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Morphologic and Taxonomic Analysis of the Weedy and Cultivated *Amaranthus hybridus* Species Complex

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Abstract—The hybridus species complex of the genus *Amaranthus* is a group of weedy and cultivated plants from the New World that are considered difficult to identify. Classification schemes have varied between a single species approach, *Amaranthus hybridus* s.l., and a five species approach that recognizes the widespread weedy *A. hybridus* s.s., the South American endemic *A. quitensis*, and the three cultivated taxa (*A. hypochondriacus, A. cruentus,* and *A. caudatus*) as distinct species. The goals of this study were to analyze patterns of floral variation within the species complex and to determine distinguishing morphological features of the species. Twenty-one pistillate and twelve staminate floral characters from 41 specimens representing all five species were analyzed morphologically. Results indicate that morphological characters split the hybridus complex into two larger groups; that the widespread weedy *A. hybridus* divides into two morphologically distinct groups, each associated with different cultivated taxa; and that staminate morphological variation may be more taxonomically informative than previously assumed.

Keywords—Grain amaranths, pigweeds, qualitative characters, quantitative characters.

The *Amaranthus hybridus* species complex (Amaranthaceae) is a group of five species including the widespread agricultural weed *A. hybridus* L. (smooth pigweed), a South American endemic *A. quitensis* Kunth, and the three cultivated grain amaranths *A. hypochondriacus* L. from central Mexico, *A. cruentus* L. from southern Mexico and Guatemala, and *A. caudatus* L. from the northern and central Andes. These five species along with *A. dubius* Mart. ex Thell. are monophyletic (Waselkov 2013), although *A. dubius*, an allotetraploid species not known to be involved in recent hybridization with the other species, was excluded from this analysis. All species of the hybridus complex are monoecious, having flowers with five sepals, lacking petals, subtended by a bract, and a circumscissily dehiscent utricle typical of the subgenus *Amaranthus* (Mosyakin and Robertson 1996). Several studies of genetic diversity have been performed with a primary goal of elucidating the origins of the grain amaranths and their relationship to each other (Hauptli and Jain 1984; Gudu and Gupta 1988; Gupta and Gudu 1991; Transue et al. 1994; Kirkpatrick 1995; Chan and Sun 1997; Xu and Sun 2001; Mandal and Das 2002; Mallory et al. 2008; Jimenez et al. 2013; and Kietlinski et al. 2013). Despite these numerous studies of genetic diversity, classification in the hybridus species complex varies widely from a single species approach that lumps all five species into *A. hybridus* s.l. to recognition of five species (*A. hybridus* s.s., *A. quitensis, A. hypochondriacus, A. cruentus,* and *A. caudatus*). Difficulties in classification stem from sampling issues and lack of morphological studies.

All studies prior to Kietlinski et al. (2013) were plagued with sampling issues in regard to *A. hybridus* and *A. quitensis*, the potential weedy progenitor species of the cultivated grain species. Sampling issues included: exclusion of *A. quitensis* from the study; underrepresentation of progenitor species samples; or representation of *A. hybridus* primarily by specimens collected from outside their American center of origin and diversity. Results indicated *A. hybridus* as the progenitor species of the grain amaranths but varied in supporting either *A. hypochondriacus* sister to *A. caudatus* (Gudu and Gupta 1988; Gupta and Gudu 1991; Transue et al. 1994; Mandal and Das 2002; and Mallory et al. 2008; Kietlinski et al. 2013) or *A. hypochondriacus* sister to *A. cruentus* (Kirkpatrick 1995; Sun et al. 1999; and Xu and Sun 2001).

Species of flowering plants are often identified based on the presence of fixed diagnosable qualitative characters; however, floral variation in the hybridus complex consists primarily of quantitative differences between minute flower parts (Kirkpatrick 1995). Traditionally, species of the hybridus complex were identified based on three qualitative characters including seed color, bract to utricle length ratios, and sepal posture (Sauer 1950; 1967), although taxonomic significance of variation in these characters has not been rigorously tested (Table 1) and pale seeds, the "diagnostic" feature of the grain amaranths, is known to be unreliable as many populations of cultivated species are dark seeded (Table 1).

Species of the hybridus complex are frequently considered difficult to identify, partly due to the general lack of reliable fixed diagnostic qualitative characters. While there are many quantitative floral characters for the species of the hybridus complex, patterns of quantitative variation have not been statistically analyzed for either pistillate or staminate flowers. Staminate floral variation has been ignored in most treatments either because staminate flowers can be difficult to find in these monoecious species (Mosyakin and Robertson 2003), or because staminate floral variation has been assumed to be plastic and uninformative (Coons 1977), although this hypothesis has not been confirmed. The single morphological study to date (Kirkpatrick 1995) does not include staminate characters and includes no rigorous statistical analysis of individual pistillate characters.

The goals of this study are to:

1. Characterize patterns of quantitative and qualitative floral variation within and among species of the hybridus complex.
2. Analyze variation in quantitative characters using ANOVA and in qualitative characters using contingency tables.

Table 1. Diagnostic features of species classified in the hybridus complex (based on Sauer 1950, 1967).

Species	Seed Color	Bract: Utricle Length	Sepal Posture
A hybridus	Dark	Bract > Utricle	Straight
A quitensis	Dark	Bract > Utricle	Reflexed
A hypochondriacus	Pale or dark	Bract = Utricle	Straight to slightly reflexed
A cruentus	Pale or dark	Bract < Utricle	Straight
A caudatus	Pale or dark	Bract < Utricle	Reflexed

3. Determine distinguishing morphological features for species of the hybridus species complex.

Materials and Methods

Sampling—Forty-one specimens from five species representing maximum geographical diversity in the New World were obtained for morphological study using voucher specimens at the Stephen F. Austin State University Herbarium (SFC) from a previous analysis of microsatellite diversity (Kietlinski et al. 2013) or from USDA (Ames, IA) accession vouchers for accessions for which specimens at SFC were not available (Appendix 1). All specimens used in this study were from USDA accessions included in the SSR study (Kietlinski et al. 2013).

Morphological Procedure—Twenty-one pistillate and 12 staminate characters were chosen for analysis including a mix of raw bract, sepal, and utricle measurements as well as ratios of measurements (Appendix 2) for a total of 33 characters. Two pistillate flowers and two staminate flowers per specimen were removed, softened in Pohl's solution (Pratt and Clark 2001), dissected, and measured at 20x magnification under a dissecting scope. The two measurements for each flower were averaged and recorded to construct a raw data matrix.

Sampled quantitative characters included utricle and pistillate bract lengths as well as the bract to utricle length ratio that have traditionally been considered diagnostic features in the hybridus complex (Table 1; Appendix 2). Bracts in the hybridus species complex consist of a long excurrent midrib and a membranous lamina. Because the midrib is the most conspicuous feature of the bract, most treatments refer to the bract midrib length as the "bract length." For ease of comparing this study to previous studies and taxonomic treatments, we use the term "bract length" to refer to bract midrib length here as well.

Three qualitative diagnostic features, seed color, bract posture, and sepal posture (Table 1), were examined under the dissecting microscope. Seed color was recorded as white or dark for all specimens. Bract and sepal posture were recorded as straight or reflexed for the 32 specimens located at the Stephen F. Austin State University herbarium (ASTC) (Appendix 1). Specimens appear polymorphic for bract and sepal posture and individuals with reflexed or straight bracts and sepals can be found on most specimens. Each specimen was recorded for posture based on overall impression of the most common type of posture for that specimen.

Data Analysis—The morphological data were analyzed using JMP Pro 10.0.0 (SAS 2012) to compute principal components (PCA) based on a correlations matrix. The first two principle components were graphed and the eigenvectors were recorded. The PCA analyses were performed separately on the staminate, the pistillate, and the combined pistillate and staminate data. Alternate classification schemes were compared to the original USDA taxonomic designations using discriminant analysis computed using JMP Pro. The number of groups and composition of group membership was explored using k-means clustering in JMP Pro for 2–10 groups.

Patterns of pistillate and staminate characters were examined using Analysis of Variance (ANOVA) at the 95% significance level using JMP Pro using the revised classification provided by PCA and k-means clustering. Student's t post-hoc test was used to determine differences between group means using JMP. Patterns of variability for the qualitative characters of seed color, bract posture, and sepal posture were analyzed using contingency analyses in JMP Pro.

Results

Reclassified Materials—Four specimens were determined to be misidentified: Ames 5320 (listed in the USDA database as a potential hybrid) and Ames 5323, both originally listed as *A. cruentus* from Sonora Mexico, are here reclassified as a hybrids; PI 490752, originally listed as *A. hypochondriacus* from Guatemala, here treated as *A. cruentus*; and PI 490694, an *A. hybridus* accession from Ecuador that was mislabeled in the greenhouse as PI 511877 (an accession of *A. hypochondriacus*) and here reclassified as *A. quitensis* (Fig. 1). Discriminant analysis –2LogLiklihood scores of the combined staminate and pistillate data for this scheme improved from 0.001 for the USDA passport data identifications to 6×10^{-8} for the reclassification scheme outlined above.

Staminate PCA—The first two principal components account for 65.8% of the variability (Fig. 1) based primarily on sepal data (Table 2). Staminate data break *A. hybridus* s.s. into two geographically widespread but discrete groups along PCA 1. "Group 1" falls along quadrants one and three with *A. cruentus* and consists of individuals from the United States, Mexico, and Colombia. "Group 2" falls along quadrants two and four along with *A. quitensis*, *A. caudatus*, and *A. hypochondriacus* and consists of individuals from Guatemala, Brazil, and Peru (Fig. 1). K-means clustering optimizes two clusters; Group 1 corresponding to *A. hybridus* p.p. (group 1 above); *A. quitensis* p.p., *A. caudatus* p.p., *A. cruentus*, and the putative hybrid Ames 5320; Group 2 corresponds to *A. hybridus* p.p. (group 2 above), *A. quitensis* p.p., *A. caudatus* p.p., *A. hypochondriacus*, and putative hybrid Ames 5323 (Figs. 1, 2).

Pistillate PCA—The first two principal components of the pistillate PCA account for 59.1% of the variability (data not shown) based primarily on sepal data along the first dimension and bract to utricle length along the second (Table 2). The specimens form a single morphological continuum with no discrete breaks. In general *A. hybridus* is extremely diverse

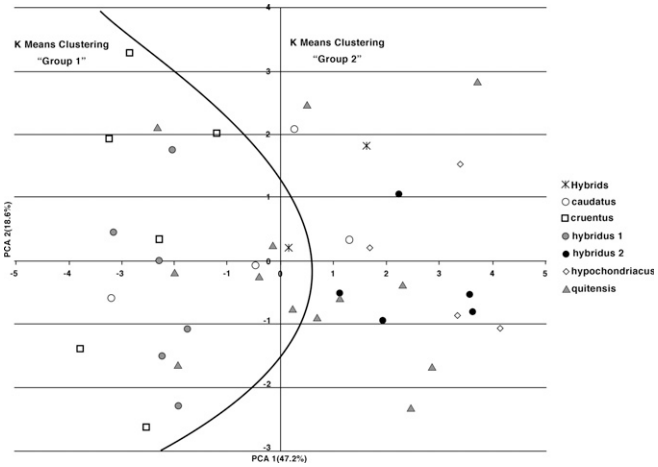

Fig. 1. Principal Components Analysis (PCA) of staminate floral characters of the hybridus species complex. Crossed X = Hybrids, open circles = *A. caudatus*, open squares = *A. cruentus*, grey circles = *A. hybridus* "group 1", black circles = *A. hybridus* "group 2", open diamonds = *A. hypochondriacus*, and grey triangles = *A. quitensis*. K-means clustering groups are indicated by the solid black line.

TABLE 2. Morphological characters contributing most to PCA 1 and 2 by data set. Characters contributing strongly to PCA 1 and 2 are listed from highest to lowest contribution based on highest absolute eigenvalues.

Staminate PCA		Pistillate PCA		Combined Data	
PCA 1	PCA 2	PCA 1	PCA 2	PCA 1	PCA 2
Outer Sepal Length	Inner Sepal Width	Outer Sepal Midrib Length	Bract to Utricle Length	Staminate Outer Sepal midrib Length	Pistillate Bract to Utricle Length
Outer Sepal Midrib Length	Outer Sepal Width	Outer Sepal Length		Pistillate Outer Sepal Length	Utricle Length
Inner Sepal Length		Inner Sepal Length		Pistillate Inner Sepal Length	Utricle Cap Length
		Inner Sepal Midrib Length		Pistillate Outer Sepal Midrib Length	Utricle Width
					Pistillate Bract to Outer Sepal Length

and covers the range of pistillate morphological variation. Although no discrete visual break occurs between the two groups of *A. hybridus* detected in the staminate data, they do conform to the staminate pattern with "group 1" located in the quadrants one and three with *A. cruentus, A. caudatus,* and *A. quitensis* and "group 2" located in quadrants two and four with *A. hypochondriacus*. *A. quitensis* groups primarily in the third and four quadrants. *A. caudatus* is scattered throughout the PCA. *Amaranthus hypochondriacus* is found along the right hand side of quadrants two and four. The k-means clustering optimizes two groups: Group 1 corresponding to *A. hybridus* p.p. as per the staminate data, *A. cruentus, A. caudatus,* and *A. quitensis*; and Group 2 corresponding to *A. hybridus* p.p. as per the staminate data, *A. hypochondriacus*, and both hybrids. Pistillate k-means clustering groups are indicated on the combined pistillate and staminate PCA (Fig. 2).

Combined Staminate and Pistillate PCA—The first two principal components of the PCA of quantitative pistillate and staminate characters account for 51.6% of the variability (Fig. 2) based on sepal, bract, and utricle data (Table 2). The largest eigenvector along the first dimension is a staminate floral character (Table 2). Pistillate and staminate floral variability of the hybridus species complex reveals a single morphological continuum (Fig. 2). Although the species visually appear to form a single cluster in PCA (Fig. 2), k-means clustering separates the species into two groups segregating along PCA 1. Group 1 corresponds to *A. hybridus* p.p. (group 1 as per staminate data); *A. quitensis* p.p., *A. caudatus, A. cruentus*, and the putative hybrid Ames 5320; Group 2 corresponds to *A. hybridus* p.p. (as per staminate data), *A. quitensis* p.p., *A. hypochondriacus*, and both putative hybrids (Fig. 2).

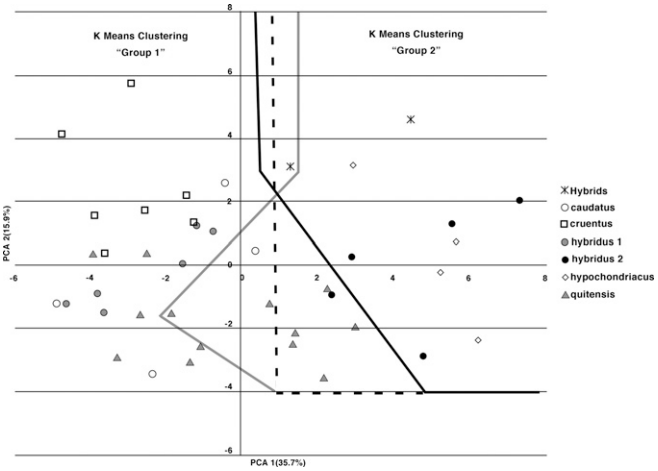

FIG. 2. Principal Components Analysis (PCA) of pistillate and staminate floral characters of the hybridus species complex. Crossed X = Hybrids, open circles = *A. caudatus*, open squares = *A. cruentus*, grey circles = *A. hybridus* "group 1", black circles = *A. hybridus* "group 2", open diamonds = *A. hypochondriacus*, and grey triangles = *A. quitensis*. K-means clustering groups are indicated by heavy lines as follows: black line = pistillate data; dashed line = combined data; and grey line = staminate data.

Character Analyses—Analysis of variation (ANOVA) performed on 33 quantitative floral characters shows that variation of 22 out of 33 characters (67%) is statistically significant (Table 3). Variation in 15 out of 21 pistillate characters (71%) is statistically significant and variation in seven out of 12 staminate characters (58%) is statistically significant (Table 3).

Contingency analysis of seed color was statistically significant (N = 41, df = 6, p = 0.0013). As predicted, *A. hybridus* and *A. quitensis* are uniformly dark seeded, and white seeds are a predictor for the cultivated species. However, all three cultivated species contain both white and dark seeded individuals, thus seed color is not a diagnostic character for the cultivated species. Bract posture was statistically significant (N = 32, df = 6, p = 0.0014). *Amaranthus quitensis* and *A. hypochondriacus* bracts were uniformly recurved. *Amaranthus caudatus* samples were mostly recurved (75% of samples). Eighty-five percent of *A. cruentus* bracts were straight, as were 66% of both types of *A. hybridus*. No species appeared to be completely fixed for straight bracts. Sepal posture does not appear to form any morphologically meaningful patterns and was not statistically significant (N = 32, df = 6, p = 0.2813).

DISCUSSION

Genetic Comparisons—The specimens examined in this study were voucher specimens of a study of genetic diversity of the hybridus complex (Kietlinski et al. 2013). As such, the results of this morphological study can be directly compared to that SSR study. Both molecular and morphological data reveal a single continuum of diversity using PCA, and both split *A. hybridus* into two distinct groups using k-means clustering of morphological data and structure analysis of genetic data. However, the studies are incongruent in the sister relationships of *A. caudatus*; number of clusters optimized by k-means clustering; and group membership of the two *A. hybridus* clusters.

TABLE 3. Statistically significant pistillate and staminate ANOVAs. *p* values and treatment averages listed. Student's t means comparisons provided as superscripts.

Pistillate Floral Characters								
Character	$F_{5, 33}$	P value	cruentus	hybridus 1	caudatus	quitensis	hypo	hybridus 2
Outer Sepal Midrib	12.8947	< 0.0001	1.70^{B}	1.57^{B}	1.51^{B}	1.68^{B}	2.64^{A}	2.60^{A}
Inner Sepal	11.1749	< 0.0001	1.28^{B}	1.30^{B}	1.34^{B}	1.41^{B}	2.05^{A}	2.06^{A}
Inner Sepal Midrib	10.5865	< 0.0001	1.33^{B}	1.35^{B}	1.42^{B}	1.46^{B}	2.11^{A}	2.19^{A}
Bract L	9.6031	< 0.0001	1.77^{D}	2.46^{BC}	2.02^{CD}	2.70^{B}	3.30^{A}	3.29^{A}
Outer Sepal	9.1160	<0.0001	1.58^{B}	1.44^{B}	1.49^{B}	1.60^{B}	2.39^{A}	2.36^{A}
Bract Lamina	7.4175	<0.0001	1.56^{C}	2.12^{BC}	1.77^{BC}	2.33^{B}	2.99^{A}	2.98^{A}
Utricle Tower	5.9055	0.0005	0.21^{BC}	0.26^{AB}	0.16^{CD}	0.13^{D}	0.18^{BCD}	0.30^{A}
Utricle Cap	5.8457	0.0006	0.86^{AB}	0.77^{BC}	0.74^{CD}	0.68^{D}	0.81^{ABC}	0.88^{A}
Bract Midrib: Utricle	5.7727	0.0006	1.12^{C}	1.70^{AB}	1.49^{BC}	1.95^{A}	1.88^{AB}	2.00^{A}
Inner Sepal Index	5.2261	0.0012	2.91^{C}	3.27^{BC}	2.82^{C}	2.98^{C}	4.01^{AB}	4.14^{A}
Utricle	4.4635	0.0032	1.60^{AB}	1.44^{BC}	1.40^{BC}	1.39^{C}	1.78^{A}	1.67^{A}
Outer Sepal Index	3.8776	0.0071	3.26^{AB}	3.01^{B}	3.15^{AB}	2.78^{B}	4.02^{A}	3.86^{A}
Bract Midrib: Outer Sepal	3.6549	0.0097	1.11^{B}	1.75^{A}	1.43^{AB}	1.70^{A}	1.38^{AB}	1.44^{AB}
Utricle: Outer Sepal	3.0051	0.0242	1.05^{A}	1.02^{A}	0.95^{AB}	0.88^{AB}	0.77^{B}	0.73^{B}
Utricle Base	2.6897	0.0381	0.79^{AB}	0.67^{B}	0.72^{B}	0.68^{B}	0.96^{A}	0.81^{AB}
Staminate Floral Characters								
	$F_{5, 33}$	P value	cruentus	hybridus 1	caudatus	quitensis	hypo	hybridus 2
Outer Sepal Midrib	12.4985	<0.0001	1.93^{C}	1.76^{C}	2.10^{BC}	2.25^{B}	2.95^{A}	2.80^{A}
Outer Sepal	11.7823	<0.0001	1.67^{CD}	1.58^{D}	1.96^{BC}	2.09^{B}	2.62^{A}	2.41^{A}
Bract Midrib	10.2082	<0.0001	1.53^{B}	1.78^{B}	1.95^{B}	2.52^{A}	2.83^{A}	2.81^{A}
Bract Lamina	9.4391	<0.0001	1.29^{B}	1.47^{B}	1.66^{B}	2.19^{A}	2.50^{A}	2.49^{A}
Inner Sepal Midrib	8.0379	<0.0001	1.54^{E}	1.64^{DE}	1.90^{BCD}	1.91^{C}	2.33^{A}	2.21^{AB}
Inner sepal	6.3287	0.0003	1.47^{D}	1.56^{CD}	1.84^{ABC}	1.86^{B}	2.17^{A}	2.08^{AB}
Inner Sepal Index	2.7794	0.0334	2.66^{B}	3.12^{AB}	3.09^{AB}	3.19^{AB}	3.81^{A}	3.72^{A}

Incongruence between morphological and molecular data sets is common (Duminil and Di Michele 2009). In general, incongruence can be ascribed to one of two phenomena: cryptic species that are morphologically indistinguishable but are genetically distinct, and morphological variation that is uncorrelated to genetic variation. Several biological processes are known to cause the second phenomenon: including local adaptation, phenotypic plasticity, and neutrality of many genetic markers (Duminil and Di Michele 2009).

Incongruence in the sister relationships of *A. caudatus* is not unsurprising, given that the relationships of *A. caudatus* have been particularly unstable with some studies showing a sister grouping with *A. hypochondriacus* while others indicate a sister grouping with *A. cruentus* (Kietlinski 2012; Kietlinski et al. 2013). Differences between the studies can be ascribed either to sampling issues or hybridization, although low sample number is likely to be a factor in this analysis.

K-means analysis of morphological data separate the species into two clusters, whereas structure analysis of molecular data indicates three. The primary difference lies in the treatment of *A. quitensis*, which forms a separate cluster in the SSR study primarily due to the presence of a nearly fixed unique allele (Kietlinski et al. 2013). The PCA and k-means morphological analyses are based solely on continuous characters and do not reflect the variation indicated by qualitative characters. The combination of short pistillate flowers based on quantitative characters reflected in PCA and k-means clustering combined with recurved bracts, a qualitative character not included in the PCA and k-means analyses, supports the findings of the SSR data indicating the distinctiveness of *A. quitensis*.

Both molecular and morphological data indicate that *A. hybridus* segregates into two distinct groups, although group assignment is inconsistent between the studies. Of the eleven *A. hybridus* specimens examined in this study, five of the specimens were consistently grouped with *A. cruentus* in both the morphological and molecular studies, one specimen was consistently placed with *A. hypochondriacus* in both studies, and placement was incongruent between studies for the remaining five specimens. Duminil and Di Michele (2009) list local adaptation, phenotypic plasticity, and neutrality as potential explanations in cases where patterns of morphological and molecular variation are incongruent. It is possible that flower sizes in the hybridus complex are under natural selection, whereas SSRs are assumed to be selectively neutral, in which case the differences in diversity patterns indicated by the types of data may reflect differences in evolutionary forces. Morphologically the species fall into two groups corresponding to flower size (Tables 3, 4). While we treat flower parts as separate characters, it is possible that our results reflect the effect of a single locus or suite of loci operating on flower size on all parts of the flower simultaneously, or that multi-locus selection is occurring for flower size. In this case all quantitative characters may be acting as a single selective unit that we have artificially subdivided.

While neutral markers such as SSRs are unaffected by selection and environment, they are sensitive to migration and effective population size. Comparison of two recent studies of genetic diversity in the hybridus complex shows an intriguing pattern. Average observed heterozygosity of USDA collections using SSRs was 2% (Kietlinski et al. 2013) whereas average observed heterozygosity in a study of South American accessions maintained by the Centro de Investigaciones de Cultivos Andinos (CICA) using SNPS was 20% (Jimenez et al. 2013). One possible explanation of this tenfold difference in observed heterozygosity is that the USDA accessions used in the SSR study may have been artificially bottlenecked during collection of the accessions. If so genetic

Table 4. Comparison of pistillate and staminate characters by group and species.

Pistillate Floral Characters					
Group 1				Group 2	
A. cruentus	*A. hybridus 1*	*A. caudatus*	*A. quitensis*	*A. hypochondriacus*	*A. hybridus 2*
Very short bracts	Long bracts	Short bracts	Long bracts	Very long bracts	Very long bracts
Long utricles	Short utricles	Short utricles	Short utricles	Very long utricles	Very long utricles
Long utricle cap	Moderate utricle cap	Short utricle cap	Very short utricle cap	Moderate utricle cap	Very long utricle cap
Moderate tower	Long tower	Short tower	Very short tower	Moderate tower	Very long tower
Seeds white or black	Seeds black	Seeds white or black	Seeds black	Seeds white or black	Seeds black
Bract Posture	Straight	Recurved	Recurved	Recurved	Straight
Staminate Floral Characters					
Group 1				Group 2	
A. cruentus	*A. hybridus 1*	*A. caudatus*	*A. quitensis*	*A. hypochondriacus*	*A. hybridus 2*
Short bract	Short bract	Short bract	Long bract	Long bract	Long bract
Short outer sepal	Very short outer sepal	Long outer sepal	Long outer sepal	Very long outer sepal	Very long outer sepal

diversity results of all studies of the hybridus complex based on the USDA collections may be suspect.

Duminil and Di Michele (2009) list phenotypic plasticity as a source of incongruence between morphological and genetic data sets. While the morphological samples were taken from vouchers of the SSR study, the sampling strategies are not truly parallel. The microsatellite sampling included 258 individuals from 56 different accessions (Kietlinski et al. 2013). In most cases only one voucher specimen was prepared per accession from the SSR study, thus the morphological sampling includes only 41 specimens, each representing one accession. Since multiple individuals per accession were not examined morphologically, the possibility of phenotypic plasticity cannot be ruled out in this study.

Species Circumscriptions—Species traditionally classified in the hybridus complex separate into two main groups in k-means clustering. Although the cluster boundaries vary by data set (Fig. 2) there are some distinct trends. In the following discussion we will follow the pistillate data circumscription for groups 1 and 2, as it is the most consistent with the traditional circumscriptions of the species in the hybridus species complex (Sauer 1967). Group 1 is geographically widespread and contains *A. hybridus* p.p., *A. cruentus, A. caudatus,* and *A. quitensis*. Morphologically both pistillate and staminate character measurements of the species of group 1 are smaller than those of group 2 (Table 4). Group 2 contains *A. hypochondriacus* and *A. hybridus* p. p. The species are characterized by very large flowers compared to group 1 (Table 4).

An interesting result of the morphological analysis is that all three data sets (staminate data, pistillate data, and the combined data) support segregating *A. hybridus* into two morphologically distinct groups (Figs. 1, 2). Both groups are geographically widespread throughout the Americas. The "*hybridus* group 1" individuals are morphologically most similar to *A. cruentus*. The "*hybridus* group 2" individuals are morphologically most similar to *A. hypochondriacus*. The segregation of *A. hybridus* into two groups is not surprising given that Sauer separated *A. hybridus* into two races he designated as "Northern" and "Tropical" (Sauer 1950). However, Sauer did not follow up his own lead, and his later work treats *A. hybridus* as a single species with no subspecies (Sauer 1967). Modern treatments of the hybridus complex have followed Sauer's 1967 publication and recognize only one species for *A. hybridus*.

Within group 1, *A. cruentus* can be distinguished by its short pistillate bracts, long utricles and utricle caps with a moderate tower, short staminate bracts and short outer sepals (Table 4). The form of *A. hybridus* (here designated as *A. hybridus 1*) associated with group 1 has long pistillate bracts, short utricles and moderate utricle caps with a long tower, short staminate bracts and short outer sepals (Table 4). *Amaranthus caudatus* can be identified by its short pistillate bracts, short utricles and utricle caps with a short tower, short staminate bracts and long outer sepals (Table 4). *Amaranthus quitensis* has long pistillate bracts, short utricles and short caps and towers, long staminate bracts and outer sepals. The long staminate bract is an anomalous feature within group 1 which otherwise has short staminate bracts (Table 4).

Group 2 contains *A. hypochondriacus* and *A. hybridus* (here designated as *A. hybridus 2*). The form of *A. hybridus* associated with group 2 is virtually indistinguishable from *A. hypochondriacus*. The main difference lies in the moderate utricle cap and tower of *A. hypochondriacus* compared to the very long utricle cap and tower of *A. hybridus 2* (Table 4).

Character Trends—Three characters have traditionally been used to distinguish the species: seed color, sepal posture, and bract to utricle ratios. While the weedy species (*A. hybridus* and *A. quitensis*) are uniformly dark seeded, and white seeds are an indicator for the cultivated taxa (*A. caudatus, A. cruentus,* and *A. hypochondriacus*), many populations of the cultivated taxa are also dark seeded, thus precluding the use of that feature as completely diagnostic. Sepal posture has been considered a diagnostic feature for amaranth species, but was here found to be statistically uninformative. Bract posture has not traditionally been considered a diagnostic feature, but appears to have some statistical significance with *A. quitensis, A. caudatus,* and *A. hypochondriacus* trending towards reflexed bracts and *A. cruentus* and both groups of *A. hybridus* being slightly polymorphic for the trait, but trending towards straight bracts. Sauer (1950; 1967) postulated that there would be human-mediated selection for short bracts on the cultivated taxa because shorter bracts are less sharp and irritating to the human hand. While this proves true for *A. caudatus* and *A. cruentus*, the bracts of *A. hypochondriacus* are long (Tables 3, 4).

Traditionally, staminate floral variation has been assumed to be plastic following the lead of Coons (1977). This assumption

may be partly due to the difficulty of locating staminate flowers on herbarium specimens (Mosyakin and Robertson 2003). Morphologic analysis of staminate characters reveals that they are taxonomically more useful than previously assumed. One character, staminate outer sepal midrib length, contributed the most heavily to the variation along PCA 1 of the combined pistillate-staminate data set (Table 2). Furthermore, the PCA and k-means clustering of staminate data both delimit two discrete groups (Figs. 1, 2), and ANOVA of staminate data reveals five characters with p values less 0.0001 (Table 3). It appears that while staminate floral variation historically has been of limited use due to the difficulty of finding staminate flowers, they are nonetheless taxonomically informative and taxonomists should not continue to ignore them in future treatments of monoecious *Amaranthus*.

Species of the hybridus complex are notoriously difficult to distinguish. Part of the difficulty lies in the fact that there are few qualitative differences between the species and most quantitative characters exhibit a range of overlapping values with few distinct or diagnostic differences between them (Tables 3, 4). Correct identification of species requires dissection of flowers and measurement of multiple features under a dissecting scope. Although this study provides some important morphological trends to help in identification of species (Tables 3, 4), the number and complexity of features needed to accurately identify species of the hybridus complex does not lend itself to easy identification using traditional dichotomous keys and will ultimately require a computer-guided polyclave key.

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Appendix 1. Sampling and voucher information. Samples listed by country of origin, locality, USDA accession number (PI or Ames), collector name and number, and herbarium (ASTC or NCRPS). Voucher specimens were grown, collected, and vouchered at SFA or were loaned from the USDA North Central Regional Plant Introduction Station (NCRPS) located in Ames, Iowa. Species designations follow USDA passport data.

Amaranthus caudatus L. ECUADOR. Pinchincha: Tabacundo, PI 608019, *Kietlinski 100* (ASTC); BOLIVIA. Cochabamba: Quillacolla, PI 490579, *Kietlinski 4* (ASTC); ARGENTINA. La Pampa: PI 490491, *Kietlinski 111* (ASTC); Corralitos: PI 511679, *Kietlinski 8*, (ASTC).

Amaranthus cruentus L. MEXICO. Sonora: Rancho Terrero Ames 5320, *Kietlinski 14* (ASTC); Sonora: Unknown. Ames 5323, *Kietlinski 11* (ASTC); Veracruz: Unknown, PI 511727, *Kietlinski 36* (ASTC); GUATEMALA. Alta Verapaz: San Pedro Carcha, PI 511715, *Kietlinski 31*, (ASTC); Baja Verapaz: Unknown, Ames 22000, *Kietlinski 25* (ASTC); Chimaltenango: Choatalum, PI 633585, *Kietlinski 33* (ASTC); Chimaltenango; San Martin Jilotepeque, PI 451825 *Kietlinski 38* (ASTC); Solola: Unknown, Ames 5277, *Kietlinski 35* (ASTC).

Amaranthus hybridus L. U. S. A. Indiana: Prairie Township, PI 603895, *Kietlinski 54* (ASTC); Ohio: Ludlow Falls, PI 603889, *Kietlinski 42* (ASTC); MEXICO. Puebla: Naupan, PI 604602, *Kietlinski 45* (ASTC); Puebla: Xochitlan, PI 604568 *Brenner s. n.* (NCRPS); Oaxaca: Ojitlan, PI 511724, *Brenner s. n.* (NCRPS); GUATEMALA. Huehuetenango: Unknown, Ames 22001, *Brenner s. n.* (NCRPS); Sacatepequez: Antigua, Ames 5267, *Kietlinski 41* (ASTC); COLOMBIA. Cundinamarca: Tibaitata, PI 636180, *Kietlinski 46* (ASTC); PERU. Ancash: Yungay, Ames 5232 *Brenner s. n.* (NCRPS); Apurimac: Andahuaylas, PI 490489, *Kietlinski 27* (ASTC). BRAZIL. Goias: Luziania, PI 652416, *Kietlinski 53* (ASTC).

Amaranthus hypochondriacus L. MEXICO. Chihuahua: Chinipas, PI 633589, *Kietlinski 106* (ASTC); Federal District: Tulyehualco, PI 643038, *Kietlinski 5* (ASTC); Puebla: Hueyapan, PI 604576, *Kietlinski 55* (ASTC); Morelos: Amayuca, PI 643041, *Kietlinski 6* (ASTC); GUATEMALA. Unknown, PI 490752, *Kietlinski 49* (ASTC).

Amaranthus quitensis Kunth. ECUADOR. Azuay: Cuenca, Ames 5247, *Brenner s. n*. (NCRPS); Pinchincha: Chaupi, PI 490708, *Kietlinski 72* (ASTC); Pinchincha: Checa, PI 490694, Tungurahua: Ambato, PI 511743, *Kietlinski 66* (ASTC); PERU. Ancash: Uranchacra, PI 649246, *Kietlinski 57* (ASTC); Apurimac: Andahuaylas, PI 490454, *Kietlinski 18* (ASTC); Cusco: Colquepata, PI 490466, *Kietlinski 70* (ASTC); Tacna: Unknown, PI 511751, *Kietlinski 62* (ASTC); BOLIVIA. Chuquisaca: Tarabuco, PI 511736, *Brenner s. n.* (NCRPS); Tarija: Tarija, PI 568154, *Kietlinski 21* (ASTC); ARGENTINA. Santa Fe: Unknown, Ames 5334, *Kietlinski 74* (ASTC); BRAZIL. Goias: Luziania, PI 652419, *Kietlinski 59* (ASTC); Federal District: Brasilia, PI 652428, *Kietlinski 102* (ASTC).

Appendix 2—Morphological characters

Character	Measurement Protocol
Qualitative Characters	
1. Seed color	Determined under dissecting scope pale= white or tan seeds; dark= black or brown seeds
2. Bract posture	Most common type per specimen as visualized under the dissecting scope Straight= bracts straight; recurved= bracts curving away from fruit
3. Sepal posture	Most common type per specimen as visualized under the dissecting scope Straight= sepals straight; recurved= sepals curving away from fruit
Pistillate Flower Characters	
1. Utricle Length	Measured in mm under dissecting scope
2. Utricle Width	Measured in mm under dissecting scope
3. Seed Width	Measured in mm under dissecting scope
4. Utricle Cap Length	Measured in mm under dissecting scope
5. Utricle Base Length	Measured in mm under dissecting scope
6. Utricle Length to Width ratio	Utricle length divided by utricle width
7. Utricle Cap Tower Length	Measured in mm under dissecting scope measured from the stigmatic cleft to the point at which the utricle widens
8. Bract Lamina Length	Measured in mm under dissecting scope
9. Bract Length	Measured in mm under dissecting scope
10. Bract Width	Measured in mm under dissecting scope
11. Outer Sepal Length	Measured in mm under dissecting scope
12. Outer Sepal Midrib Length	Measured in mm under dissecting scope
13. Outer Sepal Width	Measured in mm under dissecting scope
14. Outer Sepal Index	Outer sepal length divided by width
15. Inner Sepal Length	Measured in mm under dissecting scope
16. Inner Sepal Midrib Length	Measured in mm under dissecting scope
17. Inner Sepal Width	Measured in mm under dissecting scope
18. Inner Sepal Index	Inner sepal length divided by width
19. Bract to Outer Sepal Length ratio	Bract length divided by outer sepal length
20. Bract to Utricle Length ratio	Bract length divided by utricle length
21. Utricle to Outer Sepal Length ratio	Utricle length divided by outer sepal length
Staminate Flower Characters	
22. Bract Lamina Length	Measured in mm under dissecting scope
23. Bract Length	Measured in mm under dissecting scope
24. Bract Width	Measured in mm under dissecting scope
25. Outer Sepal Length	Measured in mm under dissecting scope
26. Outer Sepal Midrib Length	Measured in mm under dissecting scope
27. Outer Sepal Width	Measured in mm under dissecting scope
28. Outer Sepal Index	Outer sepal length divided by width
29. Inner Sepal Length	Measured in mm under dissecting scope
30. Inner Sepal Midrib Length	Measured in mm under dissecting scope
31. Inner Sepal Width	Measured in mm under dissecting scope
32. Inner Sepal Index	Inner sepal length divided by width
33. Bract Length to Outer Sepal Length ratio	Bract length divided by outer sepal length