracaena, Pleomele, and Sansevieria) has deciphered their ambiguous relationships that had continued since the 18th century. The results revealed that the newly recognized Hawaiian Chrysodracon is at the base of the dracaenoid phylogeny and the only two extant Central America Dracaena species are the next lineage in the root group with the Socotra species D. cinnabari following after that. Sansevieria is necessarily embedded into Dracaena to maintain this as a monophyletic lineage.

The species of Dracaena known Dragons’ blood are polyphyletic within the genus, but their relationship to other species also indicate potential candidates for examination of newly useful Dragon’s blood species.

The study of Chrysodracon species provides a good model to increase the knowledge into the Hawaiian Islands’ evolutionary processes.
Examination of population genetics is important to more fully understand evolutionary processes taking place in the Hawaiian flora. This knowledge can be useful in understanding these isolated systems to potentially avoid the high risk of their extinction in the wild (Frankham et al., 2004; Whittaker, 2007). In addition to publishing portions of this dissertation, the population genetics and phylogenetics results have been made available in the form of presentations and reports to private, academic, local and national conservation and land management and conservation organizations. The systematics of the dracaenoid based on this study will also be used to update the classification for those organizations that have provided samples for this work other otherwise facilitated my laboratory or field works.

Results from this work have the potential to be applied to answer a broader range of questions than those posed here. Future applications of this work should: 1) provide a revision of the dracaenoid genera to clarify their relationship and the taxonomic classification that will benefit the plant identification in the development of herbal medicine in Dragon’s blood; 2) expand the ITS (or other nuclear region) phylogenetic analysis to include all dracaenoid taxa; 3) develop microsatellites for more detailed examination of Chrysodracon and Dracaena species and use this data to examine more carefully their species boundaries and population dynamics; 4) use RAPD and ISSR analysis to examine the other Chrysodracon species to complete the island evolution research and make the comparison within the Hawaiian Islands and other islands’ system; 5) utilize the knowledge from this research in the conservation management of the rare dracaenoid species (in Hawaii and elsewhere around the world); and 6) screen species used for medicinal purposes and related species that may be potential candidates for new pharmaceutical products.
REFERENCES


