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Taxonomy and Biogeography of Washingtonia (Arecaceae) in Peninsular California

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Taxonomy and Biogeography of *Washingtonia* (Arecaceae) in Peninsular California

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Plant Biology

by

Lorena Villanueva Almanza

September 2019

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ABSTRACT OF THE DISSERTATION

Taxonomy and Biogeography of *Washingtonia* (Arecaceae) in Peninsular California

by

Lorena Villanueva Almanza

Doctor of Philosophy, Graduate Program in Plant Biology
University of California, Riverside, September 2019
Dr. Exequiel Ezcurra, Chairperson

Washingtonia is an American genus of palms currently composed of two species, *W. filifera* and *W. robusta*. Poor understanding of their morphology and distribution led initially to the description of numerous new species and later, to a simplification resulting in the current understanding of the genus. This widely distributed group of palms native to the Baja California peninsula, southern California, Sonora, and Arizona has been a taxonomic challenge due to a lack in type specimens, incomplete protologues, highly variable vegetative morphology, human dispersal, limited fieldwork in native populations, and poor representation in herbaria. In the wild, both distinct forms occur in the extremes of a 1300-km gradient, linked by morphological intermediates, suggesting that there is continuous morphological variation of the two forms instead of the long-held idea of two species. We addressed this hypothesis by taking measurements of morphological traits of stems and functional traits of leaves in 17 sites spanning the whole distribution range of the genus in Mexico and the US. We examined the relationship between the plants' traits and latitude, individually, and between all traits combined (using a Principal Component Analysis) and latitude. We compared a linear model against a non-linear model to test whether traits of *Washingtonia* change gradually along the latitudinal gradient or if there are clear geographical breaks among populations.

We also used Genotyping By Sequencing (GBS) data to understand population structure and delimit species. Using both a *de novo* and reference based approaches, we analyzed 173 *Washingtonia* individuals from 21 populations across its distribution range through multivariate and Bayesian methods.

We found strong statistical support for the hypothesis that there is a gradual change in the vegetative morphological traits of *Washingtonia* along the latitudinal gradient of Peninsular California, instead of two clear morphologically distinguishable species with a hybrid zone. However, we also found that *Washingtoni*a populations are highly structured with four major geographic regions having unique genotypes. Based on previous morphological results and the evidence herein we propose recognizing a single species of *Washingtonia* with four subspecies. Our findings suggest *Washingtonia* is composed of one highly variable species and that local differentiation of populations is related to environmental gradients. Our results provide a robust phylogenetic analysis of *Washingtonia* settling a taxonomic debate that has lasted over a century.

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Chapter 1. Introduction

The *LA Times* published a story on September 2017 about the "dying palm trees of Los Angeles" (Levine, 2017). The story was reprinted some days later by the British newspaper, *The Guardian* (Carroll, 2017). The bottom line of both publications is that LA palms are dying. Later, JSTOR publicized an exhibition to commemorate the "disappearing palm trees of Los Angeles" (Meier, 2018).

However, there are several palm species cultivated in southern California and they are certainly different—the Arecaceae is composed of around 2,600 species (Baker and Dransfield). Even when the articles mention there are several species of palms growing in LA, it is unclear to which the stories refer to since the term “palm” is used rather vaguely. A reader familiar with palms might figure out that "Los Angeles palms" of the JSTOR’s story belong to the species *Washingtonia robusta* —a name not mentioned in either of the three articles despite lining iconic streets in LA like Venice Beach or Sunset Blvd. Regardless of the species, most palms in LA are probably here for the long run, according to Donald R. Hodel from the Environmental Horticulture Advisor to the UC Agriculture and Natural Resources Office (Hodel, 2019). Instead, the articles allow for a more relevant discussion; the conservation of native stands of palms trees from where the cultivated palms came from in the first place.

The irony of *W. robusta* is almost tragic; while being Hollywood's iconic palm and one of the most widely cultivated palms in the world (Felger and Joyal, 1999; Henderson et al., 1997) it is considered an invasive species in California (California Invasive Species Council, 2005) and its distribution in the wild is poorly known. Moreover, some of the wild populations in Baja California Sur and Sonora, Mexico are under extreme threat (León de la Luz et al., 1997). Despite all this evidence, the species has not been assessed by the International Union for Conservation of Nature (IUCN) (Johnson and Group, 1996).

On the other side of the border, among the eight species of palms reported to be found in LA, only the California fan palm, also known by its scientific name *Washingtonia filifera*, is native to the state. Natural populations of California fan palms are found in secluded places of Joshua Tree and the Aguacaliente Indian Palm Canyons. Although the effects of drought on California fan palms have not been formally investigated, park rangers have reported water stress has had a negative impact on some palm populations while the IUCN considers it as a near threatened species (Johnson and Group, 1996). Neil Frakes, Vegetation Branch Chief of Joshua Tree National Park, has mentioned that, in addition to drought, the decrease in groundwater levels caused by aquifer pumping is a major threat for some palm stands. This has been happening in the historic Oasis of Mara, "a cornerstone of the Joshua Tree National Park story" and where the Park's main visitor center is found (Frakes, 2018). Although currently there are no plans for reintroducing palms in either Joshua Tree or in Palm Canyon, if palm reintroduction was eventually deemed necessary the plants would ideally have to be of the same species. However, this might not be as simple as one would suppose since its closely related species, the Mexican skyduster (*W. robusta*), has been present in southern California cities since the early 1900s and has spread close to natural populations of California fan palms. The proximity of both species has led to genetic mixing, or hybridization, between California fan palms and Mexican skydusters, mostly in the urban landscape. However, park rangers are concerned about the loss of genetic integrity of the California fan palms in natural populations, due to hybridization with its Mexican relatives (Frakes, 2018).

Although both species are easily distinguishable in cities, hybrids show intermediate traits which make them harder to identify. Therefore, park rangers need visible characteristics that allow to tell the difference between both species and their hybrids. Palms in California are a real-life example of when taxonomy, or plant identification, becomes important for the preservation of ecosystem health. Only by making proper plant identification can conservation risks and management strategies be discussed. Since palms will remain part of the LA landscape for some time, we might want to take this as an opportunity to find ways to make better palm identification and learn about caring for the places from where they came from and where they still naturally occur.

Chapter 2. A Hollywood palm icon unmasked: clinal variation in *Washingtonia* along Peninsular California

Abstract

Washingtonia is an American genus of palms currently composed of two species, *W. filifera* and *W. robusta*. Poor understanding of their morphology and distribution led initially to the description of numerous new species and later, to a simplification resulting in the genus currently being considered as composed of two species. In the wild, both distinct forms occur in the extremes of a 1300-km gradient, linked by morphological intermediates, suggesting that there is continuous morphological variation of the two forms instead of the long-held idea of two species. We addressed this hypothesis by taking measurements of 8 morphometric traits of stems and leaves and 5 functional traits of leaves in 17 sites spanning the distribution range of the genus, from the Cape Region in the Baja California Peninsula in Mexico to southern California, plus two populations outside Peninsular California in Arizona and Sonora, Mexico. We examined the relationship between the plants' traits and latitude, individually, and between all traits combined (using a Principal Component Analysis) and latitude. We compared a linear model against a non-linear model to test whether traits of *Washingtonia* change gradually along the latitudinal gradient or if there are clear geographical breaks among populations. The linear model, as revealed by the model selection analysis, shows that, although palms in each canyon have

distinct traits, there is a clear morphological continuum in *Washingtonia*, with two extreme forms. Northernmost palms have thick, blue, amphistomatic leaves and wide stems, while southernmost palms have thin, green, hypostomatic leaves and slender stems. These traits change gradually along the gradient. Leaf greenness, which decreases, and stem diameter, which increases, seem to be adaptations to cold temperature in northern canyons, allowing the plants to avoid freezing and take advantage of favorable moisture during the coldest months of the year. My work suggests the genus is composed of only one species and that its current distribution is made up of relic populations of a once widespread ancestral habitat.

2.1 Introduction

Morphological variation in the Arecaceae has mostly been addressed using a systematic approach (Henderson et al., 1997; Borchsenius, 1999; Ehara et al., 2000; Henderson, 2002, 2006, 2002; Kjær et al., 2004; Bacon and Bailey, 2006; Roncal et al., 2007, 2012; Bacon et al., 2012). Very rarely has this variation been investigated using an ecological perspective, with some exceptions hinting at the possibility of environmental gradients driving morphological variation (St. John, 1932; Read and Hodel, 1990; Bacon and Bailey, 2006).

Washingtonia H.Wendl., a North American genus in tribe Trachycarpeae (Baker and Dransfield) with highly variable morphology (Bailey, 1936), including two currently recognized species [*W. filifera* (Linden ex André) H.Wendl. and *W. robusta* H.Wendl.], is no exception. According to the most recent information on the genus, *W. filifera* occurs naturally in Arizona, southern California, and northern Baja California, while *W. robusta* is present in the Baja California Peninsula, from Cataviña at latitude 29° 45’ (Bailey, 1936; Cornett, 1987b) to the Cape Region at 23° (Minnich et al., 2011; Wiggins, 1980) and in Sonora, mainland Mexico, where it has a very narrow distribution in the Sierra del Aguaje near Guaymas (28°) (Felger and Joyal, 1999). However, there is no agreement on the northern limit of *W. robusta* (Fig. 2.1).

Palms were extensively present in the Baja California Peninsula at the arrival of the Europeans. The first casual observations of palms were made by Jesuit missionaries who made thorough explorations and descriptions of the natural history of the Peninsula. Miguel del Barco (1780) wrote about the uses of palms in the south where he distinguished "red" from "white palms", the first corresponding to *Brahea brandegeei* and the latter belonging to the genus *Washingtonia*. Wenceslaus Linck recorded his observations of the northern part of the Peninsula and noted that two types of palms were found from north of Cataviña to the south of Sierra San Pedro Mártir. One with "ashen-colored" leaves (*Brahea armata*) and a "green-colored" one (*Washingtonia filifera*) (Burrus, 1966). Actually, the introduction of *W. filifera* into cultivation is owed to Franciscan missionaries (Padilla, 1961), as supported from early-twentieth century photographs of centennial palms taken around Mission San Fernando in California (Fig. 2.2).

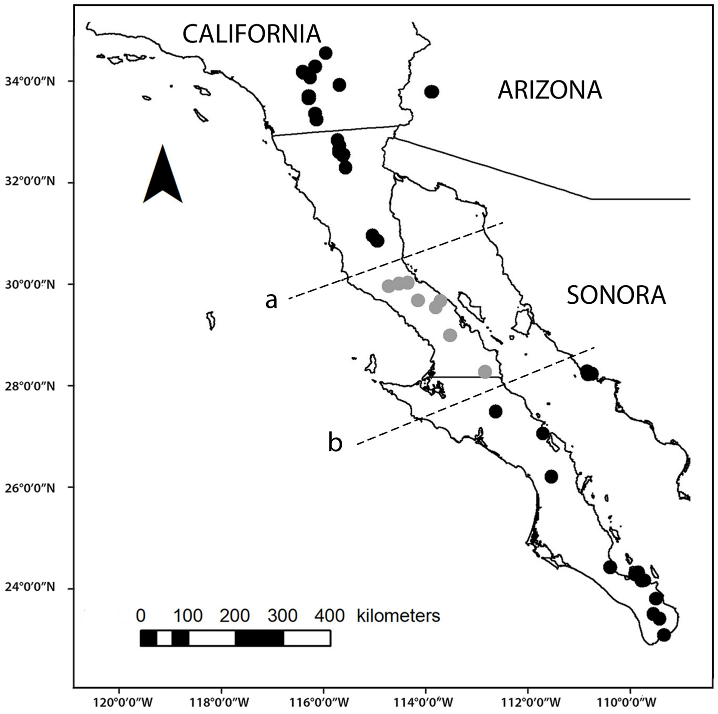

Figure 2.1: Major *Washingtonia* palm oases in the Baja California Peninsula, Arizona, and Sonora according to herbarium records and own collections.
Dashed lines mark distribution limits according to **(a)** Bailey (1936), Shreve and Wiggins (1964), Moran (1978), Wiggins (1980), Cornett (1987b), Minnich et al. (2011), Klimova et al. (2018) and **(b)** Arriaga et al. (1997), Felger and Joyal (1999), Zona (2000), Felger et al. (2001), León de la Luz et al. (2014). Grey area show oases where authors have divergently identified *Washingtonia* palms either as *W. filifera* or *W. robusta*

The genus was formally described by Hermann Wendland in 1879 from specimens of unknown origin cultivated in Ghent, Belgium (Wendland, 1879). Ever since, the distinction between both species has been a matter of taxonomic debate and calls for a modern revision (Henderson et al., 1997). While some authors have formally recognized both species in the floras of the region (Wiggins, 1980; Rebman and Roberts, 2012; Simono, 2012), others have considered *W. robusta* simply a variant of *W. filifera* (Nabhan, 1985), an idea

Figure 2.2: Mission San Fernando, California showing *Washingtonia* palms planted by Franciscan missionaries.

(A) Photograph by Isaiah W. Taber, *ca.* 1880. Courtesy of the California State Library. (B) Photograph by Frank B. Rodolph, 1905. Courtesy of the Bancroft Library, Berkeley.

supported by the fact that hybridization within the genus is possible (Hodel, 2014; Ishihata and Murata, 1971), while others have ignored *W. robusta* from their comprehensive listings (Turner et al., 1995).

The taxonomy of the group is complicated further since Wendland did not designate type material neither for *W. filifera* (Wendland, 1879), nor for *W. robusta* (Wendland, 1883). In an attempt to clarify the distinction between both species, Felger and Joyal (1999) designated a neotype for *W. robusta*, but the illustration fails to show attributes that may help to distinguish it from *W. filifera*. As for type localities, Wendland (1879) did not specify any for *W. filifera*, while he indicated the Sacramento River for *W. robusta* (Wendland, 1883). Yet, Wendland himself admitted not knowing the place from where *W. robusta* had been collected, suggesting the Colorado River Valley as the only place where both species of *Washingtonia* were found (Wendland, 1888). In fact, there is considerable evidence supporting the idea that *W. robusta* was a garden name, rather than a formally described species (André, 1900; Britton, 1904; Orcutt, 1885; Palmer, 1876; Parish, 1907; Watson, 1889; Wright, 1879) so much so, that neither Brandegee (1905) nor Goldman (1916) included the name *W. robusta* in their work. All that can be said with certainty is that the introduction into cultivation of what is currently known as *W. robusta* is owed to Kate Sessions who, around 1900, obtained seeds from San José del Cabo in the Baja California Peninsula and grew them in San Diego, California (Sessions, 1920; Fig. 2.3). Liberty Hyde Bailey collected seeds of one of the palms planted by Sessions in 1927 in San Diego, at the intersection of Maple and 3rd, and deposited them at the Bailey Hortorium Herbarium at Cornell University with number 9050 (!).

While under cultivation, the northernmost and southernmost species are clearly distinguishable from each other, wild populations show intermediate phenotypes. Existing studies have failed to find reproductive morphological traits that can be accurately used to distinguish the two species, and our own research in herbarium specimens failed to find any. Modern taxonomic keys rely exclusively on vegetative characters to distinguish *W. filifera* from *W. robusta* (Bailey, 1936; Shreve and Wiggins, 1964; Wiggins, 1980; Zona, 2000; Simono, 2012). Bailey (1936: 61) even mentions floral differences between both species are “unimportant”. Given that only vegetative morphology traits are used to distinguish the two species, the driving questions of my study are: (1) how does vegetative morphol-

Figure 2.3: Kate Sessions palm transplants

(A) Palm grove in San José del Cabo, Mexico from where Kate Sessions collected seeds in 1900. (B) Palms planted by Sessions in San Diego, California photographed by Liberty Hyde Bailey in 1927. Courtesy of Bailey Hortorium, Cornell University. (C) Palms planted by Sessions photographed by E. Ezcurra in 2018. Street cables removed using Photoshop CS6.

ogy vary in natural populations of *Washingtonia* along its entire distribution range? (2) Does the morphological variation in *Washingtonia* follow predictable patterns of temperature and precipitation? Two hypotheses can be invoked to explain the observed variation in these palms: (a) The genus *Washingtonia* is composed of two species with constant morphologies at the periphery of the species range, one in the north and one in the south, that have differentiated in disjunction and have expanded to secondary contact resulting in a possible hybrid zone (secondary intergradation), which we will call "hybrid zone model"; and (b) *Washingtonia* is composed of one highly variable species clustered in local and patchy populations in desert oases and change gradually along a clinal continuum (primary intergradation) which we will refer to as "clinal model" (Fig. 2.4).

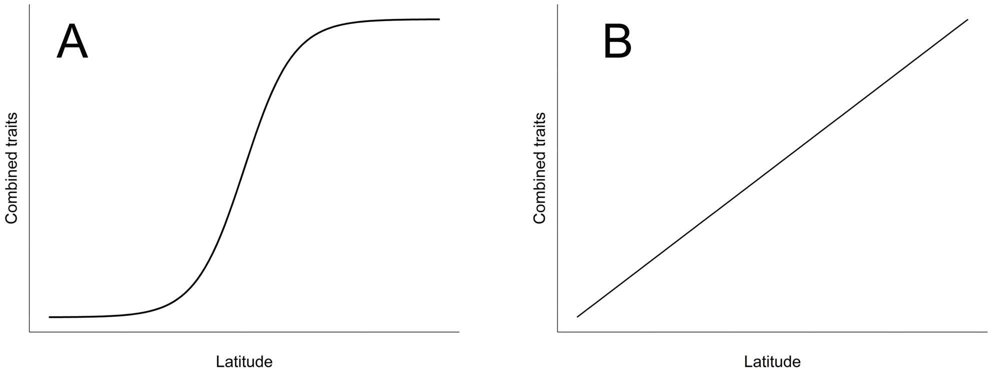

Figure 2.4: Models to explain morphological variation in *Washingtonia*

(A) Hybrid zone model showing two species joined by a hybrid zone, (B) Clinal model showing a morphological continuum along a latitudinal gradient

2.2 Materials and methods

Population sampling and environmental variables

Measurements and plant material were collected from March 2017 through October 2017 in 16 sites along a 1,300 km latitudinal transect in the Baja California Peninsula and California, and two other sites in Arizona and Sonora (Table 2.1, Fig. 2.5). Annual mean temperature, mean temperature during the coldest quarter, and annual precipitation from 1970–2000 were obtained from WorldClim version 2 with a resolution of 30 arc-seconds (~1 km) (Fick and Hijmans, 2017) as a set of rasters from where bioclimatic variables were extracted using R package version 3.3.3 (R Development Core Team, 2017) and code by Turner (2013). Minimum temperatures for each site were obtained using historic climatological (1950–2010) data from the nearest weather station from Comisión Nacional del Agua (CONAGUA, 2018) for the Mexican sites and from the National Oceanic and Atmospheric Administration (NOAA, 2018) for the American sites. To test for differences in temperature between open slopes, and the canyon trough, temperature was measured in Palm Canyon, Palm Springs, California during six weeks corresponding to the end of winter and early spring of 2018 (8 Feb–26 March) using an iButton Hygrochron (Dallas Semiconductor, Dallas, TX, USA). Temperature was measured simultaneously at the open desert slope and at the canyon trough some 200 m apart, each with three replicates. Time readings in the sensors were converted from Pacific Standard Time (PST) to Local Solar Time. Reported temperatures are averages of the three replicates.

Table 2.1: Location of populations of *Washingtonia* sampled across Peninsular California, Arizona, and Sonora, Mexico with bioclimatic variables taken from (Fick and Hijmans, 2017).

Site	Lat. Long. (d.d)	Elevation (m)	Annual mean temp. (°C)	Mean temp. coldest quarter (°C)	Annual ppt. (mm)
1. San José del Cabo (SJD)	23.05, -109.68	9.00	23.35	18.6	287
2. Sierra Cacachilas (CACA)	24.08, -110.11	754.73	20.18	14.98	438
3. Sierra San Juan de la Costa (SJCOSTA)	24.33, -110.71	71.96	22.22	17.15	181
4. Comondú (COMO)	26.06, -111.82	280.00	21.74	16.33	211
5. Mulegé (MULE)	26.88, -111.98	1.00	24.26	17.42	148
6. San Ignacio (SNIGN)	27.29, -112.88	123.08	21.71	16.33	114
7. Cañón de Nacapule (NACA)	28.01, -111.05	161.39	23.87	17.50	276
8. Santa Gertrudis (GER)	28.05, -113.08	420.03	20.77	14.90	150
9. San Francisco Borja (BOR)	28.74, -113.86	440.00	18.96	14.10	132
10. Bocana (BOCA)	29.67, -114.91	340.79	19.11	14.15	113
11. Mission Sta. María (STAMA)	29.74, -114.55	520.82	18.70	12.98	117
12. Cataviña (CATA)	29.73, -114.72	540.39	18.54	13.12	115
13. Cañón Berrendo (BERRE)	30.53, -115.13	801.69	16.70	9.93	156
14. Cañón Palomar (PALO)	31.92, -115.71	493.20	17.35	11.58	236
15. Hellhole Canyon, Anza Borrego (ANZA)	33.23, -116.44	533.76	18.99	11.08	260
16. Palm Canyon, Kofa Mountains (KOFA)	33.36, -114.09	846.26	20.00	11.32	229
17. Palm Canyon, Aguacaliente Indian Reservation (PALM)	33.73, -116.53	242.44	20.97	12.73	180
18. 49 Palms, Joshua Tree (JOSH)	34.10, -116.10	874.01	17.40	8.50	196

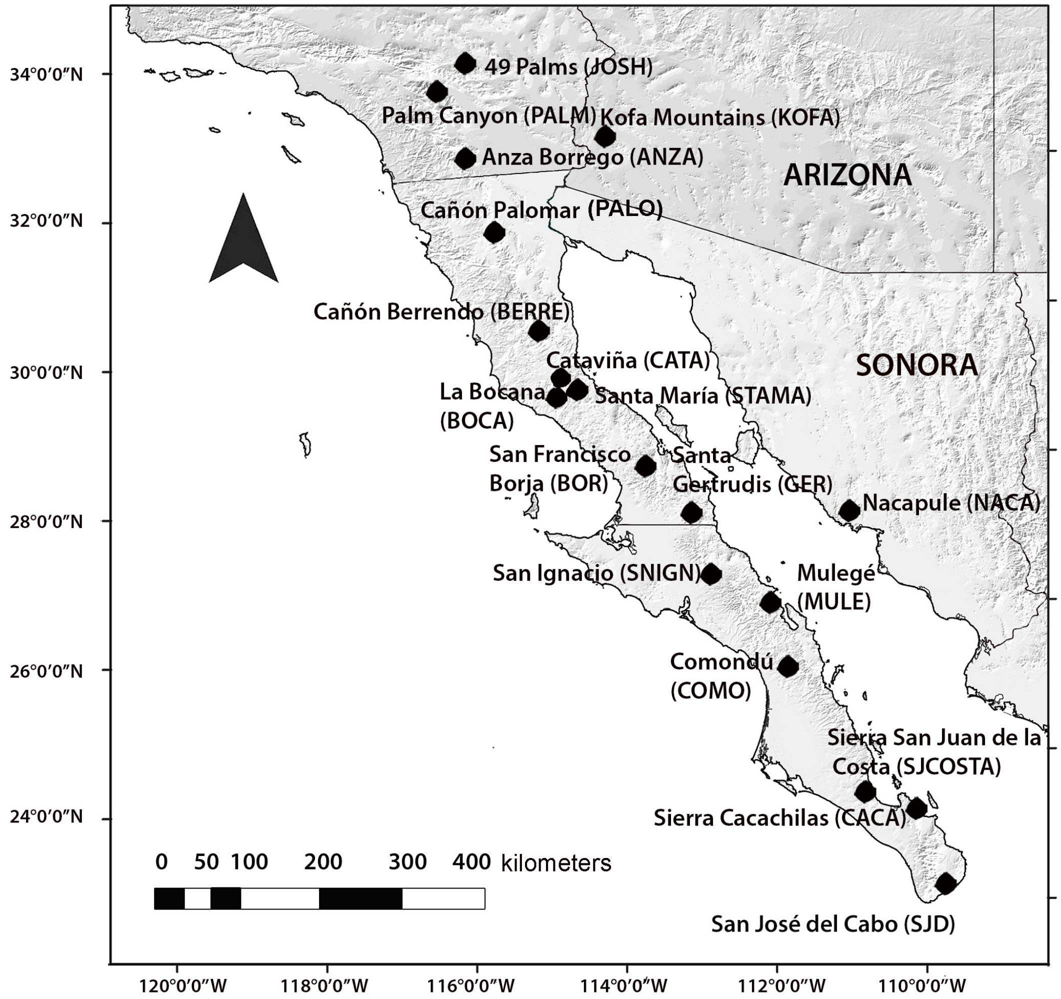

Figure 2.5: *Washingtonia* populations sampled across Peninsular California, Arizona, and Sonora. N=17

Morphological traits

Thirteen variables for each individual were measured whenever material was available (Table 2.2). Vegetative morphological traits were selected since they are used in taxonomic literature and keys to distinguish between both species (Bailey, 1936; Felger and

Joyal, 1999; Rebman and Roberts, 2012; Simono, 2012) and we were interested in testing their validity as useful characters for species identification. Careful examination of herbarium material where flowers/fruits were available—most of which came from the extreme populations or from cultivated specimens—did not reveal any distinguishing differences in reproductive morphology between the two species. The herbaria consulted were Bailey Hortorium Herbarium at Cornell (BH), Harvard University Herbaria (HUH), National Herbarium at the Biology Institute at UNAM (MEXU), Rancho Santa Ana (RSA-POM), San Diego Natural History Museum Herbarium (SDNHM), University of California Riverside (UCR), United States National Herbarium (US). For this reason, we did not include reproductive structures in my analysis. Furthermore, collecting reproductive structures in the field can be very challenging because either they are not available at the time fieldwork is done or because it is physically impossible to collect them since only the tallest and most inaccessible specimens were fertile. Although some authors have proposed the presence and size of spines in the petiole as a distinctive character for species identification, other studies have shown that spines are unreliable traits as they may change with the plant's life history decreasing from juvenile to late adult plants (Cornett, 1987; confirmed by my own field observations). For this reason, we discarded measuring spines in the petiole as a potentially discriminant trait, and we ensured that all leaf traits measured were more or less constant throughout the plant's life history.

Table 2.2: Morphological and functional traits measured from specimens of *Washingtonia* across Peninsular California, Arizona, and Sonora. Traits marked with an asterisk show no measurements were available for Cataviña and Mission Santa María, except for carbon isotope composition.

Variable	Units	Abbreviation	Number of sites	Number of individuals
1. Height	m	height	17	326
2. Diameter at base*	m	diam1	16	275
3. Diameter at breast height*	m	diam2	16	274
4. Crown width	m	crown	17	317
5. Leaf length*	m	lflen	16	150
6. Leaf width*	m	lfwid	16	147
7. Petiole width*	cm	petwid	16	153
8. Hastula length*	cm	hastula	16	152
9. Specific leaf area*	$cm^2\ g^{-1}$	SLA	16	151
10. Relative leaf green content*	%	green	16	153
11. Carbon isotope composition	‰	delta	17	164
12. Adaxial stomatal density	mm^{-2}	adax	17	51
13. Abaxial stomatal density	mm^{-2}	abax	17	51

Functional traits such as specific leaf area (SLA), carbon isotope composition ($\delta^{13}C$), and stomatal density were added to test whether there were any physiological differences between both species and if these traits were associated with the morphological traits.

In each site, ten tall (height between 7.1–23 m) and ten short individuals (height between 5–7 m) were geo-referenced and selected for measurement. Height was measured using a laser range finder (Laser Technology TruPulse 360), diameter at the base and diameter at breast height using a diametric tape, and crown width was measured using a measuring tape in short palms, or a hand-held ruler in those where the crown was too high to reach. Crown width was then estimated by proportionality between the ruler reading and the arm length of the observer against the crown width and the height of the palm.

Leaf traits

To ensure comparability between individuals, we took one leaf from the outer canopy most exposed to sunlight from the ten short individuals at each site using a pruning pole. Selected leaves were taken from adult individuals except in the case of Berrendo where only young individuals were found. Only one full leaf per population was collected and measured in Cataviña and Santa María and therefore these measurements were not included in the morphometric analysis. Stomatal traits and carbon isotope measurements were available from leaf fragments collected in Mission Santa María in 2016. Taller *Washingtonia* palms tend to have smaller leaves (Renninger, Phillips Hodel, 2009) and palm leaf traits can vary with age (St. John, 1932). For this reason, and despite our effort to eliminate characters that vary strongly during the plants' life cycle, we restricted our morphometric analysis to traits from short individuals only. We do not discard the possibility that morphological traits in taller individuals differ from those in shorter individuals but collecting material from taller individuals in natural populations remains challenging. Length and width measurements were taken on each leaf while fresh. Leaves were then cut and photographed on the abaxial and adaxial surfaces using a camera (Nikon Coolpix P340) and a color checker reference target (X-Rite, 2014), in a dark room using the following camera settings: Flash exp comp +2.0, ISO 80, Built in filter off, White balance Auto 1, 1/30, 5.1 mm, f5.6. Hastula length and petiole width measurements were taken from dried leaf fragments. A total of 153 *Washingtonia* leaves collected along the latitudinal gradient. Voucher specimens for each locality are stored in US and Mexican herbaria.

This sample represents 51% of the total number of individuals collected in natural populations and housed at the seven main US and Mexican herbaria consulted for this study. We considered naturalized and cultivated specimens as non-natural occurrences and duplicate numbers were counted as a single individual. We did not include data from online databases to avoid identification or duplication errors.

Photographs taken in the field in NRW format were white balanced and color-standardized with X-Rite camera calibration software (X-Rite, 2014). Channel information for red, green, and blue was extracted at eight points of the processed image using Adobe Photoshop CS6. The total RGB value for each leaf surface was calculated as the sum of each channel and then used to calculate the relative value of the green channel following (Richardson et al., 2007). These images also show the presence of a tomentose patch on the abaxial side which has been used for species identification (Bailey, 1936; Ishihata and Murata, 1971; Hodel, 2014). Though we did not quantify the area of the patch, we confirmed that northernmost leaves had less tomentum than those in the southern populations.

To determine SLA, a fragment of known area was taken from the ten fresh leaves collected in each locality and subsequently dried (72 h, 50 °C) in a drying oven (Fisher Scientific Isotemp, model OV701G). Dry weight was recorded, and SLA was calculated as fresh leaf area/dry mass (cm^2 g^{-1}). Because the area of all leaf fragments was the same, differences between individual samples are inversely proportional to differences in the fragment's dry mass (the higher the dry mass of the fragment, the smaller the SLA value). Thus, SLA was used as an indirect and inverse estimator of leaf thickness. Leaf fragments of leaves from 10 different individuals collected in Mission Santa María in October 2016 and all leaf fragments (except one from San Borja) used for determining SLA were ground using mortar and pestle, and liquid nitrogen. An amount between 1.0–1.1 mg of the ground material was weighed and combusted using an elemental analyzer (Costech, Valencia, CA, USA) coupled to a Delta-V Advantage, isotope ratio mass spectrometer operating in continuous-flow mode (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at the Facility for Isotope Ratio Mass Spectrometry at the University of California, Riverside.

Epidermal peels of abaxial and adaxial surfaces of three dried leaves per locality were obtained by soaking leaf fragments in 70% HNO_3 for 8–24 hours. Epidermal peels were stained with 1% safranin, placed on microscope slides and photographed using an optical microscope (Axio Lab.A1, Zeiss, Oberkochen, Germany) and a camera (Canon Rebel SL1) at 40× magnification. Stomata were counted in three fields of view, each field with an area of 0.213 mm^2.

Statistical analyses

All statistical analyses were performed with the R package version 3.3.3 (R Development Core Team, 2017) using RStudio environment version 1.0.136 (RStudio Team, 2016). We first analyzed the trends of each individual morphological trait along the geographic gradient using as predictors the latitude where the plant was collected plus that fixed effect of each site. Because most traits were significantly correlated with latitude (and in order to avoid repeatedly testing for the same hypothesis), we used Principal Component Analysis (PCA) to merge all traits into one single combined axis and analyzed the combined axis against the latitudinal gradient.

ANOVA of individual traits. Generalized linear models were used to test for associations between each morphological trait against latitude and site, as the independent predictors. This allowed us to see which traits varied significantly with latitude, and what proportion of the residual variance was explained by the fixed, latitude-independent effect of sites. Traits in which latitude had a significant effect will be referred to as "latitude-correlated" traits (P <0.0001; Table 2.3).

Merged traits analysis. In order to summarize the variation within *Washingtonia* we performed a PCA of the sites × traits matrix using trait values averaged per site. We used the Broken-Stick distribution to test for significance of the axes (Jackson, 1993). We then used the first axis to examine how all traits combined varied with latitude. For this, we used two regression models that correspond to our two hypotheses. For the clinal variation hypothesis we tried a simple linear regression of sites against latitude, while for the two-species hypothesis we tested the fit of the first PCA axis against a logistic curve, using non-linear regression (Fig. 2.4). For each of the two models, we calculated both the Akaike Information Criterion (AIC) and the F-values of the fitted model, to select the best explanatory hypothesis (see Appendices 5 and **??**). we performed this analysis (PCA of the data matrix followed by regression of the dominant axis against latitude) in a repeated, stepwise manner: we first analyzed the whole data matrix, and the subsequent regression of the first axis and against latitude showed that the *Washingtonia* palms in the Kofa mountains of Arizona are an outlier from the otherwise continuous latitudinal trend in palm morphology (Normal distribution test of standarized residuals, $P = 0.003$). we then re-did the analysis eliminating Kofa from the data matrix and, although we found a significant fit to the morphology-vs-latitude model, the "latitude-uncorrelated" traits showed low, non-significant loadings on axis 1. In the third iteration we eliminated these latitude-independent morphological variables and, expectedly, the fit of the first PCA axis improved further. Finally, following the traditional recommendation for multivariate morphometric analysis (Huxley, 1932) in order to improve allometric linearity in the data set we log-transformed all morphometric measurements such as diameter, leaf width, or hastula length (but left untransformed all counts and ratio-type variables such as stomatal density or SLA), and performed the analysis again.

2.3 Results

Most traits measured in the 17 sites occurring along Peninsular California and Sonora were significantly associated with latitude (Table 2.3). "Latitude-correlated" traits (crown, leaf length, SLA, adaxial stomatal density, petiole width, hastula, diameter at the base, DBH, and leaf green) upon which latitude had the strongest effect had r^2 values ranging from 0.11–0.47 with an average of 0.28. Although latitude, as a predictor, yielded higher and more significant F-values, there was a significant fixed effect of site on all traits. Traits which were affected most strongly by site (adaxial stomatal density, SLA, leaf width, δ^{13}C, leaf length, and abaxial stomatal density) had r^2 values ranging from 0.41–0.74 with an average of 0.5.

Table 2.3: ANOVA of linear model of latitude and site as main effects against traits, excluding Kofa. Traits ordered by ascending significance level.

Trait	Latitude				Site			
	df	r^2	F	P	df	r^2	F	P
DBH	1	0.43	399.91	***	14	0.31	20.27	***
Diam. base	1	0.38	216.96	***	14	0.18	7.31	***
Leaf green	1	0.47	152.02	***	14	0.14	3.23	***
Petiole width	1	0.3	95.55	***	14	0.3	6.98	***
Hastula	1	0.31	83.4	***	14	0.23	4.37	***
SLA	1	0.15	46.89	***	14	0.45	9.97	***
Crown	1	0.11	44.64	***	15	0.22	6.14	***
Leaf length	1	0.13	41.65	***	14	0.47	10.46	***
Adaxial stomata	1	0.23	19.45	***	15	0.41	2.36	0.02
Leaf width	1	0.03	6.5	0.01	14	0.46	7.69	***
Height	1	<0.01	0.47	0.50	15	0.16	3.74	***
Abaxial stomata	1	<0.01	0.38	0.54	15	0.74	6.09	***
δ^{13}C	1	<0.01	0.07	0.79	15	0.47	8.15	***

Note: *** $P < 0.0001$

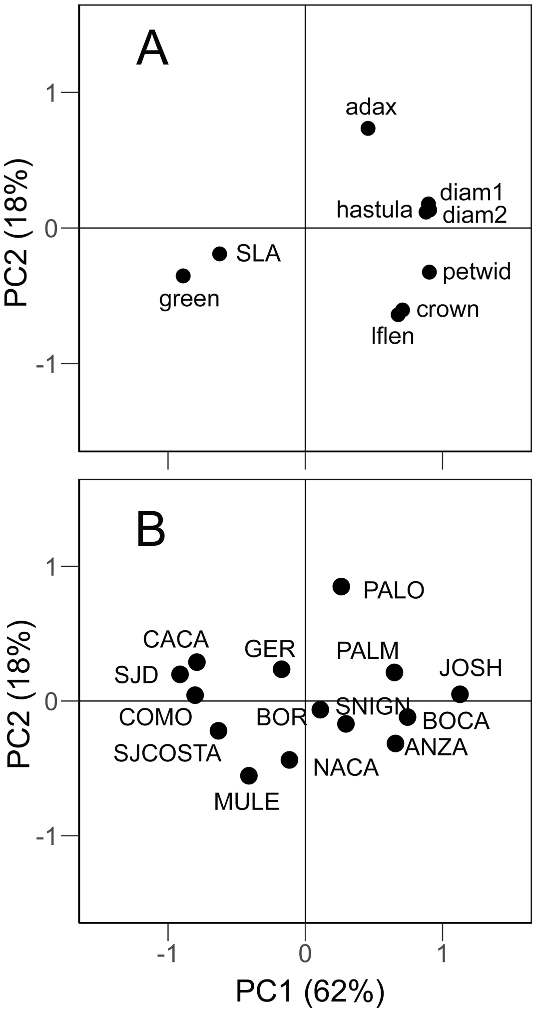

Figure 2.6: Principal components analysis (PCA) biplot.

(A) Relationship between the log-transformed "latitude-correlated" traits and (B) relationship between all sites where *Washingtonia* samples were collected, except Kofa.

Table 2.4: Comparison of the clinal model (linear model) ANOVA and the hybrid zone model (non-linear model) ANOVA of the first axis of the Principal Component Analysis obtained from five different trait matrices.

PCA matrix description		Linear model								Non-linear model							
Traits	Sites		SS	df	MS	*F* value	*P*	r^2	AIC		SS	df	MS	*F* value	*P*	r^2	AIC
All traits	All sites	Latitude	1.38	1	1.38	3.66	0.0781	0.22	29.01	Latitude	1.4	3	0.47	1.05	0.4082	0.22	32.94
		Residuals	4.89	13	0.38					Residuals	4.87	11	0.44				
		Total	6.27	14						Total	6.27	14					
										se(resid)	0.637						
All traits	All sites, except Kofa	Latitude	4.34	1	4.34	32.09	0.0001	0.73	13.85	Latitude	5.04	3	1.68	18.21	0.0002	0.85	10.51
		Residuals	1.62	12	0.14					Residuals	0.92	10	0.09				
		Total	5.97	13						Total	5.97	13					
										se(resid)	0.2897						
Latitude-correlated traits	All sites	Latitude	2.49	1	2.49	10.98	0.0056	0.46	21.92	Latitude	2.69	3	0.9	3.58	0.0503	0.49	24.95
		Residuals	2.95	13	0.23					Residuals	2.75	11	0.25				
		Total	5.44	14						Total	5.44	14					
										se(resid)	0.4788						
Latitude-correlated traits	All sites, except Kofa	Latitude	4.52	1	4.52	52.7	<0.0001	0.81	7.92	Latitude	4.56	3	1.52	15.27	0.0005	0.82	11.48
		Residuals	1.03	12	0.09					Residuals	0.99	10	0.1				
		Total	5.55	13						Total	5.55	13					
										se(resid)	0.3007						
Log-transformed, latitude-correlated traits	All sites, except Kofa	Latitude	4.65	1	4.65	59.27	<0.0001	0.83	6.75	Latitude	4.71	3	1.57	17.94	0.0002	0.84	9.82
		Residuals	0.94	12	0.08					Residuals	0.88	10	0.09				
		Total	5.59	13						Total	5.59	13					
										se(resid)	0.2821						

The final PCA model explained 62% of the total phenotypic variance along the first axis (Fig. 2.6), a value much higher than the Broken-Stick prediction of 31% . PCA results show northernmost palms have wide stems, and blue, thick, amphistomatic leaves, while southernmost palms have slender stems, and green, thin, hypostomatic leaves. PCA failed to produce clearly distinguishable groups (Fig. 2.6B). The regression of the first axis against latitude of the matrix including Kofa showed the palms at this site to be an outlier from those in Peninsular California (Fig. 2.7A).

In Peninsular California, the first axis was very strongly correlated with latitude (ANOVA, $r^2 = 0.83$). However, the regression analysis showed the simple linear model provided a better fit than the logistic model (Table 2.4) implying that there is a gradual change in the overall morphology of the palms along the latitudinal cline (ANOVA, $r^2 = 0.83$, $F = 59.27$, df = 13, $P < 0.0001$; Fig. 2.7B).

Although the non-linear model gives a slightly higher r^2 value (0.83 for the linear model *vs.* 0.84 for the logistic), the F-value of the logistic model was penalized for using four parameters to fit the data, instead of the two used in the simple linear model. The AIC estimator was also smaller in most linear models examined than in the non-linear models (Table 2.4), indicating a better fit to the data for the linear model.

Our results show precipitation during the coldest quarter increases with latitude, whereas minimum absolute temperature decreases (Fig. 2.8 A,B). At the local scale, we found that the canyon trough consistently experienced lower temperatures than those recorded

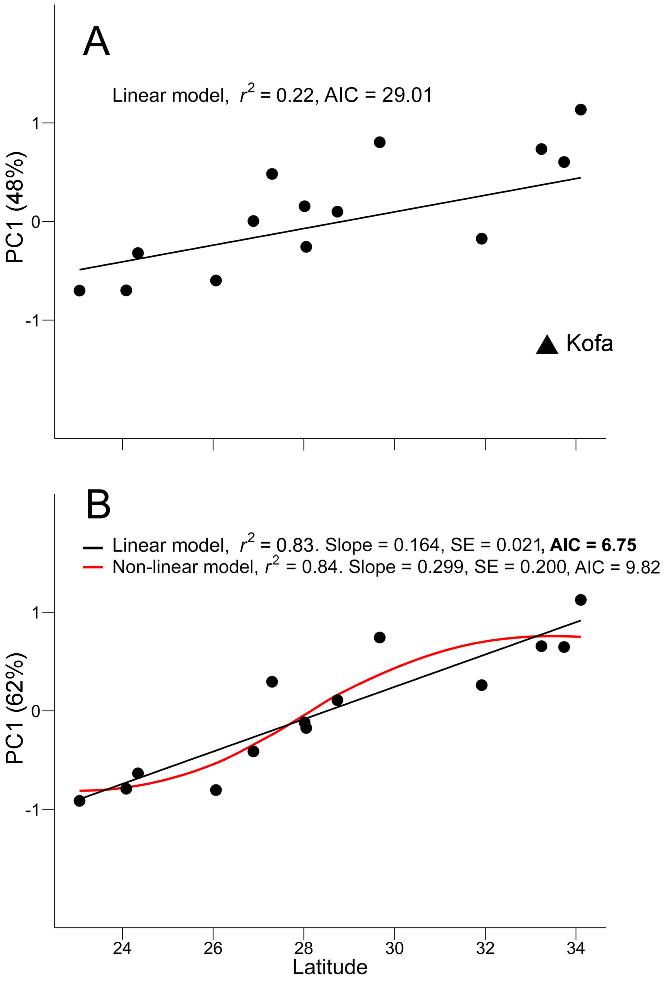

Figure 2.7: PC1 against latitude of linear and logistic models.

(A) PC1 from the PCA performed using the matrix including all traits and all sites. Kofa marked as a triangle. (B) PC1 from the PCA performed using the matrix of the log-transformed "latitude-correlated" traits and excluding Kofa.

at the desert slope outside the canyon during the coldest week of late winter, particularly at dawn and throughout the morning (Fig. 2.9).

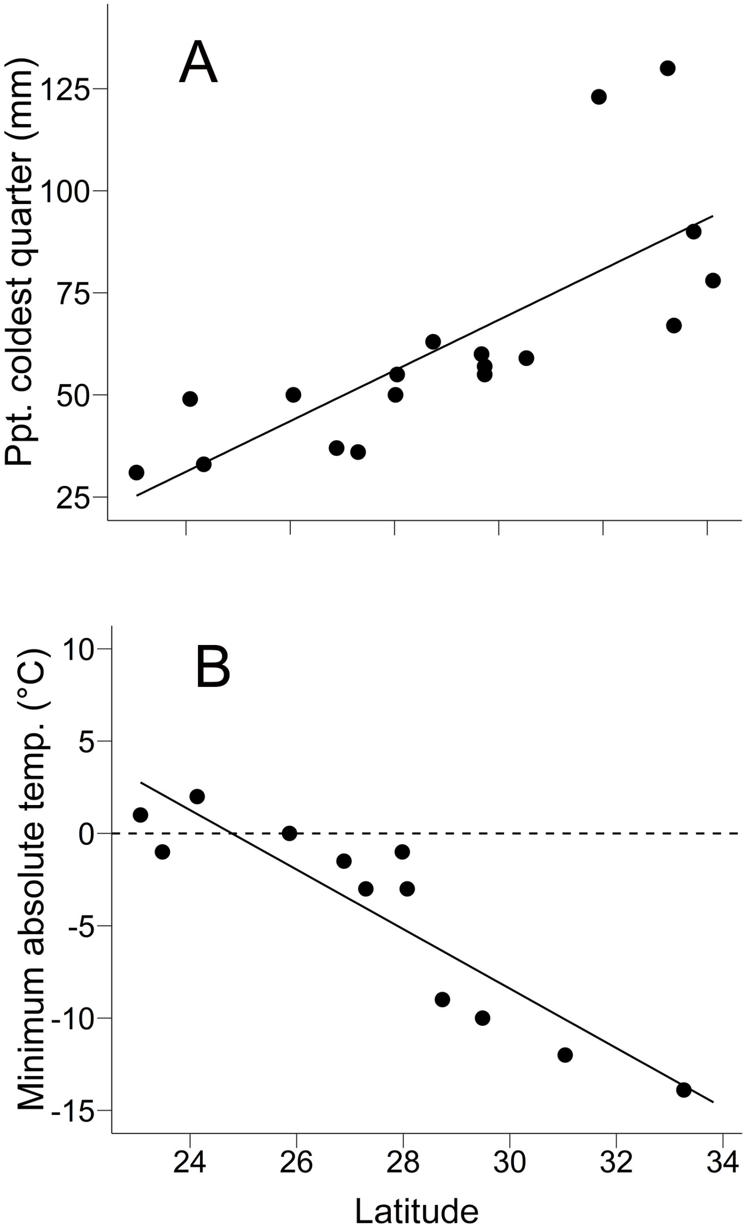

Figure 2.8: Precipitation and minimum temperature of the 17 sampled populations of *Washingtonia.*

(A) Precipitation during the coldest quarter (Fick and Hijmans, 2017). Solid black line shows regression line. (B) Minimum absolute temperature at the nearest weather station to 13 of the sampled *Washingtonia* populations. Solid black line shows the regression line. (NOAA, 2018; CONAGUA, 2018). Horizontal dashed line shows 0 °C.

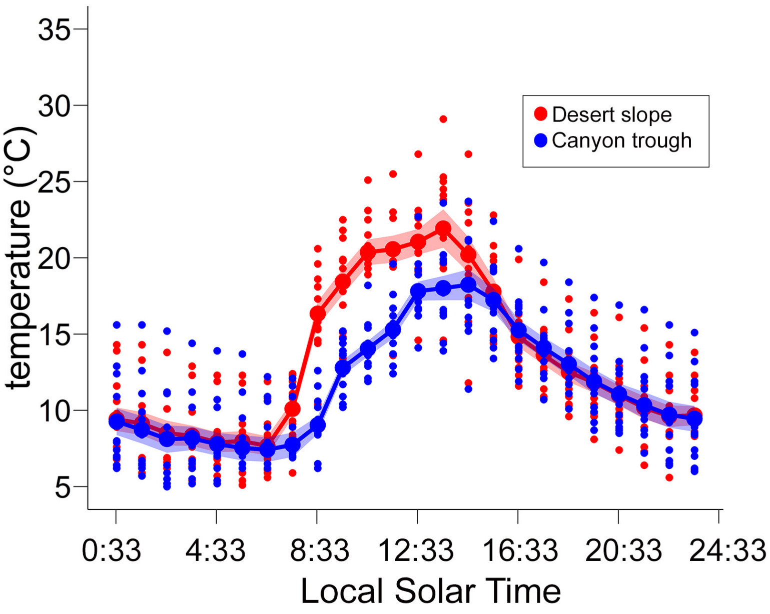

Figure 2.9: Minimum temperature within the canyons of the 17 sampled populations of *Washingtonia.*

Time series of minimum temperature at the desert slope and at the canyon trough in Palm Canyon, California during the coldest week of late winter of 2018. Shaded areas show SE.

2.4 Discussion

Clinal variation along the latitudinal transect

My results show the clinal model provides the best fit to our data either using distributional statistics (F) or information theory (AIC) tests (Table 2.4). This result strongly supports the hypothesis that there is a gradual change in the vegetative morphological traits in *Washingtonia* along the latitudinal gradient of Peninsular California, instead of two clear morphologically distinguishable species with a hybrid zone. Furthermore, the slope values of the linear model (0.164, SE = 0.021) and inflection-point slope of the non-linear model (0.299, SE = 0.200) did not differ significantly (t-test, t = 0.673, d.f. = 22, $P < 0.05$), also indicating there is no sharp differentiation between both extremes (Endler, 1977). Our results add evidence to the idea that clinal variation could be a common phenomenon in Arecaceae (Henderson, 2005).

Island-like biomes in Peninsular California

Although there is no recent information on the fossil record of *Washingtonia*, previous authors have mentioned fossils from the Miocene and Pliocene (Bailey, 1936; Vogl and McHargue, 1966; McClenaghan and Beauchamp, 1986). However, distinguishing *Washingtonia* fossils from other closely related genera such as *Sabal* Adans., *Geonoma* Willd. and *Thrinax* L.f. ex Sw. without reproductive structures or other traits is nearly impossible (Read and Hickey, 1972). More recently, and without making specific reference to *Washingtonia* fossils, Bacon (2011) discussed the fossil record of Trachycarpeae, the tribe to which *Washingtonia* belongs.

As concluded by Axelrod (1950) *Washingtonia* had a more widespread distribution during the Miocene and Pliocene, indicating that the current *Washingtonia* spp. did not differentiate in disjunction, but rather that the local populations seen today are the result of habitat contraction during the Pleistocene due to tectonic and climatic changes.

Although trait variation is largely continuous from south to north, a significant proportion of the observed variation was attributable to the fixed effect of the sites. It is yet to be tested if the populations of *Washingtonia* constitute real ecotypes. For this, a series of common-garden experiments and transplants would have to be conducted (Briggs and Walters, 2017). However, plants from the Cape Region planted in southern California and those from southern California planted in La Paz, Mexico, maintain their phenotypes, despite being grown under different environmental conditions from those in which they occur naturally, implying that the populations of *Washingtonia* have limits to their phenotypic plasticity and suggesting the existence of ecotypes.

Thus, *Washingtonia* palm oases can be considered an archipelago: habitat ‘islands’ with specific environmental conditions surrounded by a ‘sea’ of desert scrub. The marked and significant phenotypic differences between oases, apart from the latitudinal effect, could be the result of evolutionary adaptation to local environmental conditions, as has been suggested for clinal variation (Mayr, 1954). Nonetheless, due to the isolation between populations, chance mechanisms like random dispersal, which is mainly done by coyotes and foxes in southern California (Bullock, 1980), followed by founder effects and genetic drift, cannot be discarded either as a cause of morphological differences between oases (Endler, 1977). At the larger scale of the whole peninsular gradient, differences in temperature and rainfall patterns between the geographic extremes of the distribution range of the palms support the idea that variation in some morphological traits in *Washingtonia*, including stem diameter and leaf color, is of an adaptive value.

Along the 1300-km range of the *Washingtonia* oases in Peninsular California, the environment changes from dry subtropical with summer rains to arid temperate dominated by winter rains (Vogl and McHargue, 1966; Garcillán and Ezcurra, 2003). As a result, precipitation in winter increases steadily from south to north (Fig. 2.8A), making the highest water availability in the northern sites coincide with winter. On the other hand, the intensity of extreme freezing events (and consequently the risk of freeze damage) also increases towards the north (Fig. 2.8B). Some authors have proposed stem diameter in desert plants as an adaptive trait that allows them to withstand extreme minimum temperatures by protecting the apical meristem from freezing (Felger and Lowe, 1967; Nobel, 1980). It has been reported that variation in DBH in the palm *Metroxylon sagu* Rottb. is not correlated with genetic variation (Kjær et al., 2004) giving further support to the idea that stem diameter variation might be affected by environmental conditions to some degree. Like other species of the Sonoran Desert (Niering et al., 1963; Felger and Lowe, 1967; Nobel, 1980) our results show that DBH, overall, increases towards the north. Maximum DBH was recorded at Bocana located 37 km from Pacific Ocean, where minimum temperature can get as low as 10 °C (CONAGUA, 2018), and low temperatures can be maintained throughout the day by the marine layer of north-western Baja California coming in from the Pacific (Vanderplank and Ezcurra, 2015). The next highest DBH was from palms at Forty-Nine Palms, in Joshua Tree National Park, the northernmost boundary of the genus distribution, where the recorded minimum temperature is 10.4 °C (CONAGUA, 2018), putting the palms at risk of freezing, as reported by previous authors (Cornett, 1987a). It should also be noted that, although we could not collect diameter measurements in Berrendo, because the only adult individuals we observed had their stems covered in a shag of dead leaves, these palms were massive. This is not surprising given that the minimum recorded temperature is 12 °C (CONAGUA, 2018), not only a result of latitude, but also of local topography. Berrendo is located at the southern base of the Sierra San Pedro Mártir, where cold katabatic winds often descend. Over a century ago, Shreve (1912) noted that this phenomenon, known as cold air drainage (Yoshino, 1984), plays an important role in plant distribution in foothill canyons throughout the Sonoran Desert. The palms in Nacapule (Sonora), found at a similar latitude than those at Santa Gertrudis and San Ignacio, have more slender stems than their Peninsular counterparts. Minimum temperatures at Santa Gertrudis and San Ignacio have reached subzero temperatures 33 and 50 times, respectively, in the last 50 years (CONAGUA, 2018), whereas temperature during the same period has only once dropped to −1 °C at Nacapule (CONAGUA, 2018). Palms at Kofa, Arizona (latitude 33.36), are nearly as slender as those from more southern latitudes, despite experiencing temperatures as low as −13.9 °C (NOAA, 2018). Slender stems in Kofa may be due to topographical position more than latitude. Unlike palms in the other oases, these are the only individuals growing on a south-facing slope of the canyon, rather than at the bottom where winter temperatures are lower than on the open slope and remain so for a longer time (Fig. 2.8C). It has been reported that the palm *Euterpe edulis* Mart. is absent from valley bottoms, where subzero temperatures are observed, whereas the medium and higher elevation sites never experience subfreezing temperatures (Renninger and Phillips, 2016). Thus, our results support the hypothesis that larger stem diameters at the northern edge of the distribution of *Washingtonia* prevent apical freezing.

Leaf color differences have been noticed in other plant groups of the Sonoran Desert and used to distinguish among species in the same genus (Bailey, 1937; Turner et al., 1995; Felger and Joyal, 1999) including *Washingtonia* (Bailey, 1936; Turner et al., 1995). The bluest leaves of *Washingtonia* are from individuals in Joshua Tree, the northernmost and coldest site. Since leaf color is a good indicator of leaf wax deposits (Essig et al., 2000; Lee, 2007), these populations possibly have the waxiest leaves making them resistant to leaf freezing. Because winter rains dominate in the northern part of Peninsular California (Garcillán and Ezcurra, 2003), the risk of freezing coincides in these oases with the time of highest water availability. Epicuticular wax, as a protective mechanism, has been reported in leaves of *Eucalyptus urnigera* Hook.f., in which glaucous, water-repellent leaves freeze at lower temperatures than non-waxy green leaves of the same species (Thomas and Barber, 1974). Therefore, it is possible that stem diameter and leaf waxiness act synergistically in *Washingtonia* to take advantage of favourable moisture during the coldest months of the year, while avoiding stem and leaf freezing. Alternatively, and not mutually exclusive, epicuticular wax may also help avoid water loss in waxy-leaf palms occurring in the northern sites during the warmest months (Tomlinson et al., 2011).

Site effect is most evident in Kofa, Arizona, the most geographically isolated population. The other mainland oasis, Nacapule, is part of Shreve's Gulf Coast subdivision of the Sonoran Desert, an area of high floristic similarity on both sides of the Gulf of California (Shreve and Wiggins, 1964; Turner et al., 1995). Despite being at a similar latitude as other localities, the Kofa population is a deviation from the S–N pattern. *Washingtonia* palms have high water requirements and need permanent water access (Vogl and McHargue, 1966; Minnich et al., 2011). Palms in Peninsular California grow on granitic rocks that serve as water catchments (Minnich et al., 2011), ensuring regular water access. In Arizona, palms grow on a south-facing slope of the Kofa Mountains, where the porosity of the rock, coupled with the aspect of the slope, results in less water availability. These palms are probably the most xeric-adapted and water-use efficient, as revealed by their high δ^{13}C values (-25.35‰), compared to those of the other populations (-26.43‰ to -29.31‰). This is the smallest population of *Washingtonia* included in our study, and the number of palms has apparently been decreasing in recent years. Anecdotal evidence shows the main population was composed of 65 individuals in the main part of the canyon (Bailey, 1936). The number apparently remained constant from 1936 to 1960 (Kearney et al., 1960), but then decreased to c. 21 adult individuals in 1986 (U.S. Fish Wildlife Service, 2011), 20 in 2001 (U.S. Fish Wildlife Service, 2014) and 14 in 2018, although apparently smaller trees were in the process of establishment and other adult individuals could be found in nearby canyons (U.S. Fish Wildlife Service, 2014). Considering this is the most isolated population, genetic flow with other *Washingtonia* stands is unlikely, making palms at Kofa vulnerable.

Stem diameter and leaf color as taxonomic characters

Stem diameter is a diagnostic trait currently used to distinguish *Washingtonia* spp., despite not being described in either protologue (Wendland, 1879, 1883). Given the lack of clarity in the literature on stem dimensions in *Washingtonia*, Bailey (1936); Turner et al. (1995); Simono (2012) and my results, we argue this is not a critical trait to distinguish between the species, at least in natural populations. The fact that palms in urban areas, where *Washingtonia* palms can be effectively distinguished using stem diameter, come from the extreme localities of its distribution (San José del Cabo and southern California) where morphological differences are most evident provides further support for this proposal. Therefore, palms in urban landscapes do not reflect the variation in stem diameter observed in wild populations. Leaf color has also been used to differentiate between *Washingtonia* spp. (Bailey, 1936; Ishihata and Murata, 1971; Turner et al., 1995; Simono, 2012), but my results show this is also a trait that varies continuously along the gradient. Thus, it should be used cautiously as diagnostic trait for *Washingtonia.* Highly variable characters such as the ones we have investigated might not be suitable for species identification in *Washingtonia* as shown for other palm genera (Kjær et al., 2004).

2.5 Conclusion

In summary, my results show the vegetative morphological traits measured in *Washingtonia*, some of which have been used to distinguish between the species, vary continuously along the latitudinal cline in Peninsular California, but are also influenced by fixed site effects. This evidence shows the difficulty in establishing clear morphological limits between both species currently recognized in *Washingtonia.* We defer any formal taxonomic decision until the phylogenetics of the genus is resolved using molecular markers.

Chapter 3. Intraspecific variation in leaf traits of the widely distributed palm genus *Washingtonia* (Arecaceae)

3.1 Introduction

One of the goals of community ecology is to find consistent trait-environment linkages that explain the occurrence of a group of species at a particular site (Díaz et al., 2004). Understanding how plant traits vary among species will ultimately have consequences at the community and ecosystem levels (Reich et al., 1999; Wright et al., 2004; Cornwell and Ackerly, 2009; Albert et al., 2010; Shipley et al., 2016; Funk et al., 2016). Leaves, in particular, play an extremely important role as they allow light interception and CO_2 uptake necessary for photosynthesis (Lambers et al., 2008). Leaf thickness, stomatal traits, gas exchange regime, and water use efficiency are important features of leaves that have a significant effect on the physiology of plants (Hill et al., 2014; Boer et al., 2016) and community composition (Hetherington and Woodward, 2003).

Most angiosperms possess leaves with a bifacial or dorsiventral structure. The upper (adaxial) side normally harbors a layer of palisade tissue, formed by a chloroplast-rich, columnar parenchyma cells under the upper epidermis. Between the palisade tissue and the lower (abaxial) epidermis there is a spongy mesophyll, with cells widely separated from each other so that the circulation of CO_2 entering the leaf through the abaxial stomata and diffusing on to the palisade tissue above, is enhanced. In short, most angiosperms show some level of functional specialization in their leaf sides, the upper surface being specialized in the capture of light, and the lower one being specialized in the exchange of gases with the surrounding atmosphere (Smith et al., 1997).

In arid and other high-light environments such as coastal marshes, however, it is common to observe plants that have lost the dorsiventral specialization that distinguishes the adaxial from the abaxial side of their leaves, showing instead isolateral leaves with palisade tissue on both sides. Isolaterality in dryland plants is often accompanied by amphistomaty (the presence of stomata in roughly equal density in both sides of the leaf), as well as by a vertical orientation on the leaves and increased leaf thickness (Smith et al., 1998).

Stomata play a crucial role for gas exchange across leaves (Hetherington and Woodward, 2003; Hill et al., 2014; Raven, 2014; Wang et al., 2015; Carlson et al., 2016; Boer et al., 2016). Stomatal density and size, two traits that have important consequences for the conductance to H_2O and CO_2 (Carlson et al., 2016), are influenced by different environmental factors such as temperature (Hill et al., 2014), humidity, CO_2 (Boer et al., 2016), and water availability (Fraser et al., 2009). Despite the importance that stomatal distribution has for stomatal conductance (gs) and, ultimately, photosynthesis, relatively few studies have evaluated the consequences of amphistomaty (Gindel, 1969; Clay and Quinn, 1978; Parkhurst, 1978; Mott et al., 1982; Sundberg, 1985; Taylor et al., 2011; Bucher et al., 2016).

Amphistomaty and isolaterality are strikingly visible in many dominant desert plants such as jojoba (*Simmondsia chinensis* C.K.Schneid.) in the Sonoran Desert and in the different species of creosote bush (*Larrea* Ortega) in both North and South American deserts (Pyykkö, 1966; Gibson, 1996). Indeed, many studies suggest that the xeromorphic leaf anatomy might be dominant in most drylands. Almost a century ago, Wood (1932) noted that while only 5% of plants in British woodlands are amphistomatic, 88% of plants in the sclerophyll forest in Victoria, Australia (34 out of 39 species) were amphistomatic, and a full 100% of 28 species sampled from the arid scrubland of Koonamore, Australia, were amphistomatic, concluding that amphistomaty "is possibly correlated with increasing aridity". Arambarri et al. 2011 reported that, from a sample of 32 species of shrubs from the Dry Chaco forest in Argentina, "more than half presented a xeromorphic, or isolateral, [leaf mesophyll]". Similarly, Mott et al. (1982) reported that, from a list of 119 dominant dryland species from floras of California, Arizona, and Mexico, 50% of the thinner-leaved species (100-200 microns) and over 80% of thick-leaved plants (500 microns) were amphistomatic. Hull & Bleckmann (1977) found that the stomatal density on the adaxial surface of leaflets of *Prosopis tamarugo* F.Phil. in growing chambers increased with lower relative air humidity, making the plants more amphistomatic. Finally, Gibson (1996) reported that, from a list of 301 globally-distributed desert species, 278 showed isolateral leaf mesophylls.

In short, the xeromorphic syndrome (isolateral, amphistomatic leaves, often thickened and vertically-oriented) seems to be the dominant leaf morphology in desert plants. This might seem contradictory with existing theory, as some authors contend that hypostomatous leaves are better adapted to dry conditions than amphistomatous leaves (Willmer and

Fricker, 1996). This belief has been accepted, in part, because of the few existing studies that report stomatal density for both leaf surfaces. In those cases where the relationship between stomatal density and aridity or water stress has been studied, some unexpected results have emerged. In some desert species, and contrary to what is accepted, stomatal density has been reported to increase with decreasing water availability (Penfound, 1931; Evenari, 1962; Buttery et al., 1993; Smith et al., 1998). Some authors have suggested that variation in stomatal density in desert plants depends on the carbon fixation pathway (Sundberg, 1985; Herrera and Cuberos, 1990) with non-succulent desert plants having higher stomatal density than succulent plants with CAM metabolism. Others have found that amphistomaty is related to leaf thickness, where thick leaves tend to be more amphistomatic than thin ones (Parkhurst, 1978).

Most of the studies discussed above imply the comparison of plants from arid against plants from non-arid environments, which often have different taxonomic lineages. Thus, differences in plant morphology can be attributed to both environmental influence and taxonomically-fixed traits, and separating the effect of the environment from the effect of taxonomic inheritance is often a complex task (Harvey and Purvis, 1991). A simpler approach to understand changes in leaf traits, like stomatal density, across different environments is to study the variation in a single species or in closely related species of a genus throughout their distributional range. This approach—examining intraspecific variability—has recently been encouraged by several researchers studying plant functional traits (Cornwell and Ackerly, 2009; Jung et al., 2010; Albert et al., 2010; Burns and Strauss, 2012; Shipley et al., 2016). The larger Sonoran Desert, including the Baja California peninsula, exhibits a dramatic environmental gradient characterized by arid temperate climate dominated by winter rains in the north and a dry tropical climate with summer rains in the south (Garcillán and Ezcurra, 2003; Vogl and McHargue, 1966). Latitudinally, it extends some 1300 km, from the Cape Region at latitude 23° to the Lower Colorado Valley and the Mojave, at 36° The genus *Washingtonia* comprised of two C3 phreatophytic palm species, occupies the whole gradient, from the tropical tip of the peninsula to the hot, arid oases in southern California and Arizona (Fig. 1). There is a marked latitudinal cline within the genus from the tropics to the mid-latitude deserts in morphological characters such as stem diameter, petiole width, or crown size (Villanueva-Almanza et al., 2018). Based on these results, we hypothesized that leaf functional traits in *Washingtonia* palms, and very especially those related to the bifacial or isolateral anatomy of the leaves, could also exhibit a predictable trend along the gradient and allow us to test the hypothesis that the evolution of leaf xeromorphism is correlated with increasing environmental aridity. Therefore, the research questions of our study were: (1) are there any differences in leaf mesophyll and stomatal density between both leaf surfaces of palm leaves along the latitudinal gradient? (2) Are there any environmental variables that may explain these differences?

3.2 Materials and Methods

Site selection and sampling procedure

Leaf samples of both currently recognized species of *Washingtonia*, *W. filifera* and *W. robusta*, were collected in 14 natural populations along a 1300-km latitudinal transect in the Baja California peninsula and California, and two other sites in Arizona and Sonora (Table 1; Fig. 3.1) between March 2017 and October 2017.

South-east facing leaves were taken from the outer canopy most exposed to sunlight from the ten shortest adult individuals (5.0–7.0 m) at each site using a pruning pole. Fragments were air-dried and stored in the UCR herbarium. In the sampled leaves, we measured 5 traits expected to be involved in habitat differentiation. In the microscope we estimated stomatal density and size, two strictly anatomical traits. We also estimated three other traits, leaf greenness, specific leaf area (SLA), and carbon isotopic composition, expected to have functional relevance in the performance of the plants.

Anatomical leaf traits

Epidermal peels of abaxial and adaxial surfaces of three dried fragments per locality were obtained by soaking them in 70% HNO_3 for 8–24 hours. Epidermal peels were stained with 1% safranin, placed under a light microscope (Zeiss Axio Lab.A1, Carl Zeiss, Oberkochen, Germany), and photographed using a camera (Rebel, SL1, Canon, Taiwan) at 40× magnification. Stomata were counted directly in three randomly selected fields of view, each

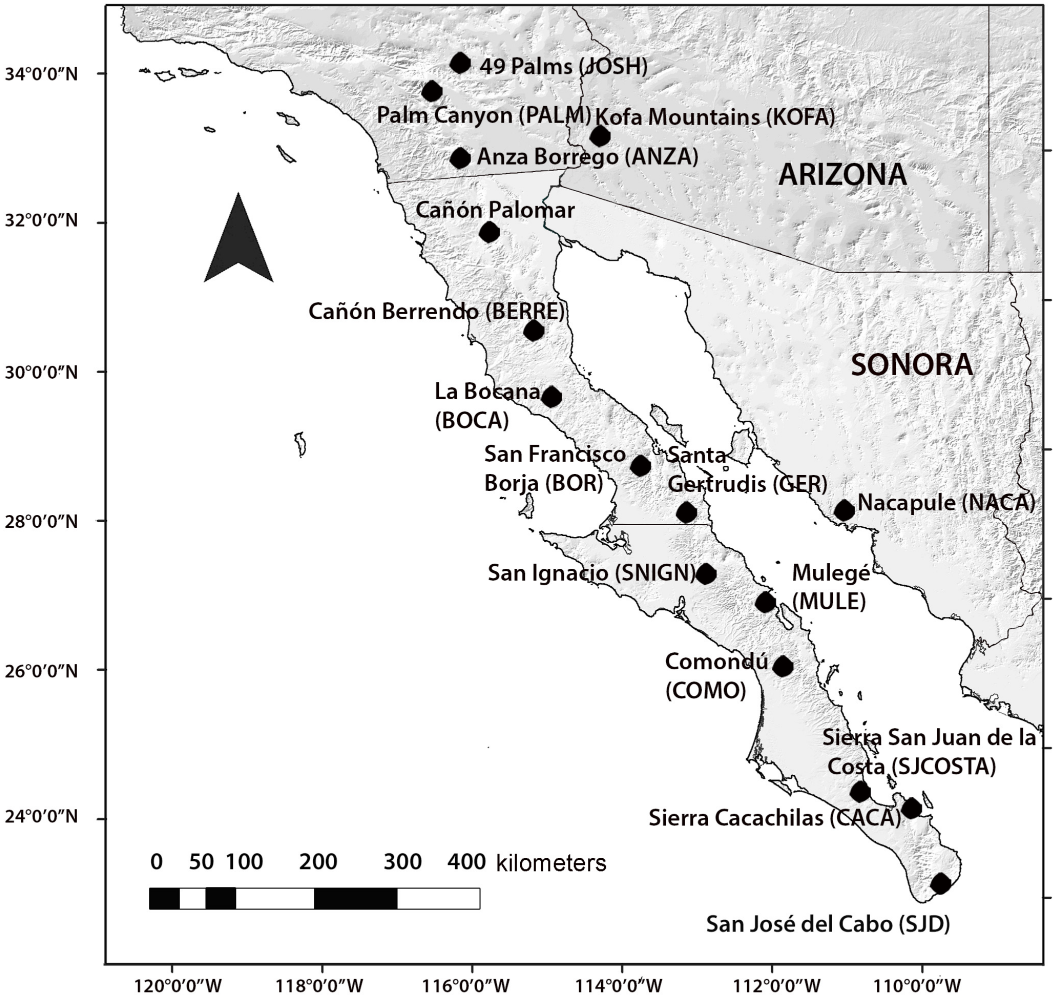

Figure 3.1: *Washingtonia* populations sampled across Peninsular California, Arizona, and Sonora. N=16

field with an area of 0.213 mm^2. Additional photographs of epidermal peels of both leaf sides were taken using an Axiocam 305 color camera (Carl Zeiss, Göttingen, Germany) adapted to the Zeiss Axio microscope. Three photographs per sample were taken at 40× magnification and stored. Pore measurements were obtained from 3 stomata per photograph using ZEN 2.3 SP1 blue edition software (Carl Zeiss, Jena, Germany) to total 9 stomata per site. For leaf mesophyll observations, fresh leaf samples of cultivated specimens of *W. filifera* and *W. robusta* were collected at the University of California Riverside campus during early spring 2018. Transversal leaf sections were cut by hand using a razor blade, dyed with 2% toluidine blue for 2–3 minutes, observed at 10× magnification, and photographed using a camera (Rebel, SL1, Canon, Taiwan).

Functional traits

Fresh leaves were cut into 30 cm × 30 cm squares and photographed on the adaxial surface using a camera (Nikon Coolpix P340, Wuxi, China) and a color checker reference target (X-Rite, 2014), in a dark room using the following camera settings: Flash exp comp +2.0, ISO 80, Built in filter off, White balance Auto 1, 1/30, 5.1 mm, f5.6. Photographs, taken in NRW format, were white balanced and color-standardized with X-Rite camera calibration software (X-Rite, 2014). Channel information for red, green, and blue was extracted at eight points of the processed image using Adobe Photoshop CS6. The total RGB value for each leaf surface was calculated as the sum of each channel and then used to calculate the relative value of the green channel following (Richardson et al., 2007). Since leaf color is a good indicator of leaf wax deposit (Essig et al., 2000; Lee, 2007), we performed scanning electron microscopy (SEM) using a scanning electron microscope (Hitachi TM4000, Tokyo, Japan) at 80× magnification. Air-dried leaf fragments of two sites were cut into small squares and mounted with the adaxial surface facing upwards onto the sample holder with double-sided adhesive Leit tabs.

To determine SLA, a fragment of known area was taken from the ten fresh leaves collected in each locality and subsequently dried (72 h, 50 °C) in a drying oven (Isotemp, model OV701G, Fisher Scientific Inc., Dubuque, IA, USA). Dry weight was recorded and specific leaf area was calculated as fresh leaf area/dry mass (cm^2g^{-1}).

All leaf fragments (except one from San Borja) used for determining SLA were ground using mortar and pestle, and liquid nitrogen. An amount between 1.0–1.1 mg of the ground material was weighed, rolled in tin capsules (5 × 9 mm), and combusted using an elemental analyzer (Costech, Valencia, CA, USA) coupled to a Delta-V Advantage, isotope ratio mass spectrometer operating in continuous-flow mode (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at the Facility for Isotope Ratio Mass Spectrometry at the University of California, Riverside.

Environmental variables

To explore if changes in leaf traits were associated with environmental drivers, we obtained 19 bioclimatic variables, solar radiation, and water vapor pressure data by intersecting GPS coordinates for all populations taken in the field with climate layers using R package version 3.3.3 (R Development Core Team, 2017) and code by Turner (2013) available at `https://gist.github.com/kgturner/6643334`.

Climate layers were derived from WorldClim version 2 with a resolution of 30 seconds (~1 km^2) for the period 1970–2000 (`http://worldclim.org/version2`; Fick and Hijmans (2017)). Solar radiation and water vapor pressure values reported are yearly averages calculated from a set of 12 rasters, each for every month of the year (Table 3.1).

Table 3.1: Location of populations of *Washingtonia* sampled across Peninsular California, Arizona, and Sonora, Mexico with bioclimatic variables taken from (Fick and Hijmans, 2017).

Site	Lat. Long. (d.d)	Elevation (m)	Mean temp. (°C)	Mean temp. coldest quarter (°C)	Annual ppt. (mm)	Prec. driest quarter (mm)	Annual mean water vapor pressure (kPa)	Annual mean solar rad, (MJ m^{-2} day^{-1})
1. San José del Cabo (SJD)	23.05, -109.68	9.00	23.35	18.60	287.0	1.0	2.03	18.93
2. Sierra Cacachilas (CACA)	24.08, -110.11	754.73	20.18	14.98	438.0	5.0	1.57	18.87
3. Sierra San Juan de la Costa (SJCOSTA)	24.33, -110.71	71.96	22.22	17.15	181.0	0.0	1.82	18.96
4. Comondú (COMO)	26.06, -111.82	280.00	21.74	16.33	211.0	2.0	1.66	19.00
5. Mulegé (MULE)	26.88, -111.98	1.00	24.26	17.42	148.0	1.0	1.81	18.91
6. San Ignacio (SNIGN)	27.29, -112.88	123.08	21.71	16.33	114.0	0.0	1.76	19.04
7. Cañón de Nacapule (NACA)	28.01, -111.05	161.39	23.87	17.50	276.0	5.0	1.61	18.38
8. Santa Gertrudis (GER)	28.05, -113.08	420.03	20.77	14.90	150.0	4.0	1.59	19.01
9. San Francisco Borja (BOR)	28.74, -113.86	440.00	18.96	14.10	132.0	5.0	1.53	19.12
10. Bocana (BOCA)	29.67, -114.91	340.79	19.11	14.15	113.0	3.0	1.52	19.25
11. Cañón Berrendo (BERRE)	30.53, -115.13	801.69	16.70	9.93	156.0	4.0	1.20	19.30
12. Cañón Palomar (PALO)	31.92, -115.71	493.20	17.35	11.58	236.0	9.0	1.12	19.21
13. Hellhole Canyon, Anza Borrego (ANZA)	33.23, -116.44	533.76	18.99	11.08	260.0	15.0	0.93	19.04
14. Palm Canyon, Kofa Mountains (KOFA)	33.36, -114.09	846.26	20.00	11.32	229.0	14.0	0.74	19.82
15. Palm Canyon, Aguacaliente Indian Reservation (PALM)	33.73, -116.53	242.44	20.97	12.73	180.0	11.0	0.98	19.13
16. 49 Palms, Joshua Tree (JOSH)	34.10, -116.10	874.01	17.40	8.50	196.0	15.0	0.69	19.55

Statistical analysis

All statistical analyses were performed with the R package version 3.5.1 (R Development Core Team, 2017) using RStudio environment version 1.1.456 (RStudio Team, 2016).

Selection of environmental variables

Linear models were used to test for associations between each leaf trait and each of the 19 bioclimatic variables, solar radiation, and water vapor pressure as independent predictors. Solar radiation and water vapor pressure showed the highest association with adaxial stomatal density and were also significantly correlated with the other leaf traits. For this reason, and because they are a clearly interpretable measure of aridity, we used them to explore environment-leaf traits correlations in the rest of our study.

Leaf trait analysis

We used simple linear models and regression ANOVAs to examine the response of SLA, leaf greenness, δ^{13}C, stomatal size, and both adaxial and abaxial stomatal density to latitude, site, water vapor, and solar radiation. Models were tested for normal regression assumptions (homoscedasticity, normality, and independence of the residuals). In the case of adaxial and abaxial stomatal counts, We first tried Generalized Linear Models for frequency counts (Poisson regression) but, because the model residuals had high overdispersion, we decided to use normal linear regression. In all cases, model selection was done using both traditional parametric tests (F-ratios) and information-theory selection (Akaike Information Criteria or AIC).

3.3 Results

Stomatal density in the adaxial side of the palms' leaves increased significantly with latitude ($r^2 = 0.31$, df 1, $P < 0.0001$, Figs. 3.2A, 3.3A), but abaxial stomatal density remained constant along the N–S cline ($r^2 = 0.03$, df 1, $P = 0.2$).

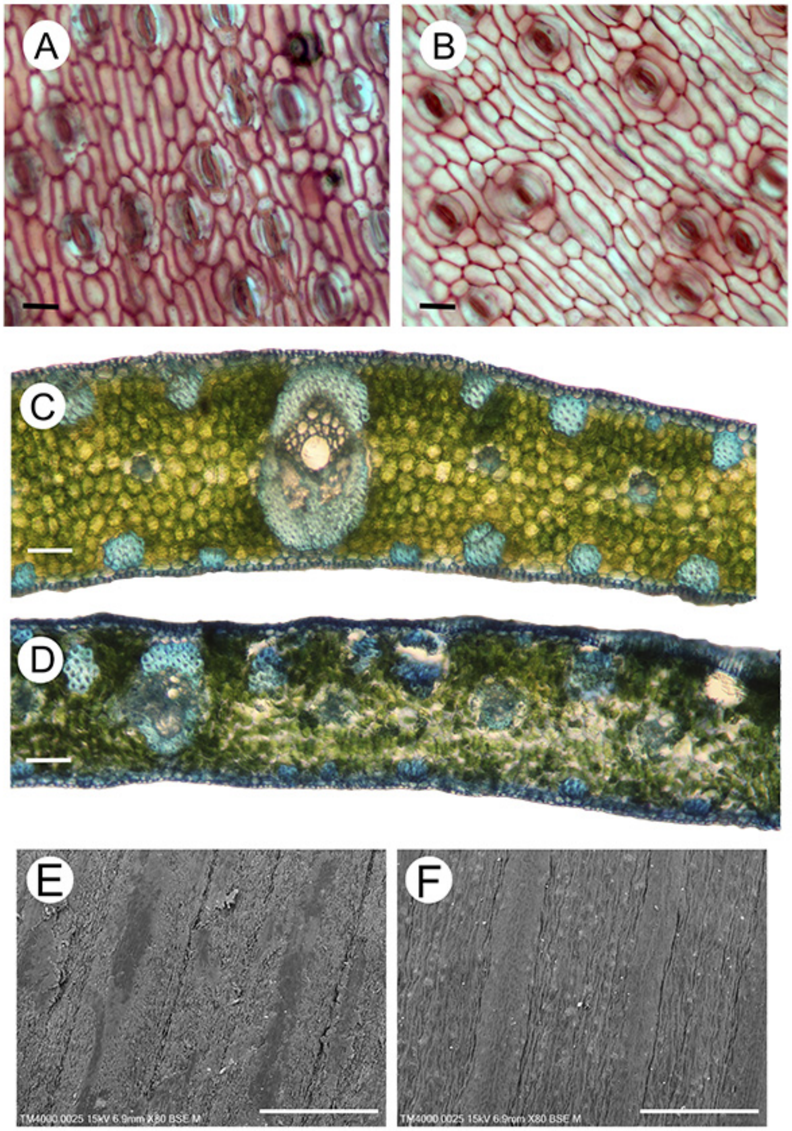

Figure 3.2: Anatomical traits of both species of *Washingtonia.*

(A, C, E) *Washingtonia filifera* (B, D, F) *Washingtonia robusta*. (A, B) Adaxial epidermis peels stained with safranin from (A) Palm Canyon, California, USA and (F) Sierra Cacachilas, Baja California Sur, Mexico. (C, D) Lamina in transverse section, adaxial surface oriented toward top of page. Both leaves from cultivated specimens grown at the University of California Riverside stained with toluidine blue and safranin. (E, F) Adaxial leaf surface from (E) Palm Canyon, California, USA showing thick epicuticular wax deposits, (F) Sierra Cacachilas, Baja California Sur, Mexico showing no epicuticular wax deposits. Bars: (A, B) 20 µm; (C, D) 50 µm; (E, F) 500 µm.

Most other variables were significantly related to latitudinal change: SLA decreased with latitude ($r^2 = 0.15$, df 1, $P = 0.0003$, Fig. 3b), implying that as palm populations move north the individuals develop thicker leaves (Figs. 3.2C, D). The greenness of the leaves decreased very significantly ($r^2 = 0.41$, df 1, $P < 0.0001$, Fig. 3.3C), showing that leaves become consistently more grayish or glaucous as palms move away from the tropics. The $\delta^{13}C$ values also increased significantly northwards but geoform (seep vs. wash oases) also had a very significant effect ($r^2 = 0.21$, df 1, $P = 0.0004$ for the effect of latitude and $r^2 = 0.15$, df 1, $P < 0.002$ for geoform, Fig. 3.3D). That is, although palms on both geoforms increased in their $\delta^{13}C$ values as the plants move north, palms growing in the seasonally dry wash oases had $\delta^{13}C$ that were 1.7‰ higher than those in the moister seep oases. Finally, stomatal pore length did not show a significant latitudinal trend ($r^2 = 0.04$, df 1, $P = 0.16$) suggesting that, like abaxial stomatal density, the size of the stomata (in terms of pore length) is constant along the N–S gradient. Additionally, the number of stomata relative to the mass of the leaf (calculated as product of total stomatal density × specific leaf area) also showed no relationship with latitude, a fact that indicates that the number of stomata per unit mass of leaf mesophyll is invariant along the gradient.

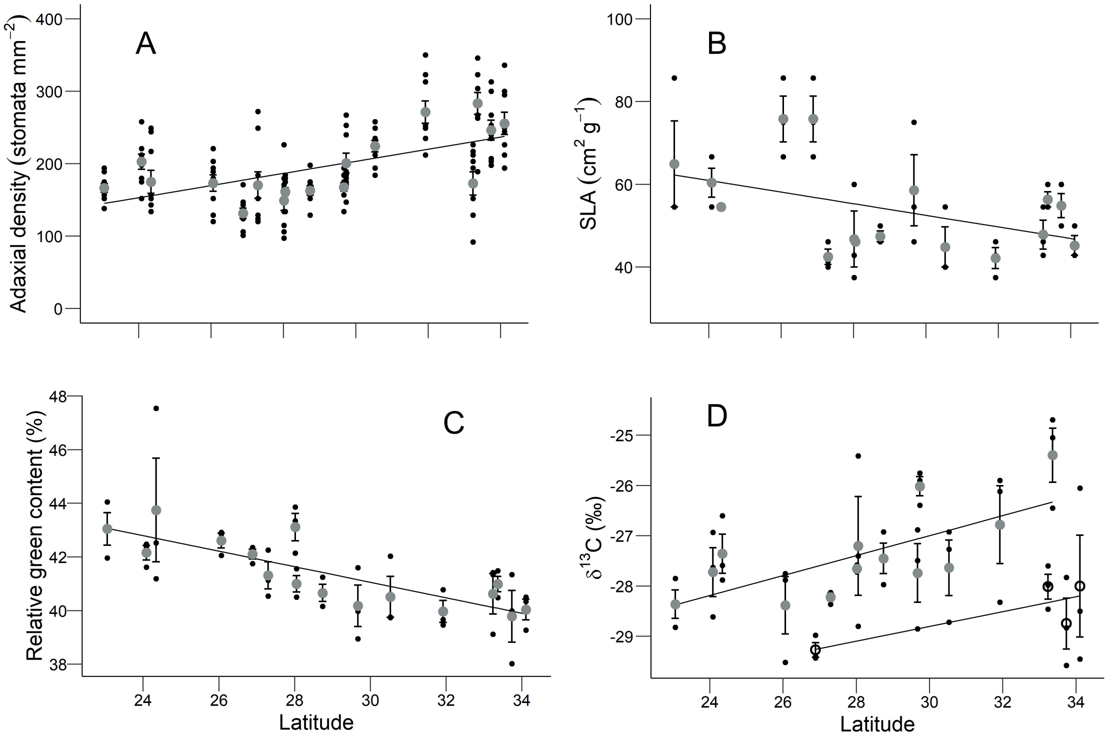

Figure 3.3: Leaf traits of *Washingtonia* as a function of latitude from 16 sites in Peninsular California, Arizona, and Sonora.

(A) Adaxial stomatal density, (B) SLA (C) relative leaf green content, and (D) δ^{13}C. Wash oases marked with solid gray circles, seep oases marked with open black circles. Means in grey circles ± SE. n = 3 individuals per locality.

In each trait, with the exception of greenness and δ^{13}C, there was a significant fixed effect of site, indicating that, despite the general N–S trend in each trait, individual palm oases may have particular characteristics that distinguish that site from the overall trend. In brief, my results show that palms at northern sites have waxy, glaucous, thick, leaves and exhibit evident xeromorphic traits like amphistomaty and isolaterality. Palms at the more tropical southern sites have glossy green, thin leaves that do not show xeromorphic traits. Instead these leaves have a lower adaxial:abaxial stomata ratio and more dorsiventral leaves (Figs. 3.2A).

Environmental variables

Adaxial stomatal density was very significantly related water vapor pressure (r^2 = 0.45, $F_{1,46}$ = 37.35, P <0.0001) and solar radiation (r^2 = 0.38, $F_{1,46}$ = 28.62, P <0.0001). However, because these two environmental variables are themselves correlated, if both are included simultaneously in step-wise regression procedure, water vapor emerges as the best predictor and the residual variance explained by solar radiation is only marginally significant (r^2 = 0.05, P =0.04; Table 3.2.)

Water vapor pressure was a better predictor than solar radiation for other trait variables like greenness (r^2 = 0.31, P <0.0001), SLA (r^2 = 0.12, P = 0.01), and δ^{13}C (r^2 = 0.08, P = 0.01). My results show that adaxial stomatal density increases with decreasing water vapor pressure, while greenness decreases with decreasing water vapor pressure.

3.4 Discussion

As palm populations transition from the tropical environments of the Cape Region, at latitude 23°N, to the arid deserts of the Mojave and the Lower Colorado Valley, at latitude 34°N, their leaves gradually change. In the northern deserts their leaves are perfectly isolateral and amphistomatic, they develop a glaucous hue resulting from the accumulation of wax in their surface, they become thicker due to the development of palisade tissue on

Table 3.2: Generalized linear models (GLMs) of leaf traits with latitude, site, water vapor pressure, and solar radiation as predictors.

Trait	Predictors	partial r^2	df	*P*	significance
adaxial stomatal density	latitude	0.31	1	<0.0001	***
	site	0.40	14	0.0039	*
abaxial stomatal density	latitude	0.03	1	0.204	
	site	0.72	14	<0.0001	***
SLA	latitude	0.15	1	0.0003	**
	site	0.55	14	0.0003	**
greenness	latitude	0.41	1	<0.0001	***
	site	0.19	14	0.4064	
δ^{13}C	latitude	0.21	1	0.0004	**
	site	0.15	14	0.0024	*
stomatal pore length	latitude	0.04	1	0.0435	
	site	0.65	14	<0.0001	***
adaxial stomatal density	water vapor pressure	0.45	1	<0.0001	***
	solar radiation	0.05	1	0.04	
abaxial stomatal density	solar radiation	0.18	1	0.0031	*
	water vapor pressure	0.01	1	0.5394	
SLA	water vapor pressure	0.12	1	0.0143	
	solar radiation	0.03	1	0.2512	
greenness	water vapor pressure	0.31	1	<0.0001	***
	solar radiation	0.02	1	0.2851	
δ^{13}C	water vapor pressure	0.08	1	0.0140	*
	solar radiation	0.05	1	0.0637	
	geomorph	0.29	1	<0.0001	
stomatal pore length	water vapor pressure	0.08	1	0.0514	
	solar radiation	0.00	1	0.6856	

both sides, and their carbon isotopic discrimination during photosynthesis is reduced, as indicated by their higher $\delta^{13}C$ values. In this study, all these traits varied simultaneously, a fact that suggests that the hypothesis that leaf isolaterality evolves together with other morphological and anatomical traits to form a xeromorphic syndrome is confirmed.

Stomatal traits and leaf thickness

Stomata were found on both abaxial and adaxial surfaces of both *Washingtonia* palms, but while adaxial stomatal density increased significantly along the peninsular cline, abaxial density remained constant. This observation supports the hypothesis that desert plants increase their adaxial stomatal density in response to increased aridity (Gindel, 1969; Dunlap and Stettler, 2001). Because leaf thickness also increases towards the northern deserts, the total stomatal density relative to unit mass of leaf mesophyll is constant, that is, the increase in adaxial stomata in the desert environments only compensates the increase in leaf mass per unit leaf area (Parkhurst, 1978; Smith et al., 1997).

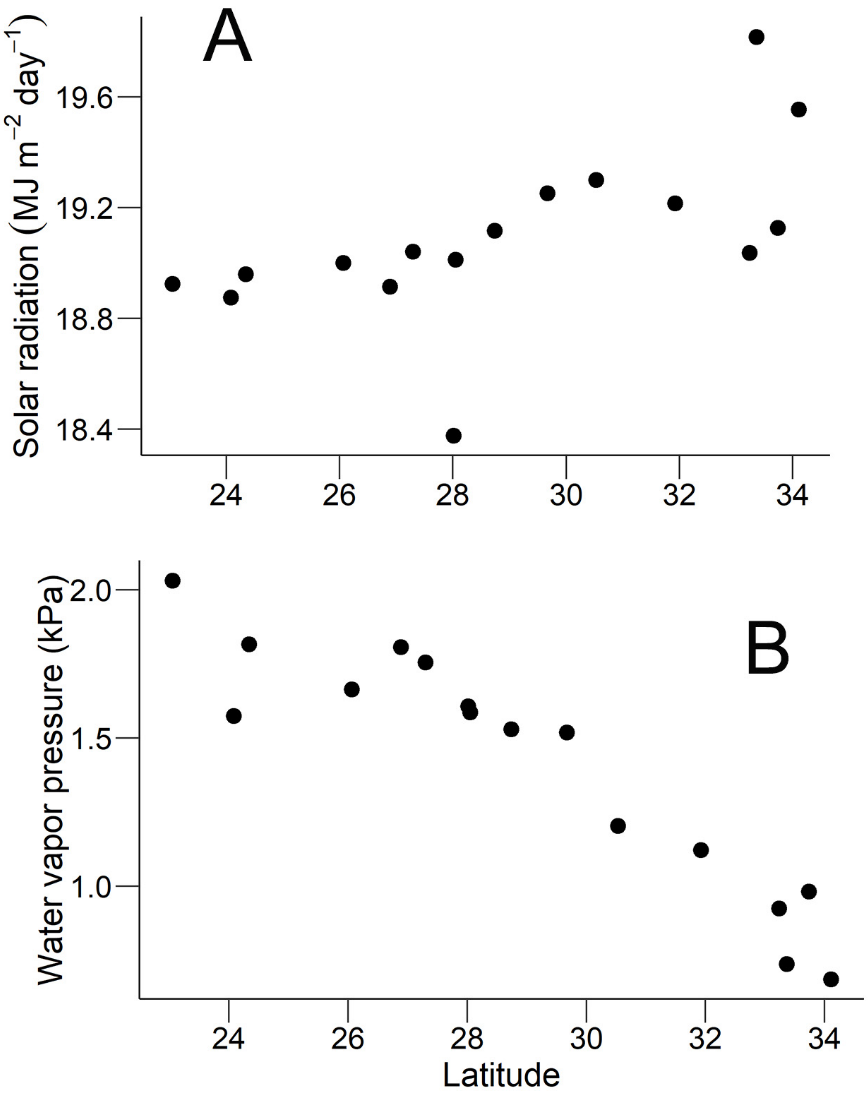

Figure 3.4: Solar radiation and water vapor pressure of *Washingtonia* oases.

(A) Mean annual solar radiation, a measure of radiative loading, and (B) mean annual water pressure deficit, a measure of transpirative demand, plotted against latitude for the 16 study sites.

Increased adaxial stomatal density towards the arid deserts was also correlated with increasing solar radiation and decreasing water vapor pressure towards these very dry environments (Figs. 3.4, 3.5). Leaves of palms in Palomar, Kofa, and Joshua Tree have the highest adaxial stomatal densities and also receive the highest annual mean solar radiation. Relatively low adaxial density for the latitude of the site was recorded in Nacapule, a site where the lowest amount of solar radiation is received (Fig. 3.4A). Because mean water vapor pressure decreases with latitude (Fig. 3.4B), palms in the more northern desert sites are experiencing drier air than those in southern tropical locations, and hence a higher evaporative demand. Increased adaxial stomatal density will result in higher transpiration rates per unit leaf area (Lambers et al., 2008) and greater evaporative cooling (Gibson, 1996), but, as increased stomatal density in the northern deserts is compensated by increased leaf thickness, transpiration rates per unit leaf mass possibly remain constant and do not increase towards the drier environments. The thickening of leaves as palm populations approach the dry deserts is consistent with previous findings reporting high light and temperature, combined with low water stress, favor the evolution of thick leaves (Smith et al., 1997). As with many other xeromorphic plants with isolateral leaves, mesophyll thickening seems to be accompanied by the development of more erect leaf blades. Over 80 years ago, Bailey (1936) had already noted that the upper leaves of the northern, waxier *Washingtonia* palms are upright, whereas those of the southern *Washingtonia* (except for the terminal ones) are more horizontal. Apart from potentially contributing to leaf overheating during midday, vertically-oriented leaves shift maximum photosynthesis to the early morning hours (and also the late afternoon), when diurnal shoot water potential and vapor pressure of the air are highest (Gibson, 1998).

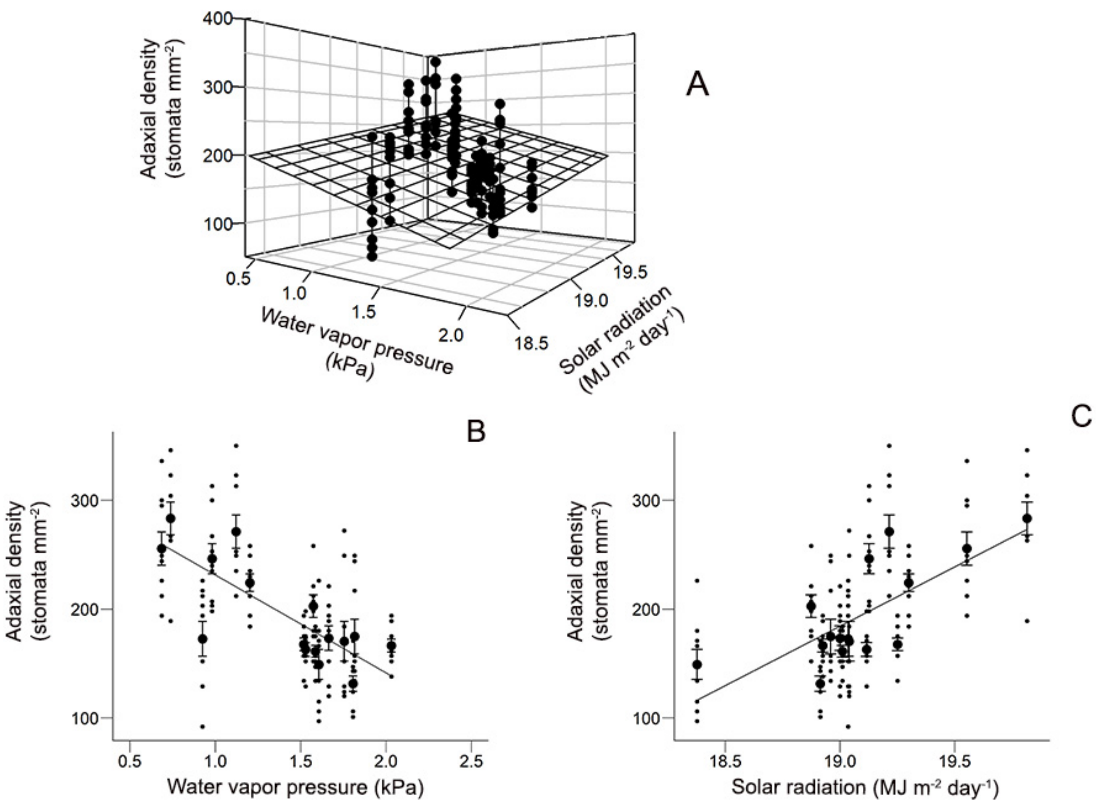

Figure 3.5: Stomatal density of *Washingtonia* as a function of environmental factors.

(A) Adaxial stomatal density as a function of mean annual water vapor and mean annual radiation at each study site with regression pane. Adaxial stomatal density as a function of (B) water vapor pressure and (C) solar radiation. Means per site with black solid circles.

Leaf greenness

The northwards increase in adaxial stomatal density, correlated with an increase in solar radiation, was paralleled by a decrease in leaf greenness and an increase in wax content in the leaf surface (Figs. 3.3A, C, 3.4A). While supra-epidermal wax might be preventing excessive transpiration or leaf damage caused by UV radiation (Gibson, 1996; Lambers et al., 2008), it could also be reducing photon supply and limiting carbon fixation. Studies have shown wax removal in plant leaves resulted in reduced reflectance and increased photosynthesis (Shepherd and Wynne, 2006). It has also been shown that photosynthetic rates of *Washingtonia filifera* were higher than those of the waxier, grayish-glaucous *Brahea armata* in the Central Desert of Baja California (Schmitt et al., 1993)

Carbon isotope composition

The relationship between carbon isotopic discrimination ($\delta^{13}C$) and water use efficiency (WUE) in C3 plants has been well known for some decades (e.g., Lajtha and Marshall 1994). In dryland plants, where stomata are often nearly closed and/or stomatal conductance is reduced, almost all of the intercellular CO_2 reacts with Rubisco during photosynthesis and there is little isotopic discrimination. Thus, reduced stomatal conductance in arid-land plants implies a reduction in water loss and also a lower discrimination against and the isotope ^{13}C and, for this reason, there should be a correlation between $\delta^{13}C$ and WUE in any given group of plants. Two clear trends appeared in our data: on the one hand, $\delta^{13}C$ was consistently higher in wash oases than in seep oases, where surface water tends to be more constant and its supply more reliable. On the other, there was a consistent trend in both wash and seep oases to increase towards the northern deserts, possibly as a result of increasing leaf-to-air vapor deficit. In short, our data suggest that, together with adaxial stomatal density, leaf thickness, and cuticular waxes, water use efficiency in *Washingtonia* palms tends to increase from the tropical southern edge of its distribution into the northern dry deserts.

3.5 Conclusion

Using a single genus along an environmental gradient in the coastal deserts of North America, we have shown that *Washingtonia* leaves receiving high rates of solar radiation and exposed to low water vapor pressure develop thick, waxy, isolateral leaves, with similar stomatal density on both sides and higher water use efficiency side than those in the southern, tropical and less arid environments, which show thinner, non-waxy, bilateral leaves with lower water use efficiency and occurring in sites with lower solar radiation and high water vapor pressure. As described by other authors for different desert taxa, these traits seem to vary simultaneously in *Washingtonia*. Like other desert plants, leaf xeromorphism, formed by thick, isolateral, amphistomatic, and waxy, vertically-oriented leaves with high water use efficiency seems to constitute a syndrome more than a set of independently-occurring traits.

Chapter 4. Reviving a century-old palm (*Washingtonia* H.Wendl.) mystery using the power of Genotyping by Sequencing (GBS)

4.1 Introduction

The advent of newer genomic technologies has allowed us to obtain a large number of molecular markers to address questions regarding the biogeographic history, population structure, phylogenetic relationships, and conservation of taxonomically challenging, non-model organisms in diverse groups such as birds (Toews et al., 2015; Winger et al., 2015), molluscs (Razkin et al., 2016), and plants (Massatti et al., 2016; Ahrens et al., 2017; Mu et al., 2017; Klimova et al., 2018; Haselhorst et al., 2019). However, despite 10% of American palm species constituting "species complexes" (Henderson et al., 1997), markers obtained through reduced-representation molecular techniques are only starting to be used for examining the population structure and systematics of the Arecaceae (Klimova et al., 2018).

Washingtonia is a North-American genus of widely cultivated diploid (n = 18) palms. High morphological variability initially led to the description of numerous species in the genus, many of which later became synonyms of the two currently recognized species *Washingtonia filifera* (Linden ex André) H.Wendl. or *W. robusta* H.Wendl. The genus is highly variable along Peninsular California presenting a taxonomic challenge (Henderson et al., 1997) which has only been partially clarified. The most recent study using single nucleotide polymorphisms (SNPs) obtained through Genotyping by Sequencing (GBS; Elshire et al. 2011) of the Mexican populations of *Washingtonia* found strong support for both currently recognized species (Klimova et al., 2018). However, these findings may not be robust given the omission of several populations found in the US. Besides, a morphological study of *Washingtonia* over its whole distribution range suggests the genus is composed of one highly variable taxon in Peninsular California (Villanueva-Almanza et al., 2018) providing support to the idea that *W. robusta* could be a variant of *W. filifera* (Nabhan, 1985).

Because species delimitation in *Washingtonia* has been problematic, accurate geographic distribution is missing and, at times, contradictory. According to the latest information on the genus, *W. filifera* occurs naturally in Arizona, southern California, and northern Baja California (Turner et al., 1995), while *W. robusta* is present in the Baja California peninsula from Cataviña at latitude 29°45’ (Bailey, 1936; Cornett, 1987b) to the Cape Region at 23° (Wiggins, 1980; Minnich et al., 2011), and in Sonora, mainland Mexico, where it has a very narrow distribution in the Sierra del Aguaje near Guaymas (28°) (Felger and Joyal, 1999). However, careful examination of bibliographic sources reveals there is no consensus on the northern limit of *W. robusta* (Fig. 4.1).

The problem in delimiting the geographic distribution of *W. robusta* is not recent. André (1900) mentioned: “the home of *W. robusta* is Baja California” apparently referring to northern Baja California, though the exact place is unclear. Clarifying the geographic limits of *W. robusta* also comes from a misinterpretation in modern literature. In *Los oa-*

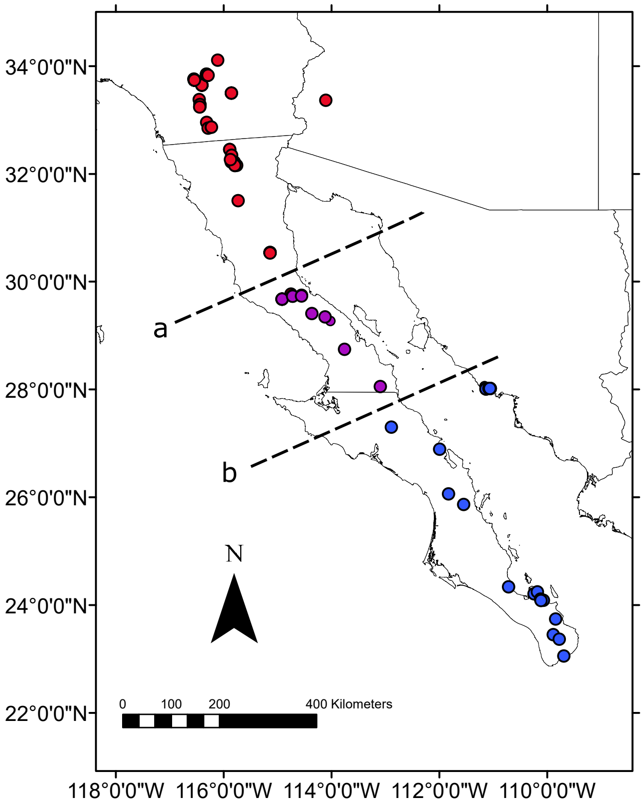

Figure 4.1: Major *Washingtonia* palm oases in the Baja California Peninsula, Arizona, and Sonora according to herbarium records and own collections.
Dashed lines mark distribution limits according to **(a)** Bailey (1936), Shreve and Wiggins (1964), Moran (1978), Wiggins (1980), Cornett (1987b), Minnich et al. (2011), Klimova et al. (2018) and **(b)** Arriaga et al. (1997), Felger and Joyal (1999), Zona (2000), Felger et al. (2001), León de la Luz et al. (2014). Purple area show oases where authors have divergently identified *Washingtonia* palms either as *W. filifera* or *W. robusta*

sis de la península de Baja California (Arriaga et al., 1997) —the groundbreaking work for the study of desert oases in the Peninsula—*W. robusta*'s distribution range is reportedly Baja California Sur. Though the authors acknowledge following the *Flora of Baja California*, Wiggins does not mention *W. robusta* only occurring in Baja California Sur (Wiggins, 1980), but rather *W. robusta*'s range extending as far north as Cataviña, in the northern state of Baja California, as others had previously reported (Bailey, 1936; Shreve and Wiggins, 1964; Moran, 1978). Some authors later accepted that *W. robusta* is only found in Baja California Sur (Felger and Joyal, 1999; Zona, 2000; Felger et al., 2001; León de la Luz et al., 2014), while others have left the distribution of *W. robusta* open to interpretation. The *Jepson Manual* and the *Field guide to the Palms of the Americas* ambiguously mention *W. robusta* being native to Baja California (Henderson et al., 1997; Simono, 2012). Understanding the natural distribution of *W. robusta* is further complicated by possible human dispersal since it is a widely cultivated palm in the Baja California peninsula, southern California, and around the world (Johnson and Group, 1996; Felger and Joyal, 1999). Regardless of where the northern limit of *W. robusta* is placed, the populations of *Washingtonia* are distributed more or less discontinuously as habitat "islands" within a "sea" of desert scrub along a 1300 km-range environmental gradient (Villanueva-Almanza et al., 2018). Southern populations experience dry subtropical climate with summer rains while northern populations are subject to arid temperate climate dominated by winter rains (Vogl and McHargue, 1966; Garcillán and Ezcurra, 2003). Despite both species apparently not overlapping, close proximity between the populations of *Washingtonia*, apparent synchronicity in flowering from May through June (Wiggins, 1980; Rebman and Roberts, 2012; Felger et al., 2001), and genetic compatibility between both species under cultivation (Ishihata and Murata, 1971; Hodel, 2014) would suggest there are no inherent barriers to gene flow. Species of *Washingtonia* are pollinated by insects (McClenaghan and Beauchamp, 1986) and the fleshy, edible fruits are dispersed mostly by coyotes (Vogl and McHargue, 1966; Felger et al., 2001) and foxes (Bullock, 1980).

We had previously offered two hypotheses to explain the observed morphological variation in *Washingtonia* palms. (a) *Washingtonia* is composed of two clearly distinguishable species at the periphery of their geographic range, one in the north and one in the south, that have differentiated in disjunction and have expanded to secondary contact resulting in a possible hybrid zone (secondary intergradation), which we called the "hybrid zone model"; and (b) *Washingtonia* is composed of one highly variable species clustered in local and patchy populations in desert oases that change gradually along a clinal continuum (primary intergradation) which we referred to as "clinal model". Our morphological results showed the vegetative traits vary continuously along the latitudinal cline in Peninsular California giving support to the clinal model hypothesis. Therefore, the goals of this chapter are: (1) determine if morphological and genetic markers reveal similar patterns by examining if there is a hybrid zone connecting northern and southern populations, (2) identify the population structure along the latitudinal cline of *Washingtonia* and determine if there is an association with environmental factors and whether it corresponds to the geographic distribution of currently recognized species, and (3) discuss the nomenclature of *Washingtonia*.

4.2 Materials and Methods

Sample collection

We collected 174 leaf samples of *Washingtonia* from 18 natural populations in Mexico and the US between March 2017 and October 2017 (Table 4.1). We also included 10 samples from the Bailey Hortorium Herbarium at Cornell University (BH). Using herbarium material was the only way to include populations which were not visited during fieldwork due to inaccessibility (Guadalupe and Tajo canyons). We also included 4 samples of plants grown in the greenhouse from seeds collected in San Javier, Baja California Sur, Mexico in 2015. All together, these collections cover the entire range of *Washingtonia*. Fresh material from the field and cultivated specimens was stored in silica gel until DNA extraction. We did not assign taxonomic determination since we were interested in elucidating phylogenetic relationships among populations. Four samples of *Brahea armata* S.Watson cultivated at the University of California Riverside campus were used as an outgroup.

We downloaded 19 bioclimatic variables from WorldClim version 2 with a resolution of 30 arc-seconds (~1 km) (Fick and Hijmans, 2017) as a set of rasters from where bioclimatic variables were extracted using R version 3.3.3 (R Development Core Team, 2017) and code by Turner (2013).

Table 4.1: Location of populations of *Washingtonia* sampled across Peninsular California, Arizona, and Sonora, Mexico

Source	Locality	Acronym	n	Lat. Long. (d.d)
Field	San José del Cabo	SJD	9	23.05, -109.68
	Sierra Cacachilas	CACA	10	24.08, -110.11
	San Juan de la Costa	SJCOSTA/ROFO	10	24.33, -110.71
	Comondú	COMO	10	26.06, -111.82
	Mulegé	MULE	10	26.88, -111.98
	San Ignacio	SNIGN/ALA	10	27.29, -112.88
	Nacapule	NACA	10	28.01, -111.05
	Gertrudis	GER	10	28.05, -113.08
	San Francisco Borja	BOR	8	28.74, -113.86
	Cataviña	CATA	11	29.73, -114.72
	Bocana	BOCA	10	29.67, -114.91
	Mission Santa María	STAMA	11	29.74, -114.55
	Cañón Berrendo	BERRE	10	30.53, -115.13
	Cañón Palomar	PALO	9	31.92, -115.71
	Hellhole Canyon, Anza Borrego	HELL/ANZA	7	33.23, -116.44
	Palm Canyon, Kofa Mountains	KOFA	9	33.36, -114.09
	Palm Canyon, Agua Caliente Indian Reservation	PALM	10	33.73, -116.53
	49 Palms, Joshua Tree	JOSH	10	34.10, -116.10
Herbarium	Cañón Guadalupe	GUA	1	32.15, -115.79
	Cañón Tajo	TAJO	3	32.26, -115.88
	Sunset Blvd., San Diego	CULT	1	32.75, -117.18
	Gaskills Tanks	GAS	2	32.45, -115.88
	Cataviña	CATA	2	NA
	Palm Canyon, Kofa Mountains	KOFA	1	33.36, -114.09
Greenhouse	Mission San Javier	JAV	4	25.86, -111.54
Cultivated	UCR Lot 4	BRA	4	33.97, -117.33

DNA extraction and library preparation

Individual samples were randomized into two plates, each corresponding to the pools of final libraries sequenced. Total genomic DNA was extracted using a modified CTAB method (Doyle and Doyle, 1987) from the 192 samples. DNA was checked for degradation using a 1.5% agarose electrophoresis gel. Samples were standarized following quantification using the Quant-It PicoGreen assay (Thermofisher, catalog number P11496). Libraries were prepared following (Rowan et al., 2017). Each DNA extract was digested using the restriction enzyme KpnI to which sequence adapters and sample-specific barcodes were ligated for sample identification. A total of 96 unique barcodes were used. All samples were sequenced at the University of California Davis using 1×150 Illumina HiSeq4000, one library per lane.

Bioinformatics analysis

After sequencing, raw reads in FASTQ format were processed using Stacks software version 2.3 (Catchen et al., 2013). Pooled reads were separated by unique barcodes and trimmed to a uniform read length of 125 bp due to lower read quality towards the end using the program process_radtags. Demultiplexed data were assembled using both *de novo* and reference-based methods using the date palm (*Phoenix dactylifera* L.) genome as a reference since *Washingtonia*'s genome is not available and date palm is the closest palm to *Washingotnia* whose genome has been sequenced. All analyses were performed using a computer cluster from the UCR High-Performance Computing Center.

de novo and reference-based SNP discovery

For the *de novo* assembly, the wrapper program denovo_map.pl was used to identify SNPs. Optimal parameters were identified by running the denovo.pl wrapper several times following the suggestions of Paris et al. (2017) and Rochette and Catchen (2017). For all runs, the minimum number of raw reads required to create a stack in ustacks remained fixed (m = 3), but the number of nucleotides between alleles (M) varied from 2 to 3, and the number of mismatches allowed between loci for building the catalog in cstacks (n) varied from 1 to 4. These various settings resulted in different numbers of loci and SNPs, but our final data set was obtained by setting M = 2 and n = 2. Our decision followed Paris et al. (2017) of using low values for M and n parameters if the genome shows low levels of polymorphism as has been reported for *Washingtonia* (McClenaghan and Beauchamp, 1986; Klimova et al., 2018). Data was filtered using VCFtools version 0.1.15 (Danecek et al., 2011) to require a minimum allele frequency of 0.05 and to remove loci with more than 30% missing data, monomorphic loci, samples with at least 50% missing data and two samples with negative inbreeding coefficients and unusually high read depths suggestive of contaminated sequencing.

The SNP set was also pruned for linkage disequilibrium using Plink version 1.90b3.38 (Purcell et al., 2007) with a 5,000 bp sliding window and a r^2 value of 0.5. The final SNP data set was then re-read into Stacks using the populations command to generate the necessary input files for downstream analyses.

Prior to calling SNPs in Stacks for the reference guided approached, reads for each individual were mapped to the date palm reference using BWA-MEM (version 0.7.12; Li 2013) with associated read groups. Created SAM (Sequence Alignment Map) files were converted to BAM (Binary Alignment Map) and then sorted using Samtools (version 1.3; Li et al. 2009). Sorted BAM files were then read into the ref_map.pl wrapper of Stacks using default parameters and exported with the populations command. Filtering was done in VCFtools using the same parameters as for the *de novo* SNP calling and LD pruned with Plink. The final VCF file was then re-read to produce the appropriate files for downstream analyses.

Analysis of population structure

Statistical analyses were performed with the R version 3.3.3 (R Development Core Team, 2017) using RStudio environment version 1.0.136 (RStudio Team, 2016). Pairwise F_{ST} values were calculated using the StAMPP package version 3.5.3 (Pembleton et al., 2013) with 100 bootstrap replicates. The *de novo* data set with 21,746 SNPs was used to calculate Nei's genetic distance among individuals through the R package poppr (Kamvar et al., 2014). The genetic distance matrix was then used to construct UPGMA (Unweighted Pair Group Method with Arithmetic means) and NJ (Neighbor-Joining) dendrograms. Alternatively to the genetic distance dendrograms, we used Non-Metric Multidimensional Scaling (NMDS) using Nei's genetic distance matrix through the isoMDS function of the MASS package in R version 7.3-50 (Venables and Ripley, 2002) to visualize genetic similarity of sampled individuals. Genetic distance was plotted in a 2-dimensional space.

Multivariate analysis

Principal Component Analysis (PCA)

In order to summarize the genetic variation within *Washingtonia* we performed a centered and non-centered PCA of the individuals × SNPs matrix using the glPCA algorithm from the adegenet version 2.1.1 R package (Jombart and Ahmed, 2011). We decided to perform non-centered PCA because this type of data transformation reveals discontinuities in a sample better than centered PCA making it more appropriate for identifying taxonomic structure (Feoli, 1977). We used the Broken-Stick distribution to test for significance of the axes (Jackson, 1993). Since the first and second axes are related by a quadratic distribution known in multivariate analyses as the "arch effect" (Novembre and Stephens, 2008; Matthew, 2019), we merged both axes following Hill and Gauch (1980) by detrending and rescaling the arms of the "U" shape data cluster projecting the data points on the reduced major axis of each arm. We then regressed the PC scores against latitude using the linear and logistic models corresponding to our two hypotheses (Villanueva-Almanza et al., 2018). For each model we calculated both the Akaike Information Criterion (AIC) and the F-values of the fitted model.

ANOVA of non-centered PCA scores

Generalized linear models were used to test for associations between the non-centered

PCA dominant axis against latitude and site, as independent predictors. This allowed us to see how all SNPs combined varied significantly with latitude, and what proportion of the residual variance was explained by the fixed, latitude-independent effect of sites. We also used simple linear regression with each bioclimatic variable as a predictor to test its association with the same dominant PCA axis mentioned above.

Mixed distribution model

When viewed as a histogram, the PC scores revealed a clear multi-modal pattern formed by a mixture of distributions. In order to find the number of distributions and the parameters that best described the first axis, we used a Hooke Jeeves direct search method (Himmelblau, 1972) implemented in compilable QBX-Basic. First, the number of *k* bins used to fit the models was calculated by the 2k rule. We then fit five different distribution models: (1) sample frequency along the first PC axis is constant (null model), (2) sample frequency had a normal distribution, (3) sample frequency had a bi-modal distribution and so forth. To compare distributions, we used the observed and expected frequencies, as well as the log likelihood deviance and significance of the residuals of each model. On the other hand, we used the AIC to determine the optimal number of modes in the mixed distribution model. The partitioning of individuals into populations was calculated as the area under the curve of the best-fitting model.

Chi-square tests on two-way contingency-tables were performed to examine the concordance between the classifications obtained from the mixed distribution model and the genetic distance dendrograms (UPGMA and NJ). χ^2 statistics were used to test the null hypothesis that there is no significant association between classification methods. We then calculated the Cramér's V statistic to test the strength of association between both classification methods (Cramér, 1999). Cramér's V statistic is used to measure the association between nominal variables and is equivalent to the coefficient of determination used for continuous variables. The values range from 0 to 1, with 0 indicating no relation and 1 indicating a perfect association.

Bayesian coalescent analysis

Structure

Population structure was implemented in the R package LEA (Frichot and François, 2015). Determination of best K was done using cross-entropy criterion from 1-25. Barplots from K 1-12 were generated.

TreeMix

Using both the filtered SNP set via the *de novo* approach and the reference guided approach, historical migration events were inferred using TreeMix version 1.13 Pickrell and Pritchard (2012). For each data set, 0-10 migration events were tested, and the optimal value was determined using a likelihood ratio test in BioGeoBEARS version 0.2.1 (Matzke, 2013). Results for the optimal number of migration events was plotted in R using the plotting_funcs.R script packaged with the TreeMix software. For the reference guided approach, the *Brahea* population was set as the outgroup. For the *de novo* analyses, NACA was set as the outgroup based on the positioning of this population in the distance dendrogram and SNAPP tree (see below).

SNAPP

Species tree inference was done using SNAPP (SNP and AFLP Package for Phylogenetic analysis) (Bouckaert et al., 2019). Due to the computationally intensive nature of the program, three individuals from each population were included when available, except for SNIGN, which all samples were included due to the polyphyletic nature of the population. The SNP data set was further pruned using vcftools max-missing command with a value of 0.97. The VCF file was then converted to a nexus format using the vcf2phylip program (Ortiz, 2019). Due to the non-monophyletic nature of SNIGN, multiple runs were performed to test the relationships with this population and all other populations. The first run consisted of all populations except SNIGN and contained 57 individuals, the second analysis had three accessions of SNIGN constrained as monophyletic, and consisted of 60 individuals total. The third analysis consisted of a single population of SNIGN represented by SNIGN 13 based on the distance dendrogram; this data set had 58 individuals. The fourth analysis consisted of two groups of SNIGN, one represented by SNIGN 13 and the second represented by SNIGN 12 and 18. The fifth analysis consisted of three populations of SNIGN, the two previously described and a third with SNIGN 10, 14, and 15. All five data sets were ran for 10 million generations with default parameters to achieve large enough ESS values. Trees were visualized with DensiTree version 2.0, part of the BEAST 2.5.1 package (Bouckaert et al., 2019).

4.3 Results

Bioinformatics and SNP discovery

We performed GBS on 188 *Washingtonia* samples and 4 of *Brahea* resulting in 649,071,324 high-quality reads. From these data, 368,507 variant sites were called using the *de novo* pipeline and 177,567 variant sites called with the reference based approach. We removed 17 individuals having at least 50% missing data (13 of *Washingtonia* and 4 of *Brahea*) and two *Washingtonia* samples with unusually high read depths (Palm19 and Ger17). Mean read depth was 16.627× with values ranging from 8.08×–34.75×. After filtering, our final data set was composed of 173 *Washingtonia* individuals and 21,746 SNPs. For the reference-based approach, from the initial 177,567 variants called, our final data set consisted of 13,459 high quality SNPs. Mean read depth was 17×, with values ranging from 5.1×–38.8× for 180 individuals. In the *de novo* approach the *Brahea* samples were removed due to high missing data. In the reference-based approach, all four samples were kept, with a median SNP call of 8,600 out of 13,459 SNPs and a median read depth of 25.2×. Four additional samples (GAS1, GAS2, JAV3, and COMO16) that were removed in the de novo approach were retained in the reference guided approach, albeit with less than 10,000 SNPs each.

Population structure

Our results from multivariate analysis, including PCA and NMDS, and Bayesian coalescent methods showed great consistency in the discovery of four genetic groups: (1) Southern Peninsular: San José del Cabo, Sierra Cacachilas, San José de la Costa, San Javier, Comondú, and Mulegé; (2) Mid Peninsular: San Ignacio, Santa Gertrudis, San Francisco Borja, Bocana, Cataviña, Santa María Mission, and Berrendo; (3) Northern Peninsular: Palomar, Guadalupe, Tajo, Anza, Palm Canyon, Joshua Tree, Kofa; and (3) Sonoran: Nacapule Canyon. The geographic limits to these differentiated regions are shown in Fig. 4.2.

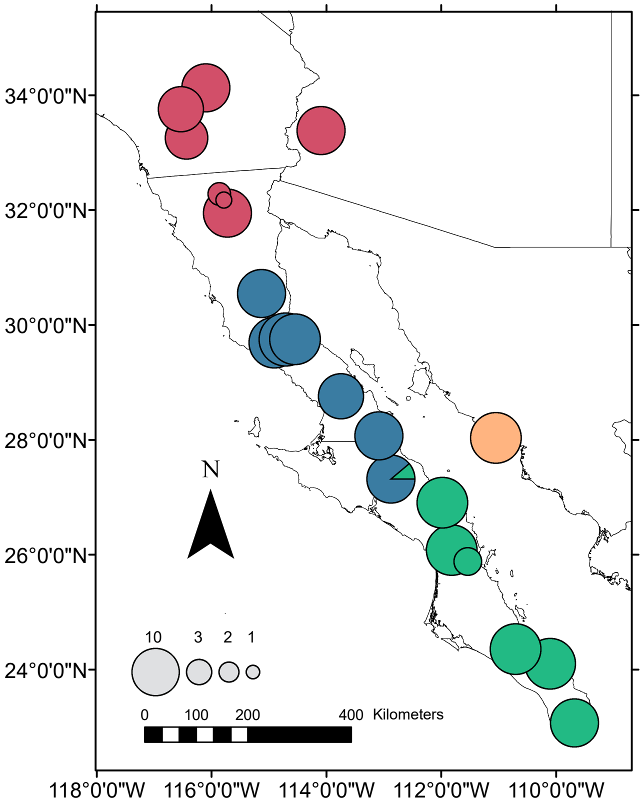

Figure 4.2: Genotype frequency distribution of the major *Washingtonia* palm oases in the Baja California Peninsula, Arizona, and Sonora.

Geographic distribution of sampled populations and their genotypes derived from Nei's genetic distance and the UPGMA algorithm calculated using 21,746 SNPs via GBS using a *de novo* approach. The area of each pie slice occupied by that color represents the proportion of individuals sharing the same genotype.

Pairwise F_{ST} values among populations ranged from 0.001 between Joshua Tree and Anza Borrego to 0.96 between Anza Borrego and Nacapule (Table 4.2). In fact, Nacapule has the highest F_{ST} values ranging from 0.68–0.96 with average 0.88. F_{ST} values between northern and southern peninsular populations were above 0.81. Populations within the same region had F_{ST} values ranging from 0.001–0.7. In addition, the NJ dendrogram of genetic distances showed high population structure within regions except for San Ignacio and Santa Gertrudis.

Table 4.2: F_{ST} estimates of genetic differentiation among *Washingtonia* populations

	SNIGN	BERRE	BOCA	BOR	CACA	CATA	COMO	GER	GUA	ANZA	JAV	JOSH	KOFA	MULE	NACA	PALM	PALO	SJCOSTA	SJD	STAMA	TAJO
SNIGN	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
BERRE	0.33	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
BOCA	0.26	0.39	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
BOR	0.22	0.45	0.32	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
CACA	0.57	0.82	0.77	0.78	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
CATA	0.23	0.34	0.16	0.27	0.74	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
COMO	0.54	0.81	0.76	0.77	0.47	0.73	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
GER	0.23	0.50	0.40	0.36	0.80	0.35	0.79	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
GUA	0.40	0.75	0.62	0.64	0.84	0.57	0.82	0.71	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
ANZA	0.62	0.84	0.74	0.78	0.90	0.71	0.88	0.81	0.70	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
JAV	0.46	0.82	0.74	0.75	0.49	0.71	0.32	0.79	0.86	0.94	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
JOSH	0.66	0.85	0.77	0.81	0.91	0.73	0.90	0.83	0.69	0.00	0.94	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
KOFA	0.65	0.85	0.77	0.80	0.90	0.73	0.89	0.83	0.39	0.71	0.94	0.70	NA	NA	NA	NA	NA	NA	NA	NA	NA
MULE	0.59	0.84	0.79	0.80	0.35	0.76	0.53	0.82	0.87	0.91	0.56	0.92	0.92	NA	NA	NA	NA	NA	NA	NA	NA
NACA	0.69	0.89	0.83	0.86	0.85	0.81	0.83	0.87	0.96	0.97	0.90	0.97	0.96	0.87	NA	NA	NA	NA	NA	NA	NA
PALM	0.65	0.85	0.77	0.80	0.91	0.73	0.90	0.83	0.75	0.02	0.95	0.02	0.73	0.92	0.97	NA	NA	NA	NA	NA	NA
PALO	0.66	0.86	0.78	0.81	0.91	0.74	0.90	0.84	-0.01	0.74	0.95	0.73	0.45	0.92	0.97	0.76	NA	NA	NA	NA	NA
SJCOSTA	0.54	0.80	0.75	0.76	0.36	0.72	0.34	0.78	0.80	0.87	0.38	0.89	0.88	0.39	0.82	0.89	0.89	NA	NA	NA	NA
SJD	0.53	0.80	0.75	0.76	0.45	0.73	0.40	0.78	0.81	0.88	0.20	0.89	0.89	0.50	0.83	0.89	0.90	0.39	NA	NA	NA
STAMA	0.15	0.33	0.23	0.17	0.75	0.19	0.74	0.25	0.56	0.72	0.71	0.75	0.75	0.77	0.82	0.75	0.76	0.73	0.73	NA	NA
TAJO	0.45	0.77	0.64	0.67	0.85	0.60	0.83	0.73	-0.05	0.73	0.88	0.71	0.43	0.88	0.96	0.78	0.05	0.82	0.83	0.60	NA

Multivariate analysis

Principal Component Analysis (PCA) and Principal Components Regression

Principal components analysis of SNP data found four significant axes that account for 70.5% of the total variance. The fourth axis is only slightly significant (0.046 vs 0.022 broken stick) and explains genetic variation unique to Nacapule (Fig. 4.3).

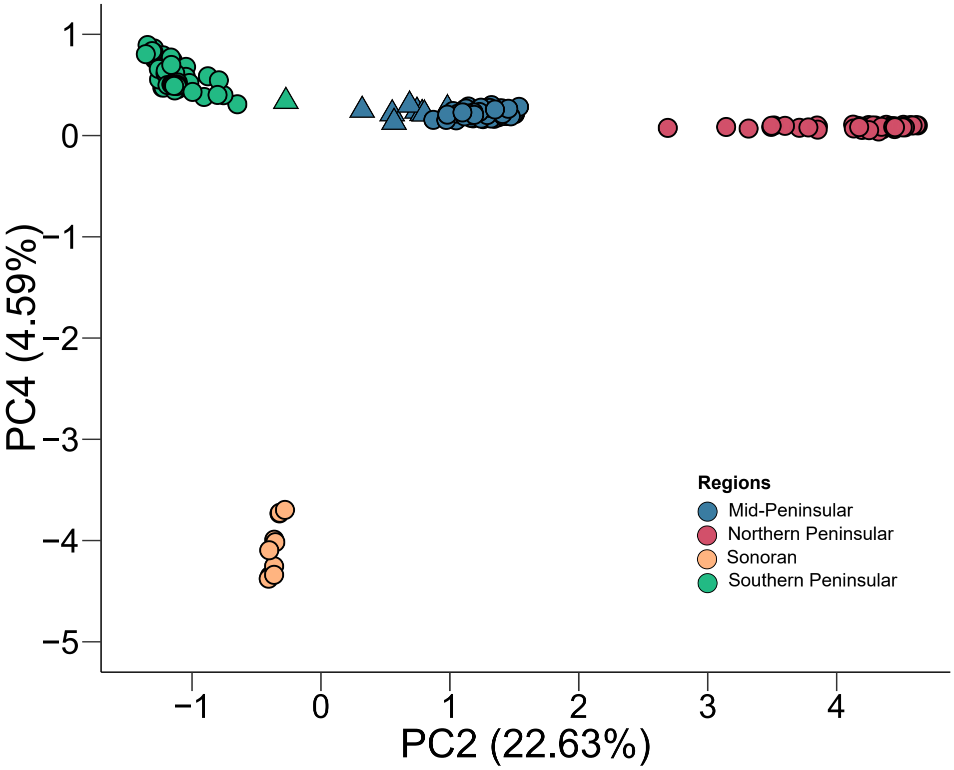

Figure 4.3: PC4 of non-centered detrended PCA showing unique genetic variation in Nacapule

Populations color coded by their genotype following UPGMA classification. Triangles are individuals from San Ignacio.

Table 4.3: Comparison of the clinal model (linear model) ANOVA and the hybrid zone model (non-linear model) ANOVA of the first informative axis of the Principal Component Analysis obtained from SNP data

PCA scores	Fit method	r^2	AIC	F
Centered	linear	0.87	459.98	1101.17
Centered detrended	linear	0.87	476.4	1126.44
Non-centered	linear	0.88	376.62	1226.23
Non-centered detrended	linear	0.89	389.71	1316.53
Centered	non-linear, hyperbolic tangent	0.89	427.89	460.11
Centered detrended	non-linear, hyperbolic tangent	0.89	448.2	458.66
Non-centered	non-linear, logistic	0.88	380.66	403.96
Non-centered detrended	non-linear, logistic	0.89	393.75	433.71

The second detrended axis of the non-centered PCA was very strongly correlated with latitude ($r^2 = 0.89$). The regression analysis showed the simple linear model provided a better fit than the logistic model (Table 4.3) implying that there is a gradual change in genotype along the latitudinal cline (ANOVA, $F_{1,171} = 1316.53$, $P < 0.0001$; Fig. 4.4).

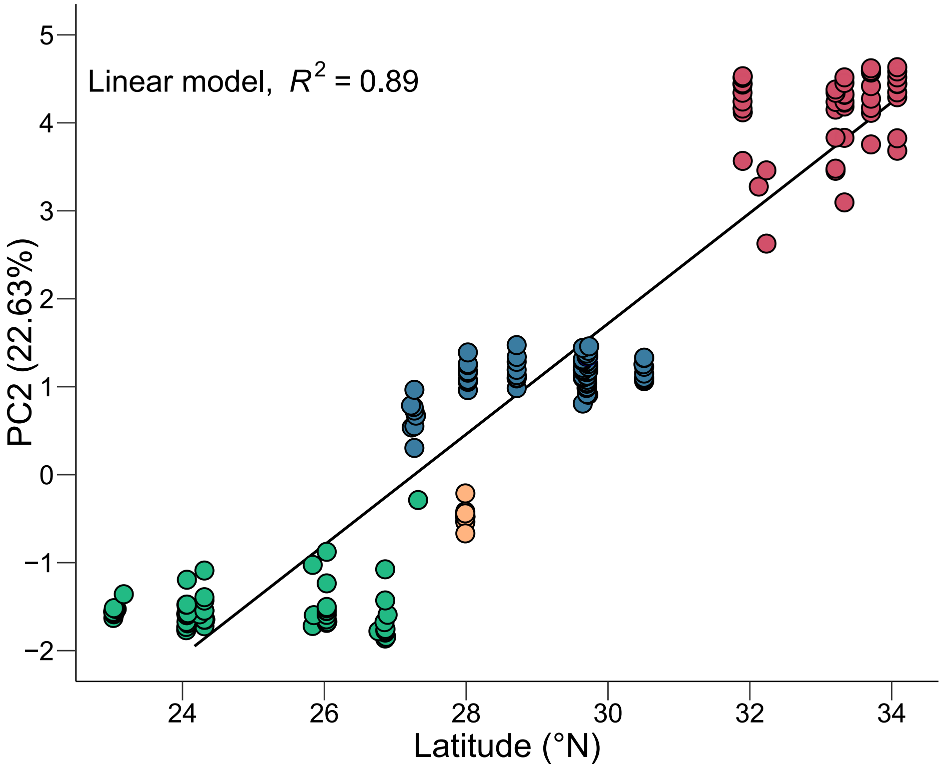

Figure 4.4: Principal Component Regression (PCR) of detrended PC2 obtained through non-centered PCA against latitude.

Logistic curve not shown. Populations and individuals are color-coded by inferred phylogeographic regions from UPGMA genetic distance dendrogram. Color codes. Green: Southern Peninsula; blue: Mid-Peninsula; pink: Northern Peninsula; yellow: Sonora.

Both linear and non-linear models give the same r^2 value (0.885), but the F-value of the logistic model was penalized for using four parameters to fit the data, instead of the two used in the simple linear model. The AIC estimator was also smaller than that of the non-linear model providing further support for the linear model. Although latitude, as a predictor, yielded higher and more significant F-values, there was a significant fixed effect

Table 4.4: ANOVA of linear model of latitude and site as main effects against SNP data

	SS	df	MS	F	P
Latitude	724.8749	1	724.87	11964.68	<0.0001
Site	85	20	4.25	70.15	<0.0001
Residuals	9.15	171	0.05		
Total	819.02	172			

of site on SNP variation. The generalized linear model with latitude and site as predictor variables had a r^2 value of 0.98 (Table 4.4).

Precipitation during the driest quarter, mean temperature during the coldest quarter, and precipitation seasonality were the the bioclimatic predictors that had the strongest association with SNP variation as evidenced by their high F-values.

Non-metric multidimensional scaling (NMDS)

The ordination plot revealed a strong grouping of individuals by region (Fig. 4.5).

Mixed distribution model

The histogram of the PC2 scores was best fit using a four-modal frequency distribution showing that *Washingtonia* populations have four distinct genotypes each with varying number of individuals (Fig. 4.6). The Southern Peninsular class is composed of 56 individuals from 7 populations; the Mid-Peninsular region comprises 61 individuals from 7 populations; the Northern Peninsular class includes 48 individuals from 7 populations; and the Sonoran region includes 8 individuals from one population. Nacapule is the only

Table 4.5: ANOVA of linear models of bioclimatic variables as independent predictors against SNP data. Traits ordered by ascending significance level.

Bioclimatic variable	df	*F*	*P*	r^2
Prec. driest qt.	1	390.16	***	0.70
Mean temp. coldest qt.	1	370.19	***	0.68
Prec. seasonality	1	290.23	***	0.63
Prec. coldest qt.	1	277.73	***	0.62
Min. temp. coldest month	1	231.01	***	0.57
Isothermality	1	206.37	***	0.55
Temp. seasonality	1	192.54	***	0.53
Prec. warmest qt.	1	138.10	***	0.45
Mean temp. wettest qt.	1	121.72	***	0.42
Annual mean temp	1	112.13	***	0.40
Temp. annual range	1	105.98	***	0.38
Prec. driest month	1	101.37	***	0.37
Prec. wettest month	1	53.97	***	0.24
Prec. wettest qt.	1	39.63	***	0.19
Annual precipitation	1	8.14	0.0048	0.05
Mean temp. warmest qt.	1	4.61	0.0331	0.03
Mean diurnal range	1	4.55	0.0342	0.02
Max. temp. warmest month	1	1.50	0.2214	0.00
Mean temp. driest qt.	1	0.13	0.7119	0.00

Note: *** P <0.0001

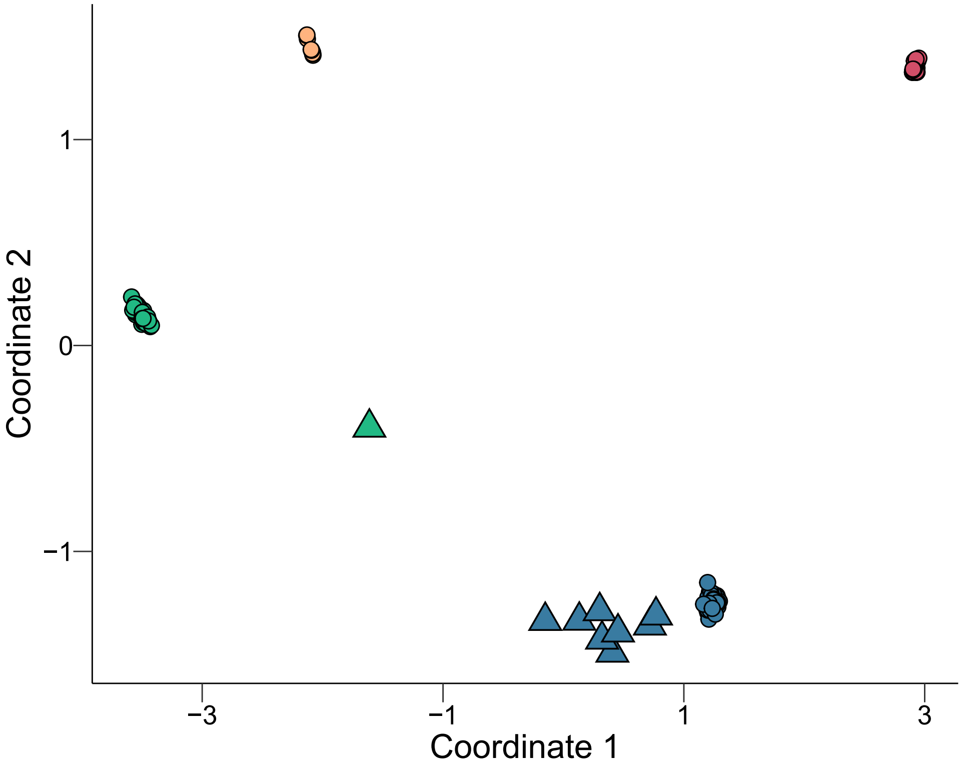

Figure 4.5: Ordination plot of Nei's genetic distance along non-metric multi-dimensional scaling (NMDS) coordinate 1 and coordinate 2.
Triangles are individuals from San Ignacio. Populations are color-coded by inferred phylogeographic regions from UPGMA genetic distance dendrogram. Color codes. Green: Southern Peninsula; blue: Mid-Peninsula; pink: Northern Peninsula; yellow: Sonora.

population whose individuals belong to two different classes in the frequency distribution: Sonoran and southern peninsular.

All comparisons between the classification methods used yielded significant correlations (Table 4.6). The correlation was between UPGMA and the mixed distribution model (χ^2 = 455.46, df = 15, P = 0.000000; *Cramér's V* = 0.94), where a single individual from San Ignacio was included in the Southern Peninsular region in the UPGMA or in the

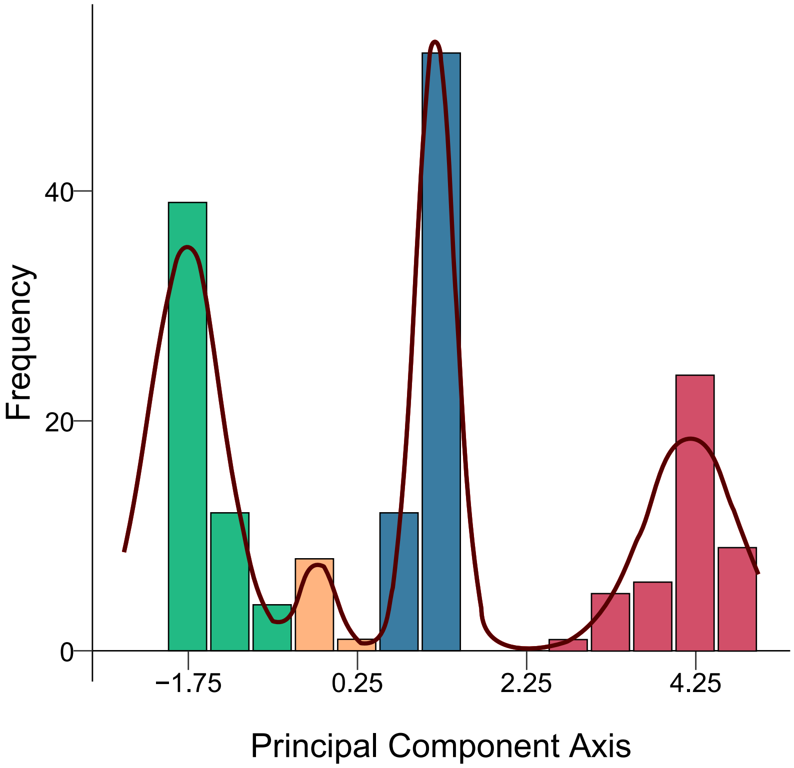

Figure 4.6: Histogram of individuals present in each genotype class calculated as the area under of the curve of the cumulative frequency distribution.

Frequency distribution of PC2 scores were obtained from a non-centered PCA of 21,746 SNPs from 173 individuals of *Washingtonia* of 21 populations. Four-modal density curve adjusted to the distribution frequency. Populations are color-coded by inferred phylogeographic regions. Color codes. Green: Southern Peninsula; blue: Mid-Peninsula; pink: Northern Peninsula; yellow: Sonora.

Mid-Peninsular region in the mixed distribution model. Concordance between NJ and the mixed distribution model was also very high (χ^2 = 479.27, df = 19, P = 0.000000; *Cramér's V* = 0.96). Discrepancies between NJ and the mixed distribution method came from three populations; Nacapule, from the Sonoran region, Santa Gertrudis and San Ignacio, from the Mid-Peninsula. Neither Santa Gertrudis nor San Ignacio nested with any of the main geographic regions in the genetic distance dendrogram and are consid-

Table 4.6: Correlations expressed as *Cramér's V* between classification methods of *Washingtonia* genotypes

	Mixture	UPGMA	NJ
Mixture	–	0.000000	0.000000
UPGMA	0.94	–	0.000000
NJ	0.96	0.99	–

ered as "unplaced" in the NJ dendrogram. However, all individuals of both populations are included in the Mid-Peninsular class of the distribution frequency. Nacapule, on the other hand, comprises a unique group based on genetic distance, while two individuals are grouped in the Southern Peninsular group in the distribution frequency.

Bayesian coalescent analysis

For the *de novo* approach cross-entropy criterion reached a plateau when the number of ancestral populations (K) increased from 1 to 4 indicating that genetic contribution from 4 ancestral populations of *Washingtonia* optimally predicts all genotypes (Fig. 4.7). While for the reference-based approach the plateau was reached at K = 5. Of the nine individuals from San Ignacio included in the admixture analysis, all show levels of mixed ancestry between the Mid-Peninsular and the southern-Peninsular regions ranging from 0.1–0.6.

Figure 4.7: STRUCTURE results for 13,459 SNP dataset for K = 4 obtained through a reference based approach. Each column represents a single individual. The length of each colored segment is the proportion of admixture from each of the K regions. Individual names color coded according to their classification in the UPGMA dendrogram. Color codes. Green: Southern Peninsula; blue: Mid-Peninsula; pink: Northern Peninsula; yellow: Sonora.

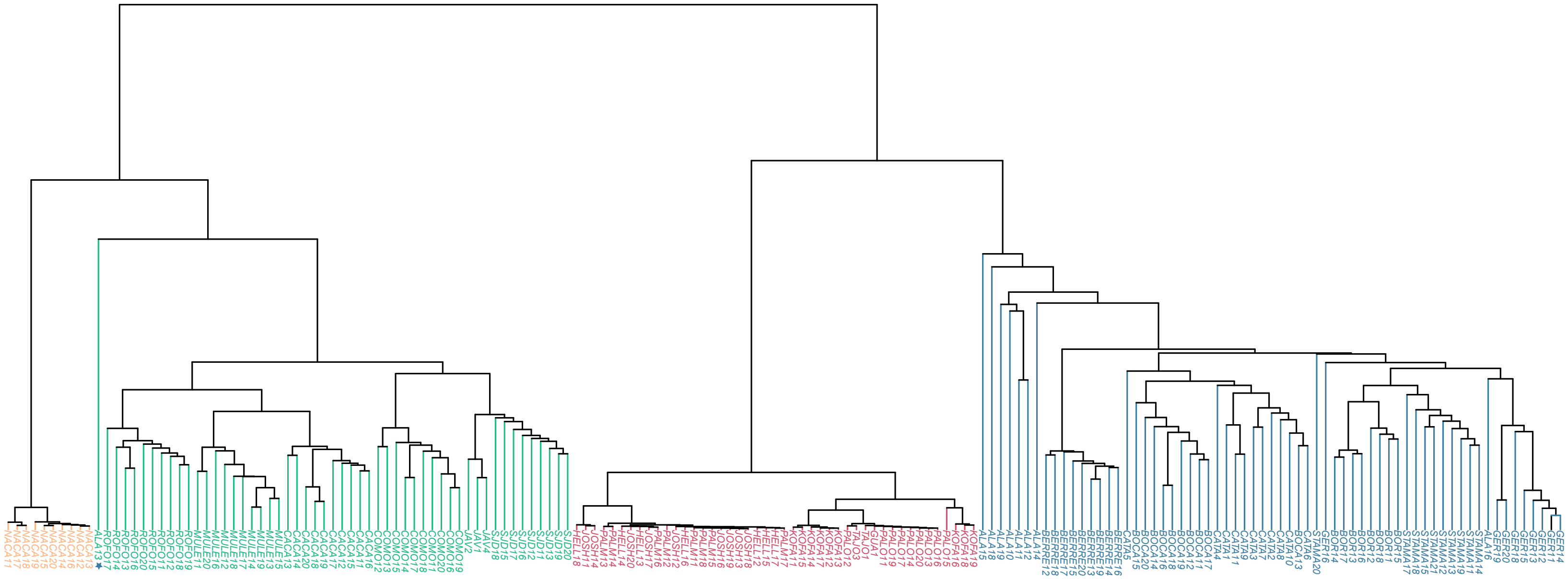

Figure 4.8: UPGMA dendrogram based on Nei's genetic distance obtained *de novo* from 21,746 SNPs. Starred individual shows admixture between southern and mid-peninsular regions. Color codes. Green: Southern Peninsula; blue: Mid-Peninsula; pink: Northern Peninsula; yellow: Sonora.

TreeMix

For the reference based approach, two migration events were favored over three events (*P*=3.21e-14), but three events was not favored over four (*P*=1). For the *de novo* approach, a single migration event was preferred compared to two events (*P*=7.99e-22). Both data sets suggest the migration event involving JAV/COMO and GER, likely resulting in the formation of SNIGN, but the *de novo* approach is missing the inferred migration event of the Northern-Peninsular populations to *Brahea*, since *Brahea* was removed from that data set. The tree with the best log-likelihood contained the subtree composed of the four phylogeographic regions proposed here (Fig. 4.9).

SNAPP

The SNAPP results highlight the fact that individuals from San Ignacio are polyphyletic. When no individuals are included, the resulting tree is simlar to the genetic distance dendrogram with the existence of three clearly distinguishable groups and one group (Nacapule) as sister to the southern peninsular region. The mid-peninsular populations and northern-peninsular populations share a more recent common ancestor with each other than they do with the southern peninsular populations. A similar pattern is observed with only one population of SNIGN (SNIGN 13), with that individual now sister to all the other populations. However, when two or three populations of SNIGN are added, and especially when they are treated as monophyletic, the broad relationships between popula-

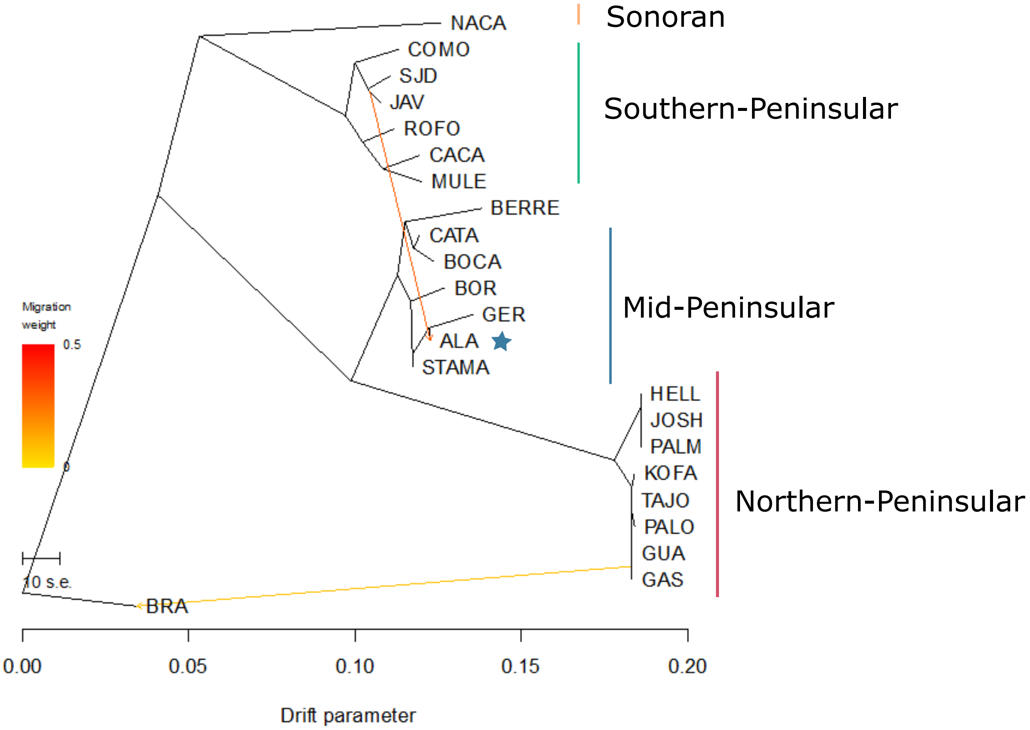

Figure 4.9: TreeMix analysis with one migration event.
Brahea armata used as the outgroup. *Washingtonia* populations included are listed in table 4.1. The starred population indicates individuals at this site show admixture between southern and mid-peninsular regions. The scale bar shows ten units of standard error (s.e.), and the amount of drift is plotted along the x axis.

tions change. Now the southern- and mid-peninsular populations are more closely related to each other than they are to the northern-pensinsular populations.

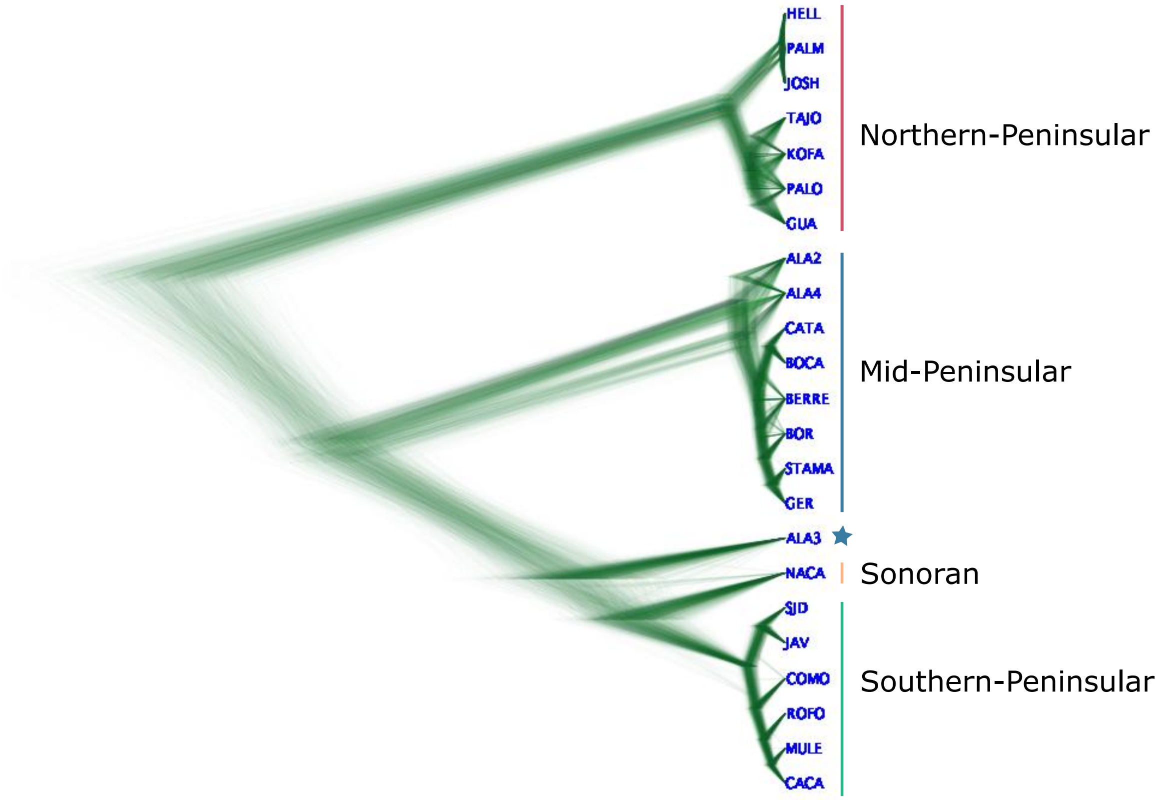

Figure 4.10: Phylogenetic relationships of *Washingtonia* from the Baja California Peninsula, Arizona, and Sonora according to Bayesian methods.
Colored lines are proposed phylogeographic regions. Starred individual shows admixture between southern and mid-peninsular regions.

4.4 Discussion

Population structure and phylogenetic relationships

Our results of multivariate (Figs. 4.5, 4.6) and Bayesian coalescent methods (Figs. 4.7, 4.10, 4.9), as well as those of population statistics (Fig. 4.8) consistently show four well-differentiated genetic regions that do not follow current species boundaries. The breaks between regions are approximately at 31°N between the northern and the mid-peninsular regions, at 27°N between the mid- and southern peninsular regions, and in the Sea of Cortez between the Nacapule and the Peninsular populations (Fig. 4.2). Of the three peninsular genotypes identified in this study, two are consistent with vicariant events proposed for the Peninsula. Our results show that palms in Nacapule comprise a genetically unique group as had been previously shown (Klimova et al., 2018). Divergence between Nacapule is evidenced by the high F_{ST} values of pairwise comparisons with peninsular populations (Table 4.2). Genetic differences between palms in mainland Mexico and peninsular California are the result of the separation of the Peninsula from mainland Mexico about 5.5 million years ago (Riddle et al., 2000).

The phylogeographic break between the mid- and southern-peninsular regions at latitude 27 °N is consistent with a putative seaway near the Vizcaíno Desert that existed ca. 1 Ma isolating northern Baja California from the rest of the Peninsula (Upton and Murphy, 1997). This seaway has been used to explain genetic differences in other groups of plants such as species in the Euphorbiaceae and Viscaceae (Garrick et al., 2009; Lira-Noriega et al., 2015).

Although we suspected the existence of a hybrid zone between latitude 28 °N and 29.7 °N encompassing the area between Santa Gertrudis and Cataviña, respectively (Fig. 4.1), we did not find evidence of admixture except in San Ignacio, where all individuals show genetic influence of Southern Peninsular and Mid-Peninsular regions (Fig. 4.7). San Ignacio has been a Mission since 1728 and our sampling site is close to the town making introduction of plant material from the south highly likely as our TreeMix results suggest (Fig.

4.9). The area has also been subject of species turnover studies in the context of environmental factors. While the north and the south of the Peninsula experience rainfall either during winter or summer, respectively, sites at approximately 27 °N like San Ignacio and Nacapule, experience bi-seasonal rainfall (Turner et al., 1995). Thus, environment could likely be playing a role in the genetic strucuture of these populations.

The phylogeographic break at latitude 31 °N separating the northern-peninsular from the mid-peninsular regions does not coincide with any vicariant event. However, high pairwise F_{ST} values between populations at the edges of these two inter-regional breaks would suggest little genetic flow which cannot be explained by linear distance alone. For example, F_{ST} values between Berrendo and Palomar is 0.85 despite being 163 km apart. In contrast, F_{ST} between San Juan de la Costa and San José del Cabo, found 176 km apart is 0.39. This suggests gene flow is not random, but rather that there is clear genetic distinctiveness in these regions. Although this could be the result of limited pollen dispersal, unsuccessful colonization, asynchronous phenology, or historic bottlenecks, the topography of the region could represent a major barrier to gene flow. Berrendo, is located in a deep and inaccessible canyon of the Sierra San Pedro Mártir, while Palomar is nestled in the Sierra Juárez. Another possibility is that regions are genetically differentiated because of environmental factors. Precipitation during the driest quarter, mean temperature during the coldest quarter, and Precipitation seasonality—winter/summer rainfall regime—could all be driving regional transitions (Table 4.5). This is especially interesting given our morphological results and the evolutionary hypotheses proposed there (Villanueva-Almanza et al., 2018).

Genetic structure at the sub-clade level is also interesting because it shows more complex patterns that cannot be explained by vicariant events alone. Contrary to what has been found in other plant groups (Nason et al., 2002; Garrick et al., 2009; Lira-Noriega et al., 2015), we did not find any evidence to support population structure by a break at the Isthmus of La Paz. In the southern-peninsular region two groups can be distinguished; one made up of Cacachilas, Mulegé, San Juan de la Costa, and another made up of San José del Cabo, San Javier, and Comondú. This is not in accordance to what would be expected under the phylogeographic break due to the inundation of the Isthmus of La Paz which would result in isolation of the Cape from other peninsular populations. This pattern could be evidence of human and animal long-distance dispersal. The Cape has been an area of human occupation since before the arrival of Europeans (del Barco, 1780) and transportation of plant material is likely to have occurred some time ago allowing genetic variation to become uniform across the region.

While Hodel (2014) suggests there is no genetic impediment for gene flow between *Washingtonia* species in the cultivated landscape, our results show that regions maintain their genetic identity and that hybrids are not frequent in natural populations. This could be explained by several biological factors that have not been properly studied such as genetic incompatibility in natural populations, unknown flowering times, unknown pollinator behavior, or limited pollen dispersal due to geographical barriers as mentioned above.

Phenology could be an important aspect for understanding apparent reduced gene flow among *Washingtonia* populations as in other palms. For example, speciation in the palm genus *Howea* Becc. is due to asynchronous flowering between *H. belmoreana* (C.Moore F.Muell.) Becc. and *H. forsteriana* (F.Muell.) Becc., driven by soil differences (Savolainen et al., 2006).

Even slight differences in flowering times could potentially lead to reduced gene flow (Ellstrand, 2014), but flowering times in *Washingtonia* remain uncertain as revealed by herbarium specimen labels and inconsistencies across references. While the *Jepson Manual* reports *W. filifera* flowers from February through June (Simono, 2012), the *Flora of Baja California* and the *Baja California Plant Field Guide* record flowering for *W. filifera* occurring between May and June (Wiggins, 1980; Rebman and Roberts, 2012). However, herbarium specimens show flowering of *W. filifera* could be as early as April in Tajo Canyon (*C.F. Harbison Apr. 1, 1953*, SD) or as late as August in Murray Canyon in the Agua Caliente Indian Reservation (*O. F. Cook Aug. 6, 1906*, BH), although recent reports from park rangers at this site mention flowering occurs in June (pers. comm. Justin Conley, 2019). Flowering of *W. filifera* in Baja California has been recorded as late as October in Valle de las Palmas (*R. Moran 25147*, SD).

The mid-Peninsular region is the most understudied of the phylogeographic regions identified in this study. For example, information on the phenology of Mid-Peninsular populations comes from a single flowering herbarium specimen collected in Santa Gertrudis in July (*R. Domínguez Cadena 4355*, SD).

Flowering times for *W. robusta* are also different depending on the source consulted. While some report that *W. robusta* flowers from April to June (Simono, 2012), others report flowering from May through June (Wiggins, 1980; Felger et al., 2001), and herbarium specimens report flowering in *W. robusta* may occur as early as March in Sonora (*L.H. Bailey 16*, BH).

Geographic distribution and extent of sampling

Since there is little genetic differentiation among *Washingtonia* populations from the same region (McClenaghan and Beauchamp, 1986) sampling should aim to cover a wider geographical area rather than sampling multiple populations within the same region to uncover genetic patterns. This is also evidenced by the the most recent study using SNP markers on *Washingtonia* where no clear differentiation between both currently recognized species was found (Klimova et al., 2018). This pattern might be the result of limited geographic extent, number of individuals per population, and number of markers used.

Taxonomy and nomenclature of Washingtonia

While aware of the need to make the classification of *Washingtonia* as simple as possible, we also consider important for the taxonomy and nomenclature of the group to reflect its population structure and phylogeny. Based on our extensive sampling and the different methods used to analyze sequence data, we have strong evidence to propose *Washingtonia* is composed of four subspecies: *W. filifera* subsp. *filifera*, *W. filifera* subsp. *cataviñensis*, *W. filifera* subsp. robusta, *W. filifera* subsp. *sonorae*. The specific epithet *W. filifera* is chosen here to comprise all taxa within *Washingtonia* adhering to the principle of priority contained in article 11.3 of the *International Code of Nomenclature for algae, fungi, and plants* (Turland et al., 2018).

Our results lend support to those of Klimova et al. (2018) in the existence of four groups within *Washingtonia*. The reasons for them to continue recognizing *W. filifera* and *W. robusta* as two distinct species remain unclear to us, especially when they mention: "Our data therefore lead us to the conclusion that *W. filifera* is more likely to represent the northernmost population of *W. robusta* than a separate species" (Klimova et al., 2018; p. 5883). Although we know that renaming of ornamental species might be unsettling for horticulturalists, our results evidence that *Washingtonia* palms in the urban landscape are only a glimpse of the genetic and morphological diversity found in natural populations. Therefore, we must insist that the usage of names should follow the results instead of forcing names to fit convention. In fact, this is probably the reason why the taxonomy of *Washingtonia* has been so confusing in the first place.

Clarifying a century-old palm mystery: the becoming of W. robusta

Between 1876 and 1889 extensive postal correspondence was held between a group of botanists in an attempt to clarify the distinction between two species of palms that would later be described as *W. filifera* and *W. robusta*. Despite being a validly and effectively published name, botanists were unable to know what *W. robusta* really was—the name lacked a type specimen and had a very poor description. Most accounts of *W. robusta* describe it as being more slender than *W. filifera*. However, stem diameter changes gradually along latitude and varies even within the same site (Villanueva-Almanza et al., 2018). Even when it is unclear what the original *W. robusta* looked like, the fact is that a slender palm was brought into cultivation and soon became highly desirable. These conditions, together with the "tropicalization" of southern California, generated a real need to find the palm that would become the "mythical" *W. robusta*. The time coincided with the description and later introduction of *W. sonorae* into cultivation by Kate Sessions, who collected seeds in San José del Cabo around 1900 and grew them in San Diego. Bailey later photographed the palms planted by Sessions in 1927 (Villanueva-Almanza et al., 2018). The images show that the palms we now consider as *W. robusta* are the same as those then referred to as *W. sonorae*. Understandably, Bailey (1936) considered *W. sonorae* a synonym of *W. robusta*, hence providing the perfect solution: the discovery of a slender palm to bestow the name *W. robusta*. Since the the name *W. robusta* was available, it was favored and *W. sonorae* faded into oblivion as is evidenced in a nurserymen's account: "It is certain that a part of the material offered by nurserymen under the name of *Washingtonia sonorae* is really *W. robusta*" (Davy, 1902). Bailey's subsuming of *W. sonorae* as a synonym of *W. robusta* is the correct nomenclatural decision—*W. robusta* was described in 1883, *W. sonorae* in 1889—were it not for the fact that they are different. The root of the problem is the lack of type specimens. Wendland's original description is all we have to make an interpretation of the name *W. robusta* (Wendland, 1883, 1888). His work shows there is little evidence to support the idea this was a name originally intended for the palms of Baja California Sur. Wendland himself admitted not knowing the original place where *W. robusta* grew (Wendland, 1888). He mentioned the Colorado River, in southern California and Arizona, as the only place where both species of *Washingtonia* were found ("*Die Gattung, von der bis jetzt zwei arten bekkannt sind, ist dem Rio Colorado-Gebiete des südlichen Kaliforniens und Arizonas eigen*").

On the other hand, Nacapule constitutes a distinct genetic cluster that is not reflected by its current taxonomic placement within *W. robusta*. Because genetic diversity tends to be overlooked when designing conservation policies (Laikre, 2010; Ahrens et al., 2017), we consider important to recognize the genetic uniqueness of the *Washingtonia* palms in Nacapule even when they are not easily recognizable from the southern-peninsular populations using morphological traits (Villanueva-Almanza et al., 2018). The name we are assigning here is a new combination of the old name *W. sonorae*.

Washingtonia sonorae

Sereno Watson described *W. sonorae* in 1889 using plant material collected by Edward Palmer in 1887 in Sonora. He later gave additional details on the flower morphology using Palmer's collection from La Paz, Baja California Sur, Mexico (Watson, 1889a).

Nomenclature

Historical documents show there was great confusion regarding the name *W. robusta*. Some botanists believed the name was actually used to refer to several groups of palms east of San Bernardino (Palmer, 1876; Wright, 1879; André, 1900; Britton, 1904; Parish, 1907). If there was indeed a distinct group of palms within *W. filifera* that merited using the name *W. robusta* this raises the question regarding variation within *W. filifera*.

The northern peninsular group—traditionally recognized as *W. filifera*—shows very little levels of polymorphism as been previously noted (Vogl and McHargue, 1966; Klimova et al., 2018). However, we found enough genetic variation within this cluster as to recognize two subgroups (Fig. 4.10, 4.8). One group is composed by the strictly Californian populations Anza Borrego, Palm Canyon, and Joshua Tree, what we consider to be *W. filifera sensu stricto*. The other group, composed of Kofa, Palomar, Guadalupe, and Tajo, includes populations which have been considered different from *W. filifera*, *W. filifera* var. *arizonica*, from the Kofa Mountains in Arizona, and what we consider to be the original *W. robusta* from Tajo Canyon in northern Baja California. Therefore, this clade is what we refer to as *W. filifera sensu lato* (s.l.).

Possible morphological variability due to genetic distinctness of *W. filifera* s.l. in northern populations, from where the first seeds were collected (Palmer, 1876; Wright, 1889), could have lead botanists to apply different names to the same species. Parish (1907; 416) mentions: "[...] the seeds supplied to the market as 'true *W. robusta*' belong in reality to an undescribed species". While others argued it was a mere garden name. By interpreting historical documents with molecular data, we argue that *W. robusta* was originally used to refer to a particular group of *W. filifera*.

4.5 Conclusion

Using a widely distributed palm genus of the Sonoran Desert, we have shown that *Washingtonia* populations are highly structured in four phylogeographic regions that do not correspond to current species. Our results did not reveal the existence of a hybrid zone, giving further support to our morphological results in that *Washingtonia* is composed of a single, highly-variable species along the latitudinal transect we examined. Given this evidence, we propose using the name *Washingtonia filifera* for all four subspecies identified here.

Chapter 5. Conclusions

Between 1876 and 1889 extensive postal correspondence was held among a group of botanists in an attempt to clarify the distinction between two species of palms in the genus *Washingtonia* which would later be described as *W. filifera* and *W. robusta*. This widely distributed group of palms native to the Baja California peninsula, southern California, Sonora, and Arizona has been a taxonomic challenge due to a lack in type specimens, incomplete protologues, highly variable vegetative morphology, human dispersal, limited fieldwork in native populations, and poor representation in herbaria. We used functional leaf traits and morphological and molecular markers to answer the long-withstanding mystery of what is a species in *Washingtonia*?

We have found that vegetative morphological traits, some of which have been used to distinguish between both species, vary continuously along the latitudinal cline in Peninsular California, but are also influenced by fixed site effects. This evidence shows the difficulty in establishing clear morphological limits between both species currently recognized in the genus *Washingtonia*.

We also found that *Washingtonia* leaves receiving high rates of solar radiation and exposed to low water vapor pressure develop thick, waxy, isolateral leaves, with similar stomatal density on both sides and higher water use efficiency side than those in the southern, tropical and less arid environments, which show thinner, non-waxy, bilateral leaves with lower water use efficiency and occurring in sites with lower solar radiation and high water vapor pressure. Like other desert plants, leaf xeromorphism, formed by thick, isolateral, amphistomatic, and waxy, vertically-oriented leaves with high water use efficiency seems to constitute a syndrome more than a set of independently-occurring traits.

We found that *Washingtonia* populations were highly structured with four major geographic regions having unique genotypes. Based on previous morphological results and the evidence herein we propose recognizing a single species of *Washingtonia* with four subspecies. Our results provide a robust phylogenetic analysis of *Washingtonia* settling a taxonomic debate that has lasted over a century.

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Appendix A. Fitting sigmoid functions: the hyperbolic tangent is a rescaled logistic function

The logistic function, also known as the inverse logit function, is

$$lg(x) = \frac{e^x}{1+e^x} \tag{A.1}$$

Its values range from 0 to 1; when x$\rightarrow \infty$, $lg(x) \rightarrow 1$; and when $x \rightarrow -\infty$, $lg(x) \rightarrow 0$. For this reason the function is often used to model probabilities in logistic regression. Similarly, the hyperbolic tangent function, also frequently used to model sigmoid-curve response, is

$$tanh(x) = \frac{e^x - e^{-}x}{e^x + e^{-}x} \tag{A.2}$$

Its values range from -1 to 1;when x$\rightarrow \infty$, $tanh(x) \rightarrow 1$ and when $x \rightarrow -\infty$, $tanh(x) \rightarrow -\infty$. Multiplying the numerator and the denominator in equation A.2, by e^x we get:

$$tanh(x) = \frac{e^{2x}}{1+e^{2x}} - \frac{1}{1+e^{2x}} \tag{A.3}$$

Now, adding and subtracting e^{2x} to the second term in equation A.3, we get:

$$tanh(x) = \frac{e^{2x}}{1+e^{2x}} + \frac{e^{2x}}{1+e^{2x}} - \frac{1+e^{2x}}{1+e^{2x}} \tag{A.4}$$

So that

$$tanh(x) = 2lg(2x) - 1 \tag{A.5}$$

Equation A.5 shows that both the hyperbolic tangent function and the logit function can be used to fit geometrically identical. curves. The values of the parameters, however, will not be identical but will differ by a factor of 2.

Appendix B. Derivation of the formula of the Akaike Information Criterion for the particular case of regression modeling

The Akaike Information Criterion (AIC) for any statistical model is defined as $AIC = 2k - 2ln(\hat{L})$, where k is the number of estimated parameters in the model and $\hat{L}$. If we fit a regression model to a set of paired data x and y, assuming that the error $\epsilon = y_i - \hat{y_l}$ has mean zero, constant variance, and is Normally distributed, then the likelihood of the model can be calculated from the Normal distribution:

$$\hat{L} = \prod_{i=1}^{n} \frac{1}{\sqrt{2\pi s^2}} exp \frac{(y_i - \hat{y}_i)^2}{2s^2} \tag{B.6}$$

where s is the estimated standard error of the distribution, and $\hat{y}_i$ is the value predicted by the regression model predicted from the value of $\hat{x}_i$. The log-likelihood function used to calculate the AIC values can then be written as:

$$ln(\hat{L}) = -\frac{1}{2} \sum_{i=1}^{n} ln(2\pi) - \sum_{i=1}^{n} ln(2s) - \frac{1}{2s^2} - \sum_{i=1}^{n} (y_i - \hat{y}_i)^2 \tag{B.7}$$

Because the expected value of the standard error of the residuals (s) is constant for all values of he regression range, the sums in the first two terms can be simplified to a product so that:

$$ln(\hat{L}) = -\frac{n}{2}ln(2\pi) - nln(s) - \frac{1}{2s^2} - \sum_{i=1}^{n}(y_i - \hat{y}_i)^2 \tag{B.8}$$

Finally, recalling that the term $\sum_{i=1}^{n}(y_i - \hat{y}_i)^2$ is simply the residual sum of squares (RSS, also called Sum of Squares of the Error term), and that the standard error of the residuals is calculated as $\frac{SSE}{(n-k)} = \frac{\sum_{i=1}^{n}(y_i-\hat{y}_i)^2}{n-k}$, then equation B.8 can be written as:

$$ln(\hat{L}) = -\frac{n}{2}ln(2\pi) - \frac{n}{2}ln\frac{RSS}{n-k} - \frac{(n-k)RSS}{2RSS} \tag{B.9}$$

And hence,

$$ln(\hat{L}) = -\frac{n}{2}ln(2\pi) - \frac{n}{2}ln(RSS) + \frac{n}{2}ln(n-k) - \frac{n-k}{2} \tag{B.10}$$

$$ln(\hat{L}) = -\frac{n}{2}(ln(2\pi) + ln(RSS) - ln(n-k) + \frac{n-k}{n}) \tag{B.11}$$

$$ln(\hat{L}) = -\frac{n}{2}(ln(\frac{2\pi RSS}{n-k}) + \frac{n-k}{n}) \tag{B.12}$$

From equation B.12 we can now calculate the value of the AIC function for a regression model as:

$$AIC = 2k + n(ln(\frac{2\pi RSS}{n-k}) + \frac{n-k}{n}) \tag{B.13}$$