

Tracing the native ancestors of the modern *Theobroma cacao* L. population in Ecuador

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Abstract The native *Theobroma cacao* L. population from Ecuador, known as Nacional, is famous for its fine cocoa flavour. From the beginning of the twentieth century, however, it has been subjected to genetic erosion due principally to successive introductions of foreign germplasm whose hybrid descendants gradually replaced the native plantations, implying a decrease in cocoa quality. We attempted to trace this native cacao within a wide pool of modern Ecuadorian cacao population. Three hundred and twenty-two cacao accessions collected from different geographical areas along the pacific coast of Ecuador and maintained in two living collections were analysed using 40 simple-sequence repeat markers. Most of Ecuadorian cacao accessions displayed a high diversity and heterozygosity level. A factorial analysis of correspondence (FAC) showed a continuous variation among them, with a few ones,

grouped at an extreme side of the FAC cloud, showing higher levels of homozygosity and lower introgression level by foreign cacaos. A paternity analysis revealed that these highly homozygous individuals are the most probable ancestors of the modern Nacional hybrid pool. These particular accessions studied could represent the native Nacional cacao present in Ecuador before the foreign introductions. Their identification will help to conserve valuable genetic material and to improve cocoa quality in new cacao varieties.

Keywords *Theobroma cacao* · Native Nacional · Arriba flavour · Cocoa quality · Ecuador · SSR

Introduction

Theobroma cacao L. ($2n=20x=20$) is a perennial species member of the Malvaceae family which has its putative centre of origin and diversity in the upper basin of the Amazon River tributaries (Napo, Putumayo and Caqueta) at the foot of the Ecuadorian Andes (Cheesman 1944). The greatest diversity of wild cacao is located in the Amazonian region. During the course of cacao domestication, a small part of this primary cacao diversity was exported to various destinations to be cultivated with three main genetic groups being spread and cultivated around the world before 1950. These genetic groups have been traditionally described as Criollo, Forastero and Trinitario.

The *Criollo* group was originally cultivated by the Mayas in Central America and represents the first domesticated cacao. Its cacao beans have been used in food for more than 2,600 years, as shown by archaeological evidence in cacao residues found in Mayan cooking vessels dating back to approximately 600 years B.C. (Hurst et al.

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2002). The ancestral Criollo genotypes have been identified, revealing a very narrow genetic base associated to a high homozygosity level (Motamayor et al 2002).

The *Forastero* group includes several very diverse populations found in all Amazonian areas from Colombia to Guyanas. During an outbreak of witches' broom disease in the 1930s, several expeditions were made in Upper Amazonian regions to collect material resistant to this disease (Pound 1938, 1945). The high diversity of these cacao populations was confirmed by molecular markers analyses (Laurent et al. 1994; N'Goran et al. 1994, 2000; Motamayor et al. 2003), and they generally conclude a strong geographic structuration for this population.

The *Trinitario* group is a hybrid population developed from natural hybridisations between Criollo and Lower Amazon *Forastero* imported to the island of Trinidad when the Criollo plantations from Trinidad were partly destroyed by a natural disaster in the late eighteenth century (1727).

In addition to these three main cacao groups, a primitive cultivated population of *T. cacao* known as "Nacional" existed in the Pacific Coast Region of Ecuador. Although its exact origin is unknown, it is considered indigenous to this country. Throughout the years, its classification has varied: It was classified as *Forastero* by Cheesman (1944) and Soria (1970) but placed among Criollo by Enriquez (1992). More recently, it is considered as a group different from Criollo or *Forastero*, but genetically closer to the later (Lerceteau et al. 1997a). According to Allen and Lass (1983), the Nacional cacao might have been derived from a local wild population which could now have completely disappeared together with the original forest cover of the coast region. Soria (1970) and Vera (1987, 1993) suggested as its centre of origin the oriental declivities of the Andes mountain in the Amazonian area of Ecuador.

In early 1600s, small plantations of Nacional cacao existed along the Guayas River shores located in a region called "ARRIBA" (Fig. 1) that spread in its tributaries to the Daule and Babahoyo upward rivers. The Nacional cacao trees develop a strong floral aroma known as "arriba" flavour in international markets. This cocoa flavour is highly appreciated to make specific chocolate products and is exclusively produced in Ecuador. With the exception of the province of Esmeraldas, the Nacional cacao was the only one grown in the Western coastal plain of Ecuador until 1890 (Van Hall 1932) when, for the first time, pods of cultivars called "Venezuela" were introduced from Trinidad. With the appearance in 1916 of *Moniliophthora roreri* (Rorer 1926) and, in 1919, of *Moniliophthora perniciosa* (Pound 1938), fungi that cause the frosty pod and witches' broom diseases, respectively, foreign materials were introduced in larger quantity in Ecuador. During the expeditions undertaken by Pound in the Upper Amazon region (Pound 1938, 1945), cacao material from the Scavina family was

collected and used from 1940 onwards in the cacao breeding programs of Ecuador because of its known high level of resistance to witches' broom (Ampuero 1960). Consequently, more than 95% of the original area previously planted with Nacional cacao has been replaced by hybrid material involving foreign clones, especially Trinitario types (Loor et al. 2002). This genetic mixing has led to a dilution of the "arriba" flavour in the modern Nacional cacao population.

In an effort to preserve the Nacional cacao genetic resources and to broaden its genetic basis, a substantial amount of germplasm was collected along the Pacific Coast Region of Ecuador from several expeditions made since the 1940s. Today, an important fraction of these accessions are maintained as ex situ collections in the two main cacao germplasm banks of Ecuador (Fig. 1): Estación Experimental Tropical Pichilingue (EET-P) and Centro de Cacao Aroma Tenguel (CCAT). Although the majority of the passport information was lost in the case of CCAT, these gene banks still appear attractive to cacao breeders. The understanding of their patterns of genetic diversity will help to optimise breeding strategies to produce a new improved Nacional varieties with a conserved "arriba" flavour.

Microsatellite markers or simple sequence repeat (SSR) is one of the most powerful molecular marker types to reveal the genetic diversity of natural populations. For cacao, a large number of microsatellite (SSR) markers were developed by Lanaud et al. (1999), Risterucci et al. (2000), Lanaud et al. (2004) and Pugh et al. (2004). However, few genetic studies have been carried out on the Nacional cacao of Ecuador (Lerceteau et al. 1997b; Quiroz 2002; Loor et al. 2002), and of those studied, only a small number of samples have been used.

In the present study, SSR genotyping technology was applied to study the diversity of a large number of samples (a) to assess the genetic structure and diversity present in modern Nacional cacao, (b) to determine the genetic relationships between the different cacao accessions and (c) to identify individuals that could represent the native Nacional cacao, which could be used as source of genes for a new cacao breeding program of aromatic varieties.

Materials and methods

Plant material

Leaves were collected from 322 cacao trees (Table 1) conserved at CCAT and EET-P germplasm banks (Fig. 1). All trees were characterised for their agronomical performances and, in some cases, for their flavour through sensorial evaluation by an expert panel. The CCAT collection is the result of samplings of cacao trees along the coast region

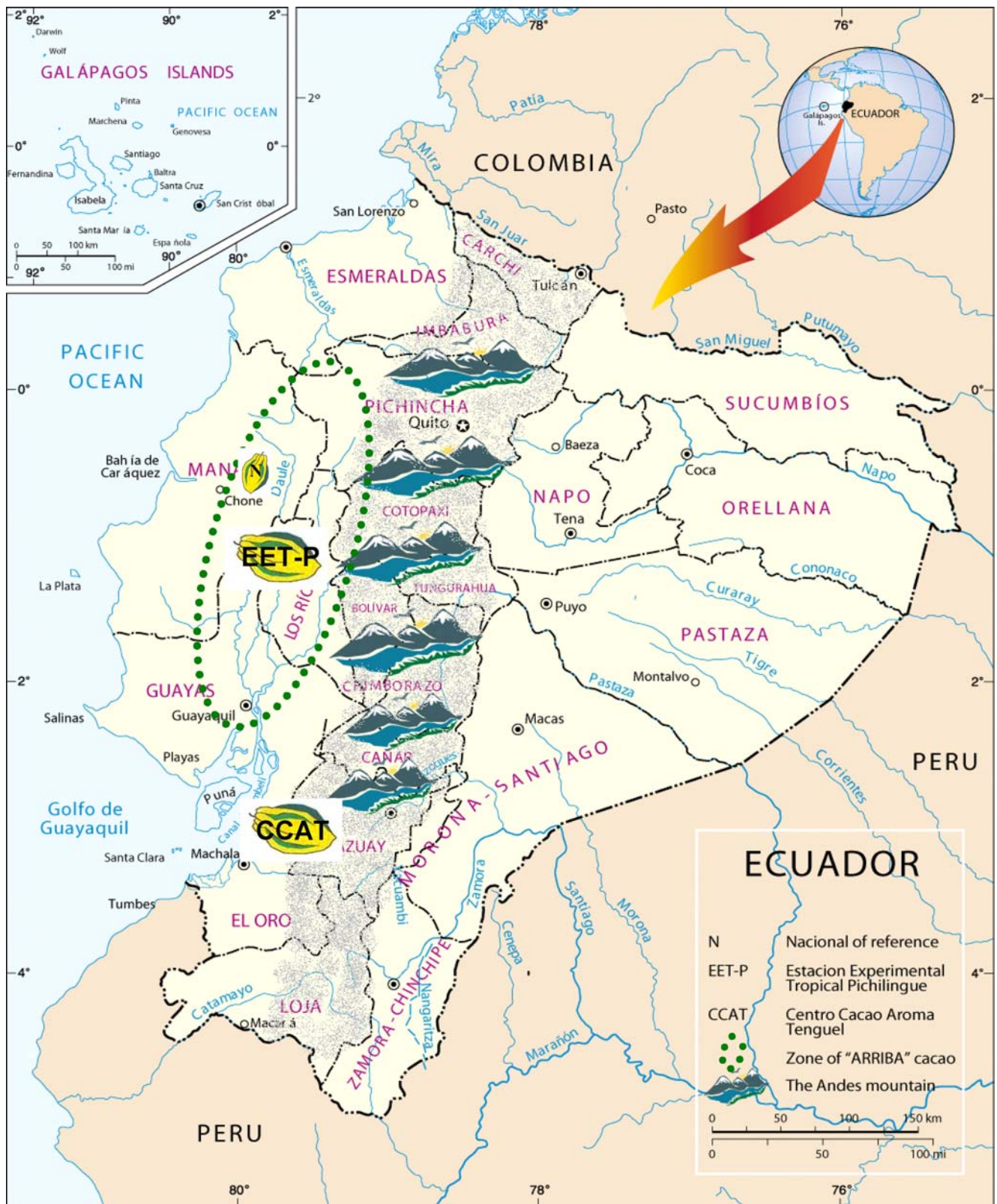


Fig. 1 Map of Ecuador showing the different ecological zones, the geographic locations of Nacional germplasm banks and the zone called ARRIBA where the first Nacional cacao population in Ecuador was cultivated

Table 1 List of Ecuadorian cacao accessions used in this study and level of heterozygosity (% heter.) at the 40 loci studied

No.	Name	Origin, region	%heter.
1	L27-H68	CCAT	52.6
2	L28-H46	CCAT	87.5
3	L29-H47	CCAT	42.5
4	L29-H48	CCAT	57.5
5	L30-H47	CCAT	80.0
6	L28-H48	CCAT	87.5
7	L26-H60	CCAT	30.0
8	L25-H60	CCAT	72.5
9	L25-H62	CCAT	72.5
10	L24-H62	CCAT	53.8
11	L24-H64	CCAT	30.0
12	L25-H57	CCAT	76.3
13	L25-H64	CCAT	72.5
14	L21-H56	CCAT	57.5
15	L31-H48	CCAT	47.5
16	L21-H54	CCAT	80.0
17	L14-H44	CCAT	45.0
18	L30-H01	CCAT	82.5
19	L33-H49	CCAT	72.5
20	L33-H47	CCAT	72.5
21	L33-H27	CCAT	72.5
22	L33-H24	CCAT	68.4
23	L33-H23	CCAT	72.5
24	L34-H57	CCAT	36.0
25	L34-H58	CCAT	85.0
26	L34-H59	CCAT	37.5
27	L34-H61	CCAT	45.0
28	L34-H66	CCAT	46.0
29	L34-H67	CCAT	50.0
30	L31-H54	CCAT	72.5
31	L31-H68	CCAT	72.5
32	L32-H72	CCAT	67.6
33	L32-H69	CCAT	72.5
34	L13-H46	CCAT	45.8
35	L31-H45	CCAT	42.5
36	L30-H06	CCAT	85.0
37	L15-H39	CCAT	78.0
38	L14-H48	CCAT	82.5
39	L33-H10	CCAT	47.5
40	L34-H46	CCAT	85.0
41	L32-H48	CCAT	72.5
42	L33-H65	CCAT	55.0
43	L33-H58	CCAT	72.5
44	L33-H55	CCAT	72.5
45	L20-H43	CCAT	50.0
46	L28-H34	CCAT	62.5
47	L36-H61	CCAT	47.5
48	L20-H07	CCAT	52.5
49	L47-H75	CCAT	46.2
50	L29-H63	CCAT	53.0
51	L48-H73	CCAT	62.0
52	L23-H53	CCAT	45.0
53	L23-H49	CCAT	55.3
54	L23-H48	CCAT	57.9
55	L23-H42	CCAT	41.4

Table 1 (continued)

No.	Name	Origin, region	%heter.
56	L23-H41	CCAT	52.8
57	L21-H61	CCAT	55.0
58	L20-H57	CCAT	67.5
59	L39-H64	CCAT	38.5
60	L39-H70	CCAT	75.0
61	L39-H54	CCAT	72.5
62	L46-H83	CCAT	77.5
63	L29-H59	CCAT	45.0
64	L36-H37	CCAT	44.9
65	L35-H60	CCAT	45.0
66	L36-H53	CCAT	37.5
67	L47-H78	CCAT	77.5
68	L16-H36	CCAT	55.6
69	L16-H26	CCAT	77.5
70	L39-H46	CCAT	40.6
71	L40-H12	CCAT	85.0
72	L23-H38	CCAT	51.5
73	L22-H39	CCAT	57.5
74	L21-H53	CCAT	77.5
75	L30-H20	CCAT	40.0
76	L30-H43	CCAT	77.5
77	L30-H46	CCAT	77.5
78	L23-H63	CCAT	59.3
79	L39-H61	CCAT	85.0
80	L39-H51	CCAT	74.4
81	L48-H99	CCAT	60.0
82	L38-H31	CCAT	32.5
83	L39-H13	CCAT	40.0
84	L37-H31	CCAT	40.0
85	L37-H53	CCAT	77.5
86	L37-H68	CCAT	57.5
87	L36-H28	CCAT	35.0
88	L36-H17	CCAT	34.5
89	L36-H27	CCAT	67.5
90	L35-H46	CCAT	67.5
91	L35-H62	CCAT	37.5
92	L35-H56	CCAT	52.5
93	L45-H76	CCAT	76.9
94	L46-H92	CCAT	61.0
95	L43-H15	CCAT	42.5
96	L46-H63	CCAT	79.5
97	L46-H81	CCAT	82.1
98	L43-H59	CCAT	50.0
99	L19-H27	CCAT	31.6
100	L18-H49	CCAT	65.0
101	L18-H58	CCAT	64.5
102	L17-H46	CCAT	29.7
103	L17-H42	CCAT	52.5
104	L46-H45	CCAT	56.4
105	L45-H75	CCAT	80.0
106	L45-H78	CCAT	79.0
107	L45-H83	CCAT	72.5
108	L46-H51	CCAT	47.4
109	L45-H85	CCAT	71.8
110	L44-H63	CCAT	74.4
111	L45-H74	CCAT	73.7

Table 1 (continued)

No.	Name	Origin, region	%heter.
112	L44-H88	CCAT	71.8
113	L40-H90	CCAT	47.4
114	L43-H92	CCAT	65.0
115	L43-H71	CCAT	71.0
116	L10-H25	CCAT	47.5
117	L10-H28	CCAT	47.5
118	L10-H29	CCAT	50.0
119	L13-H34	CCAT	62.5
120	L13-H37	CCAT	77.5
121	L13-H41	CCAT	60.0
122	L14-H34	CCAT	42.5
123	L14-H39	CCAT	45.0
124	L14-H40	CCAT	47.5
125	L14-H45	CCAT	58.0
126	L15-H30	CCAT	80.0
127	L15-H31	CCAT	77.5
128	L15-H32	CCAT	50.0
129	L15-H33	CCAT	80.0
130	L15-H38	CCAT	82.5
131	L15-H43	CCAT	57.5
132	L16-H29	CCAT	77.5
133	L16-H43	CCAT	77.5
134	L17-H53	CCAT	77.5
135	L18-H38	CCAT	65.0
136	L18-H46	CCAT	50.0
137	L18-H59	CCAT	65.0
138	L19-H29	CCAT	52.5
139	L19-H32	CCAT	52.5
140	L19-H34	CCAT	52.5
141	L19-H37	CCAT	52.5
142	L20-H35	CCAT	52.5
143	L20-H46	CCAT	75.0
144	L21-H38	CCAT	75.0
145	L21-H44	CCAT	80.0
146	L21-H46	CCAT	50.0
147	L21-H47	CCAT	80.0
148	L21-H48	CCAT	77.5
149	L22-H38	CCAT	55.0
150	L22-H43	CCAT	42.5
151	L22-H58	CCAT	52.5
152	L22-H62	CCAT	52.6
153	L23-H44	CCAT	55.0
154	L23-H56	CCAT	55.0
155	L24-H41	CCAT	35.0
156	L24-H60	CCAT	65.0
157	L24-H65	CCAT	47.5
158	L25-H46	CCAT	42.5
159	L25-H61	CCAT	72.5
160	L26-H64	CCAT	72.5
161	L27-H51	CCAT	32.5
162	L27-H53	CCAT	45.0
163	L27-H58	CCAT	42.5
164	L27-H67	CCAT	65.0
165	L28-H47	CCAT	50.0
166	L28-H59	CCAT	52.5
167	L29-H45	CCAT	52.5

Table 1 (continued)

No.	Name	Origin, region	%heter.
168	L30-H54	CCAT	55.0
169	L30-H59	CCAT	55.0
170	L30-H62	CCAT	52.5
171	L31-H47	CCAT	75.0
172	L32-H46	CCAT	52.5
173	L32-H56	CCAT	60.0
174	L32-H58	CCAT	45.0
175	L32-H65	CCAT	72.5
176	L33-H45	CCAT	85.0
177	L33-H68	CCAT	55.0
178	L35-H40	CCAT	48.7
179	L35-H51	CCAT	32.5
180	L36-H42	CCAT	47.5
181	L36-H49	CCAT	47.5
182	L36-H65	CCAT	47.5
183	L37-H41	CCAT	57.5
184	L37-H55	CCAT	35.0
185	L38-H51	CCAT	47.5
186	L39-H52	CCAT	72.5
187	L39-H62	CCAT	47.5
188	L39-H63	CCAT	45.0
189	L39-H66	CCAT	10.0
190	L40-H64	CCAT	57.5
191	L41-H64	CCAT	62.5
192	L41-H75	CCAT	55.0
193	L42-H73	CCAT	57.5
194	L42-H83	CCAT	65.0
195	L42-H87	CCAT	45.0
196	L42-H90	CCAT	37.5
197	L42-H96	CCAT	57.5
198	L42-H97	CCAT	55.0
199	L43-H78	CCAT	32.5
200	L43-H90	CCAT	35.0
201	L43-H96	CCAT	37.5
202	L44-H66	CCAT	73.7
203	L44-H68	CCAT	72.5
204	L44-H72	CCAT	42.5
205	L44-H84	CCAT	55.0
206	L46-H70	CCAT	79.5
207	L46-H74	CCAT	80.0
208	L46-H75	CCAT	79.5
209	L46-H85	CCAT	80.0
210	L46-H88	CCAT	80.0
211	L46-H90	CCAT	80.0
212	L48-H77	CCAT	53.9
213	L48-H93	CCAT	45.0
214	L49-H74	CCAT	46.2
215	L49-H84	CCAT	70.0
216	L49-H87	CCAT	50.0
217	L49-H93	CCAT	80.0
218	L49-H98	CCAT	80.0
219	L49-H100	CCAT	41.1
220	L50-H65	CCAT	35.9
221	Sa8	Rio Chone	60.0
222	Sa16	Rio Chone	10.0
223	SNA101	EET-P	66.7

Table 1 (continued)

No.	Name	Origin, region	%heter.
224	SNA104	EET-P	47.5
225	SNA107	EET-P	41.4
226	SNA203	EET-P	35.7
227	SNA204	EET-P	57.9
228	SNA305	EET-P	35.7
229	SNA403	EET-P	40.0
230	SNA405	EET-P	50.0
231	SNA407	EET-P	40.0
232	SNA409	EET-P	50.0
233	SNA409a	EET-P	15.0
234	SNA412	EET-P	59.0
235	SNA418	EET-P	45.7
236	SNA423	EET-P	37.5
237	SNA425	EET-P	38.5
238	SNA428	EET-P	37.5
239	SNA433	EET-P	59.4
240	SNA438	EET-P	77.5
241	SNA503	EET-P	22.5
242	SNA504	EET-P	37.5
243	SNA505	EET-P	33.4
244	SNA512	EET-P	48.3
245	SNA602	EET-P	60.5
246	SNA604	EET-P	15.0
247	SNA608	EET-P	40.0
248	SNA610	EET-P	56.5
249	SNA613	EET-P	60.9
250	SNA614	EET-P	40.7
251	SNA701	EET-P	61.3
252	SNA707	EET-P	45.0
253	SNA718	EET-P	40.0
254	SNA901	EET-P	57.5
255	SNA903	EET-P	48.6
256	SNA907	EET-P	47.5
257	SNA1001	EET-P	10.0
258	SNA1003	EET-P	10.0
259	L11H19	EET-P	64.0
260	L12H01	EET-P	57.5
261	L19H16	EET-P	50.0
262	L19H28	EET-P	35.0
263	L19H30	EET-P	50.0
264	L21H43	EET-P	80.0
265	L22H40	EET-P	46.7
266	L30H50	EET-P	75.7
267	L30H61	EET-P	69.7
268	L32H60	EET-P	61.9
269	L34H07	EET-P	67.5
270	L42H60	EET-P	66.7
271	L42H65	EET-P	75.0
272	L43H64	EET-P	67.5
273	L45H84	EET-P	74.4
274	L50H64	EET-P	52.0
275	L51H36	EET-P	52.5
276	L52H06	EET-P	55.0
277	L52H12	EET-P	55.0
278	L54H77	EET-P	45.0
279	EET-19	EET-P	76.9

Table 1 (continued)

No.	Name	Origin, region	%heter.
280	EET-48	EET-P	75.0
281	EET-62	EET-P	75.0
282	EET-62LL	EET-P	76.9
283	EET-95	EET-P	80.0
284	EET-96	EET-P	67.5
285	EET-103	EET-P	79.5
286	EB-01-04	Machala, El Oro	35.9
287	EB-04-01	EET-P	36.9
288	EB-04-02	Chontillal, El Oro	37.5
289	EB-05-01	Cone, Guayas	65.0
290	EB-10-10	Astudillo, Guayas	53.4
291	EB-10-11	Milagro, Guayas	54.9
292	EB-10-13	Milagro, Guayas	50.0
293	EB-12-03	El Triunfo, Guayas	37.5
294	EB-15-16	P. Viejo, Los Rios	80.0
295	EB-16-17	EET-P	35.2
296	EB-19-15	Vinces, Los Rios	42.5
297	EB-19-22	Vinces, Los Rios	55.0
298	EB-20-09	Zapotal, Los Rios	43.6
299	EB-21-02	Mcache, Los Rios	62.5
300	EB22-22	Camreta, Manabí	60.5
301	EB-22-33	Calceta, Manabí	45.0
302	EB-22-36	Canuto, Manabí	42.1
303	EB-22-37	Canuto, Manabí	42.5
304	EB-501	Cone, Guayas	42.5
305	BCH-9	Balao, Guayas	48.7
306	BCH-14	Balao, Guayas	65.8
307	FIDENCIO	Zapotal, Los Rios	50.0
308	EET-34	EET-P	67.0
309	EET-38	EET-P	45.3
310	EET-50	EET-P	54.5
311	EET-58	EET-P	78.8
312	EET-59	EET-P	62.0
313	EET-64	EET-P	74.0
314	EET-65	EET-P	48.0
315	EET-67	EET-P	65.0
316	EET-69	EET-P	39.7
317	EET-71	EET-P	67.5
318	EET-73	EET-P	58.6
319	EET-117	EET-P	35.0
320	Nac-2	EET-P	43.6
321	Nac-4	EET-P	53.9
322	Espe-2	EET-P	36.4
Ref. 1	B240	Manabí, Ecu	25.0
Ref. 2	LAN28	Mexique	0.0
Ref. 3	MAT-1-6	Costa Rica	0.0
Ref. 4	SCA6®	Loreto, Peru	43.6
Ref. 5	UF676	Costa Rica	100.0

Ref. 1 = Nacional (B240)

Ref. 2 = Criollo genotype (LAN28)

Ref. 3 = Forastero from Lower Amazon (MAT-1-6)

Ref. 4 = Forastero from Upper Amazon (SCA-6)

Ref. 5 = Trinitario genotype (UF676)

CCAT (1-220), EET-P (221-332)

Table 2 Summary of genetic parameter values for the 40 microsatellite loci evaluated on the cacao populations collected in the coast region from Ecuador

SSRs ^a	Linkage group	Allele		Genotype		Gene diversity (H_e)	H_o	PIC
		N_e	F_a	N_g	F_g			
mtcCIR15	1	6	0.5729	11	0.5186	0.5492	0.6475	0.4678
mtcCIR84	1	2	0.9476	3	0.9021	0.0994	0.0909	0.0945
mtcCIR275	1	3	0.5859	6	0.5219	0.5408	0.6768	0.4597
mtcCIR94	1	5	0.5530	12	0.5166	0.5780	0.7119	0.5052
mtcCIR230	2	4	0.5932	7	0.6122	0.4960	0.6274	0.3889
mtcCIR268	2	5	0.3848	12	0.2695	0.6839	0.6680	0.6245
mtcCIR100	2	5	0.4860	8	0.5979	0.5635	0.7343	0.4671
mtcCIR73	2	4	0.3639	10	0.3836	0.6887	0.7410	0.6282
mtcCIR82	3	4	0.9400	6	0.8867	0.1143	0.1067	0.1107
mtcCIR167	3	4	0.5214	7	0.6335	0.5108	0.6512	0.3925
mtcCIR175	3	5	0.4966	12	0.5593	0.5613	0.6678	0.4657
mtcCIR204	3	4	0.8704	7	0.7807	0.2287	0.1827	0.2081
mtcCIR57	4	3	0.5419	6	0.5940	0.5289	0.6644	0.4241
mtcCIR107	4	3	0.5201	6	0.4818	0.6040	0.7445	0.5306
mtcCIR32	4	3	0.4850	6	0.6113	0.5360	0.6545	0.4277
mtcCIR242	4	3	0.9521	5	0.9175	0.0922	0.0693	0.0901
mtcCIR109	5	5	0.4946	8	0.6304	0.5342	0.6957	0.4255
mtcCIR267	5	3	0.8490	4	0.7083	0.2570	0.2813	0.2248
mtcCIR265	5	6	0.4443	11	0.3716	0.6473	0.7500	0.5766
mtcCIR80	5	3	0.5261	6	0.5765	0.5450	0.6678	0.4453
mtcCIR238	6	4	0.5811	9	0.4570	0.5526	0.5894	0.4767
mtcCIR136	6	7	0.3656	17	0.2143	0.7309	0.7755	0.6839
mtcCIR291	6	6	0.5286	13	0.5724	0.5398	0.6397	0.4385
mtcCIR290	6	7	0.4785	12	0.5033	0.6192	0.6490	0.5443
mtcCIR56	7	5	0.7374	9	0.5863	0.3996	0.3094	0.3381
mtcCIR93	7	5	0.4710	12	0.4539	0.6391	0.7235	0.5716
mtcCIR110	7	3	0.5446	6	0.3927	0.5997	0.8152	0.5328
mtcCIR186	7	3	0.5524	5	0.5804	0.5037	0.5874	0.3870
mtcCIR163	8	3	0.5409	4	0.6548	0.4983	0.6584	0.3759
mtcCIR189	8	7	0.4589	15	0.3980	0.6071	0.6020	0.5280
mtcCIR225	8	4	0.4908	8	0.4890	0.5954	0.7096	0.5142
mtcCIR258	8	2	0.8221	3	0.6577	0.2924	0.3289	0.2497
mtcCIR30	9	3	0.5964	6	0.5179	0.5189	0.5964	0.4290
mtcCIR79	9	4	0.5212	9	0.3497	0.6177	0.6928	0.5519
mtcCIR157	9	4	0.5470	9	0.6342	0.5160	0.6678	0.4054
mtcCIR58	9	5	0.5033	13	0.3679	0.6435	0.7124	0.5867
mtcCIR31	10	3	0.8364	4	0.6909	0.2743	0.2909	0.2377
mtcCIR91	10	3	0.6071	4	0.5170	0.4861	0.5408	0.3790
mtcCIR220	10	5	0.8163	9	0.7789	0.3178	0.1020	0.2965
mtcCIR229	10	6	0.5447	10	0.5704	0.5416	0.6735	0.4452
Σ		169		330				
Mean		4.22	0.5918	8.25		0.4963	0.5675	0.4232

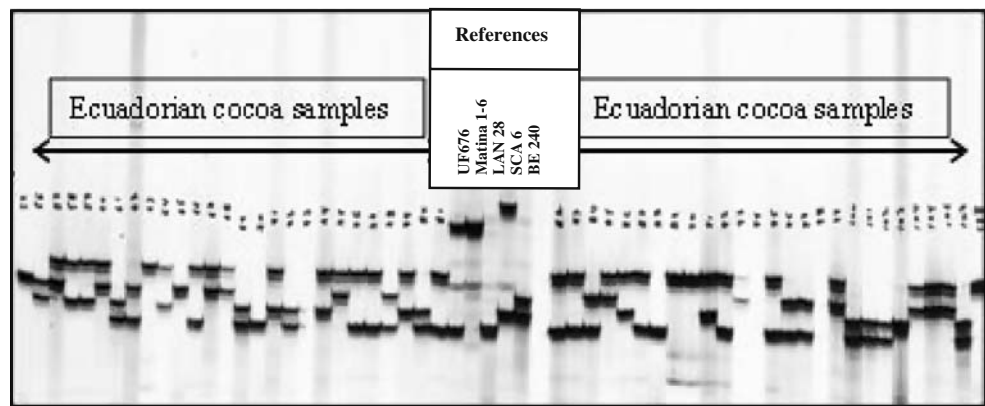
Allele parameters: N_e number of allele per locus, F_a frequency of the most common allele; genotype parameters: N_g number of effective genotype, F_g frequency of the most common genotype; gene diversity: H_e Nei's expected heterozygosity, H_o observed heterozygosity, PIC polymorphism information content

^a More information on the mapped SSRs markers used in this study can be found in Lanaud et al. (1999), Risterucci et al. (2000) and Pugh et al. (2004)

undertaken by the United Fruit Company in the 1940s. In the case of the EET-P collection, trees were collected in different expeditions and with broader objectives from 1940 to 2000. The CCAT is presently managed by the Uni-

versidad Técnica Estatal Quevedo and is located in the South coast region of Ecuador. The EET-P is located in the Central coast region of Ecuador and is managed by the Ecuadorian Agricultural Research Center (INIAP).

Fig. 2 Polymorphism profile of modern Ecuadorian cocoa samples using mtcCIR 136 located on chromosome 6



One old cacao tree with “arriba” flavour selected in a traditional farm (B240) was used as reference for Nacional (Fig. 1). Additionally, four genotypes representing a typical Trinitario (UF676) and its two probable ancestors, LAN28 (Criollo) and MAT-1-6 (Lower Amazon) (Motamayor et al. 2003), and the clone Scavina 6 (SCA6), a Forastero genotype resistant to witches’ broom and *Phytophthora* diseases originating from the Upper Amazon region of Peru, were included in this analysis (Table 1).

DNA isolation

Adult fresh leaves were collected and stored at -20°C until DNA extraction was performed. Genomic DNA was isolated following the method described by Risterucci et al. (2000) with some slight modifications. The DNA was purified by a silica-based anion-exchange resin as recommended by the supplier (NucleoBond® AX, Macherey-Nagel), and the concentration was estimated using a fluorometer (Fluoroskan Ascent 2.5) according to the manufacturer’s instructions. The proper DNA work dilution was established at 0.2 ng/ μl .

PCR amplification and SSR analysis

Polymerase chain reaction (PCR) amplification and SSR analyses were carried out following the methods described by Risterucci et al. (2000) and Pugh et al. (2004). A total of 40 SSR primers pairs (Table 2) were chosen to analyse the genetic diversity based upon their position in the most recent cacao genetic map (Pugh et al. 2004). Four loci per chromosome were selected. The PCR products were denatured and separated by electrophoresis in 0.5% TBE buffer at 60 W for approximately 2 h. The gel was dried for 25 min at 80°C and the results were revealed by autoradiography. SSR loci were scored individually and alleles were recorded by the presence of polymorphic DNA fragments (alleles) among the individuals of each population. Only those alleles that showed consistent amplification were used in the analysis of results and smeared or weak bands were ignored.

Data analysis

The SSR bands were scored as alleles and the genetic diversity was represented using multivariate analysis. A factorial analysis of correspondence (FAC; Benzecri 1973) was carried out using GENETIX V.4.05 software (Belkhir et al. 2004) and a graphic representation of the variation among individuals was obtained (Dervin 1992; Phillips 1995). POWER MARKER V.4.03 (Liu and Muse 2005) was used to calculate the allelic and genotypic frequencies. Based upon genetic dissimilarity indices and neighbour-joining method, a diversity tree was constructed using the DARWIN-5.0 software (Perrier et al. 2003). The following parameters were also computed: (1) Nei’s genetic parameters (Nei 1972, 1978): genetic distance, observed heterozygosity (H_{o}) and gene diversity, often referred to as expected heterozygosity (H_{e}); (2) genetic polymorphisms (Hartl and Clark 1997) was calculated by the effective number of alleles/locus (N_{e}); (3) F statistics were measured at different hierarchical levels according to Weir and Cockerhan (1984): Wright’s F_{IS} (correlation of alleles within individuals of one population), F_{ST} (correlation of alleles between individuals of a population compared to the whole populations) and F_{IT} (correlation of alleles within individuals “inbreeding”). In addition, to identify the two most

Table 3 Summary of statistic genetic parameters evaluated in the two Ecuadorian cacao germplasm banks

	CCAT	EET-P	Overall
$H_{\text{(exp)}}$	0.4853	0.4805	0.4829
$H_{\text{(obs)}}$	0.5922	0.5255	0.5588
$P_{0.95}$	0.9500	0.9750	.9625
$P_{0.99}$	1.0000	1.0000	1.0000
Alleles/locus	3.6500	4.2000	3.9250
F_{IS}	-0.2181	-0.0604	-0.1393
F_{IT}			-0.1169
F_{ST}			0.0369

$H_{\text{(exp)}}$ expected heterozygosity, $H_{\text{(obs)}}$ observed heterozygosity, $P_{0.95}$ and $P_{0.99}$ proportion of polymorphic loci when the most frequent allele does not exceed 95% and 99%.

likely parents of each sample, paternity inference was conducted using CERVUS V.2.0 software (Marshall et al. 1998), a Windows-based maximum likelihood program designed for use with co-dominant markers. Paternity assignment using likelihood techniques was determined at 95% confidence level and with 10,000 simulated offspring as suggested by Marshall et al. (1998).

Results

Level of polymorphism revealed in “modern” Nacional genotypes

Forty SSR primers were used to reveal polymorphism among a subset of 322 “modern” Nacional cacao accessions. As shown in Table 2, genetic variability was detected for all loci in the natural cacao populations covering most of the Pacific Coast Province in Ecuador. A total of 169 alleles were scored, ranging from two alleles at mtcCIR84 or mtcCIR258 to seven alleles at mtcCIR136, mtcCIR290 and mtcCIR189. The frequency of the most common allele was also determined per locus and ranged from 0.36 (mtcCIR73) to 0.95 (mtcCIR242). The number of effective genotype ranged from three at mtcCIR84 and mtcCIR258 to 17 genotypes at mtcCIR136. On the other hand, the frequency of the most common genotype ranged from 0.21 at mtcCIR136 to 0.92 at mtcCIR242. High levels of gene diversity ($H_e > 0.50$) were observed for 28 SSR markers among the 40 SSR studied (Table 2). Indeed, for most SSR loci, gene diversity (Nei's H_e) was apparently lower than the observed heterozygosity (H_o). Polymorphism information content (PIC) ranged from 0.09 (mtcCIR242) to 0.68

(mtcCIR136; Table 2), with the polymorphism profile illustrated in Fig. 2.

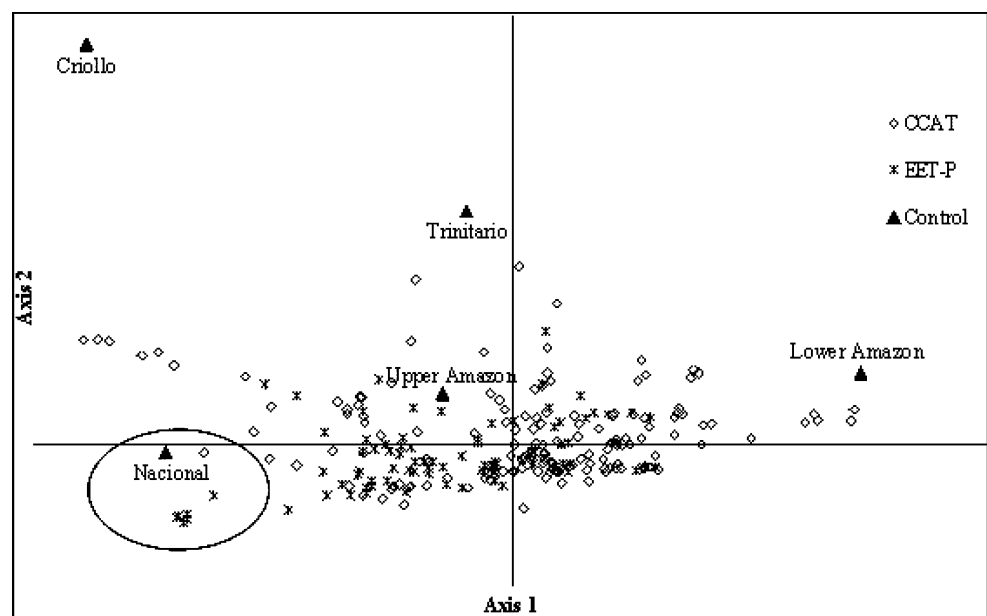
Genetic structure and diversity in modern Ecuadorian cacao population

Based on polymorphic SSR, eight accessions showed a higher level of homozygosity (>75%). Among these, four accessions showed 90% of homozygosity: L39-H66, Sa16, SNA1001 and SNA1003. Other accessions with high level of homozygosity were SAN409a and SNA604, both with 85% of homozygosity, SNA503 (77.5%) and B240 with 75% of homozygosity (Table 1). Individuals characterised by a higher level of homozygosity will be referred to as HoN in the further results or discussions reported in this paper.

No significant genetic distance was observed (0.031) between the collections from EET-P and CCAT on the basis of Nei's distance (1972). The genetic structure of the studied population was also analysed in term of F statistics. A summary of genetic statistics values measured for each population and the overall sample is shown in Table 3. In each population, Wright's F_{IS} measured the heterozygous deficiency within each sample, and negative values were obtained in each group collected. Overall, negative values were obtained for F_{IS} (−0.1393) and F_{IT} (−0.1169) in both cases, reflecting high values of heterozygosity level. The result of F_{ST} showed low level of differentiation among individuals of each population ($F_{ST}=0.0369$).

A FAC gives a visual representation of the relationships and variation among individuals (Fig. 3), and an analysis of genetic dissimilarity allowed the construction of a neighbour-joining tree (Fig. 4). As shown in Fig. 3, the

Fig. 3 Multidimensional scaling plot of a factorial analysis of correspondence (FAC) showing the genetic diversity among the Ecuadorian cacao samples. The first and second axes accounted for 8.14% and 6.82% of the variance, respectively



FAC (circled in Fig. 3). The diversity among HoN individuals was very low, although these trees have been collected from diverse geographic zones along the coast provinces of Ecuador

The results obtained in FAC (Fig. 3) and the neighbour-joining trees (Fig. 4) are quite consistent. Based on both analyses, we found that with the exception of the sample L39H36, all HoN individuals clustered together and with the Nacional reference genotype B240, showing a high genetic similarity among them, and the highest genetic divergence with the three reference genotypes representing the first potential foreign introductions: the Trinitario (UF676) and their two potential ancestors LAN28 and MAT-1-6.

Identification of potential parents of modern hybrid Nacional genotypes

Using paternity inferences from Cervus software, we attempted to identify the potential parents of the actual Nacional cacao population in Ecuador. Thus, the two most likely parents were identified for each individual. The first most likely parent was identified at 95% confidence for 78% of individuals. According to the two most likely parents assigned for each individual from our paternity analysis, we could clarify the genetic structure of the cacao population analysed in this study. Based on this analysis, we could propose several classes of genetic crosses which probably occurred during the last century in Ecuador and from which the modern Nacional genotypes belong to:

- crosses between two HoN genotypes (22.91%);
- crosses HoN × foreign genotypes (3.94%);
- backcrosses (HoN × foreign genotypes) × HoN (11.48%);
- backcrosses (HoN × foreign genotypes) × foreign genotypes (40.01%);
- crosses (HoN × foreign genotypes) × (HoN × foreign genotypes) (13.45%);
- crosses between only foreign genotypes (8.21%).

About 12% of the individuals, distributed in the several classes, involve SCA6 as one of their probable parents.

Our paternity results indicated also that the HoN cacao samples, Sa16, SNA1001, SNA1003, SNA409a, SNA604, SNA503 and B240, could be at the origin of more than 67% of the modern Nacional cacao population associated to various levels of introgression of foreign genotypes. They were also identified as the most likely parents at the origin of 23% of the population which result only from crosses between HoN, with a statistical confidence of 95% (Marshall et al. 1998). The next step was to determine the proportion of alleles shared between the HoN individuals previously identified. Table 4 shows that the proportion of alleles shared among HoN individuals ranges from 0.955 to

0.800, confirming the high genetic relationships between these cacao materials.

Discussion—conclusion

The cacao accessions used in this study were originally sampled in the main cocoa-producing areas of the Ecuadorian Coast Region and are maintained as living collections at EET-P and CCAT. No significant level of genetic differentiation between these two collections was evidenced. As also demonstrated by *F* statistics, the genetic structure of modern Nacional cacao population is characterised by a high level of heterozygosity in agreement with the historical events of introduction of foreign germplasm, since the end of 1890s, and its subsequent gene flow with the native Nacional cacao population (Van Hall 1932; Fowler and Lopez 1949). From our results, most of the cacao trees in the germplasm banks of Pichilingue (EET-P) and Tenguel (CCAT) appeared as hybrids that mainly shared alleles with the typical Trinitario type UF676 and with the HoN individuals, confirming the hybrid nature of modern Nacional cacao.

The modern Nacional genotypes presented a wide range of variations. Among them, a few highly homozygous cacao accessions (HoN) were also identified, in contrast with the hybrid nature of most of them. These HoN accessions showed the lowest level of introgression of Trinitario alleles, but shared the same alleles, at most loci, between them and with the reference genotype of Nacional (B240). Such highly homozygous individuals have been also identified by Lerceteau et al. (1997b) and Loor et al. (2002) using restriction fragment length polymorphism and SSR markers, respectively. The “arriba” flavour is present in all HoN accessions identified in this study (Deheuvels et al. 2004) with the exception of accession L39-H66 which is yet to be evaluated. Using paternity analyses, these genotypes appeared to be at the origin of more than 90%

Table 4 Proportion of shared alleles among the HoN putative parents at the origin of the modern Nacional cacao population analysed in this study

	SNA 1003	SNA 1001	SNA 604	SNA 503	SNA 409a	Sa 16	B 240
SNA1003	1.000						
SNA1001	0.932	1.000					
SNA604	0.955	0.910	1.000				
SNA503	0.890	0.910	0.890	1.000			
SNA409a	0.932	0.955	0.955	0.837	1.000		
Sa16	0.910	0.890	0.910	0.800	0.850	1.000	
B240	0.910	0.910	0.932	0.837	0.935	0.910	1.000

of the population studied. This last result suggests that these HoN individuals, among which some are more than 80 years old, could represent a few remaining samples from the native Nacional cacao cultivated in Ecuador before foreign introductions.

Despite the wide geographical area where the samples were collected, the HoN genotypes showed a high genetic similarity. This result could indicate a pronounced bottleneck effect on the original Nacional cacao populations along of the Coast Provinces of Ecuador. We can therefore assume that at the origin of Nacional, the size of the ancestral population was very small. The differentiation of this homozygous group may have occurred through allele fixation (genetic drift). Subsequently, intervention of man, through cultivation, may have fixed and maintained some phenotypes, as hypothesised by Motamayor et al. (2002) for the Criollo group. A very low diversity associated with a high homozygosity was also observed for the “ancient” Criollo trees from Central America, in contrast to the higher diversity found in modern Criollo and Trinitario populations (Motamayor et al. 2002).

The high level of homozygosity found in some Ecuadorian cacao accessions could be explained by the self-compatibility characteristic of the oldest Nacional cacaos from Ecuador (Quiroz 1990; Pastorelly 1992; Enriquez 1992; Loor 1998). However, an incompatibility system is present in *T. cacao*, but with varying effect depending on the genetic origins. The compatibility of modern hybrid forms of Nacional appears to vary, most likely according to the introgressed part of the Trinitario genome.

According to the history of foreign cacao introduction in Ecuador, approximately one century separates the first hybridisation events of foreign cacao with the native Nacional from the modern Ecuadorian cacao populations. Thus, probably no more than four generations of recombination occurred during this period. Paternity analyses showed that different hierarchic levels of crosses have occurred during the last century in the Ecuadorian cacao plantation from direct crosses between foreign genotypes and the native variety to more advanced hybridisation generations. Thus, paternity analyses allowed us to refine our understanding of the genetic structure of modern Nacional. From our results, we estimate that more than 65% of the cacao population analysed in this study corresponds to crosses of second generation. It is important to indicate that almost the totality of cacao accessions from Tenguel were collected in the 1950s because of their similarity with the Nacional. Currently, the majority of traditional cacao plantations are most likely established with crosses of fourth or fifth generation.

As a consequence of the introduction of foreign germplasm and the widespread cultivation of varieties unrelated to Nacional, like the CCN-51, the fine flavour

quality of Ecuadorian cocoa and the number of native trees of Nacional have decreased alarmingly (Loor et al. 2002). Consequently, 25% of the cocoa production from Ecuador has been downgraded from “fine flavour” cocoa to “bulk cocoa” with a lower price in the international market. The present study provides important information for long-term conservation and management of *T. cacao* in Ecuador, especially with regard to identifying non-introgressed Nacional genotypes, with the aim to incorporate these genotypes in new cacao breeding strategies.

The hybrid forms between Nacional and Trinitario genotypes currently present in the Ecuadorian germplasm banks offer a segregating population adapted to carry out association mapping studies to identify the genetic bases of “fine cocoa flavour” of Nacional. This knowledge will facilitate the improvement of new varieties associating Nacional quality traits and disease resistance provided by some foreign cacao genotypes using a marker-assisted strategy.

The origin of Nacional *T. cacao* cultivated in Ecuador from 1600 is currently unknown. The identification of wild ancestors at the origin of still existing cultivated Nacional types could allow for the enlargement of the narrow genetic base of non-introgressed Nacional trees identified in this study, favouring the breeding strategies for the modern Nacional population.

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References

- Allen BJ, Lass AR (1983) London cocoa trade Amazon project. Final report phase 1. Cocoa Grow Bull 34:1–72
- Ampuero E (1960) Progresos alcanzados en el Ecuador en el estudio de selección para resistencia de la Escoba de Bruja. Proceeding of the Inter American Cacao Conference. Bowen, Trinidad, pp 166–173
- Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F (2004) GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations, Laboratoire Génome, Populations, Interactions; CNRS UMR 5000. Université Montpellier II, France
- Benzecri JP (1973) L'analyse des données. Tome 2: L'analyse des correspondances. Dunod, Paris, France
- Cheesman EE (1944) Notes on the nomenclature, classification and possible relationships of cacao populations. Trop Agric 21:144–159
- Deheuvels O, Decazy B, Perez R, Roche G, Amores F (2004) The first Ecuadorean Nacional cocoa collection based on organoleptic characteristics. Trop Sci 44:23–27

- Dervin C (1992) Analyses des Correspondances. Comment interpréter les résultats? Collection STAT-ITCF. Paris, France
- Enriquez GA (1992) Characteristics of cacao “Nacional” of Ecuador. International Workshop on Conservation, Characterisation and Utilisation of Cocoa Genetic Resources in the 21st century. Port of Spain, Trinidad 13–17th September. The Cocoa Research Unit, The University of the West Indies, pp 269–278
- Fowler RL, Lopez GH (1949) The cacao industry of Ecuador. Foreign Agriculture Report No. 34, US Department of Agriculture, Washington, USA
- Hartl DL, Clark AG (1997) Principles of population genetics, 3rd edn. Sinauer, Sunderland, Massachusetts, USA
- Hurst WJ, Tarka SM, Powis TG, Valdez F, Hester RT (2002) Archaeology: cacao usage by the earliest Maya civilization. *Nature* 418:289–290
- Lanaud C, Risterucci AM, Pieretti I, Falque M, Bouet A, Lagoda PJJ (1999) Isolation and characterisation of microsatellites in *Theobroma cacao* L. *Mol Ecol* 8:2141–2152
- Lanaud C, Risterucci AM, Pieretti I, N’Goran JAK, Fargeas D (2004) Characterization and genetic mapping of resistance and defence gene analogs in cocoa (*Theobroma cacao* L.). *Molecular Breeding* 13:211–227
- Laurent V, Risterucci AM, Lanaud C (1994) Genetic diversity in cocoa revealed by cDNA probes. *Theor Appl Genet* 88:193–198
- Lerceteau E, Robert T, Pétiard V, Crouzillat D (1997a) Evaluation of the extent of genetic variability among *Theobroma cacao* L accessions using RAPD and RFLP markers. *Theor Appl Genet* 95:10–19
- Lerceteau E, Flipo S, Pétiard V, Crouzillat D (1997b) Genetic differentiation among Ecuadorian *Theobroma cacao* L. accessions using molecular and morphological analyses. *Euphytica* 95:77–87
- Liu K, Muse VS (2005) Power marker: integrated analysis environment for genetic marker data. *Bioinformatics* 21:2128–2129
- Loor RG (1998) Obtención de híbridos de cacao tipo Nacional provenientes de materiales de alta productividad y resistentes a enfermedades. Tesis de Ingeniero Agrónomo. Universidad Técnica de Manabí, Portoviejo, Ecuador
- Loor RG, Saunders JA, Amores F (2002) Characterization of Ecuadorian cacao (*Theobroma cacao* L.) Mid Atlantic Plant Molecular Biology Society Proceedings. Beltsville, Maryland, USA 19:18
- Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Mol Ecol* 7:639–655
- Motamayor JC, Risterucci AM, Lopez PA, Ortiz CF, Moreno A, Lanaud C (2002) Cacao domestication I: the origin of the cacao cultivated by the Mayas. *Heredity* 89:380–386
- Motamayor JC, Risterucci AM, Heath M, Lanaud C (2003) Cacao domestication II: progenitor germplasm of Trinitario cacao cultivar. *Heredity* 91:322–330
- Nei M (1972) Genetic distance between populations. *Am Nat* 106:283–292
- Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89:583–590
- N’Goran JAK, Laurent V, Risterucci AM, Lanaud C (1994) Comparative genetic diversity of *Theobroma cacao* L. using RFLP and RAPD markers. *Heredity* 73:589–597
- N’Goran JAK, Laurent V, Risterucci AM, Lanaud C (2000) The genetic structure of cocoa populations (*Theobroma cacao* L.) revealed by RFLP analysis. *Euphytica* 115:83–90
- Pastorelly RD (1992) Evaluación de algunas características del cacao tipo Nacional, en la zona de Tenguel. Tesis Ingeniero Agrónomo. Universidad Agraria del Ecuador. Guayaquil, Ecuador
- Perrier X, Flori A, Bonnot F (2003) Data analysis methods. In: Hamon P, Seguin M, Perrier X, Glaszmann JC (eds) Genetic diversity of cultivated tropical plants. Enfield, Science Publishers, Montpellier, France, pp 43–76
- Phillips D (1995) Correspondence analysis. Social research update. Department of Sociology, University of Surrey, Guildford GU2 7XH, England
- Pound FJ (1938) Cacao and witches’ broom disease (*Marasmius perniciosus*) of South America, with notes on other species of *Theobroma*. Yuille’s Printerie, Port of Spain, Trinidad and Tobago, 9–49. Reprinted 1982. *Arch Cocoa Res* 1:21–64
- Pound FJ (1945) A note about the cacao populations of South America. Report and Proceedings Cocoa Research Conference, London. *Colonial* 192:95–7. Reprinted 1982. *Arch Cocoa Res* 1:93–97
- Pugh T, Fouet O, Risterucci AM, Brottier P, Abouladze M, Deletrez C, Courtois B, Clement D, Larmande P, N’Goran JAK, Lanaud C (2004) A new cacao linkage map based on codominant markers: development and integration of 201 new microsatellite markers. *Theor Appl Genet* 108:1151–1161
- Quiroz J (1990) Estudio de la compatibilidad en algunos cultivares de cacao (*Theobroma cacao* L.). Tesis de Ing. Agr. Universidad Técnica de Babahoyo, Ecuador
- Quiroz J (2002) Caracterización molecular y morfológica de genotipos superiores de cacao nacional (*Theobroma cacao* L.) de Ecuador. Tesis Ms.Sc. Centro Agronómico Tropical de Investigación y Enseñanza. Turrialba, Costa Rica
- Risterucci AM, Grivet L, N’Goran JAK, Pieretti I, Flament MH, Lanaud C (2000) A high-density linkage map of *Theobroma cacao* L. *Theor Appl Genet* 101:948–955
- Rorer JB (1926) Ecuador cacao. *Trop Agric (Trinidad)* 3:69–69
- Soria JV (1970) Principal varieties of cocoa cultivated in tropical America. *Cocoa Grow Bull* 19:12–21
- Van Hall CJ (1932) *Cocoa*, 2nd edn. MacMillan, London, pp 304–320
- Vera BJ (1987) Antecedentes históricos. Manual del cultivo de cacao. INIAP, EET-Pichilingue, Quevedo, Ecuador, pp 6–9
- Vera BJ (1993) Botánica del cacao. Manual del cultivo de cacao 2da edición. INIAP, EET-Pichilingue, Quevedo, Ecuador, pp 10–15
- Weir BS, Cockerhan CC (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution* 38:1358–1370