

Incomplete domestication of South American grain amaranth (*Amaranthus caudatus*) from its wild relatives

Markus G. Stetter, Thomas Müller and Karl J. Schmid

Institute of Plant Breeding, Seed Science and Population Genetics, University of Hohenheim, Fruwirthstr. 21, 70599 Stuttgart / Germany

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Corresponding Author

Name: Karl Schmid, Prof. Dr.

Address: Institute of Plant Breeding, Seed Science and Population Genetics (350)

University of Hohenheim

Fruwirthstraße 21

D-70599 Stuttgart / Germany

Tel: +49 711 459 23487

Fax: +49 711 459 24458

E-Mail address: karl.schmid@uni-hohenheim.de

Running title: Incomplete domestication of *A. caudatus*

Incomplete Amaranth domestication

1 **Abstract**

2 Grain amaranth is a pseudo-cereal and an ancient crop of Central and South America. Of the
3 three species of grain amaranth, *Amaranthus caudatus* is mainly grown in the Andean region.
4 Several models of domestication were proposed including a domestication from the wild rela-
5 tives *A. hybridus* or *A. quitensis*. To investigate the domestication history of *A. caudatus* and
6 its relationship to the two wild relatives, we used genotyping-by-sequencing (GBS) to geno-
7 type 119 amaranth accessions from the Andean region. We determined the genome sizes of
8 the three species and compared phenotypic variation in two domestication-related traits, seed
9 size and seed color. We show that the population genetic analysis based on 9,485 SNPs
10 revealed very little genetic differentiation between the two wild species, suggesting they are
11 the same species, but a strong differentiation between wild and domesticated amaranths. *A.*
12 *caudatus* has a higher genetic diversity than its wild relatives and about 10% of accessions
13 showed a strong admixture between the wild and cultivated species suggesting recent gene
14 flow. Genome sizes and seed sizes were not significantly different between wild and domesti-
15 cated amaranths, although a genetically distinct cluster of Bolivian accessions had significantly
16 larger seeds. Taken together our analysis suggests that grain amaranth is an incompletely do-
17 mesticated species, either because it was not strongly selected or because high levels of gene
18 flow from its sympatric wild relatives counteract the fixation of key domestication traits in the
19 domesticated *A. caudatus*.

Incomplete Amaranth domestication

20 Introduction

21 The genus *Amarantus* L. comprises between 50 and 75 species and is distributed worldwide
22 (Sauer, 1967; Costea & DeMason, 2001). Four species are cultivated as grain amaranths or
23 leaf vegetables (Sauer, 1967; Brenner, 2000). The grain amaranths *Amaranthus caudatus*,
24 *Amaranthus cruentus* and *Amaranthus hypochondriacus* originated from South and Central
25 America. Amaranth is an ancient crop, archaeological evidence in Northern Argentina sug-
26 gested that wild amaranth seeds were collected and used for human consumption during the
27 initial mid-Holocene (8,000 - 7,000 BP; Arreguez *et al*, 2013). In the Aztec empire, amaranth
28 was a highly valued crop and tributes were collected from the farmers that were nearly as high
29 as for maize (Sauer, 1967). Currently, amaranth is promoted as a healthy food because of its
30 favorable composition of essential amino acids and high micronutrient content.

31 The three grain amaranth species differ in their geographical distribution. *A. cruentus* and
32 *A. hypochondriacus* are most common in Central America, whereas *A. caudatus* is cultivated
33 mainly in South America. In the Andean region, *A. caudatus* grows in close proximity to the
34 two wild *Amaranthus* species *A. hybridus* and *A. quitensis*, which are considered as potential
35 ancestors (Sauer, 1967). Of these, *A. quitensis* was tolerated or cultivated in Andean home
36 gardens and used for coloring in historical times.

37 Past research on the domestication of major crop plants revealed that crops from different plant
38 families have similar domestication syndromes that include larger seeds, loss of seed shatter-
39 ing, reduced branching, loss of seed dormancy and increased photoperiod insensitivity (Abbo
40 *et al*, 2014; Hake & Ross-Ibarra, 2015). In addition to phenotypic changes, domestication
41 strongly affected the structure of genetic diversity of domesticated plants and created a genetic
42 signature of selection and drift because domestication is frequently associated with a strong ge-
43 netic bottleneck (Doebley *et al*, 2006; Olsen & Wendel, 2013; Sang & Li, 2013; Nabholz *et al*,
44 2014). The history of amaranth domestication is still under discussion. Sauer (1967) proposed
45 two scenarios based on the morphology and geographic distribution of the different species.
46 The first model postulates three independent domestication events, in which *A. hypochondria-*
47 *cus* originated from *A. powellii*, *A. cruentus* from *A. hybridus*, and *A. caudatus* from *A. quitensis*.
48 The second model proposes an initial domestication of *A. cruentus* from *A. hybridus* followed

Incomplete Amaranth domestication

49 by a migration and intercrossing of *A. cruentus* with *A. powellii* in Central America and an intercrossing of *A. cruentus* with *A. quitensis* resulting in *A. caudatus* in South America. Another model based on SNP markers suggested that all three domesticated amaranths evolved from *Amaranthus hybridus*, but at multiple locations (Maughan *et al*, 2011). Most recently, Kietlinsky *et al.* (Kietlinski *et al*, 2014) proposed a single domestication *A. hybridus* in the Andes or in Mesoamerica and a subsequent spatial separation of two lineages leading to *A. caudatus* and *A. hypochondriacus* or two independent domestication events of *A. hypochondriacus* and *A. caudatus* from a single *A. hybridus* lineage in Central and South America. Taken together, the diversity of hypotheses indicates either a complex domestication history or insufficient data to strongly support a single model of domestication.

59 Despite its long history of cultivation, the domestication syndrome of cultivated amaranth is remarkably indistinct because it still shows strong photoperiod sensitivity and has very small shattering seeds (Sauer, 1967; Brenner, 2000). Other crops like maize that were cultivated at a similar time period in the same region exhibit the classical domestication syndrome (Sang & Li, 2013; Lenser & Theißen, 2013). This raises the question whether amaranth has a different domestication syndrome or whether genetic constraints, a lack of genetic variation or (agri)cultural reasons led to a distinct domestication pattern compared to other crops. The phenotypic analysis of amaranth domestication is complicated by the taxonomic uncertainty of wild amaranth species. Although *A. quitensis* was suggested to be the ancestor of *A. caudatus*, the state of *A. quitensis* as a separate species is under debate. Sauer (1967) classified it as species, but later it was argued that it is the same species as *A. hybridus* (Coons, 1978; Brenner, 2000). However, until today *A. quitensis* is treated as separate species and since genetic evidence for the status of *A. quitensis* as a separate species is based on few studies with limited numbers of markers, this topic is still unresolved (Mallory *et al*, 2008; Kietlinski *et al*, 2014).

73 The rapid development of sequencing technologies facilitates the large-scale investigation of the genetic history of crops and their wild relatives. Among available methods, reduced representation sequencing approaches such as genotyping-by-sequencing (GBS) allow a genome-wide and cost-efficient marker detection compared to whole genome sequencing (Elshire *et al*, 2011; Poland *et al*, 2012). Despite some biases associated with reduced representation sequencing, GBS and related methods are suitable and powerful approaches for studying inter-

Incomplete Amaranth domestication

79 specific phylogenetic relationships (Cruaud *et al*, 2014) and intraspecific patterns of genetic
80 variation in crop plants (Morris *et al*, 2013).

81 We used GBS and genome size measurements to characterize the genetic diversity and rela-
82 tionship of cultivated *A. caudatus* and its putative wild ancestors *A. quitensis* and *A. hybridus*,
83 and compared patterns of genetic structure with two domestication-related phenotypic traits
84 (seed color and hundred seed weight). We tested whether domestication led to a reduction of
85 genetic diversity and larger seed size in domesticated amaranth, and clarified the taxonomic
86 relationship and gene flow with the close relatives. Our results indicated that *A. caudatus* has
87 a history of domestication that may be considered as incomplete and is consistent with models
88 of multiple domestication.

89 **Material and Methods**

90 **Plant material**

91 A total of 119 South American amaranth accessions of three *Amaranthus* species were ob-
92 tained from the USDA gene bank (<http://www.ars-grin.gov/npgs/searchgrin.html>). Of
93 these accessions, 89 were classified as *A. caudatus*, 17 as *A. hybridus*, seven as *A. quitensis*
94 and six as interspecific hybrids according to the passport information (Figure S5). We selected
95 the *A. caudatus* accessions based on the altitude of the collection site and focused on high-
96 altitude populations (2,200 to 3,700 m). We further subdivided the species into populations
97 according to their country of origin and included *A. caudatus* from Peru, Bolivia, *A. hybridus*
98 from Peru, Bolivia, Ecuador, *A. quitensis* from Peru and Ecuador as well as hybrids from Peru
99 and Bolivia. Accessions were planted in a field in Nürtingen (Germany), where a single young
100 leaf of one representative plant per accession was sampled. From 12 accessions, three plants
101 were sampled and sequenced individually for quality control.

Incomplete Amaranth domestication

102 **DNA extraction and library preparation**

103 Genomic DNA was extracted using a modified CTAB protocol (Saghai-Marooif *et al*, 1984). The
104 DNA was dried and dissolved in 50-100 μ l TE and diluted to 100 ng/ μ l for further usage. Two-
105 enzyme GBS libraries were constructed with a modified protocol from the previously described
106 two-enzyme GBS protocol (Poland *et al*, 2012). DNA was digested with a mix of 2 μ l DNA,
107 2 μ l NEB Buffer 2 (NEB, Frankfurt/Germany), 1 μ l ApeKI (4U/ μ l, NEB), 1 μ l HindIII (20U/ μ l,
108 NEB) and 14 μ l ddH₂O for 2 hours at 37°C before incubating for 2 hours at 75°C. Adapters
109 were ligated with 20 μ l of digested DNA 5 μ l ligase buffer (NEB), T₄- DNA ligase (NEB), 4 μ l
110 ddH₂O and 20 μ l of adapter mix containing 10 μ l barcode adapter (0.3 ng/ μ l) and 10 μ l common
111 adapter (0.3ng/ μ l). Samples were incubated at 22°C for 60 minutes before deactivating ligase
112 at 65°C for 30 minutes. Subsequently, samples were cooled down to 4°C. For each sequencing
113 lane 5 μ l of 48 samples with different barcodes were pooled after adapter ligation. Samples
114 of the different species were randomized over the 3 pools and different barcode lengths. The
115 12 replicated samples were in each pool. The pooled samples were purified with QIAquick
116 PCR purification kit (Qiagen, Hilden/Germany) and eluted in 50 μ l elution buffer before PCR
117 amplification of the pools. The PCR was performed with 10 μ l of pooled DNA, 25 μ l 2x Taq
118 Master Mix (NEB), 2 μ l PCR primer mix (25pmol/ μ l of each primer) and 13 μ l ddH₂O for 5 min
119 at 72°C and 30 sec at 98°C before 18 cycles of 10 sec at 98°C, 30 sec at 65°C and 30 sec at
120 72°C after the 18 cycles 5 min of 72°C were applied and samples were cooled down to 4°C.
121 Samples were purified again with QIAquick PCR purification kit (Qiagen) and eluted in 30 μ l
122 elution buffer. Three lanes with 48 samples per lane were sequenced on an Illumina HighScan
123 SQ with single end and 105 cycles on the same flow cell (see supporting data).

124 **Data preparation**

125 Raw sequence data were filtered with the following steps. First, reads were divided into sepa-
126 rate files according to the different barcodes using Python scripts. Read quality was assessed
127 with fastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Due to lower
128 read quality towards the end of the reads, they were trimmed to 90 bp. Low quality reads were
129 excluded if they contained at least one N (undefined base) or if the quality score after trimming

Incomplete Amaranth domestication

130 was below 20 in more than 10% of the bases. Data from technical replicates were combined
131 and individuals with less than 10,000 reads were excluded from further analysis (Table S5).
132 The 12 replicated samples were used to detect a lane effect with an analysis of variance.

133 **SNP calling and filtering**

134 Since no high quality reference genome for *Amaranthus* sp. was available for read mapping,
135 we used `Stacks 1.19`, for the *de novo* identification of SNPs in GBS data (Catchen *et al*,
136 2011, 2013). The pipeline provided for `Stacks denovo_map.pl` was used to call SNPs from
137 the processed data. Highly repetitive GBS reads were removed in the `ustacks` program with
138 option `-t`. Additionally, the minimum number of identical raw reads required to create a stack
139 was set to three and the number of mismatches allowed between loci when processing a single
140 individual was two. Four mismatches were allowed between loci when building the catalog.
141 The catalog is a set of non redundant loci representing all loci in the accessions and used as
142 reference for SNP calling. SNPs were called with the `Stacks tool populations 1.19` without
143 filtering for missing data using option `-r 0`. One individual, PI 511754, was excluded from
144 further analysis because it appeared to be misclassified. According to its passport information
145 it belonged to *A. hybridus*, but with all clustering methods it was placed into a separate cluster
146 consisting only of this individual, which suggested it belongs to a different species. Therefore,
147 we repeated the SNP calling without this individual. The SNPs were further filtered with `vcftools`
148 (Danecek *et al*, 2011), by allowing a maximum of 60% missing values per SNP position.

149 **Inference of genetic diversity and population structure**

150 Nucleotide diversity (π) weighted by coverage was calculated with a Python script implement-
151 ing the formula of Begun *et al* (2007) which corrects for different sampling depths of SNPs
152 in sequencing data. The confidence interval of π was calculated by bootstrapping the calcu-
153 lation 10,000 times. Mean expected (H_{exp}) and observed (H_{obs}) heterozygosities based on
154 SNPs were calculated with the R package `adegenet 1.4-2` (Jombart & Ahmed, 2011). The
155 inbreeding coefficient (F) was calculated as:

$$156 \quad \frac{H_{exp} - H_{obs}}{H_{exp}}$$

Incomplete Amaranth domestication

157 Weir and Cockerham weighted F_{ST} estimates were calculated with vcftools (Weir & Cocker-
158 ham, 1984; Danecek *et al*, 2011). To infer the population structure, we used ADMIXTURE for a
159 model-based clustering (Alexander *et al*, 2009) and conducted the analysis with different num-
160 bers of predefined populations ranging from $K = 1$ to $K = 9$ to find the value of K that was most
161 consistent with the data using a cross-validation procedure described in the ADMIXTURE man-
162 ual. To avoid convergence effects we ran ADMIXTURE 10 times with different random seeds
163 for each value of K . As a multivariate clustering method, we applied discriminant analysis of
164 principal components (DAPC) implemented in the R-package adegenet (Jombart *et al*, 2010;
165 Jombart & Ahmed, 2011) and determined the number of principal components (PCs) used
166 in DAPC with the `optim.a.score` method. To investigate the phylogenetic relationship of the
167 species, we calculated an uncorrected neighbor joining network using the algorithm Neighbor-
168 Net (Bryant & Moulton, 2004) as implemented in the SplitsTree4 program (Huson & Bryant,
169 2006). The Euclidean distance was calculated from the genetic data to construct a neighbor
170 joining tree, which was bootstrapped 1,000 times with the `pegas` R-package (Paradis *et al*,
171 2004). The migration between genetic groups was modeled with TreeMix (Pickrell & Pritchard,
172 2012). For the TreeMix analysis we used the groups that were identified by ADMIXTURE ($K = 5$)
173 without an outgroup, and allowed 4 migration events, as preliminary runs indicates 4 migration
174 events to be the highest number. The tree was bootstrapped 1,000 times.

175 Genome size

176 To compare genome sizes between *Amaranthus* species, we measured the genome size of
177 22 *A. caudatus*, 8 *A. hybridus* and 4 *A. quitensis* accessions. Plants were grown for four
178 weeks in the greenhouse before one young leaf was collected for cell extraction. A tomato
179 cultivar (*Solanum lycopersicum* cv Stupicke) was used as internal standard, because it has a
180 comparable genome size that has been measured with high accuracy (DNA content = 1.96 pg;
181 Dolezel *et al*, 1992). Fresh leaves were cut up with a razor blade and cells were extracted
182 with CyStain PI Absolute P (Partec, Muenster/Germany). Approximately 0.5 cm² of the sample
183 leaf was extracted together with similar area of tomato leaf in 0.5 ml of extraction buffer. The
184 DNA content was determined with CyFlow Space (Partec, Muenster/Germany) flow cytometer
185 and analyzed with FlowMax software (Partec, Muenster/Germany). For each sample, 10,000

Incomplete Amaranth domestication

186 particles were measured each time. Two different plants were measured for each accession.

187 The DNA content was calculated as:

$$188 \quad \text{DNA content 2C [pg]} = \text{genome size tomato} \times \frac{\text{fluorescence amaranth}}{\text{fluorescence tomato}}$$

189 and the genome size (in Mbp) was calculated as followed:

$$190 \quad \text{genome size 1C [Mbp]} = (0.978 * 10^3) \times \frac{\text{DNA content 2C [pg]}}{2}$$

191 The conversion from pg to bp was calculated with $1\text{pg DNA} = 0.978 \times 10^9 \text{ bp}$ (Dolezel *et al*,
192 2003). Means were calculated using R software (Team) and an ANOVA was performed to infer
193 differences in genome size for the species.

194 **Seed color and hundred seed weight**

195 For each accession we calculated the hundred seed weight (HSW) by weighting three samples
196 of 200 seeds. Seed color was determined from digital images taken with a binocular (at 6.5x
197 magnification) and by visual comparison to the GRIN descriptors for amaranth (<http://www.ars-grin.gov/cgi-bin/npgs/html/desclist.pl?159>). There were three colors present in
198 the set of accessions, white, pink, which also indicates a white seed coat and dark brown.
199 To infer how the species, assigned genetic groups or seed color influenced seed size, we
200 conducted an ANOVA. Differences were tested with a LSD test implemented in the R package
201 *agricolae* (<http://tarwi.lamolina.edu.pe/~fmendiburu/>)
202

203 **Results**

204 **SNP identification by GBS**

205 To investigate genome-wide patterns of genetic diversity in cultivated amaranth and two putative
206 ancestors, we genotyped a diverse panel of 119 amaranth accessions from the Andean region
207 that we obtained from the USDA genebank. The sequencing data generated with a two-enzyme
208 GBS protocol, consisted of 210 Mio. raw reads with an average of 1.5 Mio. reads per accession
209 (Supporting information S2). We tested for a lane effect of the Illumina flow cell, by sequencing

Incomplete Amaranth domestication

210 12 individuals on each of the three lanes used to sequence all accessions. A subsequent
211 analysis of variance (ANOVA) of the read number did not show a lane effect (Table S1). Since
212 a high-quality reference genome of an amaranth species was not available, we aligned reads
213 *de novo* within the dataset to unique tags using Stacks (Catchen *et al*, 2011). The total length
214 of the aligned reads was 16.6 Mb, which corresponds to approximately 3.3 % of the *A. caudatus*
215 genome. For SNP calling, reads of each individual were mapped to the aligned tags. SNPs
216 were called with parameters described in Materials and Methods, which resulted in 63,956
217 SNPs. Since GBS data are characterized by a high proportion of missing values, we removed
218 SNPs with more than 60% of missing values. After this filtering step, we obtained 9,485 biallelic
219 SNPs with an average of 35.3 % missing data for subsequent analyses (Figure S1).

220 Inference of population structure

221 To infer the genetic relationship and population structure of *A. caudatus* and its putative ances-
222 tors, we used three different methods, ADMIXTURE, Discriminant Analysis of Principal Compo-
223 nents (DAPC) and phylogenetic reconstruction with an uncorrected neighbor-joining network.
224 The ADMIXTURE analysis with three predefined groups ($K = 3$), which corresponds to the
225 number of *Amaranthus* species included in the study, did not cluster accessions by their species
226 origin, but grouped the *A. caudatus* accessions into two distinct clusters and combined the two
227 wild accessions *A. hybridus* and *A. quitensis* into a single cluster. This analysis indicates a
228 clear separation between domesticated and the wild Amaranths, but the two wild amaranths
229 appeared to be a single genetic group because with higher values of K did not lead to subdivi-
230 sion of the two wild species into separate clusters that corresponds to the species assignment
231 (Figure 1). Cross-validation showed that $K = 5$ was most consistent with the data (Supplemen-
232 tary Figure S2), which produced three different groups of *A. caudatus* accessions that included
233 a few wild amaranth accessions, and two wild amaranth clusters that both consist of *A. hy-*
234 *bridus* and *A. quitensis* accessions. The two wild amaranth clusters differ by the geographic
235 origin because one cluster contains both *A. hybridus* and *A. quitensis* accessions from Peru
236 and the other cluster from Ecuador. This indicates a strong strong geographic differentiation
237 among wild ancestors.

238 The *A. caudatus* accessions clustered into three groups that also showed geographic differenti-

Incomplete Amaranth domestication

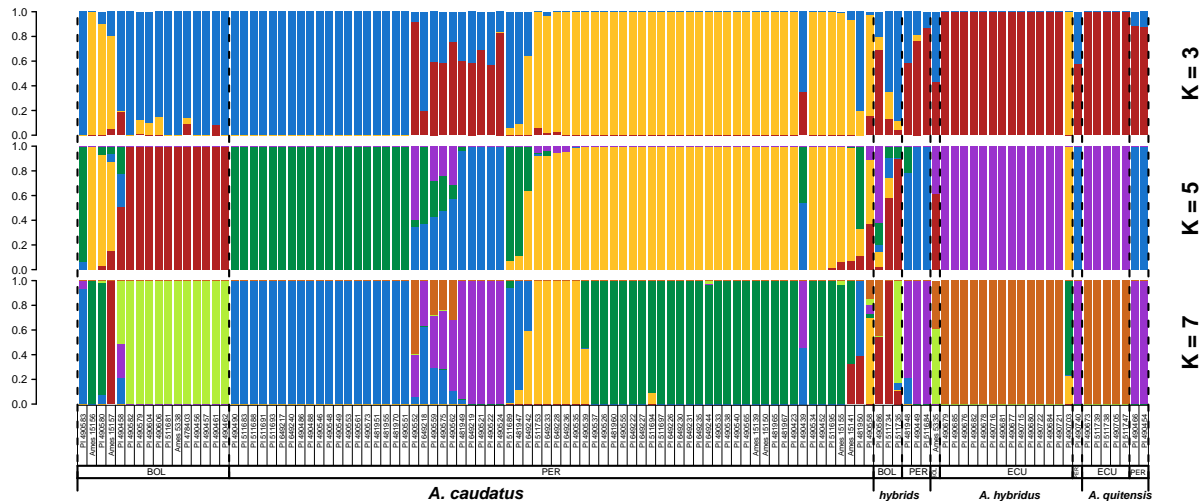


Figure 1: Model based clustering analysis with different numbers of clusters ($K=3, 5, 7$) with ADMIXTURE. The clusters reflect the number of species in the study ($K=3$), the number of single populations (species per country of origin, $K=7$) and the optimal number as determined by cross validation ($K=5$). Individuals are sorted by species and country of origin (BOL=Bolivia, PER = Peru and ECU = Ecuador) as given by their passport data.

239 ation. The first cluster consisted of individuals from Bolivia (Figures 2 and 1; $K = 5$, red color).
240 *A. caudatus* accessions from Peru were split into two clusters of which one predominantly rep-
241 resents a region from North Peru (Huari Province; Figures 2 and 1; $K = 5$, yellow color),
242 whereas the second cluster contains individuals distributed over a wide geographic range that
243 extending from North to South Peru ($K = 5$, green color). Ten *A. caudatus* accessions from
244 the Cuzco region clustered with the three accessions of wild amaranths from Peru ($K = 5$,
245 blue color). These ten accessions showed admixture with the other cluster of wild amaranths
246 and with a Peruvian cluster. Accessions that were labeled as 'hybrids' in their passport data,
247 because they express a set of phenotypic traits of different species, clustered with different
248 groups. 'Hybrids' from Bolivia were highly admixed, whereas 'hybrids' from Peru clustered with
249 the Peruvian wild amaranths (Figure 1). Taken together, the population structure inference
250 with ADMIXTURE identified a clear separation between the wild and domesticated amaranth
251 species and genetic differentiation among domesticated amaranths but also gene flow between
252 populations.

253 The inference of population structure with a discriminant analysis of principal components
254 (DAPC) and Neighbor-Joining network produced very similar results as ADMIXTURE. The first

Incomplete Amaranth domestication

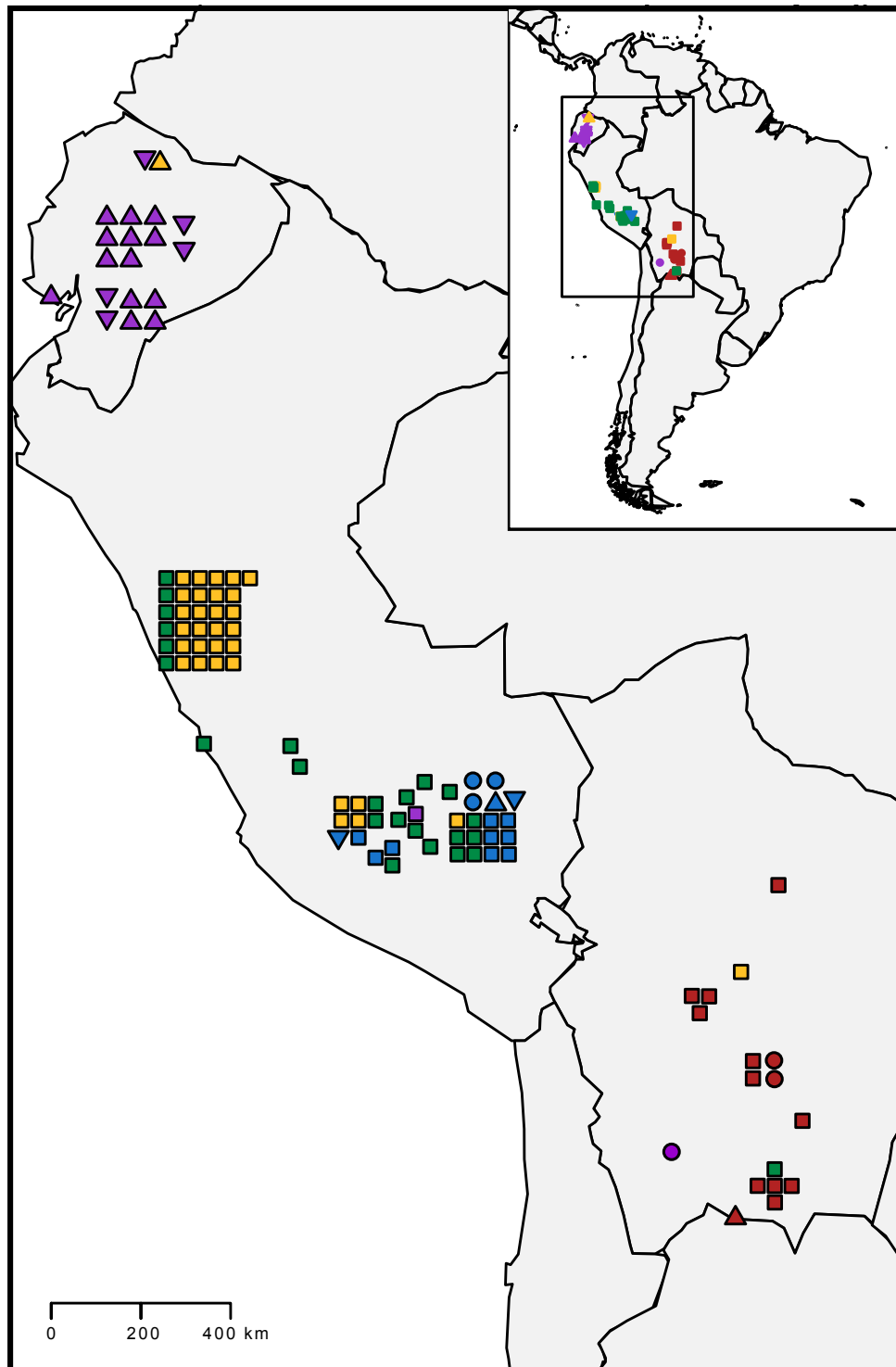


Figure 2: Geographic distribution of accessions for which data was available from passport information. Locations are not exact geographic locations because location data was given as country province . Colors are given by ADMIXTURE with K=5 (Figure 1). Species are indicated by shapes. *A. caudatus* (□), *A. hybridus* (△), *A. quitensis* (▽) and hybrids between species (○)

255 principal component of the DAPC analysis which we used to cluster accessions based on their

256 species explained 96% of the variation and separated the two wild species from the domesti-

Incomplete Amaranth domestication

257 cated *A. caudatus* (Figure S3A). In a second DAPC analysis that was based on information on
258 species and country of origin (Figure S3B) the first principal component explained 55% of the
259 variation and separated most of the wild from the domesticated amaranths. The second princi-
260 pal component explained 35% of the variation and separated the Peruvian from the Bolivian *A.*
261 *caudatus* accessions.

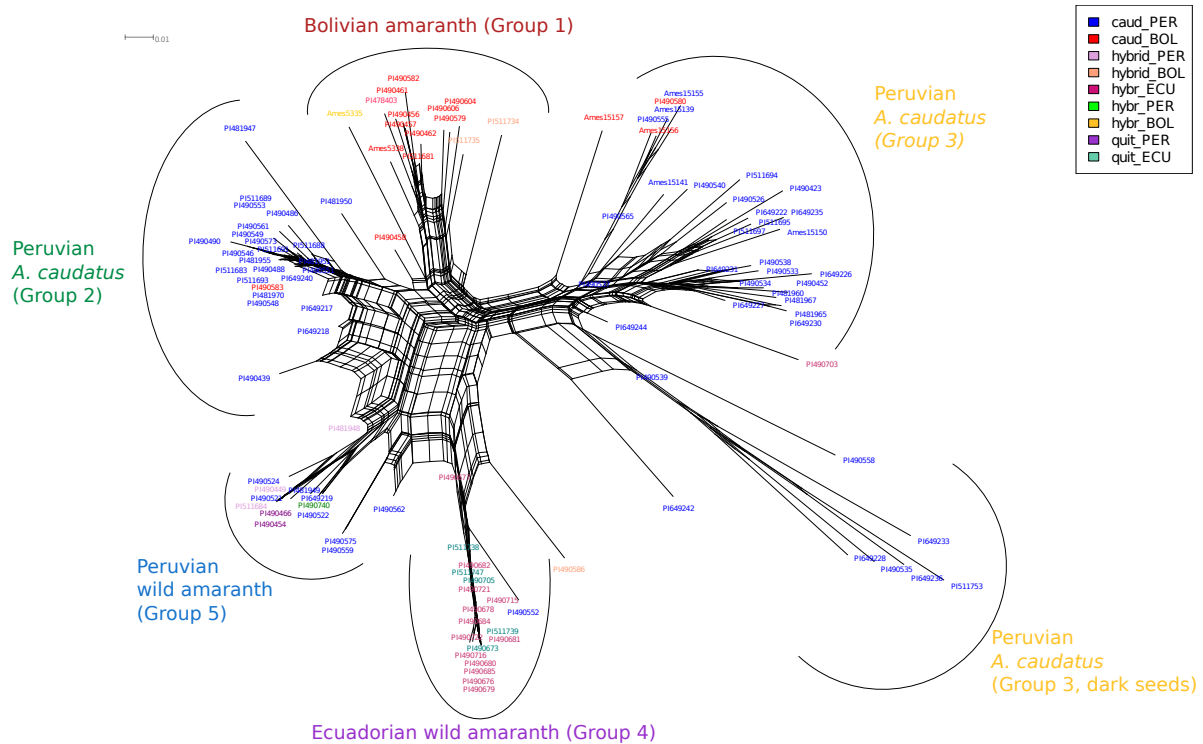


Figure 3: Neighbor-joining network of 113 amaranth accessions from six potential populations. Different colors indicate the species and origin according to gene bank information. *A. caudatus* from Peru (blue) and from Bolivia (red), *A. hybridus* from Ecuador (magenta), from Peru (green) and Bolivia (yellow), *A. quitensis* from Ecuador (turquoise) and Peru (purple) and hybrids between species from Peru (salmon) and Bolivia (light orange). Arches show genetic clusters as inferred with ADMIXTURE (K = 5).

262 The phylogenetic network outlines the relationships between the different clusters (Figure 3).
263 It shows two distinct groups of mainly Peruvian *A. caudatus* accessions and a group of ac-
264 cessions with a wide geographic distribution (Figure 2; green color). The latter is more closely
265 related to the Bolivian *A. caudatus* and the wild relatives. The strong network structure between
266 these three groups suggests a high proportion of shared polymorphisms or a high level of re-
267 cent gene flow. In contrast, the clade with *A. caudatus* accessions from Northern Peru are more
268 separated from the other clades which indicates a larger evolutionary distance, less ongoing

Incomplete Amaranth domestication

269 gene flow with the wild ancestors or stronger selection (Figure 2; yellow color). They are split
270 into two groups, of which the smaller includes only accessions with dark seeds. In a bifurcating
271 phylogenetic tree, ten domesticated amaranth accessions clustered within the same clade as
272 the wild species (Figure S4). The same clustering was also obtained with ADMIXTURE and
273 $K = 7$ (Figure 1).

Table 1: Weir and Cockerham weighted F_{ST} estimates between populations based on the taxonomic assignment of their passport data. The group of wild amaranth are *A. hybridus* and *A. quitensis* taken together.

	F_{ST}
<i>A. caudatus</i> x <i>A. hybridus</i>	0.319
<i>A. caudatus</i> x <i>A. quitensis</i>	0.274
<i>A. caudatus</i> x wild amaranth	0.322
<i>A. hybridus</i> x <i>A. quitensis</i>	0.041
<i>A. caudatus</i> (PER) x <i>A. caudatus</i> (BOL)	0.132

274 To quantify the level of genetic differentiation between the species and groups within *A. cau-*
275 *datu*s, we estimated weighted F_{ST} values using the method of Weir and Cockerham (Weir &
276 Cockerham, 1984). F_{ST} values between *A. caudatus* and the wild *A. hybridus* and *A. quiten-*
277 *sis* species were 0.31 and 0.32, respectively (Table 1), and 0.041 between *A. hybridus* and *A.*
278 *quitensis* based on the taxonomic assignment. The latter reflects the high genetic similarity
279 of the accessions from both species observed above. Within *A. caudatus* subpopulations, the
280 F_{ST} between *A. caudatus* populations from Peru and Bolivia was 0.132, three times higher than
281 between *A. hybridus* and *A. quitensis*. The above analyses suggested that some individuals
282 may be misclassified in the passport information, and we therefore calculated F_{ST} values of
283 population sets defined by ADMIXTURE. Although such F_{ST} values are upward biased, they
284 allow to evaluate the relative level of differentiation between groups defined by their genotypes.
285 The comparison of F_{ST} values showed that the three *A. caudatus* groups (groups 1-3) are less
286 distant to the Peruvian (group 5) than to the Ecuadorian wild amaranths (group 4; Table S2).
287 A tree constructed with TreeMix, which is based on allele frequencies within groups (Figure 4),
288 suggests gene flow from the Peruvian *A. caudatus* (group 2) to Peruvian wild amaranth (group
289 5) and, with a lower confidence level, between wild amaranths from Ecuador (group 4) into
290 Bolivian *A. caudatus* (group 1), as well as from Bolivian *A. caudatus* to Peruvian *A. caudatus*

Incomplete Amaranth domestication

291 (Group 2).

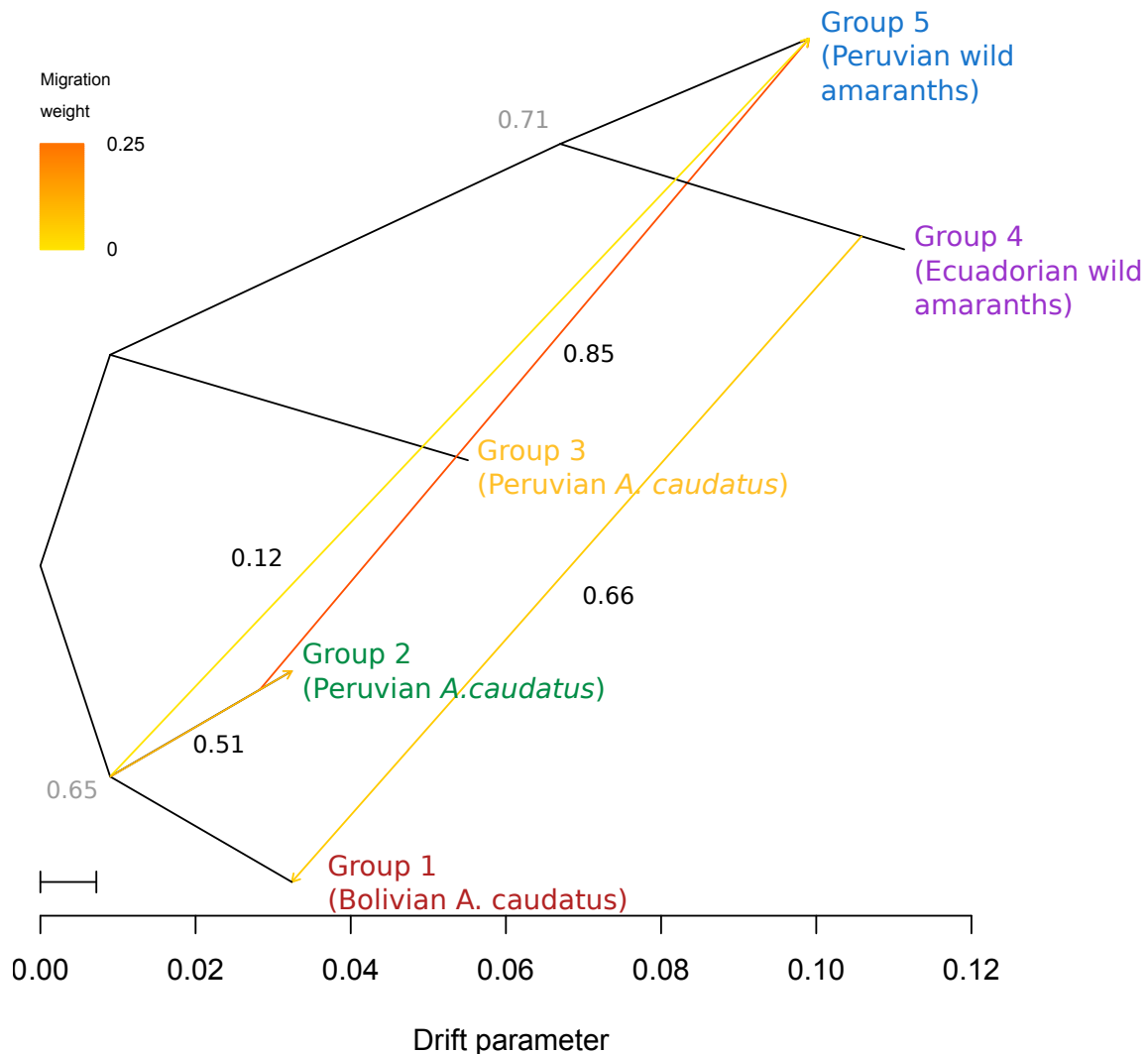


Figure 4: Tree of five genetic clusters of South American amaranths inferred with TreeMix. The genetic clusters which were used to calculate the tree were inferred with ADMIXTURE. Groups 1 to 3 represent *A. caudatus* clusters from Peru and Bolivia, group 4 represents wild amaranth from Ecuador and group 5 wild amaranth from Peru. The migration events are colored according to their weight. Numbers at branching points and on the migration arrow represent bootstrapping results based on 1,000 runs.

292 Analysis of genetic diversity

293 We further investigated whether domestication reduced genetic diversity in *A. caudatus* com-
294 pared to wild amaranths (Table 2). All measures of diversity were higher for the cultivated
295 than the wild amaranths. For example, nucleotide diversity (π) was about two times higher in

Incomplete Amaranth domestication

296 *A. caudatus* than in the two wild species combined. The diversity values of the accessions
 297 classified as hybrids showed intermediate values between wild and domesticated populations
 298 supporting their hybrid nature. The inbreeding coefficient, F , was highest in the domesticated
 299 amaranth but did not differ from the wild amaranths combined. In contrast accessions classified
 300 as 'hybrids' and *A. quitensis* showed lower inbreeding coefficients. Within the groups of acces-
 301 sions defined by ADMIXTURE, genetic diversity differed substantially. The wild amaranths from
 302 Ecuador had the lowest ($\pi = 0.00031$) while the group from northern Peru showed the highest
 303 level of nucleotide diversity ($\pi = 0.00111$; Table S3). Figure 5 shows that even though the over-
 304 all diversity of *A. caudatus* was higher a substantial proportion of sites were more diverse in the
 305 wild amaranths ($\pi_{caud} - \pi_{wild} < 0$; Figure 5).

Table 2: Genetic diversity parameters for the three putative *Amaranthus* species and the wild amaranth (*A. hybridus* and *A. quitensis*). π is the nucleotide diversity over all sites, CI_{π} is the 95% confidence interval of π , H_{exp} the mean expected heterozygosity for the variant sites and SD_{He} its standard deviation, H_{obs} the mean observed heterozygosity and SD_{Ho} its standard deviation. F is the inbreeding coefficient and SD_F its standard deviation.

Population	π	CI_{π}	H_{exp}	SD_{He}	H_{obs}	SD_{Ho}	F	SD_F	θ_w
<i>A. caudatus</i>	0.00117	± 0.00002	0.175	0.167	0.049	0.140	0.688	0.462	0.00123
<i>A. hybridus</i>	0.00061	± 0.00001	0.085	0.135	0.041	0.170	0.679	0.608	0.00073
<i>A. quitensis</i>	0.00059	± 0.00001	0.076	0.169	0.040	0.170	0.451	0.763	0.00048
Wild amaranth	0.00062	± 0.00002	0.090	0.140	0.041	0.166	0.681	0.591	0.00070
Hybrids	0.00091	± 0.00001	0.112	0.179	0.060	0.173	0.436	0.645	0.00107

Incomplete Amaranth domestication

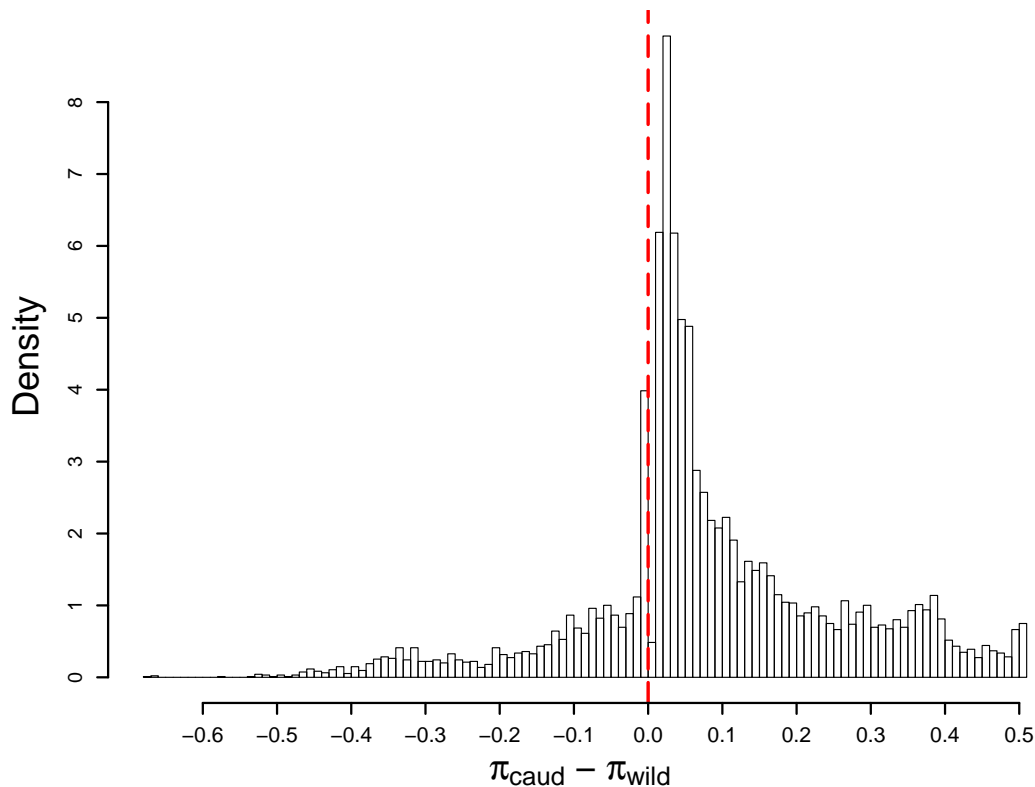


Figure 5: Per site difference in nucleotide diversity (π) between domesticated amaranth (*A. caudatus*) and wild amaranth (*A. hybridus* and *A. quitensis*)

306 **Genome size in wild and cultivated amaranth**

307 Although the genomic history of amaranth species still is largely unknown, genome sizes and
308 chromosome numbers are highly variable within the genus *Amaranthus* (<http://data.kew.org/cvalues/>). This raises the possibility that the domestication of *A. caudatus* was accom-
309 panied by polyploidization events as observed in other crops. We therefore tested whether a
310 change in genome size played a role in the context of domestication by measuring the genome
311 size of multiple individuals from all three species with flow cytometry. The mean genome size
312 of *A. caudatus* was 501.93 Mbp, and the two wild ancestors did not differ significantly from
313 this value (Table 3) indicating that polyploidization did not play a role in the recent evolution of
314 domesticated amaranth.
315

Incomplete Amaranth domestication

Table 3: Genome size of representative group of individuals for each species. There are no significant differences between genome sizes ($p \leq 0.05$). The number of individuals per population is N and SD is the standard deviation for each parameter.

	N	DNA content (pg)	SD	genome size (Mbp)	SD
<i>A. caudatus</i>	22	1.026	0.026	501.93	12.74
<i>A. hybridus</i>	8	1.029	0.025	502.96	12.20
<i>A. quitensis</i>	4	1.021	0.016	499.07	7.91

316 **Seed color and seed size as potential domestication traits.**

317 In grain crops, grain size and seed color are important traits for selection and likely played
318 a central role in domestication of numerous plants (Abbo *et al*, 2014; Hake & Ross-Ibarra,
319 2015). To investigate whether these two traits are part of the domestication syndrome in grain
320 amaranth, we compared the predominant seed color of the different groups of accessions and
321 measured their seed size. The seeds could be classified into three colors, white, pink and
322 brown. The white and pink types have both a white seed coat, but the latter has red cotyledons
323 that are visible through the translucent seed coat. A substantial number of seed samples
324 (19) from the genebank contained seeds of other color up to a proportion of 20%. One *A.*
325 *caudatus* accession from Peru (PI 649244) consisted of 65% dark seeds and 35% white seeds
326 in the sample. No accession from the two wild species or hybrid accessions had white seeds,
327 whereas the majority (74%) of *A. caudatus* accessions had white (70%) or pink (4%) seeds,
328 and the remaining (26%) brown seeds (Figure 6 A). We also compared the seed color of groups
329 defined by ADMIXTURE ($K = 5$; Figure 1), which reflect genetic relationship and may correct
330 for mislabeling of accessions (Figure 6 B). None of these groups had only white seeds, but
331 clusters that mainly consist of accessions from the wild relatives had no white seeds at all. In
332 contrast to seed color, the hundred seed weight (HSW) of the different *Amaranthus* species did
333 not significantly differ between wild and cultivated amaranths. The mean HSW of *A. caudatus*
334 was 0.056 g and slightly higher than the HWS of *A. hybridus* (0.051 g) and *A. quitensis* (0.050
335 g; Figure 6 C and Table S4). Among the groups identified by ADMIXTURE ($K = 5$), one
336 group showed a significantly higher HSW than the other groups, while the other four groups
337 did not differ in their seed size. The group with the higher HSW consisted mainly of Bolivian
338 *A. caudatus* accessions and had a 21 % and 35 % larger HSW than the two groups consisting

Incomplete Amaranth domestication

339 mainly of Peruvian *A. caudatus* accessions, respectively (Figure 6 D). An ANOVA also revealed
340 that seed color has an effect on seed size because white seeds are larger than dark seeds
341 (Table 4).

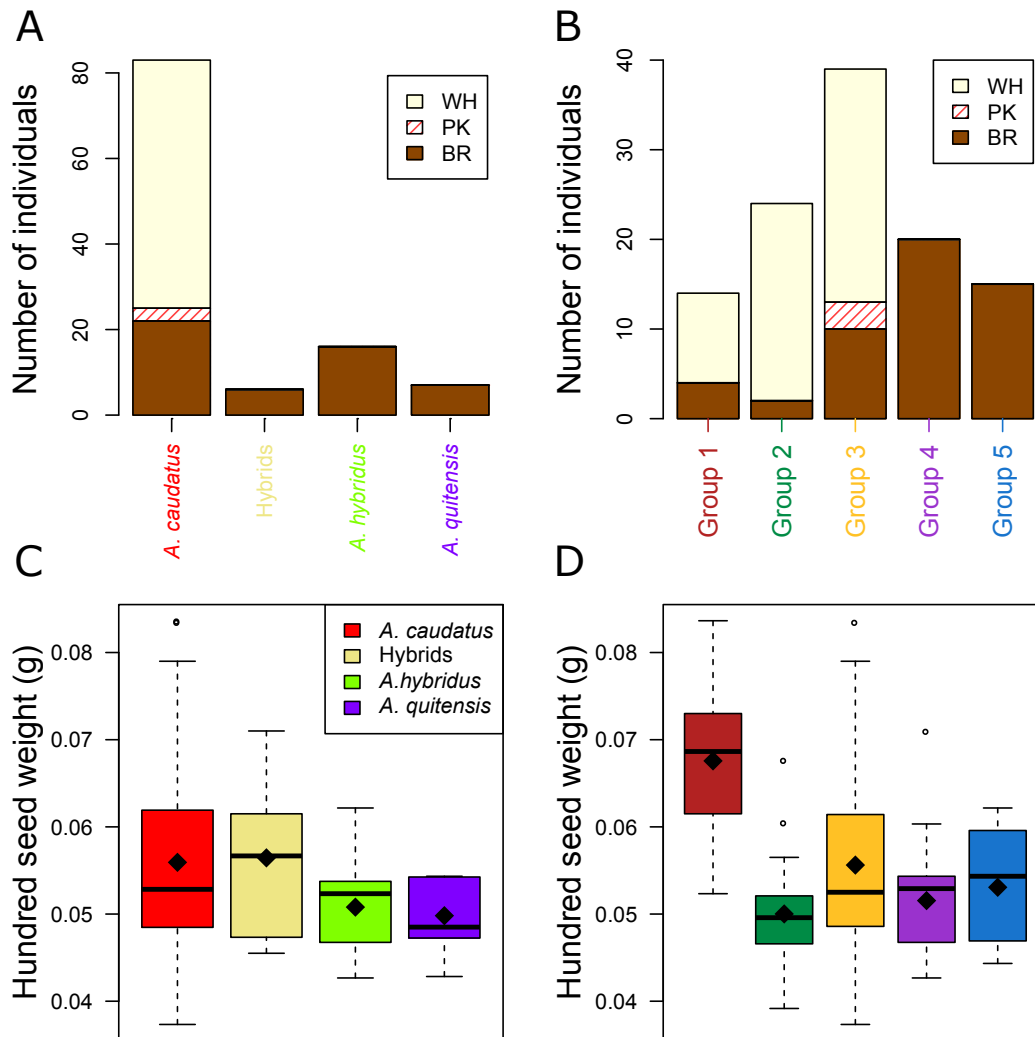


Figure 6: Hundred seed weight (**A,B**) and predominant seed color (**C,D**) by *Amaranthus* species (**A,C**) and groups identified with ADMIXTURE for K=5 (**B,D**) where group 1 (red) resembles *A. caudatus* from Bolivia, group 2 (green) and 3 (yellow) *A. caudatus* from Peru, group 4 (purple) represents wild amaranth from Ecuador and group 5 (blue) wild amaranth from Peru. Seed colors were white (WH), pink (PK) and dark brown (BR). While there were no significant differences in seed size between the species, Group 1 had significantly higher hundred seed weight ($p \leq 0.05$) than the other groups.

Incomplete Amaranth domestication

Table 4: Analysis of variance for the hundred seed weight in dependence of the Seed color and Population as determined by ADMIXTURE

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Seed color	2	0.000657	0.0003285	4.657	0.0116 *
Group	4	0.003151	0.0007877	11.165	1.46e-07 ***
Seed color:Group	2	0.000042	0.0000209	0.297	0.7440
Residuals	103	0.007266	0.0000705		

Discussion

Genotyping-by-sequencing of amaranth species

The genotyping of wild and cultivated amaranth accessions revealed a strong genetic differentiation between wild and cultivated amaranths and a high level of genetic differentiation within domesticated *A. caudatus*. We based our sequence assembly and SNP calling on a *de novo* assembly of GBS data with Stacks because currently no high quality reference sequence of an amaranth species is available. Stacks allows SNP calling without a reference genome by constructing a reference catalog from the data and includes all reads in the analysis (Catchen *et al*, 2011). Since *de novo* assembled fragments are not mapped to a reference, they are unsorted and do not allow to investigate differentiation along genomic regions but the data are suitable for the analysis of genetic diversity and population structure (Catchen *et al*, 2013). GBS produces a large number of SNPs (Poland *et al*, 2012; Huang *et al*, 2014), albeit with a substantial proportion of missing values. Missing data lead to biased estimators of population parameters such as π and θ_w (Arnold *et al*, 2013) and need to be accounted for if different studies are compared. The comparison of accessions and groups within a study is possible, however, because all individuals were treated with the same experimental protocol. We filtered out sites with high levels of missing values to obtain a robust dataset for subsequent population genomic analysis. Compared to previous studies on amaranth genetic diversity (Maughan *et al*, 2009, 2011; Khaing *et al*, 2013; Jimenez *et al*, 2013; Kietlinski *et al*, 2014), our study combines a larger number of accessions and more genetic markers, which allowed us to assess the genetic diversity and population structure on a genome-wide basis.

Incomplete Amaranth domestication

363 ***A. quitensis* and *A. hybridus* are not different species**

364 The two wild relatives *A. quitensis* and *A. hybridus* do not appear to be separate species in
365 our analyses but form two distinct subgroups of Peruvian and Ecuadorian wild amaranths that
366 both consist of accessions from the two species. It was suggested before that *A. quitensis* is
367 the same species as *A. hybridus* (Coons, 1978), but the passport information regarding the
368 species of genebank accessions was not changed and *A. quitensis* is still considered as a sep-
369 arate species in these records. The taxonomic differentiation between the two species rests on
370 a single morphological trait, namely the shape of the tepals, which are very small and prone to
371 misidentification (Sauer, 1967). The high phenotypic similarity of *A. quitensis* and *A. hybridus*
372 is supported by our analyses which showed that they are very closely related and mainly sep-
373 arated by their geographic origin, from Peru and Ecuador. The F_{ST} value between the two wild
374 species was lower than between the two *A. caudatus* groups from Peru and Bolivia (Tables 1
375 and S2). A close relationship is also supported by the highly similar genome sizes of all three
376 species, although the genus *Amaranthus* harbors species with very different genome sizes
377 due to variation in chromosome numbers and ploidy levels (Baohua & Xuejie, 2002; Rayburn
378 *et al*, 2005). In contrast to our results, a recent study found evidence for a genetic differen-
379 tiation between *A. hybridus* and *A. quitensis* (Kietlinski *et al*, 2014). This discrepancy may
380 result from the different composition of samples because our sample consists of accessions of
381 both species from the Andean region whereas Kietlinski *et al.* (2014) used *A. hybridus* and *A.*
382 *quitensis* accessions with little geographic overlap between the two species. Our F_{ST} values
383 also indicate that Peruvian and Ecuadorian wild amaranths show a high level of differentiation
384 ($F_{ST} = 0.579$; Table S2), which is similar to the differentiation between one of two Peruvian
385 *A. caudatus* groups and the wild amaranths from Peru ($F_{ST} = 0.553$). In summary, under the
386 assumption that the passport information of the wild amaranths is correct, we propose that *A.*
387 *quitensis* and *A. hybridus* are a single species. The high level of intraspecific differentiation in
388 both wild and cultivated amaranth is relevant for investigating domestication because the ge-
389 netic distance between groups of cultivated amaranth is related to the geographic distance of
390 the wild ancestors.

Incomplete Amaranth domestication

391 **Diversity of South American amaranth**

392 In numerous crops, domestication was associated with a decrease in genome-wide levels of
393 diversity due to bottleneck effects and strong artificial selection of domestication traits (Gepts,
394 2014). In contrast, the overall genetic diversity in our sample of domesticated amaranths was
395 higher than in the two wild relatives. The distribution of diversity between the GBS fragments
396 includes genomic regions with reduced diversity in *A. caudatus*, which may reflect selection in
397 some genomic regions (Figure 5). Without a reference genome it is not possible to position
398 reads on a map to identify genomic regions that harbor putative targets of selection based on
399 an inference of the demographic history. Despite the indirect phenotypic evidence for selection,
400 the higher genetic diversity of domesticated grain amaranth may result from a strong gene flow
401 between wild and domesticated amaranths. Gene flow between different amaranth species
402 has been observed before (Trucco *et al*, 2005) and is also consistent with the observation of
403 six highly admixed accessions classified as 'hybrids' in the passport data and which appear
404 to be interspecific hybrids (Figure 1 and Table 2). Gene flow between *A. caudatus* and other
405 *Amaranthus* species in different areas of the distribution range could explain a higher genetic
406 diversity in the domesticated amaranth, which is also consistent with the strong network struc-
407 ture (Figure 3) and the TreeMix analysis (Figure 4). Taken together, cultivated *A. caudatus* is
408 unusual in its higher overall genetic diversity compared to its putative wild ancestors, which is
409 uncommon in domesticated crops.

410 **Amaranth domestication syndrome**

411 Despite its long history of cultivation, diverse uses for food and feed and its high importance dur-
412 ing the Aztec period, grain amaranth does not display the classical domestication syndrome as
413 strongly as other crops (Sauer, 1967). On one hand, domesticated amaranth shows morpho-
414 logical differentiation from wild amaranths like larger and more compact inflorescences (Sauer,
415 1967) and a level of genetic differentiation (Table 1) which is comparable to the level of differ-
416 entiation of other domesticated crops and their wild relatives (Sunflower: 0.22 (Mandel *et al*,
417 2011); common bean: 0.1-0.4 (Papa *et al*, 2005), pigeonpea: 0.57-0.82 (Kassa *et al*, 2012)).
418 On the other hand, the individual flowers of a plant do not mature synchronously and produce

Incomplete Amaranth domestication

419 very small seeds that are shattered (Brenner, 2000). In contrast to wild amaranths, which
420 all have dark brown seeds, the predominant seed color of cultivated grain amaranth is white,
421 which suggests that selection for seed color played a role in the history of *A. caudatus*. How-
422 ever, dark-seeded accessions are present in all three groups of *A. caudatus* defined by the
423 genotypic data, which indicates that white seed color is not a fixed trait. Similarly, seed sizes
424 between wild and domesticated amaranths are not significantly different with the exception of
425 *A. caudatus* accessions with white seeds from Bolivia (Figure 6), which have larger seeds.
426 The increased seed size in this group and in white seeds in general indicates past selection
427 for domestication-related traits, but only in specific geographic regions or in certain types of
428 amaranth, and not in the whole domesticated crop species.

429 Possible explanations for the incomplete fixation of domestication traits in South American
430 grain amaranth include weak selection, genetic constraints or ongoing gene flow. First, weak
431 selection of putative domestication traits may reflect that they were not essential for domes-
432 tication. Although white seeds are predominant in cultivated amaranth and unambiguously
433 a domestication-related trait under selection, other seed colors may have been preferred for
434 different uses with the consequence that genes for white seed color were not fixed. Sec-
435 ond, domestication traits may experience genetic constraints that limit phenotypic variation.
436 Genes controlling domestication traits that are part of simple molecular pathways, have mini-
437 mal pleiotropic effects, and show standing functional genetic variation have a higher chance of
438 fixation than genes with high pleiotropic or epistatic interactions (Doebley *et al*, 2006; Lenser
439 & Theißen, 2013). Numerous genes with these characteristics were cloned and characterized
440 in major crops like rice, barley and maize and shown to contribute to the distinct domestication
441 syndrome such as a loss of seed shattering, larger seed size and compact plant architecture.
442 Since the molecular genetic basis of domestication traits in amaranth is unknown, the lack of
443 a strong domestication syndrome and a lack of fixation of putative domestication traits despite
444 a long period of cultivation may result from genetic constraints which limited the origin and se-
445 lection of domestication phenotypes. A third explanation is ongoing gene flow between wild
446 and domesticated amaranth that may prevent or delay the formation of a distinct domestication
447 syndrome and contributes to the high genetic diversity (Table 2), similar seed size (Figure 6 C),
448 and the presence of dark seeds (Figure 6) in cultivated amaranth. Both historical and ongoing

Incomplete Amaranth domestication

449 gene flow are likely because amaranth has an outcrossing rate between 5% and 30% (Jain
450 *et al*, 1982). In South America, wild and domesticated amaranths are sympatric over wide ar-
451 eas and the wild *A. hybridus* and *A. quitensis* were tolerated in the fields and home gardens
452 with *A. caudatus* (Sauer, 1967), where they may have intercrossed. Gene flow between wild
453 and domesticated plants has also been observed in maize and teosinte in the Mexican high-
454 lands, but did not have a major influence on the maize domestication syndrome (Hufford *et al*,
455 2013). Further support for ongoing gene flow in amaranth is given by the presence of hybrids
456 and admixed accessions in our sample with evidence for genetic admixture and dark seeds
457 that demonstrate the phenotypic effects of introgression. Since the dark seed color is dominant
458 over white color (Kulakow *et al*, 1985), dark seeds could have efficiently removed by selection
459 despite gene flow. Therefore, gene flow likely is not the only explanation for the lack of a distinct
460 domestication syndrome.

461 Our data are consistent with the model by Kietlinski *et al.* (2014) who proposed a single do-
462 mestication of *A. caudatus* and *A. hypochondriacus* in Central America followed by migration
463 of *A. caudatus* to South America. Gene flow between wild amaranths and *A. caudatus* in the
464 Southern distribution range (Peru and Bolivia) may explain the higher genetic diversity of the
465 latter despite a strong genetic differentiation. The second model of Kietlinski *et al.* of two in-
466 dependent domestication events from a single *A. hybridus* lineage that ranged from Central
467 America to the Andes is supported under the assumption that domestication occurred in South
468 Peru because of the strong differentiation between Ecuadorian and Peruvian wild amaranths
469 (Table S2). Since the Peruvian group of wild amaranths inferred with ADMIXTURE comprises
470 *A. quitensis* and *A. hybridus*, but also *A. caudatus* accessions, the latter may represent acces-
471 sions from the center of domestication.

472 Conclusions

473 The genotypic and phenotypic analysis of wild and domesticated South American grain ama-
474 ranths suggest that *A. caudatus* is an incompletely domesticated crop species. Key domestica-
475 tion traits such as the shape of inflorescences, seed shattering and seed size are rather similar
476 between wild and cultivated amaranths and there is strong evidence of ongoing gene flow from

Incomplete Amaranth domestication

477 its wild ancestor despite selection for domestication traits like white seeds. Although grain ama-
478 ranth is an ancient crop of the Americas, genomic and phenotypic signatures of domestication
479 differ from other, highly domesticated crops that originated from single domestication events
480 like maize (Hake & Ross-Ibarra, 2015). In contrast, the history of cultivated amaranth may in-
481 clude multiregional, multiple and incomplete domestication events with frequent and ongoing
482 gene flow from sympatric wild relatives, which is more similar to the history of species like rice,
483 apple or barley (Londo *et al*, 2006; Cornille *et al*, 2012; Poets *et al*, 2015). The classical model
484 of a single domestication in a well-defined center of domestication may not sufficiently reflect
485 the history of numerous ancient crops. Our study further highlights the importance of a compre-
486 hensive sampling to study the domestication of amaranth. All three domesticated amaranths,
487 *A. caudatus*, *A. cruentus* and *A. hypochondriacus*, as well as all wild relatives throughout the
488 whole distribution range should be included in further studies to fully understand and model the
489 domestication history of Central and South American amaranth.

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Incomplete Amaranth domestication

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617 **Data Accessibility**

618 The original genomic data will be available on the European Nucleic Archive (ENA). Scripts and
619 phenotypic raw data are available under Dryad (<http://datadryad.org/>).

620 **Author Contributions**

621 M.G.S. and K.J.S. designed research; M.G.S. and K.J.S. performed research; T.M. contributed
622 analytic tools; M.G.S. analyzed data; and M.G.S. and K.J.S. wrote the paper.

623 **Conflict of interest**

624 The authors declare no conflict of interest.