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Spectrophotometry of *Artemisia tridentata* to Quantitatively Determine Subspecies☆

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ABSTRACT

Ecological restoration is predicated on our abilities to discern plant taxa. Taxonomic identification is a first step in ensuring that plants are appropriately adapted to the site. An example of the need to identify taxonomic differences comes from big sagebrush *(Artemisia tridentata)*. This species is composed of three predominant subspecies occupying distinct environmental niches, but overlap and hybridization are common in ecotones. Restoration of *A. tridentata* largely occurs using wildland collected seed, but there is uncertainty in the identification of subspecies or mix of subspecies from seed collections. Laboratory techniques that can determine subspecies composition would be desirable to ensure that subspecies match the restoration site environment. In this study, we use spectrophotometry to quantify chemical differences in the water-soluble compound, coumarin. Ultraviolet (UV) absorbance of *A. tridentata* subsp. *vaseyana* showed distinct differences among *A.t. tridentata* and *wyomingensis*. No UV absorbance differences were detected between *A.t. tridentata* and *wyomingensis*. Analyses of samples from > 600 plants growing in two common gardens showed that UV absorbance was unaffected by environment. Moreover, plant tissues (leaves and seed chaff) explained only a small amount of the variance. UV fluorescence of water-eluted plant tissue has been used for many years to indicate *A.t. vaseyana;* however, interpretation has been subjective. Use of spectrophotometry to acquire UV absorbance provides empirical results that can be used in seed testing laboratories using the seed chaff present with the seed to certify *A. tridentata* subspecies composition. On the basis of our methods, UV absorbance values < 2.7 would indicate *A.t. vaseyana* and values > 3.1 would indicate either *A.t. tridentata* or *wyomingensis*. UV absorbance values between 2.7 and 3.1 would indicate a mixture of *A.t. vaseyana* and the other two subspecies.
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Introduction

Distinguishing native plant taxa is fundamental to restoring and conserving natural ecosystems and has been mandated in the policy of land management agencies (Olwell and Riibe 2016). However, the task of determining taxonomy becomes more challenging in taxa with overlapping distributions and intermediate forms. Taxonomic complexity across varying spatial scales describes the circumstances for the subspecies of *Artemisia tridentata* (big sagebrush). As a species, *A. tridentata* occupies a broad environmental niche of the semiarid cold desert and nonforested uplands of western North America. This species is of great conservation concern because of loss primarily from wildfire and invasive species. All three predominant subspecies (*A.t. vaseyana* [mountain big sagebrush], *A.t. wyomingensis* [Wyoming big sagebrush] and *A.t. tridentata* [basin big sagebrush]) fill distinct environmental niches (Mahalovich and McArthur 2004; Still and Richardson 2015). However, overlap along elevation and edaphic gradients is common and composition of subspecies varies considerably depending on environmental heterogeneity (McArthur et al. 1988; Goodrich et al. 1999; McArthur and Sanderson 1999). In addition, hybridization is known to occur between subspecies (Wang et al. 1997; McArthur and Sanderson 1999). Distinguishing subspecies (or hybrids) in these transitional areas can be problematic. Subsequently, commercial seed collection activities may cross these ecotones with little indication of the loss of subspecies homogeneity in the seed collection. Thus, *A. tridentata* seed collections encompassing many hectares can be a mixture of subspecies (Richardson et al. 2015). Seed collections consisting of subspecies mixtures do not necessarily diminish their value in restoration, but knowing the composition of subspecies in a collection is essential information for their appropriate placement on the landscape, increasing success of establishment and long-term resiliency.

Ideally, diagnostic characters for sagebrush subspecies would be compatible with seed testing procedures. Fortunately, techniques that

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distinguish *A. tridentata* subspecies can be determined from small amounts of seed and seed chaff that accompany seed harvest. For example, seed weights distinguish *A.t. wyomingensis* from basin big sagebrush (*A.t. tridentata*, Richardson et al. 2015), and the elution of plant tissues in water or ethanol has been shown to be diagnostic for *A.t. vaseyana* under an ultraviolet (UV) black light. Specifically, subspecies *vaseyana* elution's emits a brighter iridescent glow compared with those of the other subspecies (Stevens and McArthur 1974). The difference in UV fluorescence is caused by differing concentrations of coumarin compounds, producing a strong iridescent blue color when dissolved in water and illuminated by black light. Coumarin is found in all subspecies, but at higher concentrations in *A.t. vaseyana* (McArthur et al. 1988). The UV test has been shown to be effective and easy to use; however, results are subjective and based on the observer interpretation of the degree of iridescence, usually a scale from 1 − 5. An empirical value for UV testing is needed to standardize seed testing. Shumar et al. (1982) first reported on the use of a spectrophotometer to provide empirical data distinguishing big sagebrush subspecies. However, spectrophotometry results from a later study showed that large variance in UV absorbance within subspecies made distinguishing subspecies using this technique difficult (Spomer and Henderson 1988). In our current study, a similar approach is employed by using a plate spectrophotometer to assess UV absorbance spectra among subspecies of *A. tridentata*. We use *A. tridentata* plants growing in two common gardens to assess the effectiveness of UV absorbance spectra in determining subspecies and evaluate the influence from environmental effects and differences between tissue types. Techniques evaluated in this study have the potential to be used as a rapid and effective seed certification method for distinguishing *A.t. vaseyana* from *A.t. tridentata* and *A.t. wyomingensis*.

Methods and Materials

More than 600 big sagebrush samples representing 55 populations were collected from two common gardens growing at Majors Flat, Utah (lat 39.339, long − 111.520, elev 2 105 m) and Orchard, Idaho, United States (lat 43.322, long: − 115.998, elev 974 m). Common gardens served to isolate environmental and genetic effects. Orchard is a warm, dry site and Majors Flat is a cool, relatively mesic site. Seeds for these common gardens were collected in 2009 from populations distributed across 11 states in the western United States. Populations were defined as randomly collected seed from separate plants within < 1 ha. Germinants were reared in 6-inch cone-tainers for 3 mo before outplanting in the spring of 2010. A combination of genetic markers, morphology, ploidy, visual-based UV fluorescence (Richardson et al. 2012), and volatile organic compounds (Jaeger et al. 2016) were used to verify subspecies before UV spectra analysis.

For each sample, fresh leaf material was removed from the stem, weighed to 0.9 g and diced (1 mm^2) into a 1.5 mL tube. Molecular-grade water (850 μL) was added to each sample and incubated at room temperature for 3 min. Eluted samples were filtered (30-μm mesh) into a 1.5-mL tube to remove leaf debris. A 200-μL aliquot of each sample was pipetted into the well of a clear, flat-bottom, 96-well plate. One well was reserved for a negative control (water). Samples underwent an absorbance spectrum analysis in a Biotek Epoch microplate spectrophotometer (Winooski, VT), and scanning was conducted in 10-nm increments from 240 − 370 nm. The sample preparation process was repeated for sagebrush seed chaff collected from the Majors Flat garden to test for similarities or differences in the absorbance spectrum for different tissues (leaf tissue vs. seed chaff). Seed chaff included floral parts (e.g., pappus and stem) and leaf fragments that are obtained during the seed collection process and is used as inert material during seeding.

Because some seed collections are a mixture of subspecies, we evaluated the effect of varying ratios of *A.t. vaseyana* on UV absorbance values. Three replications of seven different weight ratios of *vaseyana:tridentata* leaf tissue totaling 0.9 g were analyzed. These ratios equated to 10%, 20%, 30%, 50%, 70%, 80%, and 90% *A.t. vaseyana* composition. UV absorbance was acquired using the same methods as described earlier. In addition to subspecies mixture, two hybrid plants obtained from controlled crosses were analyzed for UV absorbance (McArthur et al. 1988).

A linear mixed-effect model (LMM) was used to assess differences in absorbance spectrum at 340 nm. We chose 340 nm because this data point showed the lowest absorbance values for *A.t. vaseyana* (Fig. 1a) and low variance for all subspecies (data not shown). Absorbance spectrum data were compiled according to garden, subspecies, population, and sample name (Table S1, available online at http://dx.doi.org/10.1016/j.rama.2017.07.004). An LMM segregated explanatory variables into fixed and random effects. Fixed effects were assigned to subspecies using the methods described earlier, and random effects were assigned to garden and the interaction between garden and population. Gardens were used to assess environmental variance, and population-garden interaction determined genetic × environment interaction (G × E).

In a subsequent experiment, absorbance spectrum data at 340 nm were analyzed for differences in tissue types: leaf and seed chaff. These data were collected at one garden, Majors Flat, since environment was found to be nonsignificant. Data were compiled according to tissue, subspecies, population, and sample name (Fig. S1, available online at http://dx.doi.org/10.1016/j.rama.2017.07.004). The statistical analysis was similar as described earlier except the random effect "tissue" replaced "garden."

Analyses were conducted in R v3.1.2 (R Core Team 2016) with the packages LME4 v1.1 (Bates et al. 2015), and significance for fixed effects *P* values used Satterthwaite's approximation for degrees of freedom calculated with lmerTEST v2.0 (Kuznetsova et al. 2016). Conditional and marginal R^2 values (Johnson 2014) were calculated in both models with r.squaredGLMM function in the MuMin package (Barton 2015).

Results and Discussion

UV absorbance of leaf tissue from spectrophotometry distinguished *A.t. vaseyana* from *A.t. tridentata* and *wyomingensis*. This technique did not distinguish between the subspecies *tridentata* and *wyomingensis* (Table 1, Fig. 1a). The distinction of *A.t. vaseyana* in UV absorbance was apparent from a wavelength between 290 nm and 370 nm with the greatest separation between 340 and 360 nm, regardless of garden collection. In contrast, *A.t. wyomingensis* and *tridentata* had similar spectra curves and overlapping 95% confidence intervals across all spectra tested (see Fig. 1a). These observations were supported by an LMM. Subspecies showed a highly significant difference between *A.t. vaseyana* and the other two subspecies at 340 nm, but no significant difference was found between *A.t. wyomingensis* and *tridentata* (see Table 1, $P < 0.0001$). The mixed-effects model explained 55% of the variation (conditional $R^2 = 0.55$) in UV absorbance at 340 nm. The majority of the variation accounted by the model, 78%, was explained by subspecies (marginal $R^2 = 0.43$). A smaller proportion of the absorbance variation (12%) was explained by environment and population × environment interaction. Garden (i.e., environment) alone showed no significant variance; however, population × garden (i.e., genetic × environment interaction) was significant ($P < 0.0001$).

Conditioned sagebrush seed collections are a matrix of seed, seed chaff, and leaves. Because the matrix of leaves and chaff would likely vary in composition across seed collections, we evaluated the effect of tissue types (leaves vs. chaff, Fig. 1b) and conducted LMM on the UV absorbance at 340 nm. Tissue type accounted for a significant amount of the random effect variance in absorbance, but compared with the differences in fixed-effect estimates found between *A.t. vaseyana* and other subspecies (0.7), the variance for tissue type is very small (0.0074, Table 2). These results suggest that variable composition of leaves and chaff would not compromise the determination of subspecies composition.

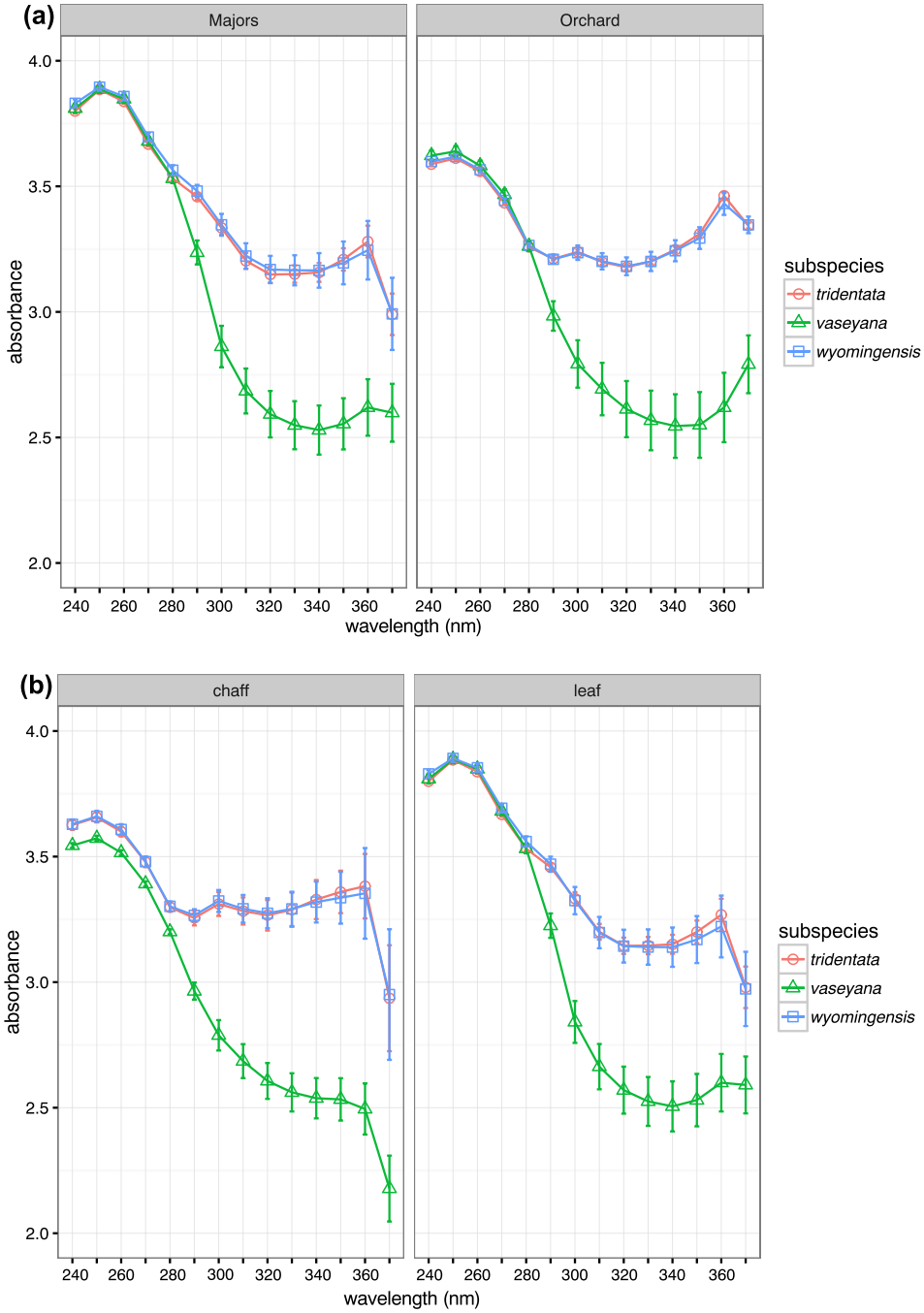

Figure 1. The absorbance spectrum for *Artemisia tridentata* from 240 nm to 370 nm. (**a**) Shows absorbance values for three subspecies (*tridentata*, *vaseyana*, and *wyomingensis*) from populations collected throughout the range of the species and planted at two common gardens (Majors Flat and Orchard). (**b**) Shows the spectrum for different plant tissues: chaff and leaf. Vertical error bars indicate the 95% confidence intervals.

Along with varying tissue composition, subspecies composition will also vary among seed collections. Spatial overlap among subspecies is common in ecotones between montane and basin habitats (McArthur et al. 1988; McArthur and Sanderson 1999). To assess how varying subspecies mixtures and hybrids affected UV absorbance values, spectrophotometry was conducted on seven different weight ratios between *vaseyana* and *tridentata* and two hybrid plants from control crosses between *A.t. tridentata* and *vaseyana* (McArthur et al. 1988). The results illustrate that coumarin from *A.t. vaseyana* can have a strong influence on the UV absorbance values. Even at low concentrations of *A.t. vaseyana* (10—20%), the UV absorbance was 2.6 at 340 nm. This value is within the 95% confidence intervals of *A.t. vaseyana* (see Fig. S1). Therefore, seed collectors targeting *A.t. wyomingensis* or *A.t. tridentata* will have to take care of the proximity to ecotones and *A.t. vaseyana* because only a small amount of this subspecies will greatly affect the absorbance results. Making several UV fluorescence assays with a handheld UV lamp at the seed collection site would help target the correct subspecies.

UV fluorescence has long been a valuable diagnostic test to determine *A.t. vaseyana*. However, the method to date is subjective, relying on a visual rating system. Shumar et al. (1982) first demonstrated the utility of spectrophotometry to assess big sagebrush subspecies. In this study, we show that determining UV absorbance through spectrophotometry provides a fast, empirical-based method to assess the composition of *A.t. vaseyana* in seed collections. We also show that this trait is insensitive to environmental effects and tissue type. In addition to determining seed weights (Richardson et al. 2015), this research

Table 1
Random effect variances and fixed effect estimates from linear mixed-effects model analysis of ultraviolet absorbance at 340 nm in *Artemisia tridentata*. Fixed effects are partitioned among subspecies, and random effects reflect different common gardens and the interaction between gardens and populations

Random effects	obs	Variance	SD	*P* value
Garden	2	0.0012	0.034	0.3
Population × garden	103	0.0142	0.120	2^{e-6}
Residual		0.0591	0.2431	
Fixed effects		**Estimate**	**SE**	***P* value**
Intercept *(tridentata)*		3.1873	0.0323	6^{e-4}
vaseyana		−0.6822	0.0423	$< 2^{e-16}$
wyomingensis		−0.0246	0.037	0.490

SD, standard deviation; SE, standard error.

Table 2
Random effect variances and fixed effect estimates from linear mixed-effects model analysis of UV absorbance at 340 nm from *Artemisia tridentata*. Fixed effects are partitioned among subspecies, and random effects reflect two tissue types: leaves and seed chaff

Random effects	obs	Variance	SD	*P* value
Tissue	2	0.0074	0.086	0.02
Population × tissue	95	0.0205	0.143	1^{e-13}
Residual		0.0594	0.244	
Fixed effects		**Estimate**	**SE**	***P* value**
Intercept (*tridentata*)		3.2295	0.0680	0.006
vaseyana		−0.7124	0.0443	$< 2^{e-16}$
wyomingensis		−0.0201	0.0456	0.660

SD, standard deviation; SE, standard error.

can be used as a seed certification step to inform managers of the subspecies composition, providing the necessary information to ensure *A. tridentata* plants are appropriately adapted to the site.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.rama.2017.07.004.

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