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Do genetic differences explain the ability of an alkaline shrub to grow in both uplands and wetlands?

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Abstract.—The hydrophyte *Allenrolfea occidentalis* (S. Watson) Kuntze (iodinebush) is a halophytic shrub of the arid southwest that is listed as a facultative wetland species on the National Wetland Plant List. This rating means that the species is usually a hydrophyte but occasionally is found in uplands. We tested for genetic (ecotypic) differences between plants sampled from wetlands versus uplands. We used the technique of genotyping by sequencing to generate data from 132 plants from 30 locations representing both wetland and upland occurrences for over 1300 loci. Analyses indicated that the strongest genetic signal is from differences in geographic distribution: samples that are in close geographic proximity tend to be more similar genetically regardless of whether they occur in wetland or upland locations. We detected no effect of habitat on overall genetic structure, and we found only 2 (of the 1381) loci with a positive association between genotype and habitat; in both cases the association was very weak. We infer that *A. occidentalis* occurrences near or in wetlands are not influenced by significant differences in genetics, and we find no evidence for wetland and upland ecotypes of this species.

Resumen.—La hidrófita *Allenrolfea occidentalis* (S. Watson) Kuntze (arbusto de yodo) es un arbusto halófilo del sudoeste árido registrado como humedal facultativo en la Lista Nacional de Plantas de Humedales. Esto significa que la especie suele ser hidrófita, aunque ocasionalmente puede ser encontrada en terrenos elevados. Evaluamos las diferencias genéticas (ecotípicas) entre las plantas muestreadas en humedales y en terrenos elevados. A través de secuenciación, obtuvimos el genotipo de 1381 loci de 132 plantas pertenecientes a 30 localidades que representaban plantas de humedales y de tierras altas. Los análisis indicaron que la señal genética más fuerte proviene de las diferencias en la distribución geográfica: las muestras que se encuentran a mayor proximidad geográfica tienden a ser más similares genéticamente, independientemente de que pertenezcan a humedales o tierras altas. En general, no detectamos ningún efecto del hábitat en la estructura genética y encontramos unicamente 2 loci (de los 1381) con una asociación positiva entre genotipo y hábitat, en ambos casos la asociación fue muy débil. Por lo tanto, inferimos que la presencia de *A. occidentalis* cerca o dentro de los humedales no es influenciada por diferencias genéticas significativas. Además, no encontramos evidencia de ecotipos de humedales y tierras altas en esta especie.

In the United States, wetlands are delineated based on the presence of 3 factors: hydrophytic vegetation, hydric soils, and wetland hydrology, using methods described in the *US Corps of Engineers Wetland Delineation Manual* (hereafter the 1987 Manual; Environmental Laboratory 1987) and the appropriate Regional Supplement (e.g., USACE 2008). To determine whether vegetation is predominantly hydrophytic or nonhydrophytic, plant species have been assessed using wetland indicator status ratings on *The National Wetland Plant List* (NWPL) (e.g., Lichvar et al. 2016). On the NWPL, plant species are rated in 5 categories, which originally represented the frequency with which plant species occur in wetlands, based on literature and field observations: obligate (OBL), >99%; facultative wetland (FACW), 67%–99%; facultative (FAC), 34%–66%; facultative upland (FACU), 1%–33%; or upland (UPL), <1% (Reed 1988, Lichvar and Minkin 2008, Lichvar and Gillrich 2011, Lichvar et al. 2012). Species rated FACW occur in wetlands most of the time but are occasionally found in uplands (Lichvar et al. 2012). The rating of the vegetation in an area is a function of the frequencies of occurrences of plants in these different categories (Reed

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1988). The result is that facultative hydrophytes (FACW) have a significant association with the wetland habitat of an area.

Much of the recent literature discussing hydrophytes has referenced older works and presented possible reasons why plants can, on occasion, occur in wetlands (Tiner 1991). For example, Tiner (1991), citing earlier work (Turesson 1922a, 1922b, 1925), discusses how genetic differences between ecotypes could enable an upland species to be found occasionally in a wetland. However, the relationship between wetland ecotypes and their genetic basis has rarely been tested. Here, we examine the relationships between geographic range and wetland occurrences on genetic variation in the FACW species *Allenrolfea occidentalis* (S. Watson) Kuntze (Amaranthaceae), iodinebush. We test whether the differences in occurrences between wetland and upland conditions are associated with genetic differences. These occurrences in both wetlands and uplands may represent a wide ecological range and tolerance of the species to both wet and dry conditions. However, an alternative scenario is that this FACW species may consist of 2 different ecotypes: one that is an obligate upland dweller and one that is an obligate hydrophyte (Tiner 1991). Typically, ecotypes will differ morphologically, but this is not always the case, and sometimes the differences are cryptic but with a clear genetic basis (e.g., Menz et al. 2015, Steane et al. 2015). If *A. occidentalis* has distinct wetland and upland ecotypes, then the FACW designation could be misleading because one of the ecotypes would be a true obligate wetland plant.

Allenrolfea occidentalis is typically found in low-lying areas and lakebeds, with salty and usually alkaline soils. The genus has only 3 species globally, and *A. occidentalis* is the only species found in North America. We chose this species because it is a facultative wetland hydrophyte (FACW), it is a diploid species, and it has no close relatives with which to hybridize. Both polyploidy and hybridization can confound population genetic analyses by obscuring relationships. Furthermore, no apparent morphological differences can be discerned between plants from different habitats or across its range.

We examined the genetic structure of *A. occidentalis* populations using the technique of genotyping by sequencing (GBS), also known as ddRAD-seq (Gompert et al. 2012, Parchman et al. 2012). This suite of methods uses a genomic library preparation method that enables multiplexing of up to several hundred individual samples. The technique samples hundreds of thousands of genomic regions in such a way as to maximize the overlap across individuals, thereby providing the researcher with a large sample of individuals and genetic loci. Genetic variants are in the form of differences in the nucleotides at homologous positions (i.e., single nucleotide polymorphisms [SNPs]).

The objectives of our study of *A. occidentalis* are to use ddRAD-seq to (1) examine the distribution of genetic variation across individuals and populations and (2) determine whether there are genetic markers that provide evidence for wetland versus upland ecotypes.

METHODS

Sampling

Allenrolfea occidentalis collections were made at 30 locations (or populations) across Nevada and Utah, USA, including both upland and wetland habitats. Distances between sites ranged from 0.02 km to 572.7 km (Fig. 1). At each of the 30 sites, we sampled 3 to 10 individuals for a total of 132 plants (Table 1). Leaves from each sample were dried and stored in silica gel at room temperature. Of the 132 samples, 20 were processed in duplicate through the subsequent protocols, starting with DNA extraction. This sample replication was performed blindly and was done as a test of data quality assurance. We used the U.S. Army Corps wetland delineation method to designate wetland and uplands in the field by the presence of hydrophytic vegetation, hydric soil indicators, and wetland hydrology indicators (Environmental Laboratory 1987, USACE 2008). A Munsell Soil Color Chart (Munsell 2000) was used to describe the hue, value, and chroma of each soil layer, when appropriate (Environmental Laboratory 1987, USACE 2008), to test for hydric soil indicators.

DNA Extraction and Genomic DNA Library Preparation

Genomic DNA was extracted using the Qiagen DNeasy 96 Plant kit instructions and

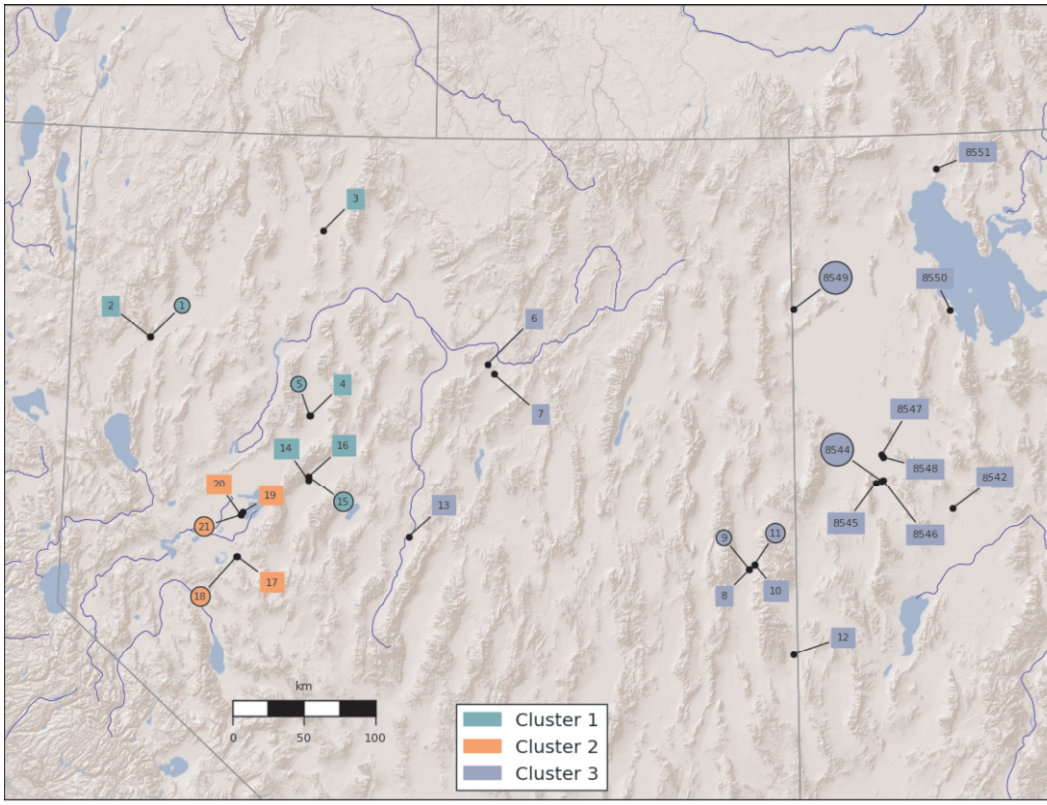

Fig. 1. Locality of *Allenrolfea occidentalis* populations sampled in western USA (see also Table 1). Colors refer to clusters from DAPC analyses. Circles with black outlines denote wetland populations, and boxes denote upland populations.

also by the CTAB method (Doyle and Doyle 1987). Extractions were assessed for quality and quantity via visualization on a 1% agarose gel and a NanoDrop instrument (Thermo Scientific, Wilmington, MA). A genomic library was generated following a standard double digestion restriction site–associated DNA sequencing (ddRADseq) protocol (Gompert et al. 2012, Parchman et al. 2012). The restriction enzymes EcoR1 and Mse1 were used to fragment the genomic DNA. Barcoded (indexed) oligonucleotides (with barcodes unique to each individual) were ligated to the EcoRI ends of DNA fragments, and a standard, nonbarcoded oligonucleotide was ligated to the MseI ends of the fragments. Samples were then PCR-amplified using iproof high-fidelity DNA polymerase (New England Biolabs Inc., https://www.neb.com) with primers that overlap the ligated oligonucleotides. To reduce stochastic variation in PCR amplification, all fragments were first mixed with only one other individual and were then further amplified in duplicate. The library was then reduced to fragments in the size range of 350–450 bp using a BluePippin (Sage Science, Beverly, MA). Quality and quantity was further verified using TapeStation 2200 (Agilent Technologies, Santa Clara, CA). The size-selected multiplexed samples were run on a single lane of Illumina HiSeq4000 with 100-bp single-end sequencing at the Genomic Sequencing and Analysis Facility at the University of Texas at Austin (GSAF).

DNA Data Processing

Raw Illumina reads were processed with ipyrad v.0.5.15 (Eaton 2014). This process was carried out twice, with the first round using the entire data set of 161 samples (including replicates) to identify low-coverage and failed reads that should be removed from further analyses, as well as to verify replication for quality control. Assessment of the replicated samples was tested by use of a neighbor-joining (NJ) tree using adegenet (Jombart

TABLE 1. Locality information for samples of *Allenrolfea occidentalis* collected for this study. Vouchers are deposited at UTC (Intermountain Herbarium, Logan, UT). Collections were made by R.W. Lichvar and associates. *N* refers to the sample size in the final analysis.

Population	Longitude	Latitude	*N*	Designation
1	−119.349	40.697	5	wetland
2	−119.349	40.697	5	upland
3	−117.945	41.405	5	upland
4	−118.013	40.240	5	upland
5	−118.018	40.239	5	wetland
6	−116.567	40.583	5	upland
7	−116.514	40.523	5	upland
8	−114.422	39.290	5	upland
9	−114.422	39.291	4	wetland
10	−114.376	39.320	5	upland
11	−114.380	39.320	5	wetland
12	−114.078	38.755	5	upland
13	−117.189	39.490	5	upland
14	−118.015	39.837	5	upland
15	−118.013	39.857	5	wetland
16	−118.013	39.858	5	upland
17	−118.576	39.342	10	upland
18	−118.573	39.342	10	wetland
19	−118.539	39.617	5	upland
20	−118.555	39.601	5	upland
21	−118.555	39.599	5	wetland
8542	−112.764	39.647	3	upland
8544	−113.339	39.834	3	wetland
8545	−113.386	39.820	2	upland
8546	−113.326	39.837	1	upland
8547	−113.331	39.998	1	upland
8548	−113.321	39.985	1	upland
8549	−114.027	40.923	2	wetland
8550	−112.734	40.896	3	upland
8551	−112.817	41.781	2	upland

2008) in R. NJ trees are a reliable tool for observing differences between recently diverged samples in a population study. All replicate samples branched most closely with their counterparts, indicating that our protocol is reliable and repeatable. The second round of analysis was performed on a reduced data set of 132 individuals and included a more stringent filtering to remove possible duplicated loci, loci with low coverage, and loci that were not in more than 100 of the 132 samples. All raw DNA sequence data plus every detail of the data processing steps and parameters used are available on Digital Commons (https://doi.org/10.15142/T35633). Using ipyrad, we generated within-sample clusters using USEARCH (Edgar 2010), and we aligned reads using MUSCLE (Edgar 2004). Error rate and heterozygosity were then estimated, and consensus bases were called and filtered. Finally, clusters were generated across samples and filters were applied to the resulting data, generating a number of genotype output formats. Due to the lack of a reference genome, ipyrad assembled the data de novo using vsearch (Enns et al. 1990). The clustering threshold was set to 90% sequence similarity.

To identify genetic subdivision, we used discriminant analysis of principal components (DAPC; Jombart et al. 2010) using adegenet in R. DAPC is a multivariate analysis that builds on the strengths of principal component analysis (PCA) and discriminant analysis (DA). PCA can identify genetic structure among individuals and accommodate large data sets. However, it does not differentiate between-group divergence and within-group variation. DA, on the other hand, differentiates these 2 groupings such that within-group variation is minimized while between-group variation is maximized, resulting in better-defined groups or clusters. The downside of DA, however, is that it can only be applied to small data sets. DAPC weds the assets of PCA and DA by first transforming the data using PCA. This initial step returns an uncorrelated and reduced data

set. Though the data are reduced at this step, information is not necessarily lost because the variation in the original data is maintained by retaining all principal components. At this point, a k-means algorithm is used to infer genetic clusters, and a statistical measure of goodness of fit using Bayesian information criterion (BIC) is taken across the number of clusters to infer the optimal clustering, which in our case was k = 3. This means that the optimal explanation for the data is that there are 3 main clusters of populations across our samples. Note that we used DAPC to cluster individuals based on genotype, but we were blind to which population they belonged.

To examine the relationship between geographic distance and genetic distance, we used adegenet to calculate Euclidean genetic distances for all pairwise comparisons of individuals and then examined the regression of Euclidean distance on geographic distance calculated by Vincenty's formula (Vincenty 1975). A positive relationship would indicate fit to an isolation-by-distance model, in which individuals are more likely to exchange genes with proximal individuals.

Environmental Association Analysis

BayPass software (Gautier 2015) was used to determine which loci, if any, were nonrandomly associated with habitat (wetland vs. upland). In identifying covariance between loci and an environmental variable, underlying complexities must be addressed. These include missing data, differences in sample sizes between populations, and underlying correlations (or differences in allele frequencies) between populations due to shared histories, location, and gene flow (Gautier 2015). BayPass addresses these problems in part by using a Bayesian method based on the model in BayEnv (Coop et al. 2010). A null model estimating underlying patterns of covariance in allele frequencies between populations is created. In estimating this covariance structure, a transformation of the population frequency of an allele across populations is assumed to have a normal distribution (Coop et al. 2010). Hence, this model accounts for populations deviating from ancestral or global allele frequencies. An alternative model is also created that reflects a linear relationship of transformed allele frequencies at a locus and the environmental variable. A Bayes Factor (BF), or measure of support, is created at each SNP. This covariance matrix is estimated using a Monte Carlo Markov chain (MCMC). Individual SNPs are then tested against this population covariance matrix using the Bayesian method. The 132 individuals were grouped into their respective 30 populations. A corresponding ecotype file was created where each population was numerically coded as wetland, 0, or upland, 1. We used the following parameter values for BayPass: 3000 post-burn-in and thinned samples (-nval), thinning of 1000 (-thin), 160,000 burn-ins (-burnin), and 20 pilot runs (-npilot). BayPass runs were repeated for a total of 96 chains. Correlation tests were made between Bayes Factor (BF) values in $10*\log_{10}$ units, BF_{10}, from the means of the first 48 chains compared to those of the second 48 chains.

RESULTS

After data quality filtering, we retained 1384 loci for which we had sufficient genotype data on 100 or more of the 132 individuals. Initial screens of genotypes indicated that in all cases, replicated samples were more similar to each other than to any other samples, indicating that our genotyping method appears to be reliable. We first examined population subdivision and relationships among individuals. Most populations contained individuals from 1 of the 3 DAPC clusters (Fig. 2), with only 4 exceptions. Populations 3 and 5 each had 4 individuals in cluster 1 and 1 individual in cluster 3; population 7 had 4 in cluster 3 and 1 in cluster 1; and population 17 had 9 in cluster 2 and 1 in cluster 1. At the individual level, most plants had more than 90% assignment to one cluster (Fig. 2), with only 8 individuals having a membership in more than one cluster by this criterion (Fig. 3), perhaps indicating recent gene flow and admixture.

Several aspects of the analysis indicated a genetic structure that reflected geographic proximity. For example, from the DAPC analysis, cluster 1 (teal) is distributed mostly in northwestern Nevada, cluster 2 (orange) is in southwestern Nevada, and cluster 3 (blue) is in Utah and eastern Nevada (Fig. 1). Furthermore, in the neighbor-joining tree, individuals from wetland sites are more similar to nearby upland plants than they are to wetland plants from elsewhere (Fig. 4). For example,

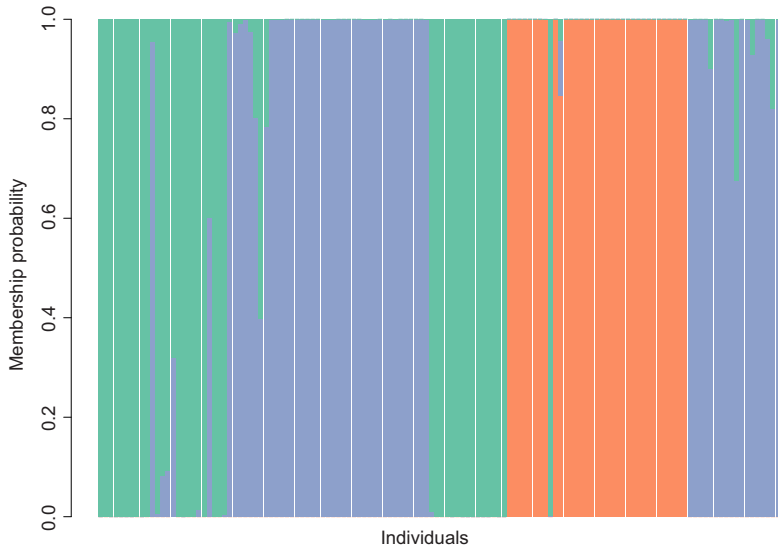

Fig. 2. Structure-like output from DAPC, showing the membership of individuals in the 3 main clusters. Each column represents a single individual, and individuals are ordered numerically (see Table 1). Colors of clusters are the same as in Fig. 1.

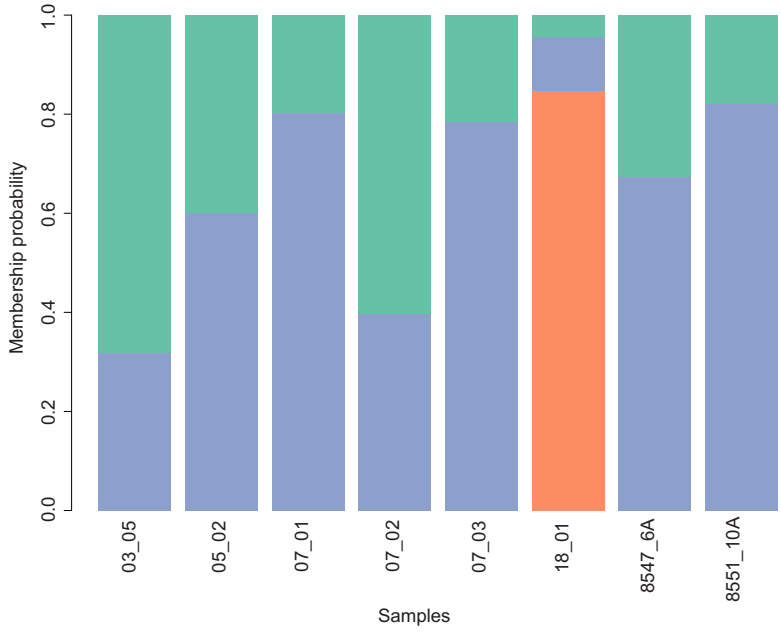

Fig. 3. Admixture proportions from the 8 samples that showed a mix of contributions from more than one cluster, where the maximum proportion from a cluster was <90%. Colors of clusters are the same as in Fig. 1.

populations 1 (wetland) and 2 (upland) are within 1 km of each other and are genetically similar (Fig. 4), whereas individuals in populations 1 and 8544 are both wetland, are 520 km from each other, and are quite distant on the tree. Thus, the strongest genetic signal is that of geographic proximity. This relationship was further explored with a regression of genetic

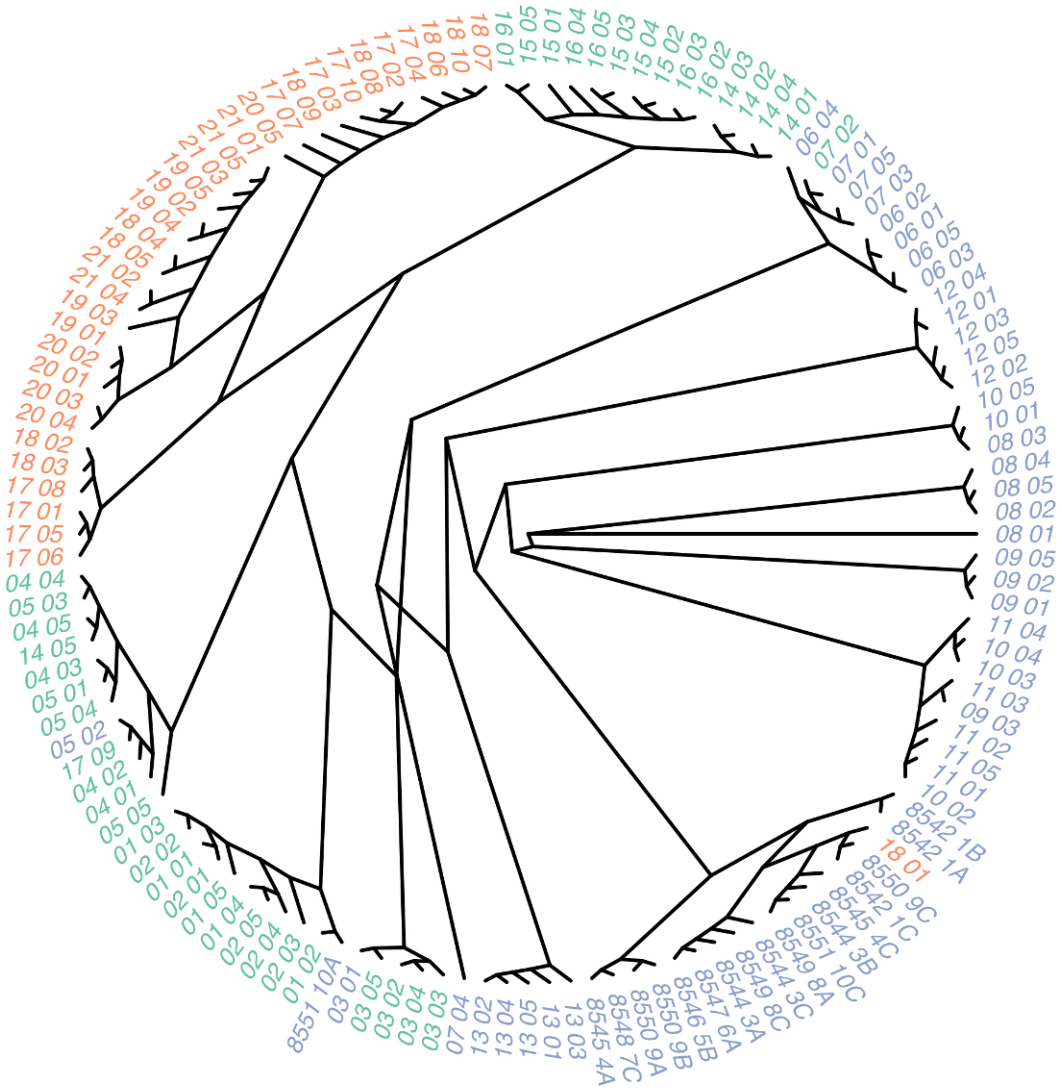

Fig. 4. Radial neighbor-joining tree of individual samples based on genetic distance. Colors refer to the 3 clusters produced by DAPC (see text).

distance on geographic distance (Fig. 5). This regression analysis shows a distinct positive relationship, consistent with an isolation-by-distance model. The adjusted R^2 was 0.269 (*P* value very close to zero) indicating a positive regression of genetic distance on geographic distance.

The correlation of Bayes factors for each locus across the 2 sets of 48 BayPass runs was 0.983, with a *P* value of effectively zero. We detected only 2 loci (of the >1300 tested) with a BF_{10} value of >10, indicating that the allelic distributions for these loci are >10 times more likely under the hypothesis of there being a genetic difference between the wetland and the upland ecotypes (versus no difference between the ecotypes). One locus had no significant similarities (based on an NCBI blastn search of the nonredundant GenBank database) to any known nucleotide sequence. The other had a close match to the NAD(P)H-ubiquinone oxidoreductase B3 in apple (*Malus;* GenBank accession XM_008370277). This gene encodes a mitochondrial membrane protein that has been linked to functions under anaerobic conditions (Igamberdiev and Hill 2009).

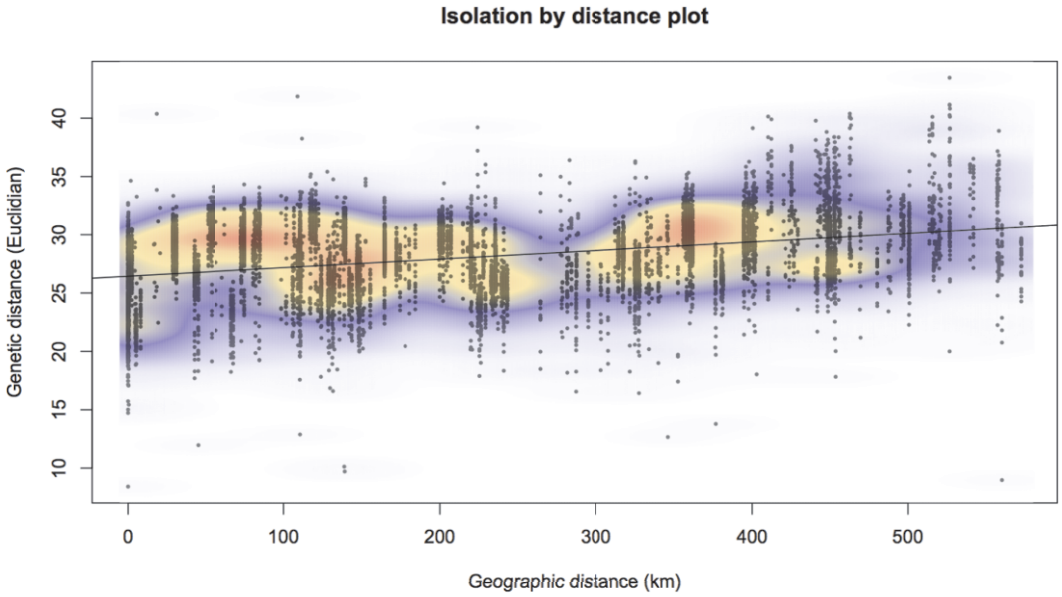

Fig. 5. Relationship between genetic and geographic distance of samples.

DISCUSSION

Patterns of genetic structure and genetic similarity among samples of *A. occidentalis* reveal that the strongest signal is geographic in nature. In general, samples that are closer in geographic proximity tend to be more similar genetically. We see no evidence of genome-wide similarities of wetland plants from distant localities.

We used a Bayesian statistical approach to test loci for which genotype is positively associated with habitat. Searching for such patterns has significant limitations. If positive associations are detected, they may be spurious false positives, resulting from small sample sizes and a large number of loci. Furthermore, even a true positive association may not point to the locus that is being affected directly by natural selection. This is because loci that are close together on a chromosome tend to have nonrandom associations of alleles in a population (i.e., linkage disequilibrium). Thus, a locus with a positive association with habitat might be as far as several recombination units from the locus that is actually under selection (Zapata et al. 2002, Koch et al. 2013). Although physical chromosome distance can vary considerably, one recombination unit is, on average, close to a million base pairs in humans and many other organisms (Yu et al. 2001). Even a failure to detect locus and habitat associations does not mean that they do not exist. In the case of *A. occidentalis*, we detected 2 loci with weak associations. The Bayes Factor for the NAD(P)H-ubiquinone oxidoreductase gene was moderate (11.3); however, the alleles were only marginally different in frequency between the 2 habitats. The frequency of the A allele at this SNP in wetlands was 0.275 and in uplands was 0.253. The other locus also had a moderate Bayes factor and a negligible difference in allele frequency. It is unlikely that either of these 2 associating loci is related to habitat adaptations, and if they were, it would be a marginal effect. It is possible that these loci are false positive associations, perhaps a result of testing so many loci. The majority of the genetic signal was clearly geographic in nature.

The goal of this research was to determine whether the FACW designation for *A. occidentalis* represents a species' ability to occur in wetlands and uplands due to genetic differences between ecotypes that are morphologically indistinguishable. Our analyses suggest that *A. occidentalis* has the ecological amplitude that allows it to occur in both wetlands and uplands, and we find no evidence for genetic-based ecotypes. If we were instead

dealing with a species that does have genetic ecotypes, what type of evidence would we find? Obviously, when ecotypic (environmental) signal is stronger than geographic signal, this would be clear evidence of ecotypes. This would be evident from both genetic distance trees and DAPC analyses, which would group samples from the same habitat but from different geographic areas. A more likely outcome is that the predominant genetic structure signal is that of geography, especially for a species with a wide geographic range, but with an additional genetic signal that reflects habitat. This could occur if only a portion of the genome has responded to selection to habitat. When this is the case, the search for loci associated with habitat would yield more positive results than we detected here for *A. occidentalis*. We would expect to find loci for which allele frequencies were quite different in the 2 habitats. In this study, we tested for ecotype occurrences to help explain a plant species' wetland rating. We found no evidence that species occurrences in uplands and wetlands are a result of genetic differences. We hypothesize that *A. occidentalis* can be found in wetlands and in uplands because of phenotypic plasticity.

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