**Title**: Population genetics of a recent range expansion and subsequent loss of migration in monarch butterflies

**Running Title**: Range expansion and migration loss in monarchs

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**Abstract**

Range expansions—whether permanent or transient—strongly influence the distribution of genetic variation in space. Monarch butterflies are best-known for long-distance migration within North America but are also established as nonmigratory populations around the world, including on Pacific Islands. Previous research has highlighted stepwise expansion across the Pacific, though questions remain about expansion timing and the population genetic consequences of migration loss. Here, we present reduced-representation sequencing data for 275 monarchs from either North America (n = 85), one of 12 Pacific Islands (n = 136), or from three locations in Australia (n = 54), with the goal of understanding (1) how the monarch’s Pacific expansion has shaped patterns of population genetic variation and (2) how loss of migration has influenced spatial patterns of differentiation. We find support for previously described stepwise dispersal across the Pacific and document an additional expansion from Hawaii into the Mariana Islands. Nonmigratory monarchs within the Mariana Islands show strong patterns of differentiation, despite their proximity; by contrast, migratory North American samples from across the continent form a single genetically panmictic population. Estimates for the timing of the monarch’s Pacific establishment have high uncertainty (approximately 100 to 1,000,000 years ago) but overlap with historical records that suggest a recent expansion. Our data support (1) a single recent expansion across the Pacific whose timing overlaps with available historical records of establishment and (2) a strong role for seasonal migration in determining patterns of spatial genetic variation. Our results are noteworthy because they demonstrate how recent evolution of partial migration can drive population differentiation over contemporary time scales.

**Key words:** Range expansion, serial dispersal, monarch butterfly, population genomics

**Introduction**

Over extended time scales, geographic range expansions generally involve decreasing relatedness and increasing contributions of genetic drift in populations further from the original source population (Hewitt 1996, Excoffier et al. 2009). This pattern is especially evident in serial stepwise expansion events, in which populations are founded in a stepping-stone fashion (Ibrahim et al. 1996, Slatkin and Excoffier 2012). Serial dispersal is characteristic of many post-glacial range expansions into temperate regions and has been shown for species including eider ducks (*Somateria mollissima*) (Tiedemann et al. 2004), ragwort (*Senecio helleri*) (Bettin et al. 2007), rough-skinned newts (*Taricha granulosa*) (Kuchta and Tan 2005), and European butterflies (Dapporto et al. 2019).

Studies on the population genetics of geographic range expansions tend to focus on expansion events that occur over extended time scales, though the same expansion processes characterize annual movements associated with seasonal migration. Unlike permanent range expansion, however, seasonal migration may involve individuals capable of making round-trip journeys and traversing the entire species range in their lifetime (Dingle et al. 2014), thereby limiting opportunities for genetic divergence in allopatry. In species that migrate seasonally, patterns of population genetic variation in space are best captured by considering migratory connectivity of breeding populations (*e.g.*, Cohen et al. 2018, Gao et al. 2020). For example, Wilson’s warbler (*Wilsonia pusilla*) has eastern and western North American summer breeding populations that are genetically distinct, despite sharing an overwintering range in Central America (Irwin et al. 2011), and anadromous Coho (*Oncorhynchus tshawytscha*) and steelhead (*O. mykiss*) salmon populations show strong genetic differentiation corresponding to the river drainages where they spawn (Waples et al. 2004, Prince et al. 2017). By contrast, Japanese eels (*Anguilla japonica*) that share common spawning grounds but migrate to disparate areas across temperate Asia show little evidence for genetic differentiation over time or space (Gong et al. 2019).

Migratory species that show evidence for partial migration, whereby species are comprised of both migratory and non-migratory populations (Chapman et al. 2011), often feature populations that are highly differentiated, both phenotypically (*e.g.*, Dingle et al. 1980, Altizer and Davis 2010) and genetically (*e.g.*, Zhan et al. 2014, Gomez-Bahamon et al. 2020). The phenomenon of partial migration is common across the tree of life and has been documented in birds (Adriaensen and Dhondt 1990), insects (Menz et al. 2019), and ungulates (Berg et al. 2019). Although the evolutionary origins of partial migration are sometimes unclear, one recently invoked scenario involves a migratory, geographically widespread lineage giving rise to one or more non-migratory descendent lineages that become genetically distinct due to mismatches in the timing and/or location of breeding. This scenario has been hypothesized to be an important contributor to patterns of speciation in tropical birds (*e.g.*, Kondo et al. 2008, Gomez-Bahamon et al. 2020) and may also contribute to diversification of other groups.

Monarch butterflies (*Danaus plexippus* (L.)) provide an intriguing system for studying the effects of both global range expansion and loss of migration on spatial population genetic structure. Migratory North American monarchs comprise a single genetically indistinguishable population (Lyons et al. 2012, Talla et al. 2020) and have a summer breeding range that covers most of the North American continent. Over recent evolutionary history, monarchs have expanded their range globally (Ackery and Vane-Wright 1984, Vane-Wright 1993, Zalucki and Clarke 2004, Zhan et al. 2014, Fernández-Haeger et al. 2015, Pierce et al. 2014a, Pierce et al. 2015), with a southern expansion into South America and the Caribbean, an eastward expansion across the Atlantic and into the Iberian Peninsula, and a westward expansion across the Pacific. In contrast to their migratory North American ancestors, these expansion populations generally form non-migratory, year-round breeding populations in areas where they become established (Zhan et al. 2014). The exceptions to this pattern are in southern Australia and New Zealand, where monarchs move seasonally and form overwintering clusters akin to those observed in western North America (Wise 1980, James 1993). In this paper, we focus exclusively on the monarch’s expansion and subsequent loss of migration in populations across the Pacific.

Little is currently known about how contemporary loss of migration has affected fine-scale patterns of population differentiation in monarchs or other taxa (but see Samarasin et al. 2017). Two studies have addressed this question in monarchs: Hughes and Zalucki (1984) used four allozyme markers and found relatively high overall FST (0.032) between monarchs in host plant patches over small spatial scales (tens to hundreds of kilometers) in Queensland, Australia. Pierce et al. (2014b) used microsatellite markers and showed that monarchs from the Hawaiian archipelago show little differentiation among islands. However, the conclusions of these studies are limited by the spatial scale of sampling and the number of loci studied. Furthermore, the timing of the monarch’s Pacific expansion remains uncertain. Demographic simulations indicate that establishment timing may have happened as long as 2000-3000 years ago (Zhan et al. 2014), although these estimates conflict with historical records, which suggest that expansion across the Pacific happened between the years 1840-1900 (Zalucki and Clarke 2004, Freedman et al. 2020). Further demographic modeling that accommodates a broad range of potential establishment scenarios—including variable establishment timing, founding population sizes, and past changes in population size—might help to resolve the discrepancy between historical and model-based estimates of expansion timing.

In this study, we sequenced 275 monarch butterflies at >70,000 highly variable genomic sites from the ancestral North American population and many Pacific Island populations, including a number of previously unsampled populations: the Mariana Islands (Guam, Rota, and Saipan) and Norfolk Island. The goals of this study were to understand (1) overall patterns of genetic relatedness among Pacific and North American populations; (2) the timing of range expansion and the amount of ongoing gene flow between North American and Pacific populations; (3) how migratory and non-migratory populations differ in their distribution of population genetic variation in space.

**Methods**

***Sample preparation and sequencing***

Monarchs were collected as either larvae or adult butterflies from various locations across their current geographic range between 1990-2017 (Figure 2a and Table S1).DNA was extracted from samples using a magnetic bead-based protocol (Ali et al. 2016) and quantified using Quant-iT PicoGreen dsDNA Reagent (Thermo Fisher Scientific) on a FLx800 Fluorescence Reader (BioTek Instruments). Restriction Associated Digest (RAD) DNA libraries were then created using the PstI restriction enzyme according to Ali et al. (2016) and sequenced using 150 bp paired-end sequencing on an Illumina Hi-Seq 4000.

*Sequence alignment, filtering, and genotype calling*

We aligned raw sequence data to version 3 of the monarch butterfly genome assembly [(Zhan](https://paperpile.com/c/tNxuHC/psiG) and Reppert 2013) using the mem algorithm implemented in Burrows-Wheeler Aligner [(Li](https://paperpile.com/c/tNxuHC/7NQQ) and Durbin 2009). Sequence data was sorted and filtered for PCR duplicates and improper pairs using **SAMTOOLS** [(](https://paperpile.com/c/tNxuHC/EXik)Li et al. 2009). We first removed potential paralogous sites using the ngsParalog tool by removing all sites within a kilobase of any SNP with a log ratio test statistic of > 10 in any population [(](https://paperpile.com/c/tNxuHC/OQZa)Linderoth 2018). From this, we created five different datasets using different filtering schemes appropriate for different downstream analysis.

***Datasets****:*

1. For use in demographic reconstruction and directionality index (*ψ*) calculation, we called genotypes using the **SAMTOOLS** genotype likelihood model [(Li](https://paperpile.com/c/tNxuHC/EXik) et al. 2009) as implemented in the **ANGSD** software package with a minimum mapping and base call quality score of 20, a SNP *p*-value of 1e-8, a uniform genotype prior, and a posterior genotype probability cutoff of 0.95 [(Korne](https://paperpile.com/c/tNxuHC/hYyB)liussen et al. 2014). In order to reduce potential bias due to linkage for demographic analyses, we randomly subsampled SNPs such that no locus was within 10,000 bp of another using a custom **R** script. The resulting SNPs were used to calculate Site Frequency Spectra (SFS) and then projected down to a sample size of 100 gene copies from North America and 10 from Hawaii for demographic analysis and down to 10 gene copies in each population for *ψ* calculation using the methods described by Gutenkunst et al. (2009). These projection numbers were picked to maximize the remaining number of SNPs in the dataset. The SFS was polarized via reference to whole genome sequence data of the best-sequenced individual of the monarch’s sister species *Danaus erippus* (Zhan et al. 2014) by alignment to the monarch genome as described above. While we did not use a Hardy-Weinburg Equlibrium (HWE) filter here, only a very small proportion of our loci were consistently not in HWE across populations (p < 1x10-6 in only 86 out of 11,384 loci, calculated using the method of Wigginton et al. [2005]).
2. For use in calculating basic diversity statistics, (the average number of pairwise differences, or π, observed heterozygosity, or HO, and the ratio of within-sample heterozygous to homozygous loci, or Het/Hom), the fixation index (FST), and Isolation-by-Distance (IBD), we called genotypes as in dataset 1, then removed individuals genotyped at less than 75% of loci. Since strong bottlenecks are likely to cause large differences in allele frequencies between populations, which, in conjunction with very different sample sizes between populations, can result in loci with very low overall minor allele frequencies having relatively high frequencies in individual populations, we did not use a minor allele frequency filter when calling genotypes. Some populations did not remain in the analysis after this filtering step.
3. For calculating Tajima’s D, we implemented the same filtering steps as described for Dataset 2, but without using a SNP p-value filter in ANGSD.
4. For analyses that did not require called genotypes (Principal Component Analysis, or PCA, NGSadmix, and a neighbor-joining tree), we used **ANGSD** as in dataset 1, but did not call genotypes and instead estimated the likelihoods with a minor allele frequency filter of 0.05. For the PCA and neighbor-joining tree, the input distance matrix was created using the Identity-by-State approach in **ANGSD** (Korneliussen et al. 2014). No individuals were removed for these analyses.

We also generated more thoroughly filtered versions of datasets 3 and 4 (see *Supplementary Methods, Filtered Datasets*), although these additional filtering steps did not meaningfully influence our inferences.

***Patterns of relatedness among monarch populations***

We calculated π, Ho, Het/Hom, and Tajima’s D (Tajima, 1989) within each population using the snpR package (Hemstrom and Jones 2021). We calculated FST between populations using the **R** implementation of the **GENEPOP** software package [(Rousset](https://paperpile.com/c/tNxuHC/UGgy) 2008) with a minor allele frequency cutoff of 0.05 and bootstrapped individuals between populations randomly 1000 times to calculate FST significance levels using the snpR package (Hemstrom & Jones, 2021). For each of these statistics, the eastern and western North American samples were pooled together, based on results from previous studies (Lyons et al. 2012, Talla et al. 2020). To determine if our relatively light filtering approach biased our results, we also re-ran the diversity statistics π, Ho, Het/Hom, and Tajima’s D, FST, and IBD analyses using more heavily filtered datasets, as described in the Supplementary Methods.

In order to describe basic population structure, we created a neighbor-joining tree (Saitou and Nei 1987) using the ape **R** package v.5.0 [(](https://paperpile.com/c/tNxuHC/jHGn)Paradis and Schliep 2019) and conducted a PCA. For comparison, NGSadmix was used to generate individual ancestry coefficients for each individual for between 1 and 9 putative population clusters (k) (Skotte et al. 2013). Each value of k was run 10 times, and the results were collapsed into consensus plots using **CLUMPP** (Jakobsson and Rosenberg 2007). The pophelper (Francis 2017) and snpR (Hemstrom and Jones 2021) **R** packages were used to run these analyses. We used the method of Evanno et al. (2005) to detect the number of clusters present from the results; however, since this method has reproducibility issues (Gilbert et al., 2012), tends to underestimate the true k unless in the context of a complex hierarchical examination (Janes et al., 2017), and generally does not improve estimates of k (Waples & Gaggiotti, 2006), we also looked at patterns of clustering in general across a range of k values.

*Serial expansion*

To quantify the direction and strength of population spread across the Pacific, we calculated *ψ* (Peter and Slatkin 2013) for each pairwise combination of North America, Hawaii, Queensland, Guam, Rota, Norfolk Island populations using the snpR package (Hemstrom and Jones 2021).

*Genetic variation across space in migratory vs. non-migratory populations*

We looked for evidence of isolation by distance (IBD) between samples from the Mariana Islands (non-migratory), Hawaii (non-migratory), Australia (partially migratory), and North America (migratory). To do so, we calculated Edwards’ angular genetic distance (Edwards 1971) between each pair of samples from the given populations, and then compared these distances to the geographic distances between samples using a Mantel Test (Mantel 1967). Here, we expect for stronger patterns of IBD within locations where monarchs have ceased seasonal migration. As with FST, we used dataset 2 with an additional minor allele frequency cutoff of 0.05.

***Demographic history of the monarch’s expansion***

To describe the patterns of establishment and migration between North America and the Pacific, the demographic reconstruction program **δaδi** (hereafter **dadi**, Gutenkunst et al. 2009) was used to estimate the demographic history of the North American and Hawaiian samples. Briefly, since demographic processes influence the frequency of common or rare alleles across loci, and the SFS describes how many individual loci fall into each possible allele rarity in each population, the SFS can be used to infer historic population processes. **dadi** therefore uses simulation to compare the SFS predicted under a specific demographic history to the SFS observed from the data in order to evaluate the likelihood of a demographic model and to optimize the parameters of that model. We chose to focus on Hawaii since prior work has suggested that this island group was likely the first in the Pacific colonized by monarchs (Zalucki and Clarke 2004, Zhan et al. 2014), and thus the timing of the monarch introduction there represents the earliest possible time for any introductions in the Pacific. The general approach that we describe here is similar to that described in Zhan et al. (2014), but with a few key differences: (1) Zhan et al. (2014) pooled samples from six distinct Pacific populations for their analysis, while we only focused on the Hawaiian population; (2) the parameter values reported in Zhan et al. (2014) represent a single instance of one focal demographic scenario, whereas our analyses considered a large set of candidate demographic scenarios and report results from multiple iterations of each scenario.

***dadi*** *model selection*

We fit a range of possible models to the observed data: (1) each of the models in the “Island Model” set described in the dadi\_pipeline (Portik et al. 2017), which contains some models originally published in Charles et al. (2018); (2) the model described by Zhan et al. (2014) for the same comparison; (3) a similar model that allowed for an additional period of growth prior to the establishment of the Hawaiian population and another following establishment (hereafter referred to as the *Three Epoch* model). The latter two models, as well as the two dadi\_pipeline models which performed well, are shown schematically in Figure 1. To allow for a more realistic population growth trajectory, we also ran each of the dadi\_pipeline models with a logistic population growth equation rather than the exponential growth variant defined in the original models. Each of the dadi\_pipeline models and their logistic growth versions were run three times: once with growth allowed in the founding population post-split, once with growth allowed in the founded population post-split, and once with growth allowed in both populations post-split. Note that in each of these (dadi\_pipeline) models, a source population splits to form two descendant populations, with an optimized parameter (*s*) controlling the portion of the population that forms each descendant population. When *s* is optimized to be very small (as it typically was), the founded population represents only a very small proportion of the ancestral population, as is likely realistic for the founding of the Hawaiian population from the North American population. Schematic depictions of the dadi\_pipeline models are available in Portik et al. (2017) and Charles et al. (2018).

To optimize the models we fit during the analysis, we used a variation of dadi\_pipeline, the sequential step-down parameter permutation approach described by Portik et al. (2017). Unlike this method, however, we set the starting parameters for each sequential run via weighting the parameters from each run in the previous iteration by the relative AIC score of that iteration, such that all but the worst runs contribute in some degree to the starting parameters for the next step. The number of runs and iterations per step are listed in Table S3. Individual optimization runs were killed if they took longer than 48 h to complete; these runs tended to take far longer to finish and often included integration errors due to extremely small population sizes resulting in extremely large amounts of genetic drift. Most runs completed in under 48 h and are included in the results.

*Parameter estimation*

To extract meaningful parameter units from the results, we assumed 0.3 years per generation and used the per-base mutation rate of 8.4x10-9 reported from *Drosophila melanogaster* (Haag-Liautard et al., 2007). We use these values to match those used by Zhan et al. (2014) for ease of comparison. We also used a potentially more realistic generation time of 7 generations per year and the slower mutation rate reported for the more closely related *Heliconius melpomene* of 2.9x10-9 (Keightley et al., 2015). In order to determine the length of the considered genomic region, we multiplied the total number of bases sequenced after quality filtering (but not SNP p-value filtering so as to count non-polymorphic sites) by the ratio of SNPs in the final allele frequency spectrum to the total number of called SNPs.

**Results**

*Sequencing results*

After paralog filtering, we were able to genotype 2,159,978 sites in at least 50% of individuals. 541,899 of these sites were polymorphic, and 71,157 had a minor allele frequency above 0.05. For dataset 1, we retained 11,384 loci after removing loci within 10kb of each other. Datasets 2 and 3 had 70,878 and 413,271 sites, respectively. The number of samples from each population after filtering is shown in Table 1. Note that some populations had no samples that passed filtering, and so are not included in Table 1.

*Overall patterns of relatedness*

Principal component analysis separated North American, Hawaiian, Mariana Islands, and southwest Pacific samples along two axes of expansion (Figure S1b). NGSadmix results showed a similar result (Figure 2b), splitting the North American samples from the Pacific samples at K = 2, the Mariana Islands at K = 3, Rota from the other Mariana Islands at K = 4, Samoa, Fiji, and New Caledonia at K = 5, Hawaii at K = 6, Saipan from Guam at K = 7, and Norfolk Island at K = 8. Notably, the Hawaiian samples were consistently classed as having ancestry partially from all other clusters until K = 6, consistent with an initial introduction into the archipelago. At K = 9, nearly all North American samples were assigned to two genetic clusters with ancestry proportions unrelated to their geographical sampling locations, which can be interpreted as the presence of a fictive cluster with no biological significance (e.g. Guillot et al., 2005; Chen et al., 2007). K = 2 and K = 5 had the highest ΔK values (Evanno et al., 2005), although we were not able to estimate ΔK for K = 3 due to very low likelihood variance between runs at K = 2 and K = 3, thereby producing an undefined ΔK (Figure S2). Eastern and Western North America were never split. Directionality index (*ψ*) scores indicated westward establishments (Figure S4). Genetic diversity (π, HO, and Het/Hom), was highest in the ancestral North American populations, followed by Hawaii, Australia, and then the remaining Pacific Island populations (Table 1, Figure S4). Tajima’s D was positive in all sites besides North America and Hawaii (Table 1). FST results also reflect the patterns we observed in the PCA and NGSadmix results (Table S2, Figure 2b, Figure S1a). These results varied slightly in the heavily filtered dataset, but followed the same general trends (with a higher diversity in North America, Hawaii, and Australia, and very similar Tajima’s D and FST results, Tables S5-6). These results did have a higher relative diversity in the Pacific populations than in the full dataset, however, and, as a natural consequence of removing loci with low minor allele frequencies, much higher overall diversity estimates.

*Patterns of differentiation within expansion populations*

Samples from the Mariana Islands (especially the well-sampled Guam and Rota populations) appear to form highly distinct populations, despite their close physical proximity (Figures 1a-b and S1a-b). By contrast, populations within Hawaii (Maui and Oahu) and Australia (Queensland, New South Wales, and Victoria) do not show strong patterns of differentiation (Figure 1a-c). Norfolk Island, the other previously unsampled population in our dataset, groups closely with samples from Australia and New Zealand (Figures 1a-b and S1a-b). IBD patterns were significant within the Mariana Island (p = 0.001, r = 0.723), North American (p = 0.001, r = 0.036), and Hawaiian (p = 0.005, r = 0.456) samples, and were present but not significant within Australian samples (p = 0.109, r = 0.122). In the heavily filtered dataset, IBD patterns were significant within all but Hawaii (p = 0.001, r = 0.593 in the Mariana Islands; p = 0.002, r = 0.054 in North America; p = 0.609, r = 0.131 in Hawaii; and p = 0.014, r = 0.238 in Australia).

*Timing of establishment and patterns of ongoing gene flow*

Among the large set of possible demographic models, the simple *Found and Grow* scenario (Figure 1b), which had a constant ancestral population size in North America, Hawaii colonization, and then population growth in both sites, produced the lowest AIC scores on the final pass of the pipeline (Figure S5). However, the *Two Epoch* model, which had a single admixture event but no consistent migration (Figure 1c), had the lowest AIC score across all passes of the pipeline. Thenew *Three Epoch* model (Figure 1a), which involved multiple rounds of demographic expansion in the ancestral North American population, followed by colonization and growth in Hawaii, had a lower AIC score than *Found and Grow* across all passes and a lower AIC score than the *Two Epoch* model on the final pass(Figure S5)*.* These were the top three models across all passes and in the final pass alone(Figure S5, Table S4). The *Three Epoch* model is a more complex version of the model specified in Zhan et al. (2014, hereafter *Zhan*). We therefore report only the results for *Found and Grow*, *Three Epoch, Two Epoch,* and *Zhan* models here.

These fourmodels gave highly variable estimates of establishment timing and founding populations size, with the *Three Epoch* model generally producing much broader estimates for these parameters. For example, while the *Three Epoch* model suggested establishment times that ranged between approximately 102 to 105 years ago, the other models suggested times between 104 and 105 years ago (Figure 3). Similarly, the latter models were more consistent in predicting a large founding population of 103 to 106 individuals, while the *Three Epoch* models suggested a broader founding population size of between 10 and 106 individuals (Figure 3). These models also differed in their estimates of the contemporary *Ne* for the Hawaiian population, with the *Found and Grow* and *Two Epoch* model suggesting a large *Ne* of around 106 and the *Three Epoch* and *Zhan* models generally producing estimates of Hawaiian *Ne* between 102 and 107 (Figure 3). For all of the models, the major discrepancy between the observed and simulated site frequency spectra tended to be that the models underestimated the number of rare derived alleles in the North American but not the Hawaiian populations (Figures 4 and S6-9). This pattern may indicate that the models did not optimize for strong enough founder effects in Hawaii, which would have caused a more drastic loss of rare alleles.

For other parameters, the four models generated similar estimates. Each of these models suggest very low levels of contemporary migration between North America and Hawaii, with the *Found and Grow* and *Two Epoch* models converging near 0 for both directions and the *Three Epoch* models generally suggesting migration rates of < 5 x 10-4 and < 2.5 x 10-4 for individuals per generation from North America to Hawaii and from Hawaii to North America, respectively, and the Zhan model suggesting migration rates of < 2.5 x 10-4 in either direction (Figure 3). Using a more accurate generation time and mutation rate than the values used by Zhan et al. (2014) produced a result with slightly more distant divergence times and larger effective sizes, but not to a substantial degree.

**Discussion**

Many geographic range expansions occur via serial stepwise dispersal, and we found strong evidence for this pattern in Pacific monarchs, consistent with a previous study (Pierce et al. 2014a). Monarchs in the Mariana Islands are the product of a distinct expansion event within the Pacific. Summary statistics support a scenario of directional dispersal from North America to Hawaii, from Hawaii to Guam, and from Hawaii to Australia. This pattern is reflected in both the positive directionality index measures (0.07, 0.08, and 0.05, respectively) (Peter and Slatkin 2013) and other summary statistics, such as the general increase in Tajima’s D across the Pacific, which is consistent with stronger or more recent population bottlenecks during successive colonization. Interestingly, monarch populations in Hawaii and Australia seem to maintain relatively high levels of genetic diversity, despite the apparent bottlenecks associated with establishment. This is especially striking in the Australian population, which was itself likely founded by individuals from a much smaller population in New Caledonia (Clarke and Zalucki 2004). The retention of genetic diversity in Hawaii and Australia may reflect rapid population growth upon establishment, which could temper the loss of allelic diversity that might be predicted with a bottleneck event, akin to the scenario described in Hawaiian *Drosophila* by Nei et al. (1975). The slightly negative Tajima’s D value in Hawaii is consistent with population growth following a bottleneck and is consistent with this hypothesis.

Within the Mariana Islands, there was a strong pattern of differentiation between islands, especially between the nearby islands of Guam and Rota. This pattern is striking because of their close geographic proximity: these islands are separated by only 40 km of open ocean. By contrast, our samples from North America, despite coming from overwintering sites nearly 2,000 km apart, formed a single genetically indistinguishable population. This pattern is apparent from the strong pattern of IBD observed within Mariana Islands samples compared to weak/no IBD within North America. Our results are similar to those of Dapporto et al. (2017) and Vodă et al. (2016), who also noted strong genetic differentiation between butterfly lineages even from nearby islands, as well as Alvial et al. (2018), who showed that a migratory dragonfly exhibits little genetic differentiation across its migratory Central and South American range but substantial genetic differentiation between non-migratory populations on Pacific islands. However, our findings are unique because the observed differences in population structure between migratory and non-migratory populations developed very recently, likely emerging over the past 150 years.

The lack of differentiation within North American monarchs corroborates other population genetic analyses of eastern and western North American monarchs (Brower and Boyce 1991, Shephard et al. 2002, Lyons et al. 2012, Zhan et al. 2014, Talla et al. 2020) and is consistent with studies that have suggested movement of individuals between eastern and western North America (Dingle et al. 2005, Morris et al. 2015, Billings 2019). The strong population genetic differentiation within the Mariana Islands but not at the scale of the entire North American continent highlights both (1) the pervasive role that long-distance migration in North America plays in collapsing any patterns of population structure that might otherwise develop, and (2) the fact that many nonmigratory Pacific monarch populations likely have extremely small effective population sizes that are susceptible to very strong genetic drift.

In contrast to populations within the Mariana Islands, Hawaiian and Australian monarchs show only modest evidence for IBD that might be expected in nonmigratory monarch populations. Within Hawaii, our samples from Maui and Oahu formed a single genetic cluster, consistent with the results of Pierce et al. (2014b). Likewise, Australian samples from New South Wales and Victoria grouped with samples from Queensland. This result differs somewhat from the results of Hughes and Zalucki (1984), who reported considerable among-site genetic variation within Queensland, but is consistent with similar later work (Zalucki et al. 1987).

For Hawaiian monarchs, it is not immediately clear why the islands of Maui and Oahu do not form clearly distinct populations. One possibility is that prevailing winds promote gene flow between islands in a way that differs from the Mariana Islands. Pacific monarchs are likely moved by wind patterns, similar to wind-driven movement patterns noted in migratory *Vanessa cardui* (Stefanescu et al. 2007), and it has been suggested that a tropical cyclone may have led to the monarch’s establishment in Australia, following an “outbreak” of monarchs shortly after establishing in New Caledonia (Clarke and Zalucki 2004). Another possibility is between-island movement of monarchs by butterfly breeders in Hawaii, who sell monarchs for release at weddings and celebrations (D. Loo-McDowell, *pers. comm.*). In the case of Australian monarchs, the lack of strong differentiation across the continent may be driven by seasonal migration patterns akin to those seen in western North American monarchs (James 1993, James and James 2019). Australian monarchs retain migration-associated behaviors such as seasonal reproductive arrest and sustained directional flight—necessary although not sufficient conditions for long-distance migration—that further support the notion that they may undergo large-scale seasonal movements (James 1993, Freedman et al. 2018, Hemstrom et al. *in prep*). Thus, the lack of continent-wide population structure seen in migratory North American monarchs may be recapitulated, albeit to a lesser extent, in Australia.

Interpreting the results of our demographic models is somewhat more complicated than interpreting basic patterns of relatedness among populations. This is due to the conflicting inferences provided by the two best-performing model structures and the wide range of parameter estimates in the *Three Epoch* models. Although we present the results of both the simpler *Found and Grow* and *Two Epoch* models, and the more complicated *Three Epoch* model, we are inclined to place more confidence in the estimates produced by the *Three Epoch* model for two reasons: (1) the demographic scenario that it specifies—recent demographic expansion in the ancestral North American population prior to geographic expansion—has empirical support from other studies (Zhan et al. 2014, Pfeiler et al. 2017, Boyle et al. 2022) and accords with our understanding of past changes in climate, and (2) this model structure produces parameter estimates that match our prior understanding for how and when monarch range expansion may have occurred. The latter point is related to the former: because a North American population expansion is not allowed until after the founding of Hawaii in the *Found and Grow* model, this model forces an ancient founding of the Hawaiian population in order to allow for the ancient growth of the North American population. As such, we focus our discussion on the estimates produced by the *Three Epoch* model.

In general, our demographic results do not exclude a recent founding of the Hawaiian population by North American monarchs (Figure 3d). While our model optimizations span several orders of magnitude for the time since establishment, many of the iterations settled on introduction estimates of less than 200 years ago for the *Three Epoch* model. Since the earliest historical records of monarchs on Hawaii date to roughly 200 years ago (1841) (Zalucki and Clarke 2004), we are inclined to accept the results of iterations with shorter estimated divergence times. Other lines of evidence supporting recent (<200 years) Hawaiian establishment include: (1) the lack of noticeable phenotypic differentiation between North American and Pacific Island monarchs, especially relative to the pronounced phenotypic differences in non-migratory populations from the Caribbean and South America (Freedman et al. 2020), which have historically been treated as separate subspecies (Ackery and Vane-Wright 1984), (2) the likely need for human-mediated transport of the monarch’s host plants (some of which are native to subtropical Africa) as a pre-condition of monarch establishment in the Pacific, and (3) recent genomic evidence showing that captive breeding of monarchs over short time scales is sufficient to generate patterns of genetic divergence comparable to those observed between North American and Pacific populations (Tenger-Trolander et al. 2019). Notably, our re-implementation of the model used by Zhan et al. (2014) produced results similar to theirs, with the majority of model iterations supporting an introduction time >1000 years ago (Figure 3). This highlights the need to run a range of possible demographic models when attempting to infer demographic history, since failing to account for underlying complexity in population histories can result in very divergent parameter estimates.

One complication for interpreting our demographic models is that they consistently underestimated the number of rare, derived alleles present in North America but not Hawaii. During very strong bottlenecks, we would expect many rare alleles to be lost, suggesting that our models may be overestimating the founding population size, and thus likely establishment date. Since **dadi** can struggle to calculate site frequency spectra when population sizes are very small due to large amounts of drift, iterations that optimize to this segment of parameter space are more likely to have integration errors or very long processing times. These uncompleted runs were not included in our model results, and so this part of the parameter space may be inadequately explored. Since small founding population sizes correlated with recent introductions across our model results, this also suggests that it would be unwise to rule out a recent introduction with a very strong bottleneck based on a demographic analysis alone.

Demographic model results were also variable in their estimates of founding population sizes in Hawaii. Some models produced estimates as high as 10,000 founding individuals, which seems implausible given the incredibly long distance (>3,500 km) between North America and Hawaii. The extremely wide range of parameter estimates for founding population size and timing may reflect that, in practice, it is difficult to distinguish between a very recent strong bottleneck versus a more distant but less severe bottleneck. Our model results are consistent with this, since the model iterations with recent establishments also tended to have smaller establishment population sizes (Figure 3).

In contrast to variable estimates of establishment timing and founding population size, demographic models were consistent in suggesting very low contemporary migration rates (on the order of 0.0001 individuals per generation from North America to Hawaii and vice versa). Our results thus contrast with those of Pierce et al. (2014a), who inferred much higher migration rates (nearly 10 individuals/generations) between North America and Hawaii. We are more confident in our results due to (1) the much larger number of sampled loci, (2) the more realistic demographic model that we used in our analysis, and (3) the absence of modern records of regular North America to Hawaii establishment events.

Understanding how migratory and non-migratory populations of monarchs differ genetically, phenotypically, and ecologically has important conservation implications. First, monarchs were recently evaluated by the U.S. Fish and Wildlife Service, who determined that a listing under the U.S. Endangered Species Act is “warranted but precluded” (USFWS 2021). This decision will be reevaluated annually, and the retention of genetic diversity in non-migratory monarch populations from outlying U.S. states/territories (Hawaii, American Samoa, the Mariana Islands, Puerto Rico, U.S. Virgin Islands) may be important in decisions regarding the adaptive capacity of the species (Freedman et al. 2021). Second, some recent evidence suggests that climate warming and planting of non-native milkweed species might tip the scales in favor of year-round breeding and loss of migratory behavior in North American monarchs, particularly in western North America (James 2021, Crone and Schultz 2021, James et al. 2021). The increased prevalence of partial migration within North America, both along the U.S. Gulf Coast and in California (Satterfield et al. 2016, Satterfield et al. 2018, James et al. 2021), may affect patterns of spatial genetic diversity: for example, if future sequencing of North American monarchs finds evidence for population structure within areas of their range where year-round breeding occurs, this would provide evidence that loss of migration is actively driving genetic differentiation. Finally, our results are also helpful for monarch conservation because they provide a relatively large sample of North American monarchs (n = 90) against which future sequencing efforts can be compared to look for evidence of contemporary losses of genetic diversity associated with population decline.

**Data Accessibility**

All scripts used for analysis are available at <https://github.com/hemstrow/F-H_2018>. Sequence data will be made available through NCBI upon publication.

**Author Contributions**

WBH, MGF, and MRM designed the research. MGF and MPZ provided samples used for sequencing. WBH and MGF performed data analysis. All authors contributed to writing and editing the manuscript.

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| **Population** | **# Samples** | **Tajima's D** | **HO** | **π** |
| **North America (NAM)** | 83 | -1.92 | 0.055 | 0.064 |
| **Hawaii (HAW)** | 9 | -0.211 | 0.048 | 0.055 |
| **Guam (GUA)** | 19 | 0.091 | 0.031 | 0.032 |
| **Rota (ROT)** | 16 | 0.388 | 0.035 | 0.038 |
| **Saipan (SAI)** | 4 | 0.326 | 0.022 | 0.025 |
| **Queensland (QLD)** | 15 | 0.349 | 0.042 | 0.044 |
| **New South Wales (NSW)** | 5 | 0.433 | 0.038 | 0.042 |
| **Victoria (VIC)** | 2 | 0.899 | 0.041 | 0.043 |

**Table 1 -** Number of samples remaining after filtering, Tajima’s D, observed heterozygosity (HO), and nucleotide diversity (π). Populations from Guam, Rota, and Saipan are all part of the Mariana Islands archipelago. Samples from Maui and Oahu are pooled into a single Hawaiian population. Queensland, New South Wales, and Victoria are all within the Australian continent. Populations from Samoa, New Caledonia, Fiji, and New Zealand are not shown because no samples remained from these populations after filtering (but see Figure 2).

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**Figure 1 –** Visual depiction of the four best performing models from **dadi** simulations. Panel (A) depicts *Three Epoch*, which allows for multiple changes in the size of the ancestral North American population prior to establishment in Hawaii, the North American and Hawaiian populations to change in size after the Hawaiian establishment, and a constant migration rate between North American and Hawaii after a brief time lag. Panel (B) depicts *Found and Grow*, which assumes a constant ancestral North American population size. Panel (C) depicts *Two Epoch*, which has a one-time admixture rather than constant migration. Panel (D) shows the model from *Zhan* et al. (2014), which allows for only a single instance of growth in the North American population prior to the establishment of the Hawaiian population. In each model, parameters are estimated jointly and correspond to the following: **Nu** = population size at a particular time, **T** = time, **m** = migration rate between populations, and **s** = founding population size. Note that occasions of population size change depicted here are allowed to freely optimize to be either population growths or declines.

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**Figure 2 –** Relatedness among sampled populations. (a) Map of sampled populations, with pie charts reflecting average population results from NGSadmix (k = 5). Note that the geographic positions of the Mariana Islands (ROT, GUA, and SAI) and the Hawaiian Islands (OAH and MAU) are shifted slightly for readability. (b) NGSadmix plots showing the proportion of ancestry across clustering values between k = 2 and k = 9. At k = 5, Hawaii reflects a mixture of ancestry comprising North American, Mariana Islands, and southwestern Pacific samples. At k = 6, Hawaii becomes its own cluster. At values beyond k = 6, populations are subdivided.

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**Figure 3 -** Results of the **dadi** optimization runs for the (left to right) *Three Epoch*, *Found and Grow, Two epoch*, and *Zhan* models demographic models. In the legend at right, “pass” refers to each iteration of the sequential step-down permutation approach (see Methods*:* **dadi** model selection), and the log10(AIC) score corresponds to the likelihood of observing a particular set of parameter values. (**Top row**) Estimated effective size of founding population in Hawaii vs. years since establishment. (**Middle row**) Estimated migration rates from North America to Hawaii and vice versa. (**Bottom Row**) Estimated current effective population sizes in North America and Hawaii. Red dots mark the runs with the lowest AIC scores in each quadrant of the respective parameter space based on the (a), corresponding to the heatmaps in Figures 4 and S6-S7 and the residuals in Figure S8. Note that the *Two Epoch* model does not include a constant migration rate between Hawaii and North America, and so is blank for the middle panel.

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**Figure 4 –** (**Left**) Observed data and (**Right**) model estimated derived site frequency spectra for the *Three Epoch* model. Cell brightness corresponds to the number of loci with derived allele frequencies in the given bin for both Hawaii (HAW) and North America (NAM). Note that spectra have been projected to a size of 100 for North America and 10 for Hawaii to match the approximate number of individuals sequenced from each population. Model-derived site frequency spectra correspond to the four points marked in red in Figure 3 for the *Three Epoch* model, which are parameter estimates from the runs with the lowest AIC score. Figures S6-S8 show comparable plots for the other candidate demographic models. (BL: bottom left, BR: bottom right, TL: top left, TR: top right).