Genetic Population Structure of Cacao Plantings within a Young Production Area in Nicaragua

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Abstract

Significant cocoa production in the municipality of Waslala, Nicaragua, began in 1961. Since the 1980s, its economic importance to rural smallholders increased, and the region now contributes more than 50% of national cocoa bean production. This research aimed to assist local farmers to develop production of high-value cocoa based on optimal use of cacao biodiversity. Using microsatellite markers, the allelic composition and genetic structure of cacao was assessed from 44 representative plantings and two unmanaged trees. The population at Waslala consists of only three putative founder genotype spectra (lineages). Two (B and R) were introduced during the past 50 years and occur in >95% of all trees sampled, indicating high rates of outcrossing. Based on intermediate allelic diversity, there was large farm-to-farm multilocus genotypic variation. GIS analysis revealed unequal distribution of the genotype spectra, with R being frequent within a 2 km corridor along roads, and B at more remote sites with lower precipitation. The third lineage, Y, was detected in the two forest trees. For explaining the spatial stratification of the genotype spectra, both human intervention and a combination of management and selection driven by environmental conditions, appear responsible. Genotypes of individual trees were highly diverse across plantings, thus enabling selection for farm-specific qualities. On-farm populations can currently be most clearly recognized by the degree of the contribution of the three genotype spectra. Of two possible strategies for future development of cacao in Waslala, i.e. introducing more unrelated germplasm, or working with existing on-site diversity, the latter seems most appropriate. Superior genotypes could be selected by their specific composite genotype spectra as soon as associations with desired quality traits are established, and clonally multiplied. The two Y trees from the forest share a single multilocus genotype, possibly representing the Mayan, ‘ancient Criollo’ cacao.

Introduction

In Central America, the cacao tree (Theobroma cacao L.), a plant of the humid neotropics, was already being cultivated by the Olmecs and early Mayas, 3000 years ago. Recent investigations on the origin of the ancient Central American cacao, traditionally referred to by its morphogeographic name ‘Criollo’, suggest that it may have been introduced from an area now in Venezuela, adjacent to the center of highest diversity of Theobroma cacao L. in upper Amazonia [1]. However, Criollo cacao represents only a small part of the allelic bandwidth of cultivated and natural cacao populations occurring in Amazonian forests where the species originated. Today’s descendants of the Mayan ancient Criollo cacao can therefore be considered as the products of multigenerational selection by Amerindian farmers [2,3]. Hybrids of Criollo and some Forastero accessions, known as ‘Trinitario or modern Criollo’ [4], and as ‘Trinidad Selected Hybrids’ (TSH), are renowned for their distinct aroma making them a preferred raw material for fine cocoa chocolate [5]. Therefore, remaining sources of ancient Criollo that can still be found in Central America, including Nicaragua, contain potentially valuable germplasm for future breeding of high quality cacao.

Types of cacao are distinguished by several partly overlapping naming schemes. There is the traditional recognition of morphogeographical groups or cultivars (Criollo from Central America, Forastero from Amazonian South America, Amelonado, a Forastero with distinct fruit shape, Trinitario from Trinidad and Tobago, and Refractario from Ecuador originally selected for its resistance to witches’ broom disease, Crinipellis perniciosa (see also http://sta.uwi.edu/cru/ictg/types.asp)). Traditional traders’ ‘varieties’ are recognized by the trade quality (e.g., Trinitario, Criollo, Amelonado, Catongo, Nacional) [4], and cocoa and chocolate are frequently graded and marketed under the name of the country (or region) of production, e.g. Amazonia, Belize, Ecuador, Ivory Coast, or Venezuela. Although modern plantations are often composed of clones (grafted trees or rooted cuttings), propagation by seed has been the simple traditional method for the multiplication of cacao trees. Cacao possesses poorly characterized sexual self-incompatibility, but many trees under cultivation are sufficiently self-fertile [6] to allow for secure yields, and to give rise to inbreeding. The use of clonally propagated, bred and selected cultivars, as are widely used with many horticultural fruit crops in temperate zones, is only just beginning.

The gourmet chocolate sector makes up 4% of the total world chocolate market (S. Vervliet, Puratos/Belcolade, 2007, pers.)
comm.) but is growing quickly. ‘Fine-flavor cocoa’ fetches a considerable price premium, up to four times of the price of standard commodity cocoa. The manufacture of gourmet chocolate depends to a large extent on intrinsic cocoa qualities which are determined by genotype, and on-farm processing including the selection of pods, fermentation, and drying of beans. This offers good opportunities for quality differentiation and value addition that would benefit the growers (S. Pechers, CATIE, 2004, pers. comm.). However, cacao is predominantly produced by smallholder farmers whose level of training and organization in the production chain is often insufficient to maximize the benefits from the production of high quality cocoa.

In Nicaragua, one of the largest cocoa production areas is found in the municipalities of Waslala and Rancho Grande, towns in the central northern part of the country. In pre-Colombian times, this area was under cultural and linguistic influences from the Mayas, and from the Aztecs further north [7]. Waslala is equidistant between the Pacific and Atlantic oceans, at an elevation of 200–740 m in a south-east facing depression adjacent to the Peñas Blancas massif. The average annual temperature ranges between 21.3 and 24.9 °C, and mean annual rainfall is between 2170 and 2660 mm [Worldclim database, www.worldclim.org [8]]. The beginnings of commercial cacao cultivation date back to 1961 (E. Ríos, first president of the cocoa producers cooperative Cacaoalina, 2007, pers. comm.). During and after the civil war in the 1980s, refugees and migrants from all over Nicaragua arrived, and cacao production has greatly expanded since 1991 with the establishment of the non-governmental organization Pro Mundo Humano, and the foundation of Cacaoalina. Cocoa has since become a popular cash crop. The area planted with cacao is now some 1700 ha, having increased during the past five years due to the attractive prices. Typically a household cultivates 0.7–1 ha of cacao, containing 300–600 trees. Plot sizes rarely exceed 2 ha because cacao cultivation is labor intensive, in particular pruning, manual removal of diseased fruits, and continual harvesting and processing. Plantings are distributed on steep slopes that are not useful for cattle pasture. Individual farms rarely yield more than 0.5 t/ha of dried cocoa beans per year, but together, the municipality’s total annual crop contributes considerably to the national cocoa production of 2650 t (in 2009). Farmers can obtain higher prices for high quality cocoa grades, especially if organically certified. There is also potential for adding value from quality differentiation based on characteristics imparted through locality-dependent (environmental), management, and genetic factors. Several commercial cocoa and chocolate companies source their raw material in Waslala, including Ritter (Germany), Cocoa S.A. (Costa Rica), Daarnhouver (the Netherlands), and Zotter (Austria).

It is believed that only a limited number of introductions contributed to the present-day germplasm in Waslala cocoa plantations, although few records are available. The Tropical Agricultural Research and Education Center (CATIE, Turrialba, Costa Rica) distributed seed (beans) in the 1980s to Central American countries including Nicaragua (W. Phillips, CATIE, 2007, pers. comm.), some of which arrived at Waslala. In addition, several farmers interviewed during this research claimed to have occasionally brought in seed and scions from other regions, and others reported finding rare pre-existing cacao trees when they arrived at their new farmland in the 1970s and 1980s. Cacao has been predominantly introduced to Waslala as seedlings, and to a lesser extent through grafted clones. The farmers themselves propagate cacao mostly through the use of seedlings.

This paper explores the genetic composition and structure of cacao populations, as a prerequisite for varietal certification and denomination of Waslala cocoa. It also assesses optimal means to improve cocoa yield and quality in this area for the benefit of the farmers and cocoa producers. For the cacao research community, it is of interest to understand how cacao populations are shaped by germplasm introductions and management. We have representatively sampled the municipality and surveyed the genotype of trees by a number of well-defined simple sequence repeat (SSR) markers. It addressed questions related to the extent of allelic diversity and the possibility of discerning the genetic structure of population, with the objective of identifying specific genetic backgrounds which can be related to geographic areas, farmers’ degree of access to germplasm, and specific environmental conditions.

Results

Descriptive statistics and genetic diversity

The 15 microsatellite primer pairs detected 116 individual alleles (with 7.73 alleles per locus on average) across all samples collected in Waslala municipality. There were no null-alleles apparent. When only one allele was detected, the individual was considered homozygous at this locus. Two trees had three individual alleles at only two SSR loci for unknown reasons. For the analyses of population genetics, the rarer alleles, relative to the entire data set, were considered in these exceptional cases. Six groups of trees sharing an identical multilocus genotype were found, and two of these genotypes were frequent (Table 1, group E with 10 members, and group D with 7 members).

Considering individual farms as separate, independent entities with individual compositions of genotypes, the average number of effective alleles present within all trees sampled at a single farm was 3.38 (range 1.0–5.4). Private alleles [9] occurred within only ten trees from seven farms (Table 2), including 8 of the 15 SSR loci investigated. The degree of expected heterozygosity (HE) averaged over all 45 sites and 15 SSR loci was 0.476 (range 0–0.638). This is equivalent to an average of almost 50% (47.6%) of all loci being heterozygous. The rate of fixed loci was less than 30%, indicating a moderate degree of inbreeding at the current state.

Estimation of the genetic diversity among farms

The degree of genetic diversity was calculated as the percentage of significant differences in all pairwise comparisons among farms, for every SSR locus in separate. Of a total of 14,064 comparisons by the G test using Shannon’s mutual information index (Hₑ(A)) as implemented in GenALEX [10], 39.4% were significant. This can be interpreted as showing considerable among-farm differences in frequency and composition of alleles at the 15 loci under study.

The existence of large among-farm differences is further supported by the large differences in the frequencies of individual alleles by farm (e.g. Figure 1).

Tracing the population structure across all cacao plantings in Waslala

Several simulations were performed in the program Structure [11] on all individuals and markers with and without consideration of the individual farms, as a factor contributing to the distribution of ‘farm subpopulations’ (LOCPRIORS option on or off, respectively). Simulations for up to K = 20 clusters were made. Each cluster was considered to represent one distinct group of ancestral genetic backgrounds that are referred to in this paper as a ‘genotype spectrum’ or ‘lineage’ (known as ‘formenkreis’ in German). In contrast, a genotype as represented by a single individual can be made up entirely of just one genotype spectrum, or from parts of several such genotype spectra.
Table 1. Trees with matching multilocus genotypes across 15 SSR markers.

| Sample | Farm/Location | No. Matches | Label |
|--------|--------------|-------------|-------|
| W042   | FBBBSB       | 2           | A     |
| W041   | FBBBSB       |             | A     |
| W356   | FJM          | 3           | B     |
| W161   | F178         |             | B     |
| W366   | F178         |             | B     |
| W327   | F165         | 2           | C     |
| W207   | F003         |             | C     |
| W305   | F084         | 7           | D     |
| W153   | F018         |             | D     |
| W102   | F022         |             | D     |
| W105   | F022         |             | D     |
| W106   | F022         |             | D     |
| W108   | F022         |             | D     |
| W141   | F027         |             | D     |
| W049   | FBBlandon    | 10          | E     |
| W132   | F006         |             | E     |
| W309   | F083         |             | E     |
| W201   | F174         |             | E     |
| W290   | F195         |             | E     |
| W299   | F225         |             | E     |
| W330   | F227         |             | E     |
| W043   | FBBlandon    |             | E     |
| W044   | FBBlandon    |             | E     |
| W046   | FBBlandon    |             | E     |
| W359   | F166         | 2           | F     |
| W325   | F166         |             | F     |

(For codes see Table S1). doi:10.1371/journal.pone.0016056.t001

The most probable number of populations (genotype spectrum clusters) was 3, as determined by a graphical method [12] as well as by the method applying Bayes’ rule [13]. The partitioning of individuals across the three clusters was stable both with and without taking into consideration the location (farm). These three groups of genotype spectra, were denominated Blue, Red, and Yellow (B, R, and Y), for further investigation. Individuals within any of the three genotype groups contained different degrees of admixture from one or both of the other lineages (Figure 2). The Y group consisted of only three trees, namely the two FBBSB orphan trees from the forest (W041 and W042), and tree W357 from farm F204 (for identities, see supplementary Table S1). Tree W357 included a 14% admixture with components from the B lineage, and 23% from the R lineage. Another 12 trees, labelled as the BY admixture group, consisted of 30–50% Y, 30–50% B, and up to 20% R shared genotype spectra. There was also a BR admixture group of inferred genotype spectra (27–66% B, 33–65% R, 0–34% Y) consisting of 81 trees. A further 107 trees possessed a majority of B lineage components (39–99% B, 0–33% R, 0–32% Y), and 114 samples were mainly R (0–33% B, 42–99% R, 0–32% Y). Subsets of samples corresponding to the B or R clusters defined in this way were subjected to clustering simulations in Structure, but all attempts to detect sub-clusters within the B or the R genotype spectrum failed, and no further separation by the genotype spectrum was applicable within this set of data.

The average genetic distance between the genotype spectra B, R, and Y was estimated by Nei’s Genetic Distance and Genetic Identity and Wright’s Fst as implemented in Genalex. Groups BR and BY with large admixtures were excluded. The results are presented in Figure 3. The closest related groups were B and R, with a Genetic Distance of 0.303, corresponding to a Genetic Identity of 73.8% and an Fst of 0.121. The Y group was most distant (Genetic Distance; 1.743 to group B and 1.141 to R), although this result must be taken with caution due to the small sample size of Y. The indices of relatedness were also calculated on reduced sets of samples restricting the portion of admixture genotypes. Allowing a minimum of 75, 85, or 95% presence of the B, Y, or R genotype spectrum (by removing samples with more than 25, 15, or 5% admixture, correspondingly), Genetic Distance increased and Genetic Identity shrunk as expected (Figure 3). This indicates that the clustering in the Structure program was successful in the detection of distinct genotype spectra.

Analysis of molecular variance (AMOVA as implemented in GenAlEx) on the B and R genotype clusters (assuming they represent founder genotype spectra) revealed 65% variation within and 35% among these clusters, and a ΦPT value of 0.354 (P<0.001). A relatively small among-cluster variation was expected due to the fact that both lineages share the same alleles and possess large Genetic Identity values.

Distribution of the three prospected founder genotype spectra at farm level

As a measure of relatedness between different farms by genotype spectra composition, the average genetic distance over
all 15 marker loci quantified by Shannon’s index was applied. The results are summarized via principal coordinates analysis in Figure 4. Except FBBSB, the two orphan trees W041 and W042 near the forest, most farms were not well separated from each other by this method. This reflects the genetic composition of farms; with every farm having trees possessing genotypes of various states of admixture, considering the lineages as detected by the Structure program. That is illustrated by the pie diagrams on the map of Waslala municipality (Figure 5), each pie plot representing the proportion of the three genotype lineages contributing to an individual farm. The majority of farms are represented by tree genotypes made up of two (B and R) or three (B, R, and Y) lineages. Only a few farms consist of nearly exclusively B genotype spectrum partitions, and only the closely spaced south-eastern plantings F083 and F084, both owned by the same single farmer, contain nearly pure B lineage trees (Figure 5).

Association among geographic characteristics of the sample sites, genotype spectra and geographic features

Pairs of the 5 continuous variables; distance to the road, altitude (m above sea level), elevation relative to nearest road (calculated as the difference between the altitude of the sample tree and that of the nearest point of the road), mean annual precipitation, and mean annual temperature, were subjected to correlation and regression analyses in a descriptive approach (Table 4). There was a small but highly significant correlation between the trees’ distance to the road, and the elevation relative to the road, altitude, and temperature. A stronger correlation ($R = 0.721$) existed for distance to the road and mean annual precipitation.
The elevation relative to the nearest road and absolute altitude were highly positively correlated (R = 0.799), meaning that trees at higher locations frequently grow on steep hills high above the neighboring roads. Consequently, the negative correlation of elevation relative to the road, and temperature, reflects the expected negative correlation between altitude and mean annual temperature (R = 0.946). This data (Table 4) also suggests that in this location, although mean annual precipitation tends to increase with increasing elevation as expected, some areas at low elevation receive much precipitation which may produce a cooling effect.

The discrete genotype spectra were used as a factor to compare geographical and climatic characteristics that they may be preferentially associated with (Table 5), in an exploratory approach. To avoid sampling bias due to grossly differing sample sizes, the under-represented groups Y (3 individuals) and BY (12 individuals) were excluded from these analyses. There were well-supported associations of individual genotype groups with the geographic distance to the nearest road, and mean annual precipitation (Table 5). The B genotype spectrum occurred more frequently at locations far from main roads (average 4.5 km) and the R and BR groups were frequently found nearer to roads (average 2.0–2.5 km). The group B was found in areas receiving the highest mean annual precipitation (2452 mm), whereas R and BR were not distinguishable in areas of 2409 mm mean annual rainfall. The elevation of R genotype spectrum trees above the nearest road was marginally but significantly above average. It is worth noting that replication, i.e., the individual trees at their given locations, also made a significant contribution to the total variance.

In summary, genotype spectrum B occurred more frequently further from the road than the R genotype spectrum. Genotype spectrum B is more frequent at lower elevations with higher mean annual rainfall, whereas R occurs preferentially at higher elevations with lower mean annual rainfall, but R is more frequent than B higher above the closest road. This could be interpreted in the way that the R lineage is found preferentially in the mountainous part of Waslala municipality, where it is planted on slopes that steeply descend from the roads. The map (Figure 5) supports this notion. This also means that in the higher elevations (the mountainous south-west), the farms are located higher above the roads than in the lowlands. These higher altitudes with slightly increased mean annual rainfall experience lower temperatures, as is suggested by the strong, negative correlation (Table 4).

The two orphan trees, FBBSB, representing the pure Y genotype lineage, are located at an average altitude of 373 m at a relatively dry area (mean annual precipitation; 2333 mm; within the lower one sixth of the range for all trees sampled), where it is relatively warm (mean annual temperature 23.8°C; compared to the maximum temperature for all sampled farms being 24.9°C). Similarly, the 12 trees representing the BY group all grew in low, relatively dry and warm places (average for this group; 266 m elevation, 2396 mm mean annual rainfall, 24.5°C mean annual temperature).

Examination of the spatial distribution revealed that several single SSR alleles occur most frequently or exclusively in locations close to the main road (Figure 6). A total of 17 alleles are unique to a buffer zone of 2 km either side of the roads. As an example, allele mTCcG1292 occurs 18 times exclusively in these farms. In contrast, only four alleles were found uniquely in the area 2–15 km away from the nearest road. The number of effective alleles is also higher close to the road (3.2 within the 2 km corridor, relative to 2.36 further away; with the degree of expected heterozygosity, He, being 0.632 vs. 0.548, respectively). These increased levels of allelic diversity nearer to the roads suggest possibly more intense introduction of genetic materials along access roads. There is, however, a possible bias in sampling frequency (206 trees near, and 104 far from, the roads) that could interfere with part of these differences.

**Genetic lineage and fruit type**

Assignment to one of three morphological fruit types, Acriollado, Común, and Híbrido, was achieved for 250 trees.

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**Figure 3. Nei’s Genetic Distance and Genetic Identity, and Wright’s Fst.**

Genetic distances between B (Blue), R (Red), and Y (Yellow) genotype spectra comprising groups of cacao trees whose admixed genotypes have certain minimum degrees of purity of the corresponding genotype spectrum (complementary to the maximum degrees of admixture with other genotype spectra, as shown in Figure 2). The increase or reduction of the parameter values throughout different degrees of purity in alignment support the clustering results shown in Figure 2.

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The elevation relative to the nearest road and absolute altitude were highly positively correlated (R = 0.799), meaning that trees at higher locations frequently grow on steep hills high above the neighboring roads. Consequently, the negative correlation of elevation relative to the road, and temperature, reflects the expected negative correlation between altitude and mean annual temperature (R = 0.946). This data (Table 4) also suggests that in
Although all types were presented in all different locations, their ratios were not equal across the genetic lineages. The Común type was confined to the B lineage, except for a single individual in the R group (Table 6). For the two large groups B and R, whose members possess at least a two thirds share of the Blue and Red genotype spectra, respectively, the ratios of the frequent Acriollado and Híbrido trees were checked with the Chi-square test for goodness-of-fit. The B group had 34 trees assigned to the Acriollado type, and the R group had 8 of these. In total, in the Acriollado and Híbrido types, 85 B and 80 R individuals were recorded, therefore, 42.5 (85/2) B and 40 (80/2) R trees were expected to be encountered with the assumption of unbiased distribution of genetic lineages across the two fruit types (Table 6). Testing the observed frequencies of 34:8, B:R individuals to fit the expected ratio of 42.5:40, revealed an unequal distribution or departure from homogeneity (Chi square = 14.28, P < 0.001; **). This allows the conclusion that the Acriollado morphotype is highly significantly underrepresented in the R lineage, and overrepresented in the B lineage. This was the most pronounced biased distribution found; with 81% of the Acriollado type within all B and R samples being present among the B lineage trees. Likewise, the Híbrido fruit type, albeit outnumbering the other varieties, was cumulated at the 5% level of marginal significance to the R genotype spectrum. Testing the observed frequency of 34:51 Acriollado:Híbrido individuals within the B genotype spectrum, and 8:72 within R, to fit the expected ratio of 21:61.5, revealed a similar bias at P < 0.01 (**) in both comparisons.

Discussion

The genetic structure of smallholder cacao plantings in Waslala was investigated. This is an economically significant Nicaraguan...
area of production, where this crop has been grown since 1961. The majority of these cacao plantings appear to possess a large diversity of tree genotypes that seems to originate from a limited number of genotype spectra. Notwithstanding, the differences in allele and genotype composition at the farm level are important.

Markers used and allelic diversity

The 15 microsatellite loci sampled in this study are dispersed across nine of the ten linkage groups (chromosomes) of *Theobroma cacao*. These loci were selected as robust, informative markers for cacao and have been characterized in detail [14]. The 15 markers have been widely used to assess the genetic diversity and redundancy among new cacao collections and within clonal collections held at genebanks [1,3,5,15]. Therefore, these markers were considered appropriate to assess the cacao genepool present at the municipalities of Waslala/Rancho Grande, Nicaragua. The markers are anonymous and unlikely to target specific expressed genes, therefore they can be considered as neutral, i.e. not under selection and thus are unbiased markers for this investigation of population structure.

To assess the allelic diversity in Waslala, the total number of alleles, and private alleles, can be used. The 116 individual alleles found within the samples are almost exactly one-half of the number of 231 alleles observed for the same loci among 548 accessions with distinct genotypes that were sampled by Zhang et al. [15] at the live cacao genebank in CATIE, Costa Rica. This means that the allelic richness in Waslala of 7.73 alleles per microsatellite locus, is approximately 50% of the richness within the CATIE collections that have 15.4 alleles per locus. The collection of the USDA-ARS Tropical Agricultural Research Station at Mayaguez, Puerto Rico, holds at the same SSR loci in total 132 alleles with 8.8 alleles per locus [16], being comparable to Waslala, although actual differences in the individual alleles are likely to exist. The level of allelic richness in Waslala is also comparable to that of a collection of semi-natural cacao from the upper Amazon, held at Universidad Nacional Agraria de la Selva, Tingo Maria, Peru [17], with allelic richness levels comparable to that of the USDA-ARS Mayaguez collection [18]. A subgroup of Ecuadorian cacao collections recognized as being the genetically narrow ‘Refractario’, had in total 63 alleles and 4.2 alleles per locus [19]. Again, the identities of the alleles may be different although the same microsatellite loci were investigated.

Cacao population structure across plantings in Waslala

Of the 15 private alleles detected by the rarefaction method (Table 2), 8 are dispersed among only 7 trees from three farms. This supports the notion of the wide dispersal of a comparatively small set of common alleles across Waslala, although there is much diversity at the genotype level (a specific combination of alleles at all loci). Evidence for this arises from the occurrence of only a few highly similar SSR genotypes. There are only 7 groups of trees with matching genotypes (Table 1), pointing towards sufficient genetic recombination, probably achieved through planned crosses. The small number of matching genotypes also indicates that during the sampling, trees of clonal origin were successfully omitted. The main method of tree propagation in Waslala is by seed, although in recent years, grafting of scions onto established rootstocks of trees that are cut due to low productivity, has become an alternative method.

The experimental station and germplasm distribution unit in Nicaragua, El Recreo, receives cacao germplasm from CATIE, and apparently, seed from crosses at El Recreo were distributed to Nicaragua’s production zones including Waslala. During 1991–96, considerable dispersal of seed from controlled Trinitario×Forastero crosses and from clonal propagation of superior Trinitario genebank accessions was recorded in Waslala (S. Thienhaus, FADCANIC, Centro Agroforestal Sostenible, Wawsang, Nicaragua, 2010, pers. comm.), and the Cacaonica cooperative was involved in the distribution of this germplasm to farms sampled in this study. Nonetheless, the data on alleles and genotypes shows that the material used may have been selected from certain parts of the genotype spectra available in cacao.

The considerable differences were observed in the frequency and on-farm composition of genotypes across farms, as witnessed
by the spatial distribution of genotypes with widely differing degrees of lineage admixture (Figure 5). This may reflect seed trade activities of the past. Nonetheless, neither differentiation-based diversity [principal coordinates analysis on mean Shannon values, Figure 4], nor probabilistic inference of population structure [20] revealed any indication of more than three distinct genotype spectra within all samples from Waslala. Likely causes for this include the preference by farmers for only a few sources of genetic material for unknown reasons, newly introduced trees of <20 years of age may not yet be among the high-yielding trees and were thus not sampled, or the parents used for the crosses were closely related. The inference of population structure applied here can only give information on the number of genotype spectra that are discernible in the existing data set. However, it cannot assess the absolute magnitude of diversity any of these single genotype spectra consists of. Likewise, at this stage it is problematic to trace any individual donors of the B and R genotype spectra due to the large number of choices that are available at the genebanks (e.g. SSR fingerprints of clonal accessions offered by the International Cocoa Germplasm Database; www.icgd.reading.ac.uk/index.php). This can be achieved by integrating the current data on the Nicaraguan populations with information on particular parental material that may have contributed to this genepool.

Figure 5. Map of Waslala municipality in central northern Nicaragua. Pie diagrams represent individual smallholder farms and the shares of putative founder genotype spectra, B, R, and Y, totalled over all cacao trees sampled.
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### Origin of the Y genotype

Of the inferred three founder genotype spectra, two, B and R, were frequent and widespread, whereas only the two cacao trees from the forest, W041 and W042 represented the pure, non-admixed Y lineage. Several instances point toward the assumption that the Y trees may indeed represent the ancient Criollo lineage. The two forest trees were growing in a wild state, and appeared significantly older than all the managed plantation trees. Farmers do not harvest fruit from such forest trees because of their low yield and small, unpigmented seed. Criollo is known to possess extremely small allelic diversity, small unpigmented seed, and exhibit low yields. The majority of Criollo trees were killed by an unknown incident in 1727 [21], and only a few plants apparently escaped by chance, with rare trees to be found at sheltered sites near ancient settlement places in this Central American region [2,3]. However, confirmation of the two orphan trees being Criollo will require additional comparative studies.

### Potential identity of the B and R genotypes and their spatial distribution patterns

The B and R lineages are present predominantly in admixed states (Figure 2), and residues of the Y lineage were detected by the probabilistic clustering method within a minority of the BR hybrids. Y-admixture could mean hybridization with Y representatives in the past, but it could also mean that intercrosses among introduced BY hybrids could have split the putative Y lineage into different lineages. Several instances point toward the assumption that the Y trees may indeed represent the ancient Criollo lineage. The two forest trees were growing in a wild state, and appeared significantly older than all the managed plantation trees. Farmers do not harvest fruit from such forest trees because of their low yield and small, unpigmented seed. Criollo is known to possess extremely small allelic diversity, small unpigmented seed, and exhibit low yields. The majority of Criollo trees were killed by an unknown incident in 1727 [21], and only a few plants apparently escaped by chance, with rare trees to be found at sheltered sites near ancient settlement places in this Central American region [2,3]. However, confirmation of the two orphan trees being Criollo will require additional comparative studies.

### Table 4. Pairwise comparisons of climatic and geographic data for the locations of 295 sampled cacao trees representing the Blue, Red, and Blue-Red lineage clusters.

| Factor                        | Elevation relative to road | Annual precipitation | Altitude | Average annual temperature |
|-------------------------------|----------------------------|----------------------|----------|---------------------------|
|                               | −28.6***                   | 72.1***              | −25.6*** | 20.2***                   |
| Elevation relative to road    | −13.5*                     | 79.9***              | −78.7*** |
| Annual precipitation          |                            | 12.1*                | −15.7**  |
| Altitude                      |                            |                      | −94.6*** |

Coefficients of correlation (in percent) and levels of significance (as determined by F tests in regression analyses) are shown.

*; P<0.05,
**; P<0.01,
***; P<0.001.

### Table 5. Summary results of general linear models for analysis of variance of climatic and geographic factors for three abundant, inferred cacao genotype spectra in Waslala municipality.

| Factor                        | Genotype spectrum P (F test) | Replication P (F test) | Multiple means comparison | Corresponding mean values |
|-------------------------------|------------------------------|------------------------|---------------------------|--------------------------|
| Distance to road              | ***                          | **                     | B BR R                    | 4492 2534 1905 m          |
| Elevation relative to road    | *                            |                        | R BR R                    | 56 51 34 m               |
| Annual precipitation          | **                          | *                      | B BR R                    | 2452 2411 2407 mm         |
| Altitude                      |                             |                         | B BR R                    | 428 438 458 m             |
| Average temperature           |                             |                         | B BR R                    | 23.5 23.4 23.4°C          |

Levels of significance of dependent variables Genotype spectrum and Replication (representing individual trees within a genotype, used for calculation of the error term). Multiple means comparisons were made with the Waller-Duncan function in SAS-GLM; items connected by an underscore are not significantly different.

Number of samples included by genotype cluster; 107 B, 111 R, 77 BR.

*; P<0.05,
**; P<0.01,
***; P<0.001; –; (not significant).

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diseases, or when more promising planting material become available. Under these circumstances, and because the majority of locally available material belong to only two basic genotype spectra, it cannot be excluded at present that microclimatic variations, in particular precipitation, may be a factor that partially determines the spatial distribution of these genotypes, alongside management practices. Again, clarity can only be obtained through additional experiments.

Remarkably, the Y lineage putatively representing the ancient Criollo type has a narrow distribution in an area that experiences relatively low annual precipitation and relatively higher mean annual temperatures. This may point to the preferred environmental conditions that facilitate the survival of this lineage under unmanaged conditions, and may help to elucidate the nature of the unknown incident that wiped out the Criollo crop in 1727 [21]. However, these findings must be treated with caution due to the small number of Y individuals.

Distribution of fruit types

Despite the great variability of morphological characteristics, the distribution of types identified by fruit shape, seed color and size (Acriollado and Común varieties) and in addition, to a limited extent the technology of production (for the Híbrido type), was unequal across the three inferred genotypes B, Y, and R. The B
Table 6. Frequencies of morphological fruit types relative to inferred genotype spectrum group.

| All trees identified                                      | B²   | BY | BR | R   | Total |
|----------------------------------------------------------|------|----|----|-----|-------|
| Acriollado                                               | 34   | 2  | 22 | 8   | 66    |
| Común                                                   | 9    | 0  | 0  | 1   | 10    |
| Hibrido                                                  | 51   | 8  | 43 | 72  | 174   |
| Total                                                    | 94   | 10 | 65 | 81  | 250   |

| B and R groups only² | B | R | Total | observed | expected | Chi squ | P    |
|----------------------|---|---|-------|----------|----------|---------|------|
| Acriollado           | 34| 8 | 42    | 34:8     | 42.5:40  | 14.28   | ***  |
| Hibrido              | 51| 72| 123   | 51:72    | 42.5:40  | 5.27    | *    |

| p                     |   | **|      |         |         |        |      |
|-----------------------|---|----|------|---------|---------|--------|------|
| observed              | 34:51 | 8:72 |       |         |         |        |      |
| expected              | 21:61.5 | 21:61.5 |   |         |         |        |      |
| Chi square            | 9.47 | 10.06 |     |         |         |        |      |

¹For codes of genotype spectrum (or founder genetic lineage) clusters, compare legend of Table 3. BY, cluster containing individuals with admixed Blue-Yellow, BR; Blue-Red, genotypes at equal proportions.

²Bottom section; fitness-to-homogeneity tests of frequencies across genetic lineage and fruit types showing observed and expected numbers of individuals, Chi square value, and corresponding probability level, P (*; P<0.05, **; P<0.01, ***; P<0.001).

Materials and Methods

Forty four cacao plantings in smallholder farms were selected to represent 14 climatic zones within the municipality of Waslala, Nicaragua. Two naturally occurring orphan cacao trees remaining from recently cleared forest were also included. This group is referred to as derived from “farm FBBSB”.

A total of 313 trees identified were selected as consistently high yielding by their owners, and two low-yielding FBBSB trees, on average 7 trees per location (range 2–20). Eight locations were represented by less than 5 trees. High yield was defined as the stable production of many fruits year-round. This ‘high yield’ of individual trees as observed by the farmer may depend on the degree of stylar self-compatibility, distance from neighboring cacao and shade trees, and degree of fertilization, rather than on the genotype, and the principle of random sampling was therefore adhered to. Care was taken to sample non-grafted seedlings. New, fully expanded adult leaves were dried on silicagel in sealed plastic bags and shipped and stored at room temperature until use. Total genomic DNA was extracted from dry leaf tissue with the Dneasy Plant Mini Kit (Qiagen) according to the manufacturer’s protocol.

Three types, mainly defined by morphological characteristics of the fruit (pod) and seed (beans) were identified. Acriollado has white beans, and pulp color and fruit shape with some resemblance to the original Criollo type. Común was used to describe trees producing fruits of one Forastero morphotype, namely Amelonado, possessing spherical pods similar in shape to honey melons (Cucumis melo). Finally, Hibrido was used to describe plants producing pods of intermediate shape and characteristics, as they occur frequently after hybridizing crosses of Forastero and Criollo. These pods often are elongated with pronounced acuminate tips and reduced seed size. The Hibrido classification was also applied to trees reported to be derived from seed programs by the Nicaraguan genebank, El Recreo, or by the Honduran Foundation of Agricultural Research (FHIA), that are creating varietal hybrids through controlled crosses.

Primers for 15 simple sequence repeat (SSR or microsatellite) markers [25] specified in Table 7 were purchased from Sigma. For each marker, one of the primers was labelled with a fluorescent dye (FAM or HEX), and the PCR amplicon was separated on ABI Prism 3100 and ABI Prism 3130xl capillary sequencers to visualize the microsatellite alleles. The data generated in the Sequencher 4
Table 7. Cacao microsatellite (simple sequence repeat; SSR) primers [27] used to fingerprint trees from plantings in Waslala, Nicaragua, 2007–2009.

| SSR code | EMBL No | 5’-Primer | 3’-Primer | Chr | Size (bp) | AT °C |
|----------|---------|-----------|-----------|-----|-----------|------|
| mTcCIR1  | Y16883  | GCCGGGAGGCCTAACATGGAAGCA | TGGGGCAACCCAGAAACGAT | 8   | 128–146   | 59   |
| mTcCIR6  | Y16980  | TTTTCTCTAAACTACCACTTTAAT | TAAAGCAAAAGCACTTACATA | 6   | 225–247   | 48   |
| mTcCIR7  | Y16981  | ATGGAATGACAACTGTGTTTGTGTTT | GCCTTCAGGCCTTTGCTTTTCT | 7   | 147–162   | 53   |
| mTcCIR8  | Y16982  | CTAGTCTCACTCTTCTCTCTCTCTC | TCTCAGGCATTCTCTCTCTC | 9   | 286–305   | 50   |
| mTcCIR11 | Y16985  | TTTGTTGATTTATATGCACTAGC | GATCTTCATTTGAGTGGAG | 2   | 287–337   | 48   |
| mTcCIR12 | Y16986  | TCTGGCTTTTTTTTTTTTTTTTCTTT | ATTCCAGTTAAAGCATC | 4   | 186–220   | 55   |
| mTcCIR15 | Y16988  | CACCGGCTCTTTGCTTATTAG | TATTGGGAGTTCTGATGAT | 1   | 231–257   | 50   |
| mTcCIR18 | Y16991  | GATAGCGTTACGAGGATTGAGA | GATGAGGAGGATGAAATAG | 1   | 272–291   | 53   |
| mTcCIR22 | Y16995  | ATTTCCGAAAAACCTTAGT | GATGGAAGGAGTGAATAG | 1   | 272–291   | 48   |
| mTcCIR24 | Y16996  | TCGTGGGATTTTCTCTCTCTCTCTC | TCTGTCCTGCTTTTGGTTGA | 9   | 185–202   | 51   |
| mTcCIR26 | Y16998  | GCATCATCAGAATACCTGCTCCTC | GCACCTCAAGCTCATACTAC | 8   | 281–306   | 46   |
| mTcCIR33 | AJ271926 | TGGTGGAGAGATTGGTGATTGGT | CAACAGAATGAAAGGGA | 4   | 271–350   | 53   |
| mTcCIR36 | AJ271942 | CTGTCGCTGTGATGATGACTGATTGAA | AATACCCCTCCACAATAAT | 10  | 133–186   | 50   |
| mTcCIR40 | AJ271943 | AATCCGAGAGTCTCTCTCTCTCTC | CTTAAGGGCAGTTATTGA | 2   | 258–294   | 51   |
| mTcCIR60 | AJ271958 | CGCTACTAAACACATCAAAA | AGAGCAACCCAGAAACGAT | 2   | 186–211   | 53   |

1The code of the SSR and corresponding EMBL accession number, PCR primers, number of the cacao chromosome (Chr), fragment size (Size), and PCR annealing temperature (AT) are indicated.

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Planned potential associations among geographic and climate variables and inferred genotypes were tested by correlation and regression analyses in Excel or by general linear models in SAS (SAS Institute Inc., Cary, USA), of the type $Y = B0 + B1X + e$, where $X$ is the discrete genotype, $e$ the error represented by the replication dependent variance, and $Y$ the individual factor of influence, where appropriate. The individual trees within one genotype group were considered as replications for this genotype.

Supporting Information

Table S1 List of the 317 cacao trees. Owner; farmer’s name. Comarca; rural district. Lineage; inferred genotype spectrum. Climate zone; defined by average temperature and precipitation. CIR1–CIR60; SSR fingerprint. The two alleles at each SSR locus are listed in two columns within one row. (XLS)

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This study has been conducted in the framework of a research and development project entitled ‘Sustainable futures for indigenous small-holders in Nicaragua: harnessing the high-value potential of native cacao diversity’. The project aims at assisting farmers in Waslala to make a gradual transition from their current production of only average quality cocoa to the production of differentiated high value cocoa based on the optimized use of cacao biodiversity, choice of locality and appropriate post-harvest procedures.

software (Gene Codes Corp., Ann Arbor, USA) was analyzed with the aid of Genotyper, Peak Scanner 1 (ABI), or Genemapper programs. The individual alleles were labeled by the size in bases of their largest repeat. The PCR was replicated up to five times to eliminate uncertainties. Together with newly shipped samples, previously analyzed control samples were included to provide the correct assignment of allele sizes. For each sampled tree, DNA was isolated once or twice. Trees were sampled during three years, from 2007 to 2009. For several trees, a second leaf was sampled in a different year.

Basic parameters on the samples’ genetic composition and allele frequencies were calculated using the GenAlEx application [10] in Microsoft Excel. Principal coordinates analysis (PCA) and analysis of molecular variance were also performed in GenAlEx. For PCA, the mean Shannon mutual information indices ($s$HuA) for pairwise farm comparisons were calculated as the fraction of Total Information index across each pair of populations, which were comprised of the weighted Allele Information indices of both populations in the pair, for each locus (compare www.anu.edu.au/BoZo/GenAlEx/new_version.php, GenAlEx Tut1, p. 35). The genotypes were further analyzed with Bayesian statistical methods in the program Structure [11] to attempt to trace the number and genetic composition of founder populations or kinships in Waslala cacao plantings. Settings for the simulations in Structure were 100000 permutations during the burnin phase and 50000 to 100000 during simulations under a model allowing for genotype admixture.

Spatial climate data were extracted from Worldclim (www.worldclim.org). This database provides detailed information on climate characteristics at 1 km x 1 km resolutions, and its estimated tolerance of annual precipitation values is 10–25 mm for this part of Central America [8]. Geographic information system (GIS) analyses and maps were made with the DIVA-GIS software (www.diva-gis.org). Administrative and access information was based on maps by MARENA, the Nicaraguan Ministry of Environment and Natural Resources [26].
Author Contributions
Conceived and designed the experiments: BT XS MH. Performed the experiments: KH-H BT AK HG. Analyzed the data: BT XS. Contributed reagents/materials/analysis tools: BT XS AK. Wrote the paper: BT XS.

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