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Morphological Assessment of Cultivated and Wild Amaranth Species Diversity

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Abstract: *Amaranthus* L. is genus of C4 dicotyledonous herbaceous plants comprising approximately 70 species, with three subgenera, which contains both cultivated and wild types, where cultivated ones are used for food grains, leafy vegetables, potential forages and ornamentals. Grain amaranth are pseudocereals from three species domesticated in North and South America and are notable for containing high amount of protein and minerals and balanced amino acid in their small seeds. Genetic diversity analysis of amaranths is important for development of core set of germplasm with widely diverse population and effective utilization of plant genetic resources. In this study, we evaluated a germplasm collection of 260 amaranth accessions from United State Department of Agriculture (USDA) and 33 accessions from Seed Savers' Exchange (SSE). We evaluated morphological traits like blade pigmentation, blade shape, petiole pigmentation, branching index, flower color, stem color, inflorescence density, inflorescence shape, terminal inflorescence attitude, plant height and yield characteristics across all 293 accessions. We compared clustering within the USDA and SSE collection and across both collections. Data analysis of morphological data showed significant difference of petiole pigmentation, stem color, blade pigmentation, blade shape and flower color across different clusters of accessions of USDA unlike among different clusters of SSE where we found significant difference of only blade pigmentation, blade shape and flower color. The relationship depicted by neighbor-joining dendogram using the morphological markers was consistent with some but not all of the differences observed between species. Some divisions were found between cultivated and weedy amaranths that was substantiated by morphological characteristics but no separation of South and Central American species was observed. Substantial phenotypic plasticity limits the use of morphological analysis for phylogenetic analysis but does show that important morphological traits such as inflorescence type and plant architecture can cross species boundaries. Similarly, color variants for leaves, flowers and seeds are not exclusive to one cluster in our study nor to one species and can be used widely for breeding any of the cultigens, but not to species identification. Our findings will help in germplasm conservation of grain amaranths and facilitate in this crop's improvement. It will also help on developing effective breeding programs involving different plant characteristics and morphological traits of Amaranths.

Keywords: *Amaranthus caudatus* L.; *A. cruentus* L.; *A. hypochondriacus* L.; ancient grains; diversity analysis; domesticates; pseudocereals

1. Introduction

Amaranths belong to the dicotyledonous genus *Amaranthus* L. which is made up of over 70 species [1], and three subgenera [2]. The word Amaranthus originated from the Greek word

amarantos meaning "one that does not wither" or "never fading" [3]. About 60 Amaranthus species are native to America while the rest originated from Asia, Africa, Australia and Europe [4].

The genus *Amaranthus* contains both cultivated and wild species. Among the cultivated species, grain amaranths have been grown for more than 8,000 years dating back to before the Pre-Colombian civilization of Central and South America [5]. The cultivated grain amaranths include *A. caudatus* L., *A. cruentus* L. and *A. hypochondriacus* L. and their parental wild species are thought to be *A. hybridus* L., *A. quitensis* Willd. ex Spreng. and *A. powellii* S. Wats. [6]. Grain Amaranths are important subsistence and commercial food crops for people living in parts of Central and South America [4,7]. They are expanding in many regions of Asia as well as Eastern and Southern Africa (www.amaranthinstitute.org). Other amaranth species like *A. dubious* L., *A. hybridus* and *A. tricolor* L. are consumed as leafy vegetables [8]. Meanwhile, *A. retroflexus* L. (redroot pigweed), *A. albus* L. (tumbleweed), *A. palmeri* S. Wats. (Palmer amaranth), *A. spinosus* L. (spiny amaranth) represent weed species [9]. While many of the latter are cosmopolitan in nature; the vegetable amaranths are commonly found in Asia and Africa while grain amaranths are native to Mexico and Peru with recent expansion around the world [10]. Genetic races have been suggested for grain amaranths with Azteca, Mercado, Mixteca, Nepal and Picos in *A. hypochondriacus*; Mexican, Guatemalan and African in *A. cruentus*; and finally South American and Edulis in *A. caudatus* [11]. The first of these two species can hybridize to each other as can all the grain amaranth with their immediate wild relatives; however, in general most cultivars tend to be self-pollinating despite being monoecious [2,10].

In terms of nutritional content, grain amaranths produce seed with high protein content (17–19% of dry weight) and well-balanced amino acid profiles [12]. The seeds of grain Amaranths possess double the amount of the essential amino acids (especially lysine, phenylalanine and threonine) and high minerals (calcium, iron and zinc) compared to wheat protein [13]. As easy to cook grains, the amaranths show promise for amelioration of protein or amino acid deficiencies, supplementing mineral content (Fe, Zn) of foods and providing protein to predominantly or completely vegetarian diets [14,15]. Grain amaranths are commonly popped or roasted before milling or mixing with other ingredients; therefore, several flours can be made from this pseudocereal and provide novel organoleptic properties and new tastes and flavors. Chemical composition analysis of grain amaranths confirms their high potential for human nutraceutical uses [16]. Amaranth seed and amaranth seed oil is high in Vitamin E and squalene, which can be beneficial for people suffering from hypertension or cardiovascular disease [3,14]. Regular consumption of grain amaranth can reduce blood pressure, cholesterol levels and improves antioxidant status and immunological parameters [17]. With increasing demand for food and current malnutrition levels, development of amaranths as an alternative food could be an important boon for people of developing countries suffering from malnutrition and hunger [10]. In summary, grain amaranth is a healthy and nutritious food crop that could benefit people if it was produced and consumed in greater quantities.

The objective of this research was to assess the morphological diversity of close to 300 cultivated grain amaranths and their wild relatives from two gene banks through field assessments of leaf, flower and grain characteristics. Another goal was to determine if morphological traits could be used for species and population identification. The two gene banks providing germplasm from this study were the United States Department of Agriculture (USDA) through the National Plant Germplasm System (NPGS) with a smaller collection provided by Seed Savers Exchange (SSE). The uncharacterized SSE collection was compared to species in the USDA collection; however, morphological analysis of whole plant traits such as leaf and petiole color blade shape or terminal inflorescent index and branching index did not distinguish species.

2. Materials and Methods

2.1. Plant Materials

A total of 293 genotypes were used in this study (Supplemental Tables S1 and S2). Of these, 260 accessions representing nine different species of *Amaranthus* and were provided by the United States Department of Agriculture (USDA) and 33 accessions of unconfirmed species were provided by Seed Savers' Exchange (SSE). The USDA set of Amaranths is maintained at the North Regional Plant Station in Ames, Iowa; while the SSE set is maintained at the Heritage Farm in Decorah, Iowa, both locations in the mid-western part of the United States. Many accessions from different parts of the world are stored in the USDA collection, while the SSE collection is mostly adapted to American growing conditions. The majority of the USDA collection were from Mexico and Peru, while all the SSE collection was from the United States. The genotypes included a majority of landraces or breeding genotypes from cultivated species, namely: 120 accessions of *A. cruentus*, 44 of *A. hypochondriacus* and 33 of *A. caudatus*. Among the wild genotypes there were 26 accessions of *A. hybridus*, 16 of *A. quitensis*, 6 of *A. powellii* (representing both sub-species), 2 of *A. retroflexus* and 1 of *A. palmeri*. A total of 44 accessions had no species identification including all of those from the SSE collection.

2.2. Greenhouse Planting and Field Transplanting

The field experiment was done to evaluate morphological characteristics of all the accessions collected from USDA and SSE. Before field planting, seed of the 33 genotypes from SSE were planted in 72-well trays in an open roof greenhouse at the Tennessee State University Agricultural Research and Education Center (AREC) on 12 May 2015. The 260 genotypes of the USDA collection were planted in the same trays on 29 April 2015. Amaranths seeds were sown at a rate of a three seeds per well, and then thinned in the greenhouse to one plant per well after germination. A total of 12 wells were planted per accession resulting in 12 seedlings. The temperature in the green house was maintained in the range of 70 °F–80 °F. Two weeks after germination, transplanting was done to a field also at the main AREC center in Nashville, TN. Rows were made with a corn planter containing no seed to mark the field in straight rows. A good plant stand was assured by transplanting individual amaranth seedlings every 20 cm within the furrow of these rows. The length of each plot was 3 m with 2.4 m filled by plants, a 0.6 m alley, and the distance between rows of 0.75 m. The site was located at 36°9′ N and 86°49′ W at an elevation of 153 m above sea level with a Byler silt loam soil type. SSE seedlings were transplanted to the field on 4 and 5 June 2015. USDA seedlings were transplanted to the field on 9 and 10 June 2015. Osmocote slow release fertilizer was applied around each seedling one week after transplanting at the dose of 1 teaspoon/plant. No insecticides or fungicides were used in the field during plant growth. One mechanical weeding was done between rows using a Husqvarna tiller/cultivator (Husqvarna Professional Products, Inc., Charlotte, NC, USA).

2.3. Phenotyping and Morphological Evaluations

Traits such as blade pigmentation, inflorescence color, petiole pigmentation, branching index, flower color, stem color and blade shape were noted as the plants grew or when the plants stared flowering (Supplemental Table S3). Inflorescence density, inflorescence shape, terminal inflorescence attitude and plant height were recorded when the plants reached maximum height. Manual Harvesting was done using pruning shears at the base of plants. SSE accessions were harvested on 15 September 2015. The harvesting of USDA accessions was started on 15 August 2015 and continued for one month due to different maturity dates of these accessions. Uniformity in maturity was seen in SSE accessions but range of maturity time was observed in USDA accessions. Harvested panicles were dried in a hoop house for two weeks and threshed manually. Threshed seeds were winnowed and evaluated for seed color.

2.4. Data Analysis of Phenotypic Data

Descriptive statistics were obtained for qualitative morphological traits. Seed color was determined from the Germplasm Resources Information Network (GRIN) database for the USDA germplasm set (Ames, IA, USA) and by a Brother DCP-7040 scanner (Brother International Inc., Bridgewater, NJ, USA) used to scan seed color of amaranths from the SSE collection using a red background for contrast. Quantitative traits were analyzed for dispersion and population distributions. SAS v. 9.4 software (SAS Institute Inc., Cary, NC, USA) was used for cluster analysis distance based on morphological data using Mahalanobis D^2 and clustering was tested by a chi-square test X^2.

3. Results

3.1. Morphological Variability in the SSE Collection

The seed scanning of the 33 genotypes from the SSE collection showed 11 white seeded accessions, 8 cream, 12 black and 2 red brown in color (Supplementary Figure S1). Once germinated, the majority of seedling and growing transplants showed green leaves, a few with marginal or vein pigmentation, some with red leaves and a very few were with dark green leaves. In all cases we evaluated the lamina instead of the petiole and amaranthine is used inter-changeably with red as the pigmentation type. Stem color were solid red, solid green, orange, amaranthine striped or pink based with green stem. Leaf shape included oval, oblong, elliptical and ovate. Petiole pigmentation varied from dark amaranthine, light amaranthine, green to yellow among different genotypes.

At flowering, plant architecture varied and included branched all along the stem, only few branches at top or without any branches. Flower color ranged from dark amaranthine to green, yellow, orange or mixed. The inflorescence could be at the terminal part of plants but many had long or short side branch. Inflorescence density were found to be high, intermediate and low among different accessions. Many of them had erect inflorescence and some of them had drooping type or arched shape inflorescence. One genotype, SSE39, did not flower and was not included in the rest of the study, making a total of 32 genotypes for dendogram construction.

Significant differences in blade pigmentation (BP), blade shape (BS) and flower color (FC) were found across different clusters of SSE germplasm (Table 1). Other morphological characteristics like Petiole pigmentation (PP), Branching index (BI), Inflorescence shape (IS), Inflorescence density (ID), Terminal inflorescence attitude (TAI) and stem color (SC) were not significantly different across different clusters.

Table 1. Significance of morphological traits on different clusters of SSE accessions.

Morphological Trait	Cluster MS	Error MS *F* Value	*p* > *F*
BP	66.10 **	3.54	<0.0001
BS	95.62 **	2.54	<0.0001
PP	6.18	1.37	0.0041
BI	7.21	3.48	0.1008
FC	18.02 **	1.58	<0.0001
IS	0.67	0.26	0.0539
ID	1.04	0.97	0.3981
TIA	0.49	0.4	0.3356
SC	11.35	2.83	0.0076

** Significant at $p < 0.0001$, Morphological Trait Abbreviations: BP = Blade pigmentation, BS = Blade shape, PP = Petiole Pigmentaion, BI = Branching Index, FC = Flower color, IS = inflorescence Shape, ID = Inflorescence density, TIA = Terminal Inflorescence Attitude, SC = Stem color.

Based on the variation among different morphological characteristics, six distinct clusters were seen for the SSE germplasm (Figure 1). The first cluster (group I) included SSE 1, SSE3, SSE34, SSE112, SSE115 and SSE117. The second cluster (II) included SSE40 and SSE108. The third cluster (III) included SSE4, SSE5, SSE6, SSE24, SSE29, SSE30 and SSE35. The fourth cluster (IV) included SSE7, SSE10, SSE15,

SSE22, SSE31, SSE38, SSE42, SSE86, SSE92, SSE93, SSE99, SSE104, and SSE132. The fifth cluster (V) included SSE8, SSE79 and SSE119. Cluster six (VI) included SSE80 alone.

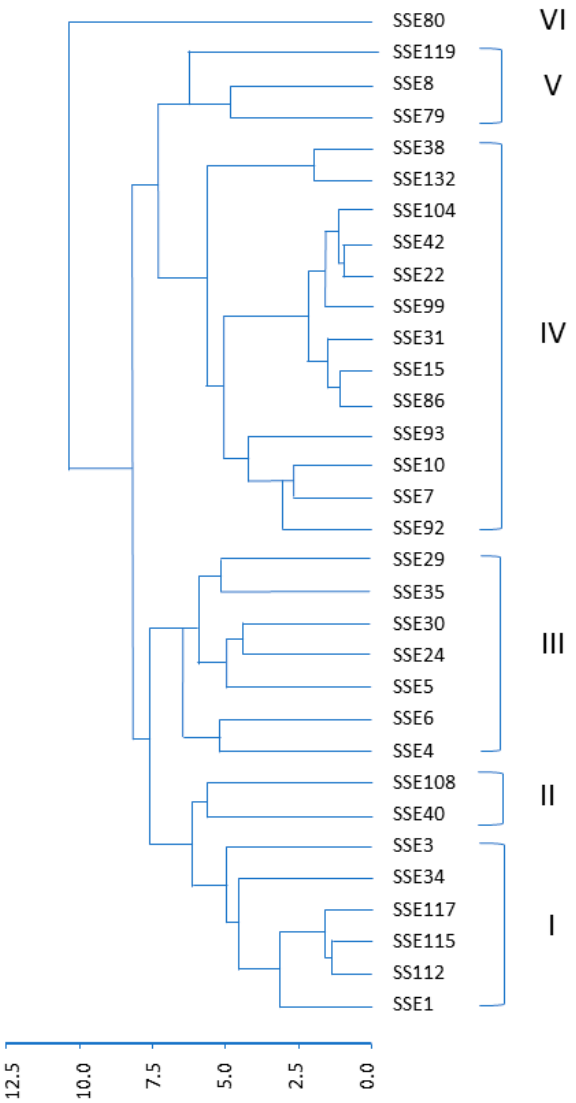

Figure 1. UPMGA (unweighted paired group method using arithmetic mean) dendrogram showing hierarchical grouping patterns of 32 SSE (Seed Savers' Exchange) Amaranths. Axis shows distance D^2 with Mahalanobis coefficient.

The distances between different clusters were calculated (Table 2). Chi square test showed the Mahalanobis D^2 distance of 14.06 as significant distance between two clusters. We did not find any significant distance between any two clusters.

Table 2. Nearest cluster analysis showing Mahalanobis D^2 distance between groups of SSE Amaranths.

Cluster	1	2	3	4	5	6
1		9.521	11.47	8.485	7.553	10.41
2	9.521		9.869	11.68	6.209	11.14
3	11.47	9.869		5.566	8.762	13.33
4	8.485	11.68	5.566		9.008	12.3
5	7.553	6.209	8.762	9.008		9.936
6	10.41	11.14	13.33	12.3	9.936	

3.2. Morphological Variability in the USDA Collection

Among 260 accessions obtained from USDA we found flowering and non-flowering genotypes. In total only 208 accessions producing noticeable inflorescence and the 52 accessions not producing noticeable inflorescence were not considered further or used for cluster analysis. Among the genotypes included, we found blade pigmentation from dark green to green to amaranthine (red) lamina with marginal or vein pigmentation. Different stem colors included solid red, pink base and green stem, green, orange or amaranthine striped. Most genotypes had oval leaves but some had oblong, elliptical and ovate leaves. Different types of petiole pigmentation were observed like amaranthine, dark amaranthine, green or yellow. At full flowering stage, the observed flower color ranged from dark amaranthine flower color, to pink, orange or mixed flower color. Both erect and drooping inflorescence shapes were seen among different genotypes.

Analysis of the morphological traits among 208 accessions of USDA resulted in 10 clusters, five of which were major and five of which were minor (Supplemental Table S4). The minor clusters contained few accessions. Overall, a variable number of genotypes were found in each cluster (Figure 2). Major Cluster 1 (V on Figure 2) contained 29 accessions; 8 from *A. caudatus*, 8 from *A. cruentus*, 4 from *A. hybridus*, 5 from *A. hypochondriacus* and 4 from *A. quitensis*. This cluster represented 12 accessions from South America, 4 accessions from North America, 2 accessions from Africa, 4 accessions from Asia and 7 accessions from Central America. They were predominantly cultivated except for the *A. hybridus* and *A. quitensis* genotypes which are direct ancestors of the cultivated species.

The other clusters similarly had a mix of cultivated and wild accessions. Major Cluster 2 (II in Figure 2) included 85 accessions representing 1 from *A. caudatus*, 61 from *A. cruentus*, 4 from *A. hybridus*, 14 from *A. hypochondriacus* and 5 from *A. quitensis*; and being predominantly of cultivated germplasm. Based on geographical origin, this cluster represented 13 accessions from Africa, 6 from Asia, 41 from Central America, 2 from Europe, 11 from North America, 11 from South America and 1 with unknown origin. This cluster had the highest number of accessions. Major Cluster 3 (III) had 31 accessions with 3 accessions from *A. caudatus*, 11 accessions from *A. cruentus*, 8 accessions from *A. hybridus* and 9 accessions from *A. hypochondriacus*, all of which were cultivated. The accessions of this cluster represented 3 accessions from Africa, 8 from Asia, 7 from Central America, 2 from Europe, 5 from North America and 6 from South America. Major Cluster 4 (IV) contained 49 accessions; among which 31 belonged to *A. cruentus*, 4 belonged to *A. hybridus*, 8 to *A. hypochondriacus*, 1 to *A. palmeri*, 2 to *A. powellii*, 2 to *A. quitensis* and 1 to *A. retroflexus*, showing this cluster to be based on weedy and cultivated accessions. Based on geographical origin, this cluster consisted of 5 accessions from Africa, 5 from Asia, 27 from Central America, 2 from Europe, 5 from North America and 5 from South America. Major Cluster 5 (or I) was found to have 8 genotypes including 3 cultivated accessions of *A. hypochondriacus* and 5 wild accessions (1 *A. palmeri*, 3 *A. powellii*, and 1 *A. quitensis*). Based on geographical origin, the cluster had 1 accession from Central America, 2 from Asia, 3 from Europe, 1 from North and 1 from South America.

Among the minor groupings, Cluster 6 (VI) comprised only 2 accession from *A. cruentus* and *A. hypochondriacus* which originated in Asia and Africa. Cluster 10 (X) also consisted of 2 accessions this time *A. hypochondriacus* which originated in Asia and Central America. Cluster 8 (VIII) represented one accession of *A. cruentus* from Europe. Cluster 9 (IX) showed only 1 accession of *A. cruentus* which originated in South America. Cluster 7 (VII) represented only 1 accession of *A. hybridus* which originated in North America.

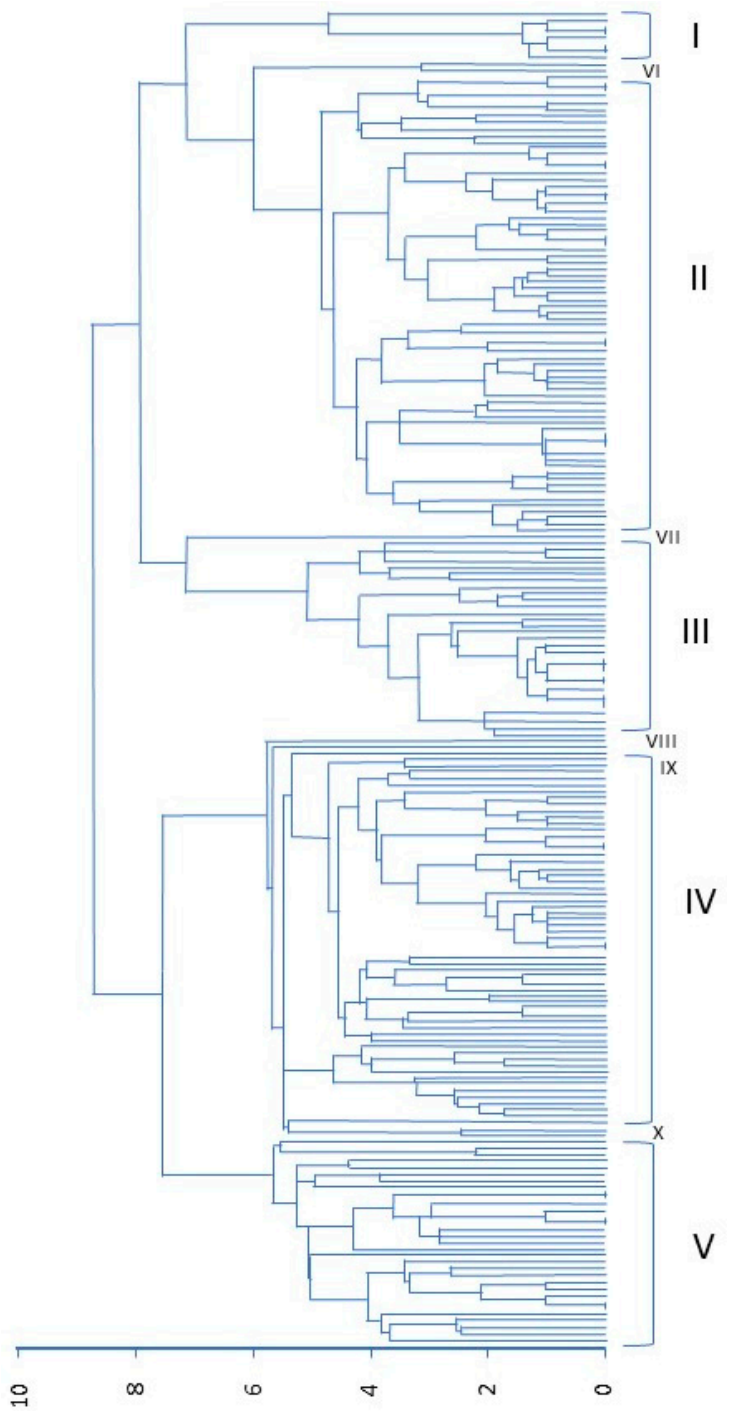

Figure 2. UPMGA (unweighted paired group method using arithmetic mean) dendrogram showing hierarchical grouping patterns of 260 Amaranths accessions from USDA.

The significance of different morphological traits among ten clusters of USDA accessions is shown in Table 3. The main morphological traits to vary between clusters were blade pigmentation, blade shape, petiole pigmentation, flower color and flower shape had significant contribution on differentiating clusters. However, inflorescence shape, inflorescence density and terminal inflorescence attitude had no significant contributions on distinguishing different clusters. The Mahalanobis D^2 distances between different USDA clusters are shown in Table 4; however, no significance distances were found. Relative distance measures showed that distance between Cluster 3 and Cluster 9 was

highest followed by distance between Cluster 2 and Cluster 10. The minor clusters 7 and 8 were also distant from the major clusters 1, 2 and 3.

Table 3. Significance of morphological traits on different clusters of USDA accessions.

Morphological Trait	Clusters	Error	*p* > *F*
BP	436.21 **	0.98	<0.0001
BS	297.44 **	2.47	<0.0001
PP	5.52 **	0.88	<0.0001
BI	7.41	4.27	0.0835
FC	17.029 **	3.21	<0.0001
IS	0.188	0.23	0.6261
ID	3.39	1.38	0.0113
TIA	0.35	0.147	0.0138
SC	48.79 **	3.87	<0.0001

** Significant at $p < 0.0001$, Morphological Trait Abbreviations: BP = Blade pigmentation, BS = Blade shape, PP = Petiole Pigmentation, BI = Branching Index, FC = Flower color, IS = inflorescence Shape, ID = Inflorescence density, TIA = Terminal Inflorescence Attitude, SC = Stem color.

Table 4. Nearest clusters analysis showing Mahalanobis D^2 distance between groups of USDA Amaranths genotypes.

Cluster	1	2	3	4	5	6	7	8	9	10
1		9.631	12.7	5.28	13.19	6.902	9.26	9.302	6.598	10.42
2	9.631		9.803	9.536	11.45	9.176	5.888	13	10.39	13.46
3	12.7	9.803		13.04	5.222	7.766	10.32	8.176	13.7	9.306
4	5.28	9.536	13.04		12.14	7.448	7.997	9.976	4.892	9.004
5	13.19	11.45	5.222	12.14		9.221	9.269	8.262	12.78	7.748
6	6.902	9.176	7.766	7.448	9.221		9.882	5.168	8.788	6.257
7	9.26	5.888	10.32	7.997	9.269	9.882		12.24	9.075	11.95
8	9.302	13	8.176	9.976	8.262	5.168	12.24		10.38	4.094
9	6.598	10.39	13.7	4.892	12.78	8.788	9.075	10.38		10.28
10	10.42	13.46	9.306	9.004	7.748	6.257	11.95	4.094	10.28	

3.3. Cluster Analysis of Full Collection from Both USDA and SSE

The morphological data analysis of all the accessions representing USDA and SSE collections together showed seven distinct clusters. Clustering was done primarily to determine if the SSE genotypes clustered apart from the USDA ones or not. If they clustered together, we were interested in seeing if there were species or morphotype associations of the SSE genotypes with a subset of the USDA collections. In general we found morphological traits to be shared among species and phenotypic analysis to constitute cross species clusters without the ability to identify unknown accessions.

Cluster 1 was comprised of 39 accessions representing 11 accessions of *A. caudatus*, 6 accessions *of A. cruentus*, 5 accessions of *A. hybridus*, 4 accessions of *A. hypochondriacus*, 4 accessions of *A. quitensis* and 9 accessions of SSE. Based on geographical origin the cluster showed 4 accessions from Africa, 2 accessions from Asia, 5 accessions from Central America, 2 accessions from North America, 17 accessions from South America and 9 accessions from SSE with unknown origin. Cluster 2 consisted of 102 accessions with 14 accessions from *A. caudatus*, 58 accessions of *A. cruentus*, 5 accessions of *A. hybridus*, 50 accessions of *A. hypochondriacus*, 2 accessions of *A. quitensis* and 1 accession of *A. retroflexus*. Among the accessions of this cluster, 12 originated in Africa, 13 in Asia, 36 in Central America, 1 in Europe, 11 in North America, 20 in South America and 9 were from SSE and of unknown origin. Cluster 3 consisted 92 accessions. The cluster represented 3 accessions of *A. caudatus*, 44 accessions of *A. cruentus*, 6 accessions of *A. hybridus*, 17 accessions of *A. hypochondriacus*, 1 accession of *A. palmeri*, 7 accessions of *A. quitensis* and 1 accession of *A. retroflexus*. Based on geographical origin, it was found that 9 accessions were from Africa, 9 accessions were from Asia, 39 accessions were from Central America, 6 accessions were from Europe, 6 accessions were from North America, 15 accessions

were from South America and 8 accessions were from SSE and had unknown origin. Cluster 4 was comprised of 39 accessions representing 5 accessions of *A. caudatus*, 12 of *A. cruentus*, 10 of *A. hybridus*, 9 of *A. hypochondriacus* and 3 of SSE. The cluster showed that 4 accessions originated in Africa, 8 in Asia, 8 in Central America, 2 in Europe, 6 in North America and 8 in South America. Cluster 5 revealed 2 accessions of *A. quitensis* which originated in South America. Cluster 6 represented 1 accession of *A. quitensis* from South America. Cluster 7 comprised 1 accession of amaranth from SSE.

Supplementary Table S4 depicts significance of different traits among various clusters. It was found that there was significant differences of petiole pigmentation, stem color, blade pigmentation, blade shape and flower color among different clusters among the 276 accessions. Mahalanobis D2 distance between different clusters is shown in Supplementary Table S5, where distance between cluster 4 and cluster 7 was found to be highly significant at the threshold Chi-square value of 14.06.

4. Discussion

Information about genetic diversity and clustering among and within crop species is important for effective utilization of plant genetic resources [18]. Analysis of genetic diversity and development of population structure have direct benefits in research related to evolution, population structure and plant breeding [19]. Clustering can also indicate phylogenetic relations. However, the clusters of amaranth accessions shown in our analysis were based on morphological traits and for the most part were either mostly cultivated accessions or mostly wild accessions; but clustering did not agree with species identification and several species accessions were found in each cluster.

Different morphological characteristics were evaluated across all the amaranths both separately by collection and together. The genotypes consisted in a total of nearly 300 accessions from two collections, SSE and USDA. Overall, the plasticity in major morphological traits like leaf and flower color did not allow us to identify species as has been discussed before [10,11]. We could not correlate the SSE collection with species identified in the USDA collection and these genotypes clustered together across species identifications in the GRIN database. Despite this, by overall race type morphology as discussed by Espitia [11], it appeared that the majority of SSE genotypes were likely to be of the cultivated species *A. cruentus* or *A. hypochondriacus* of cultivated races, given their predominantly upright architecture. The few exceptions to this were vegetable types potentially from *A. hybridus*, and one labeled as *A. gangeticus* [16].

Among the morphological traits, there was significant effects of blade pigmentation, blade shape and flower color among different clusters of amaranths accessions from SSE. In addition to these morphological traits, petiole pigmentation and stem color had significant contribution among different clusters of amaranths accessions from USDA. This showed that there was more variation in USDA collection than in SSE collection validating the points made by Brenner et al. [10] about landraces held by NPGS.

For the USDA collection, we found wide variation in morphological characteristics among and within species of amaranths. In some cases the accessions from cultivated species were clustered together however geographical origin was not important in determining clusters. In most cases, the same species was found to have variable morphological traits. This difficulty in the phenotypic identification of amaranth species has been observed before by various authors [19,20].

Variability in morphology was a widely observed factor in the accessions evaluated. The result was that morphological states were shared between species and it was hard to divide the accessions into morphotypes or to find correlation of traits with species. The one exception was level of branching in the USDA collection found to be high for wild accessions from *A. hybridus*, *A. powellii*, *A. quitensis* and *A. retroflexus* compared to the single stem of cultivated types from *A. caudatus*, *A. cruentus* and *A. hypochondriacus* in most cases. Difficult weed species identification was observed before [20,21].

In another observation, we discovered that the grain amaranth accessions from SSE were already adapted to growing condition in the Southeastern USA, even if their respective species were unknown. SSE is a non-governmental organization that works to preserve America's gardening heritage, so it

would be important to determine the species through molecular means. In most studies [1,2,22–30], molecular markers have been able to distinguish South American (*A. caudatus*, *A. quitensis*) from Central American (*A. cruentus*, *A. hypochondriacus*) species of the subgenus *Amaranthus* as well as outgroups from other subgenera. The other subgenera of the genus are subgenus *Acnida* (includes weedy amaranths such as *A. palmeri* and *A. spinosus*) and *Albersia* (includes wild and vegetable species such as *A. tricolor* and *A. viridis*).

The use of molecular markers such as Simple Sequence Repeat (SSR) or Single Nucleotide Polmorphism (SNP) seems better than morphological analysis for distinguishing species of grain amaranths [1,2,22–30]. SSR markers in the study by Oo and Park [26] did find clear clustering pattern of geographically close accessions and related species but Suresh et al. [1] did not. Vegetable species have been less well studied [31–37] at least by molecular means than grain amaranths while weedy amaranths have been well studied, especially with recent outbreaks of herbicide resistant amaranths in countries with genetically modified crops and those developing countries transitioning to mechanical weed control.

Other marker types based on isozymes, seed protein patterns or better yet next generation sequencing have proven to be effective for species identification but the first two of these methods are time consuming while the last of these methods is cost intensive. Seed protein analysis would also require producing the seed of the genotypes in either field conditions or in a greenhouse where day length and photoperiod could be controlled and allow the evaluation of seed protein variability [38] as well as provide clean tissues for isozyme analysis [39]. Perhaps most promising, the recent use of next generation sequencing technology of Genotyping by sequencing (GBS), performed by Wu and Blair [40] and Stetter and Schmid [2], was successful at differentiating cultivars from wild accessions and different species from each other.

The latter studies show that molecular marker studies can complement botanical or morphological descriptors for Amaranth species. Species separation is usually based on time consuming and growth-phase specific, reproductive traits such as bract and tepal sizes of female flowers as the traditional methods for evaluating species differences [10,11]. Meanwhile, our study showed that field assessment of major morphological traits can be successful in the grain amaranths. However, as was previously observed in species and race characterization, there is a tremendous plasticity of plant size and branching within each species [10]. Some major morphological differences, like flower and leaf color segregate across species, and most other traits like plant size depend on photoperiod and soil conditions in the site used for evaluation. A lack of flowering or seed production in many short-day photoperiod sensitive *A. caudatus* and *A. quitensis* genotypes found in the USDA collection [10], but less so in the SSE collection, prevented some morphological traits from being evaluated and is a drawback of phenotyping that would not be present in DNA studies. Common cross species traits and phenotypic plasticity in the grain amaranths make species identification difficult in an open air, field setting as compared to a greenhouse.

5. Conclusions

This is one of the first large-scale morphology field studies of a collection of grain amaranths since the study by Wu et al. [41]. In our analysis of *Amaranthus*, we were successful at planting a large number of USDA genotypes for morphological analysis in real world field conditions in Tennessee. Similarly, the SSE collection was evaluated uniformly compared to most analysis in Iowa, where each genotype was grown in isolated seed multiplication blocks over various years rather than together. Testing in one relatively homogeneous field environment is beneficial compared to testing across various fields due to plasticity in trait expression. Among the USDA accessions, significant contributions of blade pigmentation, blade shape, petiole pigmentation, flower color and stem color on differentiated different clusters of USDA accessions. Other morphological traits i.e. branching index, inflorescence shape, inflorescence density and terminal inflorescence attitude did not show any significant differences among different clusters, whereas in the SSE collection some of these were distinct and leaf blade and

petiole pigmentation, as well as flower color were important distinguishing factors. Supporting this observation, the evaluation of morphological traits showed wider variation in amaranth accessions collected by the USDA than those collected by SSE. As a result, we can conclude that the USDA amaranth collection was a better source of diversity traits but the SSE amaranth collection was a better source of adaptation traits, especially for grain cultivars. These results have implication on breeding better varieties of grain or vegetable amaranths especially for distinctiveness in leaf and flower colors while selecting for seed color traits that are commercially desirable. In addition, we showed that many field traits have promise for Genome Wide Association Study (GWAS) analysis in the future, where combining molecular marker data with agromorphology can identify the genes in grain amaranths controlling the main traits evaluated here.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/8/11/272/s1, Figure S1: Scan of the SSE collection of amaranth seeds used in this study, Table S1: Entry data for cultivated amaranth accessions from Seed Savers' Exchange (SSE), Table S2: Passport data of Amaranth accessions from USDA, Table S3: Morphological traits and number given to them, Table S4: Significance of morphological traits on different clusters of all accessions combined across USDA and SSE collections, Table S5: Nearest cluster analysis based on Mahalanobis D^2 distance between groups found among all accessions combined across USDA and SSE Amaranths genotypes.

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