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Genetic diversity and geographic pattern in early South American cotton domestication

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Abstract Amplified fragment length polymorphism fingerprinting was applied to survey the genetic diversity of primitive South American *Gossypium barbadense* cotton for establishing a possible link to its pre-Columbian expansion. New germplasm was collected along coastal Peru and over an Andean transect in areas where most of the archaeological evidence relating to cotton domestication has been recorded. Gene bank material of three diploid (*G. raimondii*, *G. arboreum*, and *G. herbaceum*) and four allotetraploid cotton species (*G. hirsutum*, *G. mustelinum*, *G. tomentosum* and additional *G. barbadense*) was added for inter- and intra-specific comparison. Eight primer combinations yielded 340 polymorphic bands among the 131 accessions. The obtained neighbor joining and unweighted pair-group method with arithmetic means are in full agreement with the known cytogenetics of the tetraploid cottons and their diploid genome donors. The four tetraploid species are clearly distinct based on taxonomic classification. The genetic diversity within *G. barbadense* reveals geographic patterns. The locally maintained cottons from coastal Peru display a distinct genetic diversity that mirrors their primitive agro-morphological traits. Accessions from the northernmost coast of Peru and from southwestern (SW) Ecuador cluster basal to the east-of-Andes accessions. The remaining accessions from Bolivia, Brazil, Columbia, Venezuela, and the Caribbean and Pacific islands cluster with the east-of-Andes accessions. Northwestern Peru/SW Ecuador (the area flanking the Guayaquil gulf) appears to be the center of the primitive domesticated *G. barbadense* cotton from where it spread over the Andes and expanded into its pre-Columbian range.

This publication is dedicated to Prof. Dr. Drs.h.c. Gerhard Röbbelen on the occasion of his 75th birthday

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Introduction

The cotton genus *Gossypium* L. consists of about 45 diploid and five tetraploid species forming a monophyletic group (Fryxell 1992; Wendel and Cronn 2003). Eight diploid genomes (A–G and K), each comprising 13 chromosome pairs, have been identified (Endrizzi et al. 1985). The native distribution of all five tetraploid cottons ($2n = 4x = 52$, AADD) is restricted to the New World, although its emergence involves a combination of an Old World A-genome (derived from an ancestor of *G. arboreum* L. and *G. herbaceum* L.) and a New World D-genome (derived from an ancestor of *G. raimondii* Ulbrich) (Stephens 1944; Phillips 1963, 1964; Seelanan et al. 1997; Cronn et al. 1999; Liu et al. 2001). The underlying trans-oceanic dispersal of the A-genome donor and the timing of the polyploidization event has been controversial, but the current view is that a single Mid-Pleistocene (1–2 mya) polyploidization event has occurred (Phillips 1963; Wendel and Albert 1992; Senchina et al. 2003). It is further assumed, that the emergence of tetraploid cotton was followed by long-distance separation into five species, three of which are truly wild species—*G. mustelinum* Miers ex Watt (limited distribution in northeast Brazil; Wendel et al. 1994), *G. darwinii* Watt (endemic to the Galapagos Islands; Wendel and Percy 1990), and *G. tomentosum* Nutall ex Seemann (endemic to the Hawaiian Islands; DeJoode and Wendel 1992). The other two species have undergone domestication—*G. hirsutum* L. (predominantly distributed in Meso America and the Caribbean) and *G. barbadense* L.(main distribution in South America and the Caribbean). Although molecular diversity within

and among the tetraploid cottons is limited (Small et al. 1999; Wendel and Cronn 2003), data indicate that *G. mustelinum* forms a basal branch in the phylogram of the tetraploids, while the remaining four species form two sister groups—one constituted by *G. hirsutum* and *G. tomentosum* and the other by *G. barbadense* and *G. darwinii* (Small et al. 1998; Liu et al. 2001).

The elongated epidermal seed trichomes of *G. herbaceum*, *G. arboreum*, *G. hirsutum*, and *G. barbadense* were recognized by humans as useful spinnable fibers and led to four independent domestication events (Wendel 1995; Brubaker et al. 1999). Archaeological evidence of cotton remains in association with human settlements dates back to the sixth millennium BC for the Greater Indus area in the Old World (Moulherat et al. 2002) and to layers dated to 6,400–5,000 years BP for the Zaña river valley in Peru in the New World (Rossen et al. 1996; Piperno and Pearsall 1998). The cotton *G. hirsutum* has received much attention in this matter due to its prime economic importance in modern cotton production (e.g., Brubaker and Wendel 1994). The South American *G. barbadense* domestication has also been studied, but mainly by means of archaeological approaches. Cotton remains along the coast of Peru and Ecuador are abundant and show a gradient in traits from wild to domesticated (Stephens 1973; Stephens and Moseley 1973, 1974). Purely wild *G. barbadense* forms are mentioned in the literature (e.g., Percy and Wendel 1990) but are not clearly defined in terms of fitness-related traits for wild survival. The wild-to-domesticated continuum is, according to Percy and Wendel (1990), delimited in four general categories in gene bank collections: (1) wild; (2) 'dooryard cottons' or 'commensals', meaning single plants found near habitations thought to be derived directly from local wild populations; (3) landraces; (4) improved modern cultivars. The maritime subsistence for the Andean civilizations depending in part on cotton fishing-nets has led to the perception that the domestication of *G. barbadense* took place along the coastline, but cotton is also found in more inland located sites like Caral (Solis et al. 2001). An allozyme study (Percy and Wendel 1990) yielded geographic clusters largely congruent with this maritime-based scenario, but only an unspecified geographic domestication region 'north-western South America west of the Andes' including Colombia, Ecuador, Peru, and Bolivia was reported.

Until now no DNA marker-based study has assessed the intra-specific genetic diversity of *G. barbadense* with the objective of describing a geographic pattern that can help in understanding the pre-Columbian domestication events of this species. To this end we conducted a new field collection of dooryard/feral cottons of *G. barbadense* in Peru and added material from the USDA Cotton Collection in order to have a representative sample from its pre-Columbian range. We included the two wild tetraploid species *G. mustelinum* and *G. tomentosum* and the domesticated *G. hirsutum* to get an inter-specific comparison at the polyploid species level. We also added the three diploid species involved in the polyploidization event so that we could directly compare the diploid and the derived polyploid cottons. We applied the widely used AFLP (amplified fragment length polymorphism, Vos et al. 1995) fingerprinting method, which has been used both to pinpoint domestication events (Heun et al. 1997) and to reveal relationships among diploid and tetraploid cottons (Abdalla et al. 2001). In addition, our study provides information on the genetic diversity of *G. barbadense*, which at the present time is grown in the 'Andean' countries in general and in Peru in particular, with a potential value for conservation strategies.

Material and methods

The cotton accessions analyzed in this investigation were obtained in two different ways. (1) Seed of 77 accessions was obtained from the Cotton Collection of the USDA-ARS, College Station, Texas, via Dr. E. Percival, who selected the accessions based on our wishes on which area to cover. (2) New material from Peru was collected by the authors. A collecting permit was obtained from the Instituto Nacional de Recursos Naturales (INRENA) to collect *Gossypium* spp. in the departments of Ancash, La Libertad, Lambayeque, Piura, Tumbes, Cajamarca, Amazonas, San Martin, Loreto, Ucayali and Huanuco. A Material Transfer Agreement (MTA) was signed to send collected material to Norway. Voucher specimens were deposited both at the Herbario Weberbauer at the Universidad Nacional Agraria La Molina and at the Herbarium Truxillense at the Universidad Nacional de Trujillo. Seed samples were deposited in the Instituto Nacional de Investigacion Agraria (INIA) Lima, Peru. Collecting data included passport information on each locality: longitude, latitude and altitude data were obtained using a global positioning system (GPS) receiver (Garmin, eTrex Summit), and phenotypic observations were added. For the DNA extraction, one small, fresh leaf was taken from each cotton plant and dried in sealed plastic bags containing dry silica gel. Seed was collected from those plants having mature bolls. Branches were cut off from a few representative plants as herbarium vouchers. In total, 100 cotton accessions were collected in this way, six of which are *G. raimondii* while the rest are *G. barbadense*.

DNA extraction

From those accessions with seed, three kernels were sown in separate pots under controlled conditions [25°C, 14/10-h (light/dark) photoperiod, pot soil kept constantly humid] at the Agricultural University of Norway (NLH), Ås, Norway, as requested by the Norwegian authorities (import permit obtained from Landbruks-tilsynet, Ås, Norway). The seeds germinated between

3 days and 3 weeks after sowing. One plant from every accession was used for DNA extraction. The cotyledons (2–6 cm^2 in size) of the plant selected were cut just above the meristem and air-brushed to avoid possible impurities. The tissue was ground in liquid nitrogen and transferred for storage to a −80°C freezer. A total of 131 accessions representing seven *Gossypium* species were included in the final analysis: 64 samples obtained from the USDA-ARS gene bank (Table 1) and 67 from the material collected in Peru (Table 2). Five samples originate from silica gel-dried material, whereas the remaining ones were obtained from freshly harvested tissue obtained from seed. DNA was extracted using a DNeasy Plant Mini kit from QIAGEN (Valencia, Calif.). The quality and quantity of the undigested DNA obtained was checked and quantified against undigested λ-DNA (Fermentas, Vilnius, Lithuania).

AFLP fingerprinting

AFLP fingerprinting was performed according to the original protocol of Vos et al. (1995) omitting the use of streptavidin beads for selecting *Eco*RI-biotinylated DNA fragments. Briefly, 500 ng DNA per sample was digested for 2 h at 37°C by 5 U *Eco*RI (Fermentas) and 5 U *Mse*I (NEB) in 1× RL buffer (10 m *M* Tris-acetate pH 7.5, 10 m *M* Mg acetate, 50 m *M* potassium acetate, 5 m *M* DTT). The ligation of adapters fitting the cutting sites was done by adding T4 DNA ligase (Fermentas), 10 m *M* ATP, 10× RL buffer and by incubating the mixture for 3 h at 37°C. Thereafter, the selective pre-amplification with primers complementary to adapter sequence with a one-base extension was performed with E01 and M02. This primer combination was chosen based on the results of Abdalla et al. (2001) and Lacape et al. (2003). The PCR reactions [5.0 µl of the above-mentioned DNA digestion/ligation mix, 1.5 µl of each +1 primer (75 ng), 1.0 µl 10 m *M* dNTP mix, 0.25 µl *Taq* polymerase (5 U/µl) (Fermentas), 5.0 µl 10× PCR-buffer, 5.0 µl 25 m *M* $MgCl_2$, in a total volume of 50 µl] were carried out on a PTC-200 thermocycler (MJ Research, Waltham, Mass.) at the temperature-time profile given by Vos et al. (1995). The resulting pre-amplification products were diluted 1:19 prior to the selective +3/+3 amplification. Primer labeling was done by phosphorylating the 5′-end of the E-primers with radioactive γ-[^{33}P] and T4 kinase (Fermentas). The selective amplification mix with +3/+3 primers [5 µl of the diluted pre-amplified DNA, 0.5 µl *E primer, 0.6 µl M primer (see below for primer sequences), 0.4 µl dNTP (10 m *M*), 2.0 µl PCR buffer (10×), 1.2 µl $MgCl_2$ (25 m *M*), 0.1 µl *Taq* polymerase (5 U/µl), in a total volume of 20 µl] was performed on a GeneAmp thermocycler (Applied Biosystems, Foster City, Calif.) according to Vos et al. (1995). Loading buffer (99% formamide, 10 m *M* EDTA, pH 8, 1.0 mg/ml xylene cyanol FF, 1.0 mg/ml bromophenol blue) was added to the final PCR products (50%), and the DNA was denatured by heat prior to running the samples (3 µl) on 5% polyacrylamide (acrylamide:bis, 19:1) gels under denaturing conditions. The electrophoresis apparatus (model S2/S2001, Gibco BRL Life Technologies, Gaithersburg, Md.) was filled with 2× TBE in the lower chamber and with 1× TBE in the upper chamber. For comparison 1.5 µl of a labeled 30- to 330-bp AFLP ladder from Invitrogen (Carlsbad, Calif.) was loaded on the polyacrylamide gel electrophoresis (PAGE). The gels were run at 80 W for 90 min and thereafter fixed, dried, and exposed to X-ray film (Kodak BioMax MR) for 8–48 h, depending on radiation intensity. X-ray films were developed with a AGFA Curix 60. The resulting images were scored by the naked eye for the presence/absence of AFLP bands using a light box. Overlapping samples as well as overlapping PCR reactions guaranteed that only reproducible AFLPs were scored and that the different PAGEs were well aligned. Based on a screening of 40 E01/M02 primer combinations with four samples (results not presented here), the following primer combinations were used to fingerprint all 131 cottons: E35/M48, E38/M48, E38/M49, E40/M50, E40/M59, E40/M60, E41/M47, E41/M60 (Invitrogen). The core sequence for the *Eco*RI primers is 5′-GACTGCGTACCAATTCNNN-3′ and 5′-GATGAGTCCTGAGTAANNN-3′ for *Mse*I. The −3′ end sequences for the primers used in this study are as follows: E01-A, E35-ACA, E38-ACT, E40-AGC, E41-AGG, M02-C, M47-CAA, M48-CAC, M49-CAG, M50-CAT, M59-CTA, M60-CTC.

Data analysis

The X-ray films were scored in a binary manner using "1" to indicate the presence of a polymorphic AFLP band and '0' to indicate its absence. Only unambiguous bands within an 80- to 800-bp range were scored. The data were assembled in a matrix and analyzed for pairwise genetic similarity (Gs) using the Dice similarity coefficient (Dice 1945; Nei and Li 1979) in the SIMQUAL option of NTSYS-PC V.2.11F (Rohlf 2000). The Dice similarity is given by the equation $Gs_{ij} = 2a/(2a+b+c)$ where Gs_{ij} expresses the genetic similarity between line *i* and *j*, *a* is the number of bands present in both accessions, *b* is the number of bands present in *i* and absent in *j*, and *c* is the number of bands absent in *i* and present in *j*. Genetic distance (Gd) measures were obtained for all Gs coefficients using the equation $Gd_{ij} = 1 - Gs_{ij}$. The Dice-based Gd matrix was used for the neighbor joining (NJ) method of Saitou and Nei (1987). The Gs data were used for calculating unweighted pair-group method with arithmetic means (UPGMA) (Sokal and Sneath 1963). The trees were calculated with the program NTSYS and PAUP (ver. 4.0b10 for Macintosh; Swofford 1998). The robustness of the obtained trees was evaluated by comparing the different data analyses [NJ, UPGMA, and principal coordinate analysis (PCA)] and by bootstrapping (Felsenstein 1985) with 1,000 re-samplings (PAUP).

Table 1 *Gossypium* accessions from the USDA-ARS Cotton Collection, College Station, Texas, included in this study

Species	Inventory no.	Accession no.	Origin country	Locality
G. hirsutum	TEX 624	PI 154104	Mexico	Chiapas
	TEX 2335	PI 607661	Costa Rica	Playa Ostinal, Nicoya Peninsula
G. mustelinum	$AD_4$7	P1 530741	Brazil	Unspecified
G. tomentosum	$AD_3$17	PI 530723	Hawaii	Unspecified
G. raimondii	$D_5$4	PI530901	Peru	Unspecified
G. herbaceum	$A_1$9	PI 175456	Turkey	Unspecified
	$A_1$17	PI 408775	Afghanistan	Unspecified
	$A_1$24	PI 408782	Uzbekistan	Unspecified
	$A_1$137	PI 630012	China	Unspecified
G. arboreum	A_2 190	PI 615699	Myanmar	Unspecified
	A_2 192	PI 615701	Iran	Unspecified
	$A_2$244	PI 615745	Pakistan	Unspecified
	$A_2$248	PI 614759	Russia	Unspecified
	A_2 256	PI 615757	Thailand	Unspecified
G. barbadense	Gb 61	PI 528320	Ecuador	Manabi
	Gb 619	PI 608062	Ecuador	Manabi
	Gb 602	PI 528118	Ecuador	Los Rios
	Gb 618	PI 608061	Ecuador	Guayas
	Gb 672	PI 608108	Ecuador	Guayas
	Gb 763	PI 608155	Ecuador	Guayas
	Gb 307	PI 528155	Ecuador	Guayas
	Gb 308	PI 528156	Ecuador	Guayas
	Gb 315	PI 528163	Ecuador	Guayas
	Gb 353	PI 528201	Ecuador	El Oro
	Gb 354	PI 528202	Ecuador	Loja
	Gb 673	PI 608109	Ecuador	Loja
	Gb 674	PI 608110	Ecuador	Loja
	Gb 675	PI 608111	Ecuador	Loja
	Gb 395	PI 528240	Ecuador	Unspecified
	Gb 594	PI528110	Ecuador	Unspecified
	Gb 596	PI528112	Ecuador	Unspecified
	Gb 63	PI528322	Ecuador	Unspecified
	Gb 314	PI 528162	Ecuador	Unspecified
	Gb 352	GB 352	Ecuador	Unspecified
	Gb 624	PI 608067	Ecuador	Galapagos Islands
	Gb 627	PI 608070	Ecuador	Galapagos Islands
	Gb 625	PI 608062	Ecuador	Galapagos Islands
	Gb 667	PI 358963	Ecuador	Galapagos Islands
	Gb 414	PI 528259	Peru	Tumbes
	Gb 819	PI 435250	Peru	Piura, S05°30/W80°30
	Gb 989	GB 989	Peru	Lambayeque
	Gb 540	PI 528056	Peru	La Libertad
	Gb 541	PI 528057	Peru	La Libertad
	Gb 758	PI 608151	Peru	Unspecified
	Gb 417	PI 528262	Peru	Unspecified
	Gb 199	PI 276476	Brazil	Saenz Pena, Chaco
	Gb 1048	PI 608347	Brazil	Federal district
	Gb 195	PI 528376	Brazil	Unspecified
	Gb 196	PI 528376	Brazil	Unspecified
	Gb 427	PI 528276	Brazil	Unspecified
	Gb 1029	PI 608345	Brazil	Unspecified
	Gb 1021	PI 543760	Bolivia	Pando, S11°00/W68°45
	Gb 347	PI 528195	Bolivia	Unspecified
	Gb 706	PI 608132	Bolivia	Unspecified
	Gb 1012	PI 608340	Venezuela	Zulia
	Gb 371	PI 528218	Colombia	Unspecified
	Gb 388	PI 528232	Colombia	Unspecified
	Gb 523	PI 528039	Colombia	Unspecified
	Gb 380	PI 528227	Dominica	Unspecified
	Gb 275	PI 528133	Haiti	Unspecified
	Gb 480	PI 527996	Haiti	Unspecified
	Gb 216	PI 528378	US	Hawaii
	Gb 444	PI 528287	US	Hawaii
	Gb 451	PI 528288	US	Hawaii

Table 2 Sixty six *Gossypium barbadense* and one (P6) *Gossypium raimondii* newly collected from Peru, included in the study

Inventory no.	Accession no.	Department	Latitude	Longitude	W/E of Andes
P1	OWMW-001	La Libertad	S.08°08'29.5''	W.79°00'07.4''	West
P3	OWVM-003	La Libertad	S.08°06'42.1''	W.79°02'17.7''	West
P6	OWCD-006	Cajamarca	S.07°26'54.9''	W.78°56'40.6''	West
P8	OWVM-008	La Libertad	S.08°08'28.7''	W.79°00'07.7''	West
P10	OWVM-010	La Libertad	S.08°08'32.2''	W.79°00'04.1''	West
P11	OW-011	Lambayeque	S.06°30'48.6''	W.79°50'51.6''	West
P15	OWMO-015	Lambayeque	S.06°41'20.2''	W.79°27'38.2''	West
P17	OWMO-017	Lambayeque	S.06°32'04.9''	W.79°35'38.1''	West
P18	OWMO-018	Lambayeque	S.06°31'36.5''	W.79°59'48.7''	West
P21	OWMO-021	Lambayeque	S.06°31'31.0''	W.79°59'34.1''	West
P22	OWMO-022	Lambayeque	S.06°31'31.0''	W.79°59'34.1''	West
P23	OWMO-023	Lambayeque	S.06°30'26.9''	W.79°59'13.0''	West
P25	OWMO-025	Lambayeque	S.06°44'17.1''	W.79°50'53.5''	West
P26	OW-026	Lambayeque	S.06°52'26.7''	W.79°52'01.7''	West
P27	OW-027	Lambayeque	S.06°52'26.7''	W.79°52'01.7''	West
P28	OW-028	Lambayeque	S.06°52'26.7''	W.79°52'01.7''	West
P29	OW-029	Lambayeque	S.06°52'26.7''	W.79°52'01.7''	West
P31	OW-031	Piura	S.05°13'27.9''	W.80°39'20.1''	West
P32	OW-032	Piura	S.05°11'48.2''	W.80°37'44.5''	West
P34	OW-034	Piura	S.05°29'35.3''	W.80°45'00.1''	West
P35	OW-035	Piura	S.04°06'03.5''	W.81°01'40.2''	West
P36	OW-036	Piura	S.04°06'51.1''	W.80°58'50.9''	West
P37	OW-037	Piura	S.04°06'51.1''	W.80°58'50.9''	West
P39	OW-039	Tumbes	S.03°38'10.2''	W.80°25'14.6''	West
P40	OW-040	Tumbes	S.03°38'10.2''	W.80°25'14.6''	West
P43	OWZH-043	Lambayeque	S.05°55'08.2''	W.79°45'16.8''	West
P44	OWZH-044	Lambayeque	S.05°36'47.8''	W.79°53'26.4''	West
P45 I	OWZH-045	Lambayeque	S.05°36'39.2''	W.79°53'35.3''	West
P45 II	OWZH-045	Lambayeque	S.05°36'39.2''	W.79°53'35.3''	West
P46	OWZH-046	Piura	S.05°10'15.4''	W.80°11'04.0''	West
P47	OWZH-047	Piura	S.05°19'22.7''	W.79°55'52.0''	West
P49	OWZH-049	Piura	S.05°25'09.8''	W.79°40'29.0''	West
P50	OWZH-050	Piura	S.05°25'14.1''	W.79°40'21.5''	West
P51	OWZH-051	Piura	S.05°24'59.2''	W.79°39'27.9''	West
P52	OWZH-052	Piura	S.05°24'59.2''	W.79°39'27.9''	West
P54	OWZH-054	Piura	S.05°24'59.2''	W.79°39'27.9''	West
P58	OWZH-058	Cajamarca	S.06°02'17.0''	W.78°54'49.4''	East
P60	OWZH-060	Amazonas	S.05°49'13.5''	W.78°44'35.0''	East
P62	OWZH-062	Amazonas	S.05°42'40.5''	W.78°34'59.0''	East
P63	OWZH-063	Amazonas	S.05°51'26.22''	W.78°13'39.3''	East
P64	OWZH-064	Amazonas	S.05°51'26.22''	W.78°13'39.3''	East
P66	OWZH-066	Amazonas	S.05°53'38.2''	W.77°58'25.6''	East
P67	OWZH-067	San Martin	S.05°44'47.9''	W.77°28'40.8''	East
P68	OWZH-068	San Martin	S.06°25'32.9''	W.76°35'37.3''	East
P69	OWZH-069	San Martin	S.06°25'32.9''	W.76°35'37.3''	East
P72	OWZH-072	San Martin	S.06°25'19.4''	W.76°31'16.4''	East
P74	OWZH-074	San Martin	S.06°27'57.3''	W.76°22'14.1''	East
P75	OWZH-075	San Martin	S.06°28'19.12''	W.76°22'14.1''	East
P76	OWZH-076	San Martin	S.06°28'00.0''	W.76°21'35.5''	East
P78	OWZH-078	San Martin	S.05°55'25.1''	W.77°18'57.3''	East
P80	OWZH-080	Amazonas	S.05°52'16.0''	W.77°57'35.8''	East
P82	OWZH-082	Amazonas	S.06°17'05.9''	W.77°56'14.4''	East
P83	OWZH-083	Amazonas	S.06°17'05.9''	W.77°56'14.4''	East
P85	OWZH-085	Amazonas	S.06°22'40.8''	W.77°54'22.4''	East
P87	OWZH-087	Amazonas	S.06°22'40.8''	W.77°54'22.4''	East
P90	OWZH-090	Amazonas	S.06°01'55.6''	W.77°56'35.5''	East
P92	OWZH-092	Amazonas	S.05°47'43.1''	W.78°20'07.3''	East
P94	OWZH-094	Amazonas	S.05°40'57.4''	W.78°29'00.1''	East
P95	OWZH-095	Cajamarca	S.05°47'02.7''	W.78°46'59.4''	East
P98	OWZH-098	Cajamarca	S.05°58'34.7''	W.79°12'15.8''	East
Ch34/36/37/39/41/42/43		Piura			West

Results

A total of 340 unambiguous, polymorphic bands were scored with eight AFLP primer combinations. Of these polymorphic AFLP bands, 185 were polymorphic among the tetraploid species, and 93 were polymorphic within the final (see below) *G. barbadense* sample. The level of intra-specific variation in *G. barbadense* ranged

from seven polymorphic bands out of a total of 44 (16%) in primer combination E38/M48 to 16 out of 60 (27%) in E35/M48. Figure 1 displays the NJ tree of the full data set. The intermediate position of the AD-tetraploids relative to the A- and D-diploids is clearly visible. *G. herbaceum*, *G. arboreum*, and *G. raimondii* are well separated and so are the four tetraploids. The average genetic distances between species (see inlet table of Fig. 1, which also contains the within-species distances) range from 0.16 (*G. barbadense* versus *G. tomentosum)* to 0.82 (*G. herbaceum* versus *G. raimondii*).

To address the tree topology of the tetraploid species, we discarded all diploid species (and 12 tetraploid accessions that had missing data in more than one primer combination) from the data matrix. The resulting Dice similarity-based UPGMA is shown in Fig. 2a. The robustness of the clustering was evaluated by bootstrapping. All species-defining branches are well supported and equal those obtained in Fig. 1. The *G. barbadense* sub-cluster in Fig. 2a displays a first split distinguishing a first subset comprising 33 coastal Peruvian accessions. The remaining accessions from northernmost coastal Peru (the departments of Piura and Tumbes) and accessions from coastal Ecuador are clustered basal to the east-of-Andes accessions and accessions from Bolivia, Brazil, Columbia, Venezuela, and the Caribbean and Pacific Islands.

	G.barb.	G.hirs.	G.tom.	G.must.	G.herb.	G.arb.	G.raii.
G.barb.	0.07	0.21	0.16	0.25	0.46	0.45	0.55
G.hirs.		0.07	0.22	0.24	0.47	0.45	0.56
G.tom.			-	0.22	0.49	0.46	0.58
G.must.				-	0.52	0.47	0.59
G.herb.					0.02	0.31	0.82
G.arb.						0.07	0.80
G.raii.							0.01

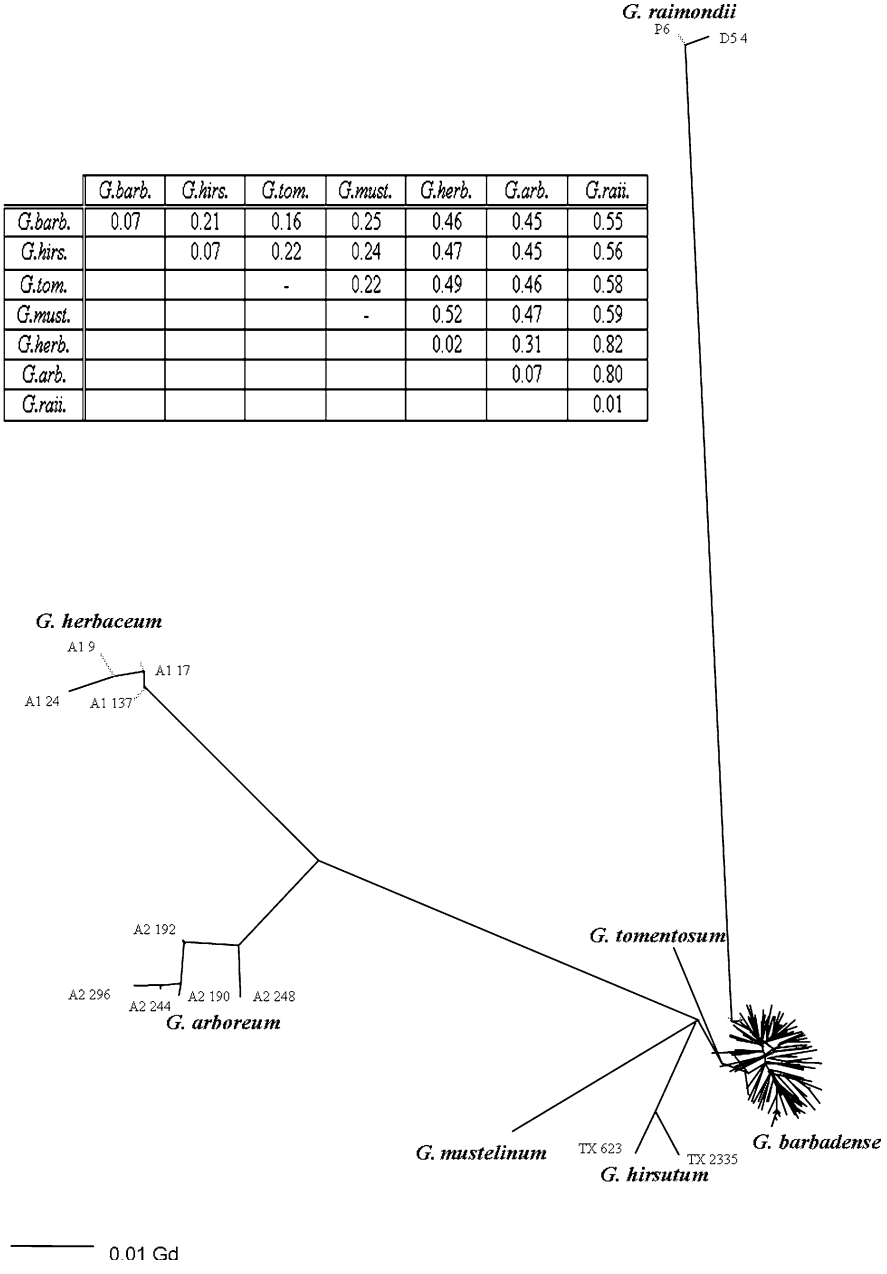


Fig. 1 NJ of seven *Gossypium* species represented by 131 accessions calculated from pairwise distances (see bar for resolution) obtained with 340 AFLPs. Inlet table shows the average genetic distance between and within species

Fig. 2 **a** UPGMA of 108 tetraploid cotton accessions obtained with 185 AFLPs. Species names and bootstrap values (>80) are indicated within the cluster. **b** NJ of 96 *G. barbadense* accessions with 93 AFLPs. Inventory no. (see Table 1 and Table 2) are colored according to their origin

To further investigate these geographic patterns a *G. barbadense* NJ tree was computed on the basis of the 96 accessions displaying no missing data in any of the primer combinations (Fig. 2b). This NJ intraspecific analysis 'zooms in' on the topology of the *G. barbadense* cluster of Fig. 2a and displays the same general pattern in an unrooted way. The highest distance between two accessions of *G. barbadense* within this subset is found between the coastal Peruvian P1 and the Hawaiian Gb480 (0.13), while the average distance is limited to 0.06. A principal coordinate analysis [not shown] revealed the same overall pattern for these 96 accessions and the two first principal coordinates accounted for 23.9% of the total variation.

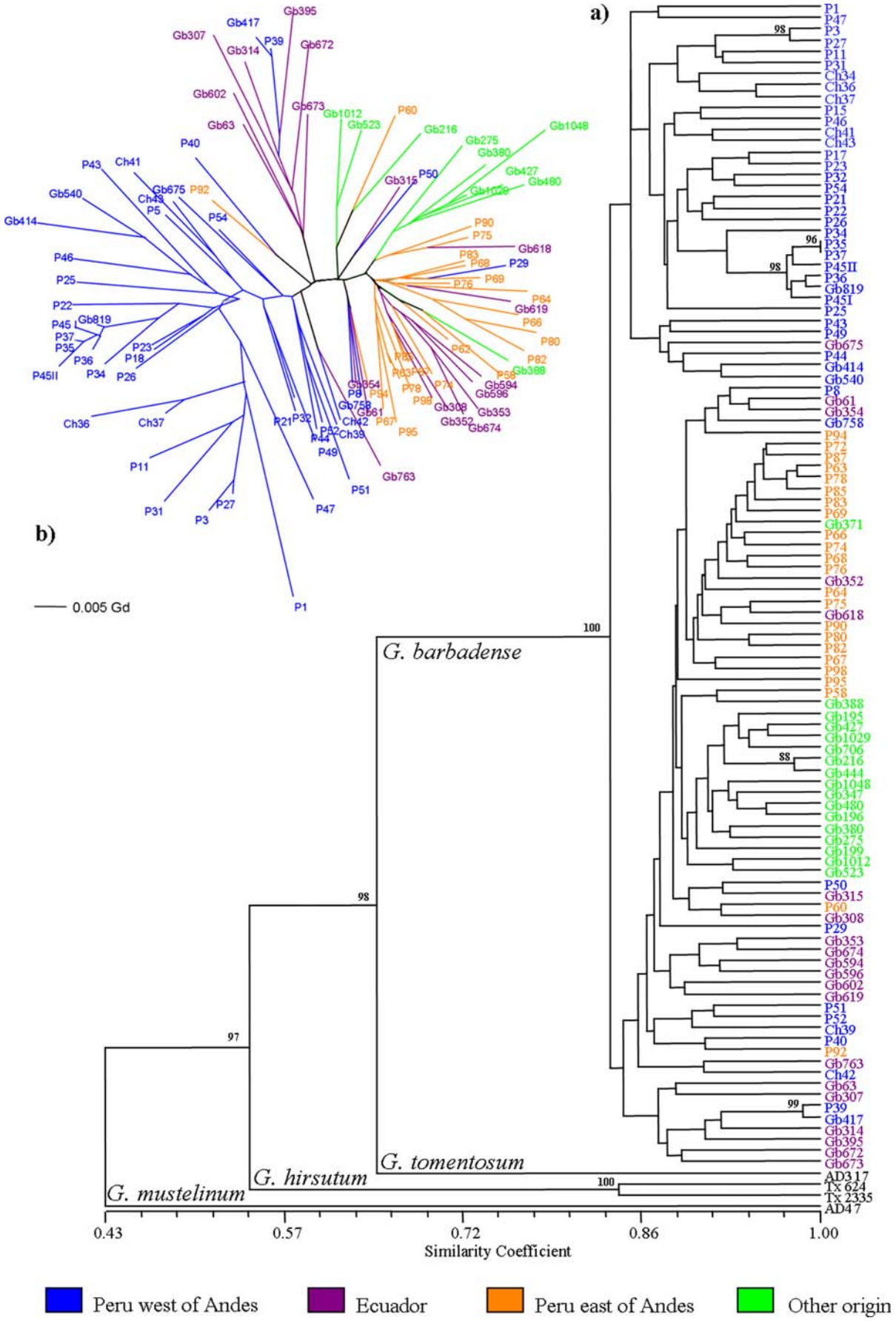
Discussion

Diploid versus tetraploid relationships

The intermediate position of the four allotetraploid AD-cotton species relative to the A- and D-diploids in the NJ analysis (Fig. 1) is in full agreement with the established cytogenetic theory (Endrizzi et al. 1985). The genetic distances between the two different diploid species (A ver. D) and the derived tetraploids are large (0.45–0.59) compared to the distance among the tetraploids (0.16–0.25), as is expected by the bottleneck effect of a single polyploidization event (Cronn et al. 1999; Wendel and Cronn 2003). It is also apparent that *G. raimondii* is less related to the tetraploids than the two A-genome species (*G. arboreum* and *G. herbaceum*), again in agreement with cytogenetic and molecular studies (Endrizzi et al. 1985; Wendel 1989; Cronn et al. 1999; Small et al. 1999; Abdalla et al. 2001; Senchina et al. 2003). This consistency of the results of AFLP analyses with cytogenetic studies is important as caution is recommended when using AFLP markers in inter-specific comparisons (e.g., El-Rabey et al. 2002), since the homology of the bands will be less well preserved the more evolutionary distant the analyzed material is. Abdalla et al. (2001) identify AFLP bands that are shared between a subset of the A-diploids and tetraploids and call these 'A-related' markers; they do the same for identifying 'D-related' markers. Applying this classification to our data, we count 136 A-related markers and 75 D-related ones, which is a proportion similar to that obtained by Abdalla et al. (2001). These numbers were subsequently used by Wendel and Cronn (2003) in supporting the relative contribution of the two diploids for the emergence of the tetraploid cottons. Similar inter-specific comparisons have shown that *Aegilops* genomes, for example, are arranged by AFLP/NJ in accordance with known cytogenetics (Sasanuma et al. 2004); this also holds true for *Avena* (Drossou et al. 2004), *Solanum* (Kardolus et al. 1998) *Musa* (Ude et al. 2002), and *Cicer* (Sudupak et al. 2004). Even in the investigation of El-Rabey et al. (2002), where the problem surrounding the homology assumption of co-migrating AFLP bands was studied, the AFLP clustering of *Hordeum* genomes was 'in full concordance with geographic origin, cytology, and taxonomic status'.

Inter-specific relations among AD-tetraploid cottons

Extensive effort has been expended on studying the molecular phylogeny of *Gossypium* (Wendel 1989; Wendel and Albert 1992; Cronn et al. 1996; Seelanan et al. 1997; Small et al. 1998; 1999; Seelanan et al. 1999; Small and Wendel 2000; Liu et al. 2001; Cronn et al. 2002). However, the phylogenetic relationship among the tetraploids has remained elusive due to their short evolutionary separation and low levels of nucleotide diversity (Small et al. 1998; 1999). The radiation of the tetraploids has only had an estimated 1.5 million years (Senchina et al. 2003), and the separation into 'good species' is confounded, as can be concluded from the detection of gene flow across the taxonomic species borders (Percy and Wendel 1990; Wendel and Percy 1990; Brubaker and Wendel 1994) and from examples of testcrosses yielding fertile F_2 progenies (e.g., Hutchinson et al. 1947; Endrizzi et al. 1985). Nevertheless, Small et al. (1998) described two main branches within the tetraploid group, with one branch leading to *G. mustelinum* and one branch leading to the remaining four species of which *G. barbadense* and *G. darwinii* form a sister group to *G. hirsutum* and *G. tomentosum*. Later studies have either not addressed the relationship between the tetraploids (Small et al. 2000; Cronn et al. 2002) or failed to resolve the relative position of *G. tomentosum* within the group (Liu et al. 2001). Our data support the basal branching of *G. mustelinum* but indicate a closer clustering of *G. tomentosum* towards *G. barbadense* than towards *G. hirsutum*. The difference in genetic distance is low (0.16 between *G. barbadense* and *G. tomentosum* versus 0.22 between *G. hirsutum* and *G. tomentosum*) and can not be taken as a final evidence for a phylogenetic revision. Yet Hutchinson et al. (1947) reported on chromosome pairing and testcross progeny studies among those tetraploids and concluded that *G. tomentosum* is closer to *G. barbadense* than to *G. hirsutum*, as also suggested by our data. Another interesting feature was observed when five *G. barbadense* accessions from the Galapagos Islands were included—two of the accessions (Gb624/Gb625) clustered separate from all other *G. barbadense*. This could have been caused by the fact that some of the Galapagos accessions in the


a)
b)
0.005 Gd
G. barbadense
G. tomentosum
G. hirsutum
G. mustelinum
0.43
0.57
0.72
0.86
1.00
Similarity Coefficient
Peru west of Andes
Ecuador
Peru east of Andes
Other origin

USDA-ARS Cotton Collection contain introgressions from *G. darwinii* growing in close proximity (E. Percival, personal communication). Due to that uncertainty, we eliminated all Galapagos accessions from our analyses. The observed bias possibly caused by the indirect involvements of the fifth tetraploid cotton (i.e., *G. darwinii*) would make future analyses of defined Galapagos material (*G. barbadense* and *G. darwinii*) important.

Domestication and genetic diversity

G. barbadense is widely distributed, covering the whole range of tropical South America including an overlapping distribution with *G. hirsutum* in the northern part of the continent and in the Caribbean (Brubaker et al. 1999; Wendel and Cronn 2003). Present-day indigenous *G. barbadense* is grown in gardens and local farmers' fields in northwestern (NW) South America and is referred to as 'dooryard' cotton or 'commensals'. The plants occur as perennial shrubs with thick basal stems up to a tree size, 4 m tall, and they produce lint in an array of brown colors. The historical record concerning the native distribution of wild *G. barbadense* is rather anecdotal, yet extant wild populations were reported from Guayas and Los Rios in Ecuador and Tumbes, Peru (Stephens and Moseley 1973, 1974; Schwendiman et al. 1986; Percival and Kohel 1990). The search for truly wild accessions is even more complicated since the wild-to-domesticated continuum in *G. barbadense* hardly allows categorical distinctions. Cotton remains obtained from archeological excavation sites from central- and northern coastal Peru show this wild-to-domesticated continuum for seed size, boll size, and fiber width. It has also been proposed that a selection for greater differentiation between fuzz and lint led to morphs with a strongly reduced fuzz layer, called 'tufted' seeds, and the so-called 'kidney-seeded' type of *G. barbadense*, as they were more easily ginned by hand (Turcotte and Percy 1990; Brubaker et al. 1999). Stephens (1975) found that the remains from the archaeological site Huaca Prieta (from about 2,500 BC) display chocolate-colored fibers and that the oldest excavation layers contain only fuzzy seeds while tufted seeds appear in more recent layers. Selection also eliminated the hard seed coat and delayed germination (Hutchinson et al. 1947). In addition, a low selection pressure was probably induced on traits like percentage lint, lint length and strength, and eventually also on color differences for artistic purposes in weaving and improved catching abilities of fishing nets (Hutchinson et al. 1947; Vreeland 1999). More specialized accessions have undergone selection for photoperiod neutrality, early maturing and fine white lint, etc., but these later domestication traits were selected in colonial times and thereafter (Hutchinson et al. 1947; Brubaker et al. 1999) and should not be considered here. In summary, for primitive cottons there exists no clear separation between wild and present-day 'dooryard' cottons. In fact, these dooryards are envisioned to be derived directly from local wild progenitors (Hutchinson et al. 1947; Percy and Wendel 1990; Brubaker et al. 1999). Despite changing climate, intermixing, and other variables, the geographic pattern of present-day dooryards might provide an insight into the pre-Columbian distribution of this plant. Thus, we focused our re-sampling on dooryard cottons to obtain (in combination with gene bank material) a representative sample of *G. barbadense* across its assumed pre-Colombian range. The most striking observation we obtained is the unique diversity in the large majority of accessions from coastal Peru. A few accessions from the NW Peru cluster with southwestern (SW) Ecuadorian accessions, and together these accessions are basal to the remaining *G. barbadense*. This indicates that primitive domesticated *G. barbadense* has its domestication center in the NW Peru/SW Ecuador region, as proposed (mainly derived from archaeological evidence) by Piperno and Pearsall (1998). Further, we also suggest, that cotton from this core area was transported across the Andes, and spread thereafter south into Bolivia, east into Brazil, and north into Colombia, Venezuela, and the Caribbean and Pacific Islands. This scenario is proposed with caution as the data do not yield high bootstrap-values on the most basal nodes of the *G. barbadense* cluster, but the pattern is evident in all the different analyses.

Our field observations (Fernandez et al. 2003) support the DNA data as they indicate a high diversity of 'primitive' types in the coastal departments of Peru. We encountered dooryard and feral plants with strongly arborescent growth habit, small bolls, and lint colors varying from reddish-brown to light yellow-brown, and purplish-brown to deep chocolate-brown. It was also apparent that the cottons east of the Andes displayed only a subset of this variation, with generally larger bolls (long and slender) and lint that varied in color from lighter shades of brown to white. In the 'Selva Alta' of San Martin, a region where the Amazonian rainforest meets the Andes, plants in the next stage of domestication, i.e., landraces, are grown in small-scale commercial productions of organic cotton. The domestication trait 'kidney-seeded' is known only from east of the Andes (Turcotte and Percy 1990) and was present in some of our accessions from Bolivia and Brazil, indicating that our geographic interpretation of the data follows an expansion towards increased domestication level.

Conclusion

Our DNA analyses confirm the relationship among diploid and tetraploid cottons. They also confirm the archeology/allozyme-derived assumption of South American cotton domestication having its origin in the coastal zone of NW Peru and SW Ecuador. We add that cotton spread out from this center (probably within the framework of domestication) over the Andes and

from there into other parts of the continent. We also observed a tremendous diversity in *G. barbadense* found along the remaining northern Peruvian coast. This diversity is facing serious threats due to habitat destruction and the replacement of local types, and both ex-situ and in-situ conservation strategies should be implemented.

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