Genetic Characterization of the Cacao Cultivar CCN 51: Its Impact and Significance on Global Cacao Improvement and Production

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ABSTRACT. Cacao (*Theobroma cacao* L.) is an important cash crop in tropical growing regions of the world and particularly for small cacao farmers. Over the past two decades, 'CCN 51' has become one of the most planted cultivars in Ecuador, mainly as a result of its high productivity and disease resistance. Intermixing of Nacional fine flavor Ecuadorian beans with beans of 'CCN 51' has become common practice, reducing overall bean quality and decreasing value. The primary goals of this study were to determine the genetic identity, structure, and allelic richness of 'CCN 51', its maternal origin and to compare 'CCN 51's' agronomic characteristics against a composite group of Nacional cultivars. To investigate the complex genetic background of this cultivar, 70 simple sequence repeat loci were used. The high heterozygosity observed (56 of 70 loci) for 'CCN 51' is not characteristic of traditional Nacional cultivars. Comparison of agronomic characteristics between 'CCN 51' and several Nacional cultivars indicates significant differences in cacao dry bean weight, yield potential, production efficiency, percent healthy pods, and witches' broom [*Moniliophthora perniciosa* (Stahel) Aime & Phillips-Mora] disease incidence. Additionally, physical, chemical, and organoleptic characteristics suggest that 'CCN 51' is different from those of Nacional lineage. Based on population structure analysis, the predominant ancestries for 'CCN 51' are Iquitos (45.4%), Criollo (22.2%), and Amelonado (21.5%) genetic groups. A lesser proportion of its genome was accounted for by genetic groups Contamana (3.9%), Purús (2.5%), Marañon (2.1%), and Nacional (1.1%) admixtures. Results of phylogenetic analyses using the unweighted pair group method with arithmetic mean yielding high bootstrap values strongly support the relatedness of 'CCN 51' with Iquitos, Criollo, and Amelonado genetic groups. Moreover, seven mitochondrial simple sequence repeat loci revealed that 'CCN 51' maternally inherited the 'IMC 67' cytotype. 'CCN 51' constitutes a valuable cacao genetic resource that is currently used not only in its country of origin, but also in many other national breeding and selection programs worldwide.

Cacao is a diploid ($2n = 2x = 20$) perennial tropical tree belonging to the family Sterculiaceae (Malvaceae *sensu lato*), order Malvales (Alverson et al., 1999; Cope, 1984). It is native to the humid neotropics between latitudes 20° S and 20° N and has been grown for consumption and commerce in the Americas since the times of Olmec, Toltec, Maya, and Aztec civilizations (Henderson et al., 2007; Hurst et al., 2002). According to Cuatrecasas (1964), modern *Theobroma cacao* resulted from hybridization of *T. pentagona* Bern. and

T. leiocarpa Bern. High levels of genetic variability are found in the upper Amazonian region, which is considered the center of highest diversity for cacao, along with greater morphological admixture and disease resistance for the species (Cheesman, 1944; Coe and Coe, 1996; Cuatrecasas, 1964; Motamayor et al., 2008).

Cacao beans (seeds) are the source of chocolate, carbohydrates, fats, proteins, natural minerals, flavonoids, and vitamins, which are the raw materials for a competitive multibillion dollar industry. Cacao is cultivated in commercial plantations around the world (Dantas and Guerra, 2010; Rice and Greenberg, 2000; Steinberg, 2002). Cacao is a major crop in west and central Africa and southeast Asia (Bartley, 2005; Hebbar et al., 2011; Hurst et al., 2002; Wood and Lass, 1985). Cacao bean production during 2011–12 was ≈4.4 million tonnes with a cultivated area over 10 million ha worldwide (Food and Agriculture Organization of the United Nations, 2013). The production chain comprises ≈5 to 6 million cacao farmers worldwide and ≈40 to 50 million people depend on cacao cultivation for their livelihood (International Cocoa Organization, 2012). Today the Americas produce nearly 13.0% of global cacao production, and Ecuador is ranked eighth in the world (International Cocoa Organization, 2012).

Cacao has been grown in Ecuador since pre-Columbian times; however, much of its history remains unknown. The history of cacao in South America suggests the idea that South American natives did not cultivate cacao or use it as in Mesoamerica and that Spaniards were responsible for bringing cacao cultivation to this part of the continent (Ogata, 2003). Historical evidence and anecdotal information indicate that the first commercial plantings in Ecuador started the early 1600s (Bartley, 2005). Until the beginning of the 20th century, Nacional genetic group cultivars were the only type cultivated in Ecuador (Soria, 1966), resulting in a limited genetic base. In the 1930s, Ecuadorian cacao production suffered a steep decline attributable mainly to a combination of aging plantations and damage caused by disease, specifically witches' broom disease, frosty pod or moniliasis disease (*Moniliophthora roreri* H.C. Evans, Stalpers, Samson & Benny), and mal de machete or ceratocystis wilt disease (*Ceratocystis cacaofunesta* Engelbrecht et Harrington) (Baker and Holliday, 1957; Phillips-Mora et al., 2005; Pound, 1938, 1945; Soria, 1966). There has been a substantial increase in Ecuadorian production since the 1930s, which is primarily the result of substitution of less productive Nacional types by more productive cultivars.

Ecuador's cacao production has become a major export commodity, particularly for fine-flavored cacao recognized for its unique flavor known as "arriba." Major production has been from cacao of the Nacional genetic group, which is renowned for its fine flavor and aroma. Traditional Nacional cultivars, belonging to a distinct genetic population, produce fine flavor cacao beans with the arriba flavor. Nacional genotypes produce large beans and a sweet-tasting pulp. The fermented beans have low acidity, low bitterness, low astringency, and a fruity, floral taste similar to violet, jasmine, lilac, or even orange blossom (Deheuvels et al., 2004; Eskes et al., 2007). However, the potential for fine flavor cacao might be largely influenced by the fermentation process and chocolate production practices (flavor attributes added), which have the capacity to either enhance or degrade the final taste, aroma, and texture of cacao products.

'CCN 51' [Colección Castro Naranjal (CCN)] is currently one of the most important genetic resources and widespread cultivars in Ecuador. 'CCN 51' was developed by H. Castro in the early 1960s in Ecuador (Castro, 1981) from the following crosses: ('ICS 95' × 'IMC 67') × 'Oriente 1'. Castro reported 'Oriente 1' as an accession that he collected on his first trip to the Canelos Valley in the Ecuadorian Amazon. H. Castro, a graduate in Agronomy from the Inter-American Institute for Cooperation on Agriculture (IICA, Turrialba, Costa Rica), was a cacao expert who did most of his research at his own farm called "Theobroma." The earliest report of 'CCN 51' being used in a plantation was in 1965 on a farm called "Sofia" in the Naranjal area, south of Guayaquil. The farm was owned by C. Baquerizo, who was an enthusiastic cacao farmer and supporter of Castro's cacao research. Initial efforts to establish 'CCN 51' clonal planting for large-scale farming dates back to 1985 (Crespo and Crespo, 1997). 'CCN 51' is a highly productive, disease-resistant, and precocious cultivar that produces large pods and beans after only 2 years of transplanting to the field. The beans have high butter content (54%), one of the highest yields for the cacao butter industry. With appropriate pre-drying before box or "yute" (sack) fermentation, the cultivar produces low acidity and acceptable flavor but does not meet the qualification of fine-flavored beans (Amores et al., 2011). 'CCN 51' accounts for ≈80,000 ha of the total planted area in Ecuador and yields an average of 1.0 t·ha⁻¹ that can be increased to over 3.0 t·ha⁻¹ under intensive management practices (Amores et al., 2011). Although the pod morphology and tree architecture of 'CCN 51' are distinct from traditional Nacional genotypes, 'CCN 51' is sometimes mistaken for being of Nacional lineage. Thus, a better understanding of the genetic constitution of 'CCN 51' is required for effective use of this valuable resource. A sustainable cacao breeding and selection program in Ecuador has been led by the Instituto Nacional de Investigaciones Agropecuarias (INIAP) where an efficient selection process of superior cacao cultivars is in place. The active participation of cacao bean producers enriches the phenotypic and genotypic variability of the cacao genebank held by INIAP at the Estación Experimental Tropical (EET) in Pichilingue, Ecuador (Amores et al., 2011). Within the program, the best hybrid trees are selected and propagated clonally to compare their performance against other high-yielding and disease-resistant cultivars, including 'CCN 51'. The hybrids are compared in multisite trials conducted at both the EET Pichilingue and in farmers' fields (Amores et al., 2011). Now most of the cultivated cacao in Ecuador are Nacional hybrids, derived from hybridization between traditional Nacional and other Trinitario cacao.

Molecular markers such as simple sequence repeat (SSR) and single nucleotide polymorphism are used to study genetic identity and evolutionary relationships in germplasm collections. In cacao, these markers have been used for establishing genotype identities, genetic relationships, identification of genetic gaps, and in marker-assisted selection in both national and international cacao breeding and selection programs (Boza et al., 2013; Irish et al., 2010; Loor et al., 2009; Motamayor et al., 2002, 2003, 2008; Schnell et al., 2005, 2007; Zhang et al., 2006a, 2006b). A study by Motamayor et al. (2008) used SSR markers to improve the understanding of the origin, classification, and population differentiation within *T. cacao*. The study proposed that cacao be classified into 10 genetic clusters as opposed to the two genetic groups traditionally recognized within the species. The major clusters or genetic groups are: Marañon, Curaray, Criollo, Iquitos, Nanay, Contamana,

Amelonado, Purús, Nacional, and Guiana. This new classification more accurately reflects the genetic diversity that is available for breeders.

We report the uniqueness of 'CCN 51', a cultivar that has been widely used in Ecuador and is currently being used as a parent in many cacao breeding and selection programs in other countries. Although 'CCN 51' is prized for its high productivity and disease resistance, it is not favored over other Nacional cultivars by the fine chocolate industry because of its less desirable organoleptic quality. The primary objectives for this study were 1) to determine the genetic identity, structure, and allelic richness of 'CCN 51' based on Motamayor's new classification using SSR markers; 2) to determine the maternal origin of 'CCN 51'cytoplasm using mitochondrial and chloroplast DNA SSR markers; and 3) to compare 'CCN 51's' agronomic characteristics against a composite group of Nacional cultivars for diverse applications in cacao genomics, breeding, and selection programs. The goal of this work is to contribute key information that will be useful to cacao breeders and industry to more effectively use 'CCN 51' in breeding and selection programs. Unraveling the genetic origin of 'CCN 51' will provide an understanding of its genetic composition. In-depth analysis of physical, chemical, and organoleptic characteristics of 'CCN 51' and comparison with those of Nacional genotypes will provide a basis for breeding strategies. An understanding of the genetic constitution of 'CCN 51' will be of great value to breeders and the industry to exploit the favorable attributes of this genotype while removing less desirable characteristics.

Materials and Methods

Plant material, DNA extraction, and SSR loci. 'CCN 51' cacao leaf material was obtained from different sources for genotype comparison. Young, fully expanded cacao leaves were collected from a single tree at EET Pichilingue in the Province of Quevedo, Ecuador; three trees (45 years old) on Hacienda Sofia, Guayaquil, Ecuador; in addition to a single tree located at the Mars Center for Cocoa Science (MCCS) farm in Bahia, Brazil. Because the original parents of 'CCN 51' as described by Castro (1981) are not available, as an alternative, the putative grandparents, 'ICS 95' (a Trinitario type that was developed from crosses between Criollo and Amelonado genetic group genotypes) and 'IMC 67' (Iquitos mixed Calabacillo, an upper Amazon genotype) as well as individuals representing the genotypes of progenitors for the putative grandparent 'ICS 95', 'LAN 21' (Criollo genetic group), and 'Original Amelonado 2' (Amelonado genetic group) were used in this study. Two trees each of cultivars ICS 95 and IMC 67 were also collected at EET Pichilingue. In addition to collection of this plant material for the purpose of obtaining SSR genotype data, the multilocus SSR genotypes of the available putative ancestors of 'CCN 51' ('ICS 95', 'IMC 67', 'LAN 21', 'Original Amelonado 2') were obtained from Motamayor et al. (2008). Additional multilocus SSR genotypes for other cultivars used in this study were provided by Motamayor et al. (2008) as noted subsequently. For all plant material, genomic DNA was extracted from 200 mg of fresh leaf tissue using a Fast-DNA SPIN kit (MP Biomedicals, Irvine, CA) as previously described (Schnell et al., 2005) except DNA was quantified on using a FL×800 Microplate Fluorescence Reader (Biotek Instruments, Winooski, VT). The study used 70 SSR markers previously reported by Lanaud et al. (1999) and Saunders et al. (2004) (Table 1). These SSR markers are highly polymorphic, robust, and repeatable in cacao (Lanaud et al., 1999; Saunders et al., 2004). Polymerase chain reaction (PCR) amplification was carried out in a DNA Engine Tetrad 2 Peltier Thermal Cycler (MJ Research, Watertown, MA) as previously described (Motamayor et al., 2008) with the following changes: PCR amplification reactions contained 4 ng of genomic DNA, 0.05 U·µL^{-1} Taq DNA polymerase (New England Biolabs, Ipswitch, MA), 1× Thermopol buffer (New England Biolabs), 0.5 µM each forward and reverse primers, and 1 mg·mL^{-1} bovine serum albumin.

SSR genotyping. Electrophoretic separation of all PCR products was conducted on an ABI Prism 3730 Genetic Analyzer (Applied Biosytems, Foster City, CA) using Performance Optimized Polymer 7 (POP 7; Applied Biosystems) as previously described by Motamayor et al. (2008). Briefly, after PCR samples were prepared by combining 1.0 µL of PCR product with 20.0 µL of distilled H$_2$O and 0.1 µL of GeneScan ROX 400HD size standard (Applied Biosystems), they were denatured at 95 °C for 5 min and immediately placed on ice. Electrophoresis was carried out using the default run module for fragment analysis with a 36-cm array. Fragment size and allelic designations of the SSR alleles were accomplished using GeneMapper™ software (Version 4.0; Applied Biosystems).

Allele origin. Allele origin was assigned to each of the 140 'CCN 51' alleles amplified across 70 SSR loci (i.e., two alleles per locus for diploid organisms such as cacao) based on observation of matching with alleles of putative grandparents ('ICS 95' and 'IMC 67') first followed by matching with alleles of putative allele donors [Amelonado genetic group ('Original Amelonado 2') or Criollo genetic group ('LAN 21')] at each of the 70 SSR loci (Table 1).

Genetic structure. Genetic structure analysis was conducted using the model-based Bayesian cluster analysis software Structure Version 2.3.3 (Falush et al., 2003; Pritchard et al., 2000, 2010). The software detects genetic structure and assigns individuals to subpopulations based on multilocus genotypes and allele frequencies (Falush et al., 2003; Pritchard et al., 2000). The Structure program estimates the natural logarithm of the probability Ln P(D) of a given genotype being part of a given population (K). An admixture model was used with 200,000 replicates after a burn-in period of 100,000 iterations. 'CCN 51' was analyzed using the prior population information option along with 20 multilocus genotype references from each of the 10 previously described cacao genetic groups (Motamayor et al., 2008). The number of clusters was set to $K = 10$ to assign the respective genetic group of correspondence for 'CCN 51'. Structure generates (K) clusters to which individuals are assigned by highest coefficient of membership. Although individuals may have membership in multiple clusters, the overall sum for the individual across clusters adds up to one.

Genetic relationship. Genetic distance among representatives of the cacao genetic groups (20 reference samples for each of the 10 genetic groups) as well as 'CCN 51', 'IMC 67', and 'ICS 95' were calculated using the Cavalli-Sforza and Edwards genetic distances method of cluster analysis (Cavalli-Sforza and Edwards, 1967). Similarly, genetic distance among cacao genetic group populations, 'CCN 51', 'IMC 67', and 'ICS 95', was calculated. The corresponding genetic distance matrices were used to generate dendrograms

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using the unweighted pair group method with arithmetic mean (UPGMA). Populations Version 1.2.32 (Free Software Foundation, Boston, MA) was used, with 1000 bootstrap replications, to test the reliability and robustness of the dendrograms. The trees were constructed using TreeExplorer Version 2.12 (Tamura et al., 2007).

Table 1. Description of 70 simple sequence repeat (SSR) loci used to investigate the genetic identity of cacao cultivar CCN 51 and results of comparison against ancestral reference genotypes for designation of allele origin to each of the alleles of CCN 51.[z]

Locus name	Linkage group no.	Distance (cM)	CCN 51 alleles (bp) Allele 1	Allele 2	Locus name	Linkage group no.	Distance (cM)	CCN 51 alleles (bp) Allele 1	Allele 2
mTcCIR1	8	1.7	126	138	mTcCIR75	8	4.9	113	119
mTcCIR6	6	—	238	246	mTcCIR77	10	39.0	280	288
mTcCIR8	9	54.6	286	302	mTcCIR80	5	71.1	99	99
mTcCIR9	6	63.1	274	286	mTcCIR81	3	78.4	204	212
mTcCIR10	5	26	203	205	mTcCIR94	1	21.86	190	192
mTcCIR12	4	45.4	187	199	mTcCIR96	9	92.6	133	133
mTcCIR15	1	—	232	232	mTcCIR107	4	27.3	111	117
mTcCIR17	4	31	271	281	mTcCIR109	5	85.4	163	165
mTcCIR18	4	25.8	331	333	mTcCIR116	7	50.0	312	314
mTcCIR19	2	4.4	371	371	mTcCIR119	5	5.2	110	122
mTcCIR21	3	11.5	142	153	mTcCIR124	9	48.6	128	132
mTcCIR24	9	29.2	184	184	mTcCIR126	9	9.9	205	211
mTcCIR25	6	36.3	132	145	mTcCIR127	5	82.5	129	141
mTcCIR26	8	36.3	296	303	mTcCIR135	3	64.7	225	247
mTcCIR32	4	53.5	191	201	mTcCIR138	1	34.8	121	126
mTcCIR35	9	36.1	237	237	mTcCIR141	7	5.0	209	215
mTcCIR37	10	4.0	146	176	mTcCIR146	3	2.18	92	117
mTcCIR49	3	0.0	196	209	mTcCIR148	5	16.0	234	234
mTcCIR54	1	40.3	155	163	mTcCIR149	5	40.0	234	234
mTcCIR64	9	81.3	166	166	mTcCIR155	10	41.8	276	276
mTcCIR73	2	104.4	114	114	mTcCIR167	3	52.8	226	236
mTcCIR168	4	0.0	174	176	mTcCIR235	6	27.6	295	297
mTcCIR181	7	30.9	197	207	mTcCIR238	6	34.8	126	126
mTcCIR184	1	0.0	116	141	mTcCIR255	6	42.6	198	201
mTcCIR186	7	49.2	145	145	mTcCIR264	1	92.1	196	206
mTcCIR189	8	50.5	150	152	mTcCIR265	5	0.0	236	246
mTcCIR190	7	5.3	151	173	mTcCIR266	9	7.0	190	196
mTcCIR193	6	33.8	125	134	mTcCIR277	7	50.3	300	302
mTcCIR194	1	99.1	179	187	mTcCIR289	3	40.3	121	131
mTcCIR204	3	17.6	128	130	SHRSTc3	1	56.9	151	175
mTcCIR222	4	5.6	219	221	SHRSTc5	3	69.8	294	311
mTcCIR227	2	55.1	139	141	SHRSTc7	3	70.0	297	314
mTcCIR230	2	89.7	234	254	SHRSTc11	5	10.2	304	307
mTcCIR233	4	72.1	209	211	SHRSTc19	5	49.0	178	188
mTcCIR234	4	77.6	121	121	SHRSTc21	2	23.8	204	206

[z]'CCN 51' alleles are color-coded by origin as follows: olive designates alleles inherited from 'ICS 95'; blue designates alleles inherited from 'IMC 67'; green designates alleles present in 'Original Amelonado 2'.

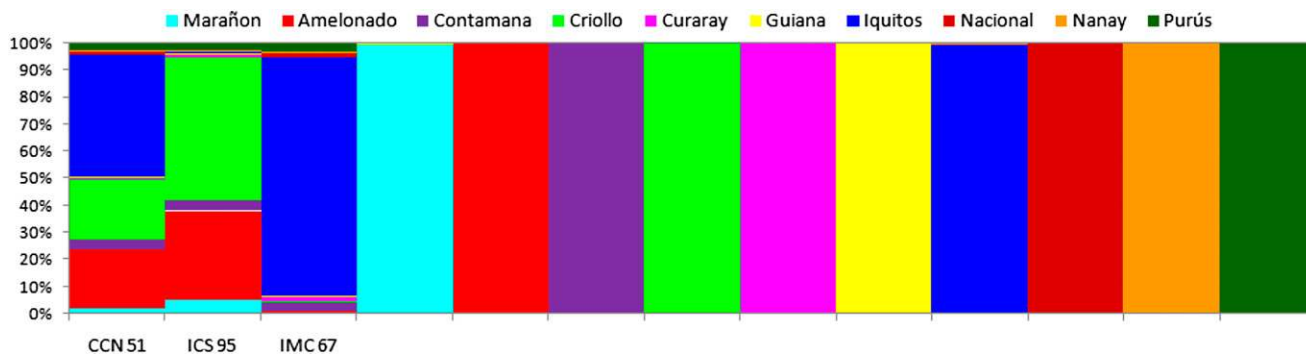Fig. 1. Population structure analysis of the cacao cultivars CCN 51, ICS 95, IMC 67, and representative genotypes of 10 cacao genetic groups (see color key) produced using Structure Version 2.3.3 (Pritchard et al., 2010). Admixed 'CCN 51' genotype is denoted with multiple colors representing specific genetic groups as are 'ICS 95' and 'IMC 67'.

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Organelle origin. Seven mitochondrial SSR markers (mtTc7, mtTc8, mtTc9, mtTc10, mtTc11, mtTc12, and mtTc13) were developed and used to determine the maternal origin of 'CCN 51' alleles (Table 3). Primers were developed using draft sequence data and assemblies for *T. cacao* mitochondrial and chloroplast genomes that were published with the 'Matina 1/6' nuclear genome sequence (Motamayor et al., 2013). The mitochondrial assembly was screened for potential microsatellites using the Simple Sequence Repeat Server available at the Genome Database for Rosaceae (2013) using default parameters. Additionally, the mitochondrial assembly from 'Matina 1/6' was BLASTed against the *T. cacao* Criollo nuclear genome sequence assembly (Argout et al., 2011); we found that a large portion of the mitochondrial sequence aligned to scaffold Tc05 of the Criollo assembly, suggesting that the mitochondrial sequence may have been positioned within the Criollo nuclear genome assembly. Potential insertions/deletions (in/dels) were identified between the sequences of the aligned regions of Criollo Tc05 and the 'Matina 1/6' mitochondrial assembly. The potential in/dels identified were compared with potential microsatellites identified from the mitochondrial assembly screening. Primers generated by the Simple Sequence Repeat Server were used for loci common to both methods of identification. Additional primers flanking in/dels were designed using Primer3 software. All primers were evaluated for polymorphism across a set of 89 cacao cultivars, representing nine of the 10 genetic groups (excluding the Guiana group) reported in Motamayor et al. (2008). Seven mitochondrial SSR markers were selected for this study and used to amplify 'CCN 51', 'ICS 95', 'IMC 67', and four additional cultivars (LcTEEN 74, LcTEEN 81, Criollo 13, and SCA 6), the DNA of which was obtained from samples used in a previous study (Motamayor et al., 2008). PCR amplification was carried out in a DNA Engine Tetrad 2 Peltier Thermal Cycler (MJ Research), as described previously for SSR loci, using an annealing temperature of 61 °C. A single chloroplast SSR marker [CaCrSSR1 (forward CATGGATTCAGCAGCAGTTC, reverse CGAGTCCGCTTATCTCCAAC), GenBank accession No JF979116], previously reported by Yang et al. (2011, 2013), was used to corroborate the results of the mitochondrial DNA data for the maternal origin of 'CCN 51' alleles. Electrophoretic separation of PCR products, fragment size, and allelic designations of the alleles were accomplished as stated previously for SSR genotyping.

Agronomic characteristics. Agronomic characteristics for 'CCN 51' were analyzed and compared against a subset of cacao cultivars of Nacional lineage using the analysis of variance procedure (SAS Version 9.2; SAS Institute, Cary, NC). Trees were planted in a randomized complete block design with four replicates evaluated over a 5-year period (2003–07) at EET Pichilingue. Cacao cultivars were previously propagated by side-grafting the selected cultivar onto cacao rootstocks obtained from open-pollinated seeds of 'IMC 67'. There were 100 trees per cultivar and 25 trees per replicate planted at a distance of 3 m. A custom-designed fertilizer with the final formula of 27N–6.6P–4.2K at a rate of 750 kg·ha^{-1} was applied at the beginning of each year. No overhead irrigation, chemical weed, or pest controls were used. After harvesting cacao pods, cacao beans were subjected to a natural wooden box fermentation process for 5 to 6 d to kill seed embryo, after which cacao beans were dried until water content was ≈8%. The percentage of healthy pods (no disease observed), total dry weight (grams), yield potential (calculation of hypothetical total yield equivalent to sum of healthy and diseased pods), production efficiency (total annual yield divided by annual transversal section growth of tree), total number of witches' brooms produced during the year, and witches' broom efficiency (total number of witches' brooms divided by annual

Table 2. Comparison of cacao cultivar CCN 51 alleles against alleles of putative ancestral reference genotypes: number of heterozygous and homozygous loci for each cultivar and number of alleles of CCN 51 originating from each putative ancestral genotype.

	ICS 95 putative parents		CCN 51 putative grandparents		
Category	LAN 21[z]	Original Amelonado 2[y]	ICS 95[x]	IMC 67[x]	CCN 51[w]
Heterozygous loci (no.)	2	1	55	51	56
Homozygous loci (no.)	65	64	15	19	14
CCN 51 alleles of IMC 67 origin (no.)					70
CCN 51 alleles of ICS 95 origin (no.)					64
CCN 51 alleles of OA 2[y] origin (no.)					6

[z]Representative Criollo genotype and putative parent of ICS 95.
[y]Representative Amelonado genotype and putative parent of ICS 95; OA 2 = Original Amelonado 2.
[x]Putative grandparents of CCN 51.
[w]Offspring.

Table 3. Cacao mitochondrial simple sequence repeat DNA primers used to determine the maternal or cytoplasmic inheritance of cultivar CCN 51 alleles.

Primer name	Forward sequence	Reverse sequence	Motif	Repeats (no.)	Product size (bp)[z]
mtTc7	tttccagtcttctcttccgc	ttgaaagatggcaaaaaccc	aaga	3	279
mtTc8	atgagtgcacctgatgcttg	tccccgggttaagaggtagt	aatt	3	275
mtTc9	cgttacgccccaaaatagaa	cttgaccgtgtctgcttcaa	ctaca	3	239
mtTc10	ctttcatggcttgaatgggt	cccggaatttcaaacaagaa	ta	5	177
mtTc11	caggcccaactgaaagaaag	ttcgcctgatagtttatggag	tgagtggagg	in/del[y]	250
mtTc12	agatgtgataggctggcgtaa	cgatgtcggattcggattat	catt	in/del[y]	270
mtTc13	gtggaaggaatgctctgctc	tctttctgtgctcgtggcta	gtaaa	in/del[y]	312

[z]Predicted 'Matina1-6' size.
[y]Insertion/deletion.

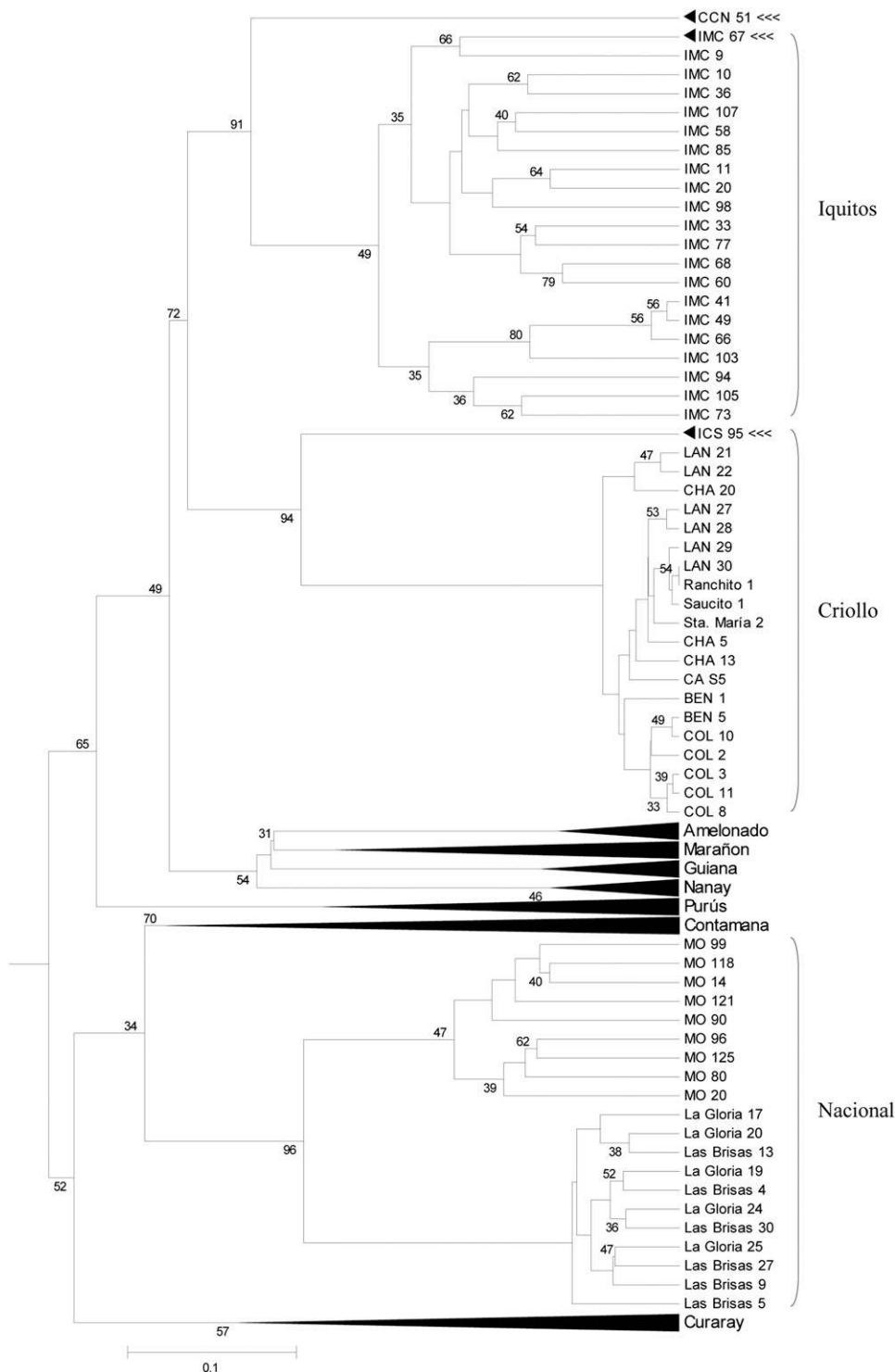*J. Amer. Soc. Hort. Sci.* 139(2):219–229. 2014.

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Fig. 2. Phylogenetic relationship of cacao cultivar CCN 51 and representative individual genotypes belonging to 10 genetic groups of cacao described by Motamayor et al. (2008) based on 70 simple sequence repeat (SSR) loci. Bootstrap values (%), based on 1000 replications, are indicated on branches. Cavalli-Sforza and Edwards (1967) genetic distance was used to generate the most parsimonious tree using unweighted pair group method with arithmetic mean (UPGMA). Tree was obtained with TreeExplorer Version 2.12 (Tamura et al., 2007).

GENOTYPING AND ALLELE ORIGIN. The 70 SSR markers used to genotype 'CCN 51', the putative grandparents 'ICS 95' and 'IMC 67' as well as putative allele donors ('LAN 21' and 'Original Amelonado 2') generated a total of 220 polymorphic alleles. The multilocus genotypes of all 'CCN 51' trees collected at EET Pichilingue station, at the Sofia farm in Ecuador, and at MCCS in Brazil were identical. No amplification products were observed for 'LAN 21' for primer sets mTcCIR35, mTcCIR81, or SHRSTc5; likewise, no amplification products were observed for 'Original Amelonado 2' for primer sets mTcCIR37, mTcCIR54, mTcCIR138, mTcCIR181, or SHSRTc11. Of the 70 SSR loci analyzed in 'CCN 51', 56 (80%) loci were heterozygous, whereas 14 (20%) were homozygous (Table 1). For the putative grandparents, 'ICS 95' and 'IMC 67', 55 (79%) and 51 (73%) of the loci were heterozygous and 15 (21%) and 19 (27%) were homozygous, respectively (Table 2). Furthermore, for the putative allele donors 'LAN 21' and 'Original Amelonado 2', two of 67 amplified loci (3%) and one of 65 amplified loci (1.5%) were heterozygous, and 65 (97%) and 64 (98%) were homozygous, respectively (Table 2). Multilocus genotype data for putative grandparents ('ICS 95' and 'IMC 67') and putative allele donors ('LAN 21' and 'Original Amelonado 2') were compared with that of 'CCN 51'. It was observed that of the 'CCN 51' alleles, 70 (50%) were of 'IMC 67' origin, 64 (45.7%) were of 'ICS 95' origin, and of the alleles not of 'ICS 95' origin, all six (4.3%) were found within 'Original Amelonado 2'.

GENETIC STRUCTURE. The structure analysis was conducted to infer the admixture composition for 'CCN 51' and to understand its genetic relationship within the cacao genetic groups reported by Motamayor et al. (2008). Results of the structure analysis indicated that the highest coefficient of genetic similarity for 'CCN 51' was for the Iquitos genetic group (45.4%) followed by the Criollo (22.2%) and Amelonado (21.5%) genetic groups (Fig. 1). A lower proportion of the 'CCN 51' genome was accounted for by Contamana (3.9%), Purús (2.5%), Marañon (1.8%), and

transversal section growth of tree) were determined. Additionally, 'CCN 51' was compared with a diverse group of seven Nacional cultivars for physical, chemical, and sensory (organoleptic) characteristics as previously described (Elwers et al., 2009; Sukha and Butler, 2005).

Nacional (1.1%) genetic groups, indicating fewer admixtures of these genetic groups.

GENETIC RELATIONSHIP. Phylogenetic trees constructed to determine the genetic relationships between 'CCN 51' and the 10 genetic groups of cacao reported by Motamayor et al. (2008) are presented in Figures 2 and 3. UPGMA clustering reflects the phylogenetic order of each accession or population. The relationship based on genetic distance among 'CCN 51', 'IMC 67', 'ICS 95', and individual cultivar representatives of the 10 genetic groups agree with results from population structure analysis (Figs. 1 and 2) by clustering 'CCN 51'and 'IMC 67' with the Iquitos population and 'ICS 95' (Criollo and Amelonado genetic groups) with the Criollo population. 'CCN 51', 'IMC 67', and 'ICS 95' also grouped with Iquitos and Criollo genetic groups when the UPGMA method was used and analyzed as a group of individual populations (Fig. 3).

ORGANELLE ORIGIN. Mitochondrial and chloroplast DNA analysis results for 'CCN 51' are presented in Figure 4. Seven mitochondrial DNA markers amplified maternally inherited 'CCN 51' alleles demonstrating them to be of 'IMC 67' origin. All 'CCN 51' alleles for each mitochondrial SSR locus were identical to those alleles amplified for 'IMC 67' suggesting cytoplasmic or maternal inheritance. Additionally, cacao cultivars LcTEEN 74, LcTEEN 81 (Nacional genetic group), Criollo 13 (Criollo genetic group), SCA 6 (Contamana genetic group), and ICS 95 (Trinitario type of Criollo and Amelonado origin) were also amplified and found to have different mitochondrial haplotypes. Moreover, chloroplast SSR marker analyses corroborated the results of the mitochondrial marker analysis. The chloroplast SSR marker CaCrSSR1 (Yang et al., 2011, 2013) amplified a 392-bp homozygous allele in both 'CCN 51' and 'IMC 67'. A phylogenetic haplotype relationship using UPGMA cluster analysis (Fig. 4) was generated for 'CCN 51', 'IMC 67', 'ICS 95', and other reference haplotypes detected in the analysis.

AGRONOMIC CHARACTERISTICS. Significant differences were observed for all the agronomic traits evaluated between 12 cacao cultivars and 'CCN 51' (Table 4). The difference in cacao bean dry weight between 'CCN 51' and several other Nacional cultivars was highly significant (Table 4). 'CCN 51' produced 89% more dry cacao beans as compared with 'EET 95' and 235% more than 'EET 19', which were the second highest producer and the least productive in the evaluations, respectively. Yield potential for 'CCN 51' was 53% higher than 'EET 574' (second in the test) and 275% over the lowest performer, 'EET 513'. Yield potential and productive efficiency of 'CCN 51' were the highest as compared with the Nacional cultivars. Additionally, 'CCN 51' had the lowest witches' broom incidence and the best witches' broom efficiency as compared with the Nacional cultivars. Differences between Nacional cultivars and 'CCN 51' for physical, chemical, and sensory characteristics were observed (Table 5). Among physical characteristics, 'CCN 51' had the highest average bean weight and lowest pH values for both testa and cotyledon. 'CCN 51' was also ranked second and third on testa (%) and number of beans per 100 g, respectively. Regarding chemical properties,
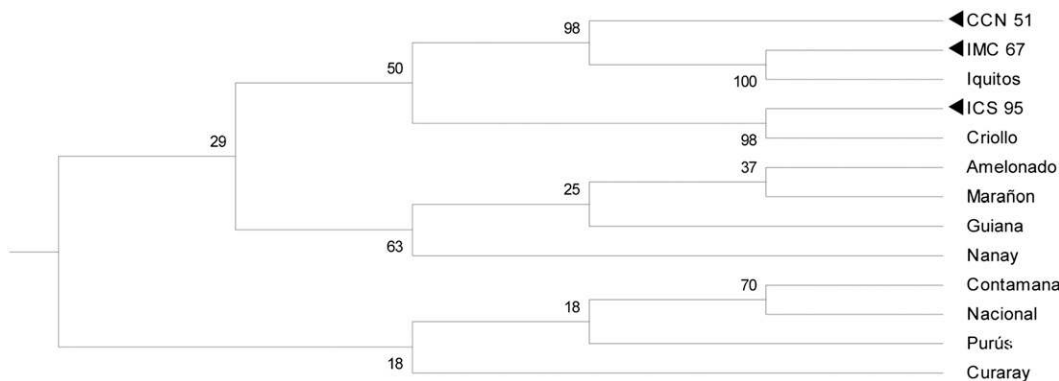

Fig. 3. Phylogenetic relationship of cacao cultivar CCN 51 and 10 genetic groups of cacao described by Motamayor et al. (2008) based on 70 simple sequence repeat (SSR) loci. Bootstrap values (%), based on 1000 replications, are indicated on branches. Cavalli-Sforza and Edwards (1967) genetic distance was used to generate the most parsimonious tree using unweighted pair group method with arithmetic mean (UPGMA). Tree was obtained with TreeExplorer Version 2.12 (Tamura et al., 2007).

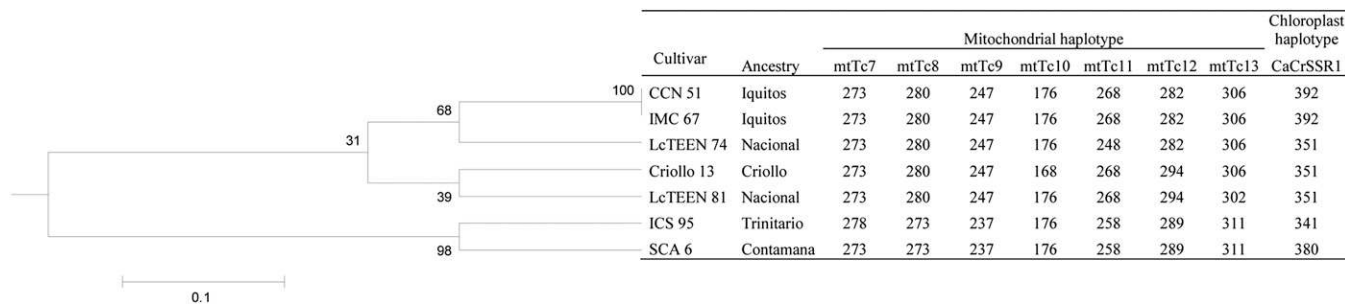| Cultivar	Ancestry	Mitochondrial haplotype							Chloroplast haplotype
		mtTc7	mtTc8	mtTc9	mtTc10	mtTc11	mtTc12	mtTc13	CaCrSSR1
CCN 51	Iquitos	273	280	247	176	268	282	306	392
IMC 67	Iquitos	273	280	247	176	268	282	306	392
LcTEEN 74	Nacional	273	280	247	176	248	282	306	351
Criollo 13	Criollo	273	280	247	168	268	294	306	351
LcTEEN 81	Nacional	273	280	247	176	268	294	302	351
ICS 95	Trinitario	278	273	237	176	258	289	311	341
SCA 6	Contamana	273	273	237	176	258	289	311	380

Fig. 4. Phylogenetic relationship, mitochondrial, and chloroplast haplotype product size (bp) for seven mitochondrial loci and one chloroplast locus for 'CCN 51' and six cacao cultivars. Cavalli-Sforza and Edwards (1967) genetic distance was used to generate the most parsimonious tree using unweighted pair group method with arithmetic mean (UPGMA). Tree was obtained with TreeExplorer Version 2.12 (Tamura et al., 2007).

Table 4. Comparison over a 5-year period between cacao cultivar CCN 51 and 12 Nacional cacao cultivars for agronomic characteristics and incidence of witches' broom disease conducted at Estación Experimental Tropical (EET), Pichilingue in the Province of Quevedo, Ecuador.

Cultivar	Healthy pods (%)	Dry wt (kg·h⁻¹)	Yield potential (kg·h⁻¹)[y]	Productive efficiency (kg·h⁻¹)[x]	Total witches' broom (no.)	Witches' broom efficiency (%)[w]
CCN-51	65.42 ab[z]	6,948.66 a	10,939.71 a	118.25 a	19.27 d	0.30 c
EET-103	58.99 bcd	2,924.44 b	5,021.33 bc	58.45 b	48.94 abc	1.01 ab
EET-19	54.10 de	2,074.38 b	3,870.91 bc	33.75 b	65.80 a	1.12 ab
EET-48	51.99 de	2,835.08 b	5,490.40 bc	52.72 b	69.42 a	1.22 a
EET-513	70.74 a	2,080.38 b	2,916.64 c	29.99 b	38.01 bc	0.55 bc
EET-547	62.49 bc	2,735.49 b	4,406.86 bc	49.11 b	57.39 ab	1.03 ab
EET-552	54.85 de	3,284.62 b	5,961.95 b	55.66 b	54.20 ab	0.80 abc
EET-574	50.03 de	3,386.17 b	7,129.05 b	57.24 b	32.25 cd	0.59 bc
EET-577	52.56 de	2,848.09 b	5,478.11 bc	57.08 b	52.93 ab	1.08 ab
EET-578	50.65 de	2,213.52 b	4,257.43 bc	42.97 b	57.47 ab	1.11 ab
EET-62	49.44 e	3,240.32 b	6,502.27 b	58.52 b	59.19 ab	1.05 ab
EET-95	56.89 cde	3,672.62 b	6,458.42 b	65.18 b	52.36 ab	0.95 ab
EET-96	53.01 de	3,261.28 b	6,180.18 c	69.84 b	56.81 ab	1.23 a
Mean	56.24	3,192.70	5,739.48	57.60	51.08	0.93
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
F calculated	10.52**	10.18**	6.84**	6.38**	6.60**	4.51**
F at $P \leq 0.01$	2.50	2.50	2.50	2.50	2.50	2.50
F at $P \leq 0.05$	1.92	1.92	1.92	1.92	1.92	1.92
cv (%)	8.72	29.75	32.08	36.17	25.78	35.25

[z]Mean separation in columns by analysis of variance procedure (SAS Version 9.2; SAS Institute, Cary, NC). Similar letters have no significant difference at $P \leq 0.05$.
[y]Calculation of hypothetical total yield equivalent to sum of healthy and diseased pods.
[x]Total annual yield divided by annual transversal section growth of tree.
[w]Total number of witches brooms produced during the year divided by annual transversal section growth of tree.
**Significant at $P \leq 0.01$.

'CCN 51' had the highest percent fat, polyphenol content, and theobromine/caffeine (T/C) ratio. In organoleptic properties, 'CCN 51' exhibited a moderate amount of cocoa flavor and higher levels of bitterness and astringency.

Discussion

Over the last few decades, great effort has been made to improve Ecuadorian cacao production for agronomic yield, disease resistance, improved genetics, and fine flavor characteristics. Ecuador was second to Brazil in total cacao production in the Americas during the period 2008–11 (Food and Agriculture Organization of the United Nations, 2013). Cacao production in Ecuador over the last 6 years increased from ≈104,000 to 180,000 t with a cultivated area of over 400,000 ha (Asociación Nacional de Exportadores de Cacao, 2013). This represents a significant increase of 73.8%; in contrast, Brazil only experienced an increase of 23% (Food and Agriculture Organization of the United Nations, 2013). This success in Ecuador was achieved by the national breeding and selection program pursuing a more efficient selection of superior cacao cultivars and hybrid trees for clonal propagation. 'CCN 51' was developed and selected by H. Castro in the early 1960s; as a result of its high yield potential and disease resistance, the cultivar has played a unique role in increasing the cacao bean production in the country. Witches' broom disease drastically reduced and impacted vast areas of cultivated cacao in Ecuador since it was first reported in the 1930s (Baker and Holliday, 1957; Bartley, 2005; Pound, 1938, 1945; Soria, 1966). However, even in areas with high witches' broom disease inoculum and

disease pressure, 'CCN 51' is still highly tolerant. These agronomic and disease severity differences were also observed when 'CCN 51' was evaluated against genotypes of Nacional origin grown in other countries (F.M. Amores, unpublished data). Similar observations have been obtained for genotypes derived from families in which 'CCN 51' was used as a parent as determined by tests conducted at the EET Pichilingue (J.C. Motamayor, unpublished data). These observed differences are therefore likely attributable to the general and specific combining ability of 'CCN 51'. This suggests the presence of both additive and dominance gene action effects with a significant role in the inheritance of superior traits, as observed in 'CCN 51', that are taken advantage of in the breeding of new cacao cultivars. The exploitation of heterosis or hybrid vigor is one of the best breeding strategies used in cacao improvement. Selection and combination of best parents in crossing schemes based on general and specific combining ability continue to be an important approach for selection of favorable genes in cacao. In addition, the information available from the two cacao genome sequence projects will expedite the selection of elite cacao trees by detecting and using mutations in the genetic code associated with cacao diseases and identifying those gene clusters with the greatest influence on desirable traits.

Despite the complexity initially suspected for the 'CCN 51' pedigree, the present study provides evidence of the origin and inheritance of each individual allele observed for the 70 assayed loci. This study assigned the origin of the majority of 'CCN 51' alleles to 'IMC 67' and 'ICS 95'. However, a small number of alleles (six) were found in 'Original Amelonado 2', the putative

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Table 5. Physical, chemical, and sensory characteristics for cacao cultivar CCN 51 and a diverse group of Nacional genotypes conducted at Estación Experimental Tropical (EET), Pichilingue in the Province of Quevedo, Ecuador.

Characteristic	CCN 51	EET 103	EET 62	EET 95	La Gloria	Las Brisas	Voluntad de Dios	Santa Lucia
Physical								
Avg bean wt (g)	1.54	1.39	1.52	1.46	1.17	1.22	1.34	1.20
Testa (%)	12.1	12.0	12.6	12.2	13.7	13.3	13.4	13.4
Beans (no./100 g)	73.6	71.4	67.7	72.4	85.1	81.1	72.7	94.0
pH of testa	4.8	5.2	5.4	5.0	5.4	5.4	5.5	5.6
pH of cotyledon	5.7	5.9	5.8	6.0	5.8	5.8	5.8	5.8
Chemical								
Fat (%)	50.3	47.4	48.4	48.3	45.8	47.2	49.3	47.9
Polyphenols (mg·g^{-1})	85.6	66.3	75.5	79.1	46.3	54.5	75.0	65.5
Theobromine (%)	2.2	2.3	2.2	2.3	1.9	1.8	2.3	2.2
Caffeine (%)	0.3	0.4	0.5	0.4	0.4	0.4	0.5	0.4
T/C^z	6.9	5.5	4.7	5.2	5.0	4.6	5.0	5.6
Glucose	0.2	0.2	0.2	0.1	0.2	0.3	0.2	0.3
Fructose	0.4	0.5	0.5	0.4	0.6	0.5	0.5	0.6
Sucrose	0.8	0.6	0.6	0.6	0.8	0.5	0.5	0.6
Organoleptic								
Cocoa	3.3	1.3	4.7	1.8	5.7	3.7	3.3	3.0
Floral	0	1.0	3.0	1.4	3.0	4.7	1.3	3.0
Fruity	0	2.0	1.3	1.1	1.3	2.3	1.0	1.3
Nutty	0	0.1	0.0	0.3	0.3	0.1	0.0	0.2
Caramel	0	0	0	0	0	0	0	0
Bitterness	4.3	3.3	4.7	3.6	4.7	3.0	4.3	3.7
Acidity	1.0	0.8	2.7	2.0	1.0	3.0	2.7	3.7
Astringency	6.3	5.8	5.0	3.9	5.0	3.7	4.3	6.0
Floral grassy	0	0	0	0	0	0	0	0

^{z}Theobromine/caffeine ratio.

progenitor of 'ICS 95'. Considerable genetic variation was detected by these 70 loci predicting a heterozygosity level of 80% for 'CCN 51', yet the presence of high heterozygosity in 'CCN 51' is not consistent with that of traditional Nacional genotypes, which are highly homozygous (Amores et al., 2011; Soria, 1966). However, the minor amount of Nacional ancestry of 1.1% that was detected in 'CCN 51' is insignificant in relation to the entire genome, and it can be accounted for by residual admixture. The Nacional lineage includes genotypes from the Amazonian side of the Andes (Morona, Nangaritza, and Zamora rivers) as well as the Pacific side; however, these two groups can be distinguished based on their allelic frequencies, likely reflecting centuries of human selection in the Ecuadorian Coast (Motamayor et al., 2008). Geographic location or local selective adaptation may also have played a role in driving divergence and structuring the Nacional population, because ecosystems in the mountain and the Pacific plain areas differ in many respects, contributing to its genetic differences. Furthermore, the evolutionary potential of the Nacional genetic group may be largely dependent on levels and patterns of genetic variation. However, the amount of that variation may have been determined by the effective population size, historical events, mating systems, and population structure. In addition, gene flow and selection pressure also contribute to population differentiation shaping the genetic structure both within and among populations.

Interpopulation differences in allele frequency distribution are also significant among Nacional, Iquitos, Criollo, and Amelonado genetic groups including those genetic groups that compose the residual admixture of 'CCN 51' (Motamayor et al., 2008). This variation in the allele frequencies for the SSR loci contributed significantly to the genetic discrimination of the lineage of 'CCN 51', and this was confirmed based on its multilocus genotype and genetic structure analysis using the bayesian method for individual assignment. The Nacional lineage is a distinctive genetic group of cacao with a fine flavor not found in other types of cacao with evidence of high homozygosity and low introgression levels (Loor et al., 2012; Zhang et al., 2008, 2012). Although fine flavor is known to be largely influenced by the fermentation process, great effort has been made over the past few years to improve the selection and breeding programs for this trait in Ecuador. However, this specific characteristic may have been eroded by successive introductions of foreign germplasm whose hybrid descendants have gradually replaced native plantations (Loor et al., 2009). The structure analysis results indicate that the origin of 'CCN 51' is based on its hybridization, suggesting the IMC type as the primary genetic source followed by Criollo and Amelonado ancestries. Substantial differentiation occurred across the geographic range of these genetic groups. Overall, the genetic differentiation within these lineages may largely have occurred as a result of spatial variation in selection pressures, genetic drift, or the joint effects of both processes.

Ecuadorian cacao is recognized internationally for its fine arriba flavor and quality. Typically, fine flavor is associated with Criollo or Trinitario genetic backgrounds. However, traditional Nacional, a genetically distinct population (Loor et al., 2012; Motamayor et al., 2008), also produces fine flavor cacao beans. Research conducted to compare 'CCN 51' and several Nacional cultivars for physical, chemical, and organoleptic characteristics suggests that 'CCN 51' is distinct from the Nacional genotypes. 'CCN 51' showed the highest

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average bean weight, fat percent, and T/C ratio; however, 'CCN 51' has less desirable traits such as a low pH for both testa and cotyledon, moderate to low amount of cocoa flavor, and high levels of bitterness and astringency. However, bitterness and astringency can be significantly reduced by a pre-drying process before box fermentation takes place. These latter characteristics make 'CCN 51' less attractive to high-end, fine-flavored chocolate makers as compared with traditional Nacional cultivars.

In plants, the direct maternal ancestor can be traced using mitochondrial and chloroplast DNA. Mitochondrial and chloroplast DNA have successfully determined genetic relationships in previous studies (Nishikawa et al., 2002, 2005; Wolfe et al., 1987). Mitochondrial DNA data reported in this study suggest 'IMC 67' as a maternal ancestor of 'CCN 51' based on the cytoplasmic inheritance of 'CCN 51' alleles from 'IMC 67' origin. The chloroplast DNA data corroborate the results of the mitochondrial DNA data. Allele origin analysis using 70 genomic DNA SSR loci reported in this study indicates the genetic pedigree of 'CCN 51' could be the product of a cross between 'IMC 67' as one of the parents and a Trinitario genotype genetically very similar to 'ICS 95' as the other parent. Therefore, because the accession 'Oriente 1' cannot be located and no information is available about its genetic constitution (with the exception that it was collected in Ecuadorian Amazon), it appears that the genetic foundation of 'CCN 51' is comprised of alleles of 'IMC 67' origin as well as those of traditional Trinitario origin (Criollo × Amelonado). Furthermore, the structure and genetic relationship analysis additionally support these findings because they reveal that 'CCN 51' has alleles belonging to the Iquitos, Criollo, and Amelonado genetic groups in its genetic lineage. Moreover, after a widespread search by INIAP researchers and other independent cacao researchers in Ecuador for the 'Oriente 1' accession, it has not been found.

'CCN 51' often is used as a parent in crosses as a result of its high general and specific combining ability and it has had a major impact on the breeding programs in Ecuador and other cacao breeding and selection programs worldwide. The present study provides and summarizes agronomic characteristic comparisons between cultivar CCN 51 and several traditional Nacional cultivars. It also presents an in-depth analysis of 'CCN 51' physical, chemical, and organoleptic characteristics. This work has significantly improved our understanding of 'CCN 51's' genetic constitution that will allow for optimizing breeding schemes for the genetic improvement of complimentary traits of inter- and intragenetic groups in cacao. One of the key goals of the genotypic characterization of cultivar CCN 51 was to dissect its genetic variation, expressed in a manner that can be useful to cacao breeders and producers. The important outcomes that have emerged from this effort highlight the future direction for their use in functional genomics. An understanding of the genetic constitution of 'CCN 51' will be of great value to cacao breeders and industry for improving unattractive characteristics of this genotype while taking advantage of the favorable traits.

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