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Gene flow and genetic diversity in cultivated and wild cacao (*Theobroma cacao*) in Bolivia[1]

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- *Premise of the study:* The role of pollen flow within and between cultivated and wild tropical crop species is little known. To study the pollen flow of cacao, we estimated the degree of self-pollination and pollen dispersal distances as well as gene flow between wild and cultivated cacao (*Theobroma cacao* L.).
- *Methods:* We studied pollen flow and genetic diversity of cultivated and wild cacao populations by genotyping 143 wild and 86 cultivated mature plants and 374 seedlings raised from 19 wild and 25 cultivated trees at nine microsatellite loci.
- *Key results:* A principal component analysis distinguished wild and cultivated cacao trees, supporting the notion that Bolivia harbors truly wild cacao populations. Cultivated cacao had a higher level of genetic diversity than wild cacao, presumably reflecting the varied origin of cultivated plants. Both cacao types had high outcrossing rates, but the paternity analysis revealed 7–14% self-pollination in wild and cultivated cacao. Despite the tiny size of the pollinators, pollen was transported distances up to 3 km; wild cacao showed longer distances (mean = 922 m) than cultivated cacao (826 m). Our data revealed that 16–20% of pollination events occurred between cultivated and wild populations.
- *Conclusions:* We found evidence of self-pollination in both wild and cultivated cacao. Pollination distances are larger than those typically reported in tropical understory tree species. The relatively high pollen exchange from cultivated to wild cacao compromises genetic identity of wild populations, calling for the protection of extensive natural forest tracts to protect wild cacao in Bolivia.

Key words: microsatellites; paternity analysis; pollen dispersal; selfing.

Gene flow is a major source of genetic variation within populations because it balances the detrimental effects of genetic drift, maintains effective population sizes, and has important implications for the management and conservation of genetic resources (Fénart et al., 2007). In tropical rainforests, where plant species are often characterized by low population densities and large distances between conspecifics, the spatial distribution of individuals has substantial consequences for the movement of their pollinators and intraspecific gene flow (Hubbell and Foster, 1983; Ashton, 1984), leading to, for example, pollen limitation (Murawski and Hamrick, 1991; Ghazoul et al., 1998). Because pollen movement is a key component of gene flow, density effects can be assumed to alter genetic structure and, especially in small populations, to increase the probability of extinction (Stacy et al., 1996; Ghazoul et al., 1998). Considering that tropical forests are experiencing high rates of deforestation, knowledge of gene flow is therefore elementary for understanding the reproductive success and management of tropical tree species.

In contrast to early theories that predicted tropical tree species to be mainly self-fertilizing or inbred (Baker, 1959; Fedorov, 1966), studies of mating systems have revealed that most species are outcrossed and that long-distance pollen dispersal is the norm rather than an exception (Ward et al., 2005). Consequently, most studies on the genetic structure of tropical tree species have found high levels of intrapopulation genetic diversity and weak-to-moderate spatial genetic structuring (e.g., Lacerda et al., 2001; Hardesty et al., 2005), although exceptions exist (e.g., Degen et al., 2004; Dutech et al., 2002). In addition, the relative rates of selfing and outcrossing seem to be highly variable, both among individuals within populations and among populations over years (Ward et al., 2005). This might be due to the influence of factors such as population density and pollinator abundance (Murawski and Hamrick, 1991; Degen et al., 2004), differences in ecological site conditions (Franceschinelli and Bawa, 2000; Ward et al., 2005), phenological asynchrony (Murawski and Hamrick, 1992), or degree of disturbance (Dick et al., 2003). This appears to be true for both canopy and understory trees (Ward et al., 2005).

The tropical tree genus *Theobroma* L. (Malvaceae) is an excellent study object to enhance our understanding of patterns of reproduction, gene flow, and speciation of tropical tree species, as well as being a plant resource of considerable economic interest. Although several of the 20 *Theobroma* species in tropical

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America are locally harvested, the cultivars of *Theobroma cacao* L. have by far the main commercial importance. Surprisingly little is known about the pollination ecology of *T. cacao*. It is mainly pollinated by tiny midges (Ceratopogonidae), but although knowledge on sexual reproduction is fairly extensive for cultivated cacao (Wellensiek, 1932; Soria et al., 1980), it is virtually nonexistent for wild populations. *Theobroma cacao* is generally assumed to be an outbreeding species, because of its floral morphology and the occurrence of a self-incompatibility system (e.g., Knight and Rogers, 1955; Falque et al., 1995). However, although self-pollination of a single flower is basically impossible in *Theobroma* because of the flower morphology (Lieberei and Reisdorff, 2007), it has been suggested that geitonogamy may be more common in the wild than has been assumed (Lanaud et al., 1987). Indeed, the low level of observed heterozygosity in wild populations in upper Amazonia might point to high rates of geitonogamous selfing (Sereno et al., 2006).

Lately, much attention has been given to gene flow from crop to wild relatives, motivated by concerns about the impacts on wild populations (Ellstrand, 2003; Armstrong et al., 2005). For gene flow between crops and wild relatives to occur, several conditions have to be fulfilled: both types must co-occur within the pollen or seed dispersal range, the flowering season must overlap, hybrid offspring must be viable and fertile, and establishment of genes in the crop or the wild relative must take place (Ellstrand, 2003). Gene flow between a crop and its wild relative can easily occur if they belong to the same species (Ellstrand et al., 1999; Snow, 2002). This phenomenon has existed since the origin of agricultural domestication, and crop–wild hybrids are often viable and fertile (Hauser et al., 1998; Snow et al., 1998). Crop-to-wild gene flow has been observed in many species, including fruit trees, for example in the genus *Prunus*, in which bidirectional gene flow between the cultivated almond tree (*Prunus dulcis*) and its wild counterparts (*Prunus orientalis*) has been reported (Delplancke et al., 2011). Considering the possible impacts on wild populations, the wild progenitor normally has higher genetic diversity, and this reservoir of genetic diversity could be threatened if swamped by depauperate domesticated types. As a consequence, extinction by displacement of native allelic diversity may occur, as has been observed in the wild walnut (*Juglans californica* Wats. var. *californica*) and in Catalina mountain mahogany (*Cercocarpus traskiae* Eastw.; Rieseberg and Gerber, 1995). Similarly, as a result of human cultivation, only a few truly wild populations of coconut (*Cocos nucifera* L.), date palm (*Phoenix dactylifera* L.), and the olive (*Olea europea* L.) remain (e.g., olive: Bronzini de Caraffa et al., 2002). However, crop–wild gene flow could also be considered positive if disease or pest-resistance genes were transferred to wild populations. Consequently, the ecological impacts in natural ecosystems and the long-term effects of gene flow between crops and their wild relatives require more research (Wolfenbarger and Phifer, 2000; Ellstrand, 2003).

In *T. cacao*, the distribution of wild populations is uncertain after millennia of cultivation and local naturalization across the Neotropics (Chessman, 1944; Cuatrecasas, 1964; Warren, 1994). It appears most likely that the species naturally occurs in western or southwestern Amazonia, including northern Bolivia (Whitkus et al., 1998; Motamayor et al., 2003; Sereno et al., 2006). However, it has also been proposed that the "wild" populations of *T. cacao* in Bolivia are the result of naturalized plants from previous cultivation (Tratado de Cooperación Amazónica, 1999). In Bolivia, cacao was first cultivated ≥200 yr ago by the native Moseten ethnic group who, encouraged by missions and Bolivian government (R. Villegas, Mayor de San Andrés University, unpublished data), grew clones from Ecuador, Trinidad and Tobago, and Costa Rica (Somarriba and Trujillo, 2005). Yet data on the genetic diversity of both wild and cultivated cacao types are lacking from Bolivia.

Our study took place in the northeastern lowlands of Bolivia, where presumed wild forms of cacao occur patchily in the natural forest. In addition, farmers cultivate commercial hybrids of both the *Forastero* and *Criollo* cultivars provided by nongovernmental organizations. The objectives of our study were (1) to establish whether wild and cultivated cacao differ genetically; (2) to quantify self- and cross-pollination; (3) to estimate pollen dispersal distance of wild and cultivated cacao trees; and (4) to test whether gene flow occurs between wild and cultivated cacao.

We hypothesized that wild and cultivated cacao plants in Bolivia differ genetically. On the other hand, we hypothesized that pollen dispersal distance of wild cacao trees is larger than that of cultivated plants and that gene flow occurs between the two cacao types.

MATERIALS AND METHODS

Study species—*Theobroma* includes about 20 species of cauliflorous trees native to tropical America (Cuatrecasas, 1964; Rondón and Cumana-Campos, 2005), most of them understory, shade-tolerant species. *Theobroma* is famed as the source of cocoa, chocolate, and vegetable butter and has been used since antiquity (Wood and Lass, 1985). Although several species are locally harvested, the cultivars of *T. cacao* L. are by far the main commercial taxa, representing the world's second most important tropical cash crop species, supporting a $5 billion industry and 40–50 million farmers (FAO statistical databases: http://faostat.fao.org). These cultivars involve partly polyembryonous forms as well as crosses with *T. bicolor* Bonpl., *T. angustifolium* D.C., and *T. grandiflorum* (G. Don f.) Schumann (Addison and Tavares, 1952; Cuatrecasas, 1964; Martinson, 1966). Wild cacao has been described as growing taller (to 18 m) than cultivated cacao (to 6 m) (Lieberei and Reisdorff, 2007), but given the difficulty of discerning truly wild populations and the different growth and management conditions in plantations and forests, this difference may not be meaningful. Wild cacao further tends to have smaller fruits with smaller seeds, more pulp, and thicker fruit shells.

Flowers are white with red nectar guides, with 5 petals ~10 mm in length, and grow in clusters on the tree trunks. The fruits are ellipsoid to elongated; segmented (10 segments); 15–19 cm in length; greenish, orange, or red when ripe; and dispersed by gravity and mammals. Anthesis occurs at dawn, and anther dehiscence takes place throughout the morning hours (Young et al., 1987). Each flower produces ~14 000 pollen grains (Massaux et al., 1976). Flowering phenology of *T. cacao* is seasonal, varying between genotypes and in response to local climatic conditions, but usually being highest at the onset of the rainy season (Paulin et al., 1983). In Bolivia, the main flowering season is in September–October for wild trees and October–November for the cultivated ones, although some flowering occurs throughout the year among the cultivated trees (C. Chumacero, unpublished data). The flowers are receptive for 1 day, and unpollinated flowers drop after ~2 days. *Theobroma cacao* is pollinated by insects and has no wind pollination (Harland, 1925). Ceratopogonid midges are considered the principal and most effective pollinators of cacao, at least in cultivars (e.g., Billes, 1941; Posnette, 1950; Brew and Boorman, 1993). Currently it is unknown how far these tiny midges transport the pollen grains and whether they accomplish gene flow between wild and cultivated cacao. Many authors have reported self-incompatibility in cacao (e.g., Pandey, 1960; Falque et al., 1995), yet the incompatibility system in cacao is not absolute but quantitative, depending on the ratio of fertilized and nonfertilized ovules. Furthermore, self-compatible genotypes exist (e.g., the commercially used ICS 1 cultivar; Smulders et al., 2008).

Study sites and sampling—We studied cultivated and wild *T. cacao* trees on the lands of the native Tacana community Napashi (67°52′17.4″S, 14°5′20.6″W) in the northeast lowlands of Bolivia. Here, presumed wild forms of cacao occur

patchily in the natural forest while farmers cultivate commercial hybrids of both the *Forastero* and *Criollo* cultivars provided by nongovernmental organizations. The farmers commonly germinate seeds from wild trees and later graft the cultivar clones on these. For our study, we selected five cultivated plantations along a 6-km path leading into the forest and identified all wild trees in the forest up to 400 m away from the path (Fig. 1). Further exploration of the area was inhibited by the inaccessibility of the area. Mean (± SD) nearest-neighbor distances were 8.0 ± 5.9 m and 63.6 ± 224.1 m for cultivated and wild trees, respectively.

We sampled leaves from both parental trees and offspring. Altogether, 603 samples of fresh leaf material were collected in 2009 and 2010. Of these, 229 samples corresponded to parental trees (86 from cultivated and 143 from wild trees), from 44 of which seeds were collected from ripe pods and from which 374 seedlings were raised (185 seeds from 25 cultivated and 189 from 19 wild trees).

***Microsatellite analysis*—**DNA extraction was performed with a standard protocol adapted from Doyle and Doyle (1987) using 20-mg silica-gel-dried leaf material and a modified extraction buffer (2% alkyltrimethylammonium-bromide, 0.1 M TRIS–HCl, 0.02 M disodium-EDTA [pH 8.0], 1.4 M NaCl, 1% polyvinylpyrrolidone). Samples were genotyped at nine microsatellite loci previously established by Lanaud et al. (1999) and Saunders et al. (2004) in one simple and four duplex reactions: mTcCir6 + mTcCir25, mTcCir11+ mTcCir 12, mTcCir15 + mTcCir21 (annealing temperature 46°C), mTcCir7 + mTcCir18 and mTcCir1 (51°C). Polymerase chain reaction (PCR) assays were set up in final volumes of 25 μL, containing 20 ng/μL of genomic DNA, 0.8/0.5 μL of primer (5 pmol/mL; Metabion International AG, Martinsried, Germany) for the locus with longer–shorter fragment length, 2.5 μL dNTPs (2 mM; Qbiogene, Montreal, Quebec, Canada), 0.2 μL Taq DNA polymerase (5U/μL; MP Biomedicals, Solon, Ohio, USA), 2.5 μL incubation mix T. Pol with 1.5 mM MgCl2 (MP Biomedicals), and 16.8 μL H2Obidest. PCR was performed in a Mastercycler gradient or Mastercycler epgradient (Eppendorf, Hamburg, Germany) under the following temperature regime: 94°C for 4 min, 32 cycles with 30 s at 94°C, 60 s at 46°C or 51°C, and 60 s at 72°C, and a final 3 min at 72°C; 2 μL PCR products (1:5 diluted) were used for separation on a MegaBace sequencer 1000 system (Amersham Bioscience, Uppsala, Sweden) with MegaBace-ET Rox 400 (Amersham Bioscience) as a size standard. The genotyping was performed with MegaBace Fragment Profiler version 1.2 (Amersham Bioscience).

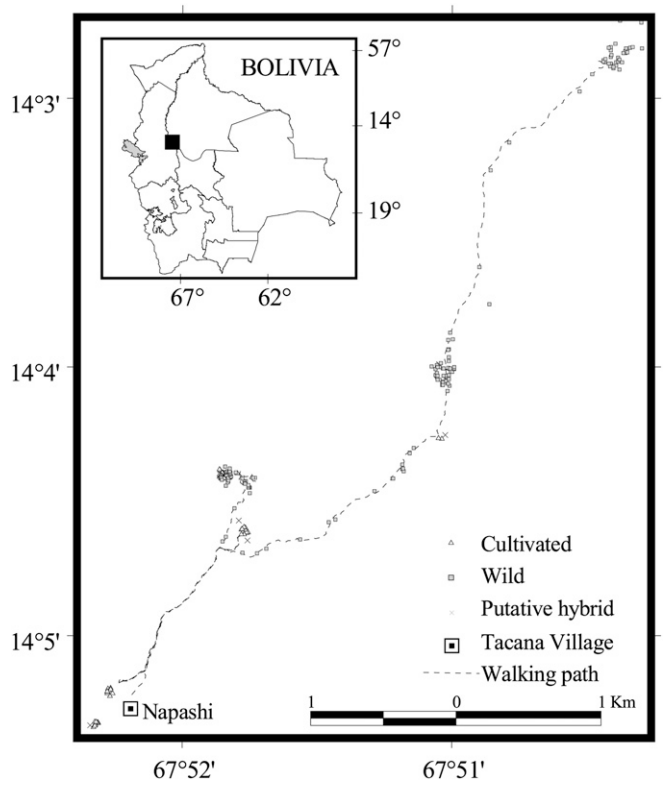

Fig. 1. Location map of the study area and the sampled trees.

***Data analysis*—**To explore overall genetic structure of wild and cultivated trees and to identify putative hybrids, we first performed a principal component analysis (PCA) calculated with GenAlEx version 6 (Peakall and Smouse, 2006). Second, we applied a Bayesian cluster approach, using Structure version 2.3.3 (Falush et al., 2007), which groups individuals into clusters representing homogeneous gene pools without a priori information about individual origin. We ran 10 replicate runs, with the number of clusters (K) ranging from 1 to 10, of an admixture model with correlated allele frequencies, with 25 000 burn-in and 50 000 subsequent Markov chain Monte Carlo repeats. The most probable K value was determined following Evanno et al. (2005). Replicate runs were averaged using Structure-sum (Ehrich, 2011). We assessed genetic variation for spatial groups of trees and estimated gene diversity (H_E), number of alleles (A), and allelic richness (AR), which is a measure of allelic variation correcting for difference of sample size using Fstat version 2.9.3.2. Diversity levels were compared between wild and cultivated cacao by resampling 1000 times in Fstat (Goudet, 2001).

To study the pollen flow of cacao, we performed a paternity analysis using Cervus version 3.0.3 (Kalinowski et al., 2007). We used multilocus genotypes of adult plants (maternal trees and candidate fathers) and seedlings of known mothers (see Supplemental Data with the online version of this article; Appendices S1 and S2). Cervus identifies the pollen donors of each seedling so that pollen dispersal distance can be deduced. The levels of gene diversity of *T. cacao* varied between 0.3 and 0.6 among loci, indicating adequate variability for pollen dispersal estimation (Ritland, 2002). To reduce the error in the paternity assignment, the samples with ≥3 mismatches with the maternal tree genotype were excluded (n = 32). Further, null allele frequency was estimated with Cervus. The primers had null allele frequencies ≤0.2 (Appendix 1). The most likely parents were determined using the Delta statistic (Marshall et al., 1998) using the allele frequency of the studied individuals as a reference. The significant critical Delta value to assign paternity was calculated through paternity simulations in Cervus. The difference between the individual with the highest Delta value and the second-most-likely candidate's should be greater than the critical Delta value to accept a paternity. For the paternity simulation, we used the following parameters: number of offspring: 10 000; number of candidate fathers: 100; proportion of candidate fathers sampled: 0.30. To test autofertilization, known mothers were tested as candidate fathers. Proportion of loci typed: 0.993; proportion of loci mistyped: 0; minimum number of typed loci: 5. We considered a null scoring error (proportion of loci mistype) taking into account the study of Oddou-Muratorio et al. (2003), who pointed out that even when scoring errors occurred at a high rate, it was better to assume a null level scoring error in Cervus to avoid the increase of Type I error (false-positive paternity).

G-tests were applied to compare self- and cross-pollination in wild and cultivated cacao trees. To test for differences in distributions of pollen dispersal distance between wild and cultivated cacao, we applied a Kolmogorov-Smirnov test

RESULTS

The PCA distinguished two main groups of *T. cacao* individuals, which correspond to cultivated and wild plants. Ten individuals were placed between these two main groups and presumably corresponded to hybrids between cultivated and wild cacao (Fig. 2). Bayesian cluster analysis with Structure fully matched the PCA analysis and revealed two clusters, representing wild and cultivated plants, respectively (Fig. 3; and see Supplemental Data with the online version of this article; Appendix S3). Two individuals turned out to be a wild genotype in a cultivated field and a cultivated genotype in a wild population, respectively. In two cultivated fields, seven individuals (i.e., 9.6% of all cultivated plants) had admixed genotypes with nearly equal contribution of wild and cultivated gene pools, suggestive of the presence of first generation hybrids between wild and cultivated genotypes (Fig. 3). Cultivated cacao had a higher level of gene diversity than wild cacao, with mean (± SD) H_E values of 0.673 ± 0.055 and 0.391 ± 0.043, respectively

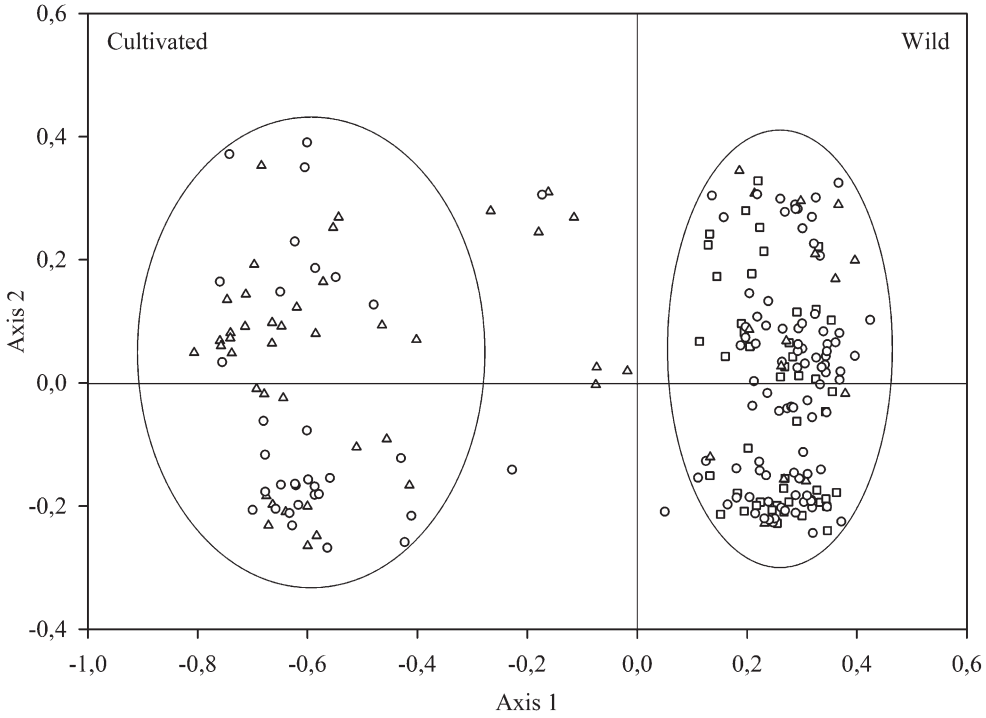

Fig. 2. Principal component analysis of microsatellite data for 229 adult trees of *Theobroma cacao* (explained variance, axis 1: 54%, axis 2: 12%), including wild cacao trees (squares), cultivated cacao trees (triangles), and unknown trees (circles).

(P = 0.001). The cultivars also showed higher allelic richness (AR = 4.84) and higher number of alleles (A = 43) than wild cacao (AR = 2.91, A = 26, P = 0.001; Table 1).

Parentage analysis revealed that self-pollination occurred in 7% of wild and 14% of crop seedlings of *T. cacao*. Accordingly, 86–93% of all seedlings were considered the result of cross-pollination (Table 2). Self-pollination events were significantly more frequent in cultivated than in wild cacao (G-test, G = 2.89, df = 1, P = 0.044).

Paternity could be assigned to 17% of the studied seedlings, considering a relaxed confidence level (Appendix 2). We found that 11 of 99 alleles were private to the offspring (Appendix 3), indicating that the overall low rate of paternity assignment was partly due to pollen immigration. The overall mean (± SD) pollen dispersal distance was 867 ± 888 m. Pollen dispersal distance of wild cacao was observed in a range of 10 to 3007 m (mean = 922 ± 1030 m), whereas pollen of cultivated cacao was dispersed over distances of 13 to 2360 m (mean = 826 ± 804 m; Fig. 4). Pollen dispersal distances were significantly larger in wild than in cultivated cacao (Kolmogorov-Smirnov test, KS = 1.323, df = 1, P = 0.03).

Considering the origin of the pollen, we found that flowers of wild cacao were mainly pollinated with pollen from wild plants (80%) and flowers of cultivated plants from cultivated plants (84%). Nevertheless, we found bidirectional pollen flow between cultivated and wild cacao, with 16% of cultivated offspring fertilized with pollen from wild trees and 20% of wild offspring resulting from fertilization by pollen from cultivated trees.

DISCUSSION

Our results show that wild and cultivated cacao populations in northwestern Bolivia are genetically distinct, with cultivated trees showing higher genetic diversity. Surprisingly, and in contrast to early assumptions (e.g., Pandey, 1960; Falque et al., 1995), self-pollination occurs at meaningful rates in *T. cacao*, especially among the cultivated trees. However, cross-pollination is prevalent and mainly takes place over distances of up to a few hundred meters. Accordingly, pollination mostly occurs within the spatially segregated cultivated and wild populations. However, pollen dispersal over several kilometers was also observed. Within the wild populations consisting of scattered *T. cacao* trees, long-distance pollen dispersal can thus play a major role in maintaining genetic connectivity. However, pollen

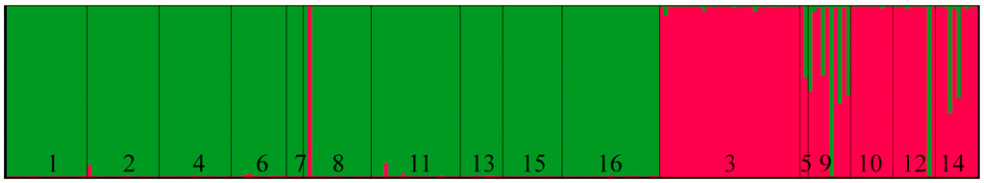

Fig. 3. Mean results of 10 runs of Structure at K = 2 for 229 *Theobroma cacao* individuals in 16 wild or cultivated groups. Note that one individual in group 9 (↓) was known to be wild.

TABLE 1. Genetic diversity of wild and cultivated *Theobroma cacao.* Allelic richness (AR) was calculated with a sample size of 220 individuals. n = sample size, H_E = expected heterozygosity, and A = number of alleles.

Group	Cacao type	n	H_E	A	AR
G01	Wild	19	0.372	24	2.67
G02	Wild	17	0.463	27	2.98
G04	Wild	14	0.374	25	2.78
G06	Wild	13	0.407	26	2.83
G08	Wild	16	0.331	27	3.00
G11	Wild	21	0.396	30	3.33
G13	Wild	11	0.415	26	2.89
G15	Wild	14	0.322	23	2.56
G16	Wild	23	0.371	29	3.22
Mean	Wild	16	0.38	26	2.92
G03	Cultivated	33	0.653	46	5.10
G09	Cultivated	10	0.756	51	5.67
G10	Cultivated	10	0.635	33	3.67
G12	Cultivated	9	0.627	38	4.22
G14	Cultivated	10	0.709	50	5.56
Mean	Cultivated	14	0.676	44	4.84

dispersal can also lead to gene flow between wild and cultivated populations. This was evidenced by a number of seedlings with mixed parentage as well as admixed adult plants.

Cacao origin—It has been proposed that the "wild" populations of *T. cacao* in Bolivia are the result of naturalized plants from previous cultivation (Tratado de Cooperación Amazónica, 1999). However, our study clearly distinguished between cultivated and "wild" cacao plants (Fig. 2), showing that the wild plants are genetically distinct from the currently cultivated ones. Although this is not conclusive evidence, it nevertheless suggests that the "wild" cacao populations of lowland Bolivia may correspond to truly wild forms or at least to ancient cultivars that have become naturalized a long time ago. This lends support to the assumption that Bolivia is part of the natural range of *T. cacao* (Soria, 1969; Whitkus et al., 1998; Arguello et al., 2000; Motamayor et al., 2003; Sereno et al., 2006). Furthermore, we found few individuals corresponding to the cultivated genotypes in the forest, which shows that currently, seed dispersal of cultivated forms into the forest (by either humans or animals) and successful establishment are infrequent.

Genetic diversity—Genetic diversity was lower among the wild than among the cultivated plants. In our study area, cultivated cacao has a higher genetic diversity, probably because the cultivated forms belong to both major cultivars (*Forastero* and *Criollo*) and, presumably, come from a variety of origins. However, genetic variation of the Bolivian populations found in our study was also lower than values reported for wild cacao populations in Brazil (Sereno et al., 2006), both for alleles (A = 26 vs. A = 49) and expected heterozygosity (H_E = 0.391 vs. H_E = 0.566). The low diversity of wild cacao populations in Bolivia has previously been deduced, also on morphological grounds, by R. Villegas (Universidad Mayor de San Andrés, unpublished manuscript) and W. July (CATIE, unpublished manuscript), who further suggest that the present-day wild populations may have derived from the propagation of a few plants present in the natural area of distribution. To which degree this reflects human activities or, e.g., natural clines of genetic variation within the natural range of cacao, which cannot be deduced with the data at hand.

TABLE 2. Paternity assignment of 342 seeds of wild and cultivated *Theobroma cacao.*

	Cultivated		Wild	
Paternity	n	%	n	%
Assigned: self-pollination	21	14	13	7
Assigned: cross-pollination	13	8	10	5
Unassigned	121	78	164	88
Total	155	100	187	100

Cross- and self-pollination—In contrast to most previous studies that have reported self-incompatibility in *T. cacao* (e.g., Pandey, 1960; Falque et al., 1995), our results revealed that self-pollination takes place in both wild and cultivated *T. cacao*, even though cross-pollination clearly predominates. Glendinning (1972) and Lanaud et al. (1987) already pointed out that the incompatibility system in cacao is not absolute, but quantitative. Lanaud et al. (1987) further suggested that geitonogamy might not be rare in the wild, even though they did not study mating systems under natural conditions. Indeed, the low rate of heterozygosity in wild populations might point to high rates of geitonogamy, in agreement with the observations of Sereno et al. (2006) in upper Amazonia.

We found self-pollination to be more common in cultivated than in wild cacao trees, despite the close proximity of cultivated plants, which should increase cross-pollination. The higher degree of self-pollination in cultivated cacao may be a result of human selection toward high crop production in cultivated plants. A similar reduction of self-incompatibility in cultivated forms has also been found (e.g., in *Brassica oleracea*; Thompson and Taylor, 1966).

Paternity assignment and pollen dispersal distance—The rate of paternity assignment in our study was low (17%). This may be attributable to three factors. First, a comparative lack of standing genetic variation precludes discrimination among potential fathers. Second, pollen immigration from outside the sampling area is indicated, in that 11% of the alleles were private to the offspring. Third, unsampled fathers within the sampling area may have contributed. Thus, the results of paternity assignments and pollen dispersal distances may be biased to a certain degree. Still, however, both self-pollination and long-distance pollen dispersal events are clearly indicated and consistent with the hybrid seed and adult individuals found.

Most pollination events documented in our study took place over distances of less than a few hundred meters, as is typical for many tropical species (e.g., Cloutier et al., 2007; Carneiro et al., 2009). Yet the distance and frequency of long-distance pollen dispersal are strongly relevant in that they connect populations and isolated groups of individuals across the landscape, increase effective population sizes, and, thus, maintain genetic variability and weakening genetic drift (Oddou-Muratorio et al., 2005). Therefore, many authors have stressed the importance of understanding mating systems and gene flow in the context of sustainable management and conservation of tropical forests (e.g., Bawa, 1990; Cloutier et al., 2007).

The mean pollen dispersal distance found by us in *T. cacao* (867 m) was higher than that reported for other insect-pollinated tropical understory tree species. Typically, such species have dispersal distances <600 m, as observed, for example, in *Carapa guianensis* and *Sextonia rubra* (Cloutier et al., 2007), *Miconia affinis* (Jha and Dick, 2010), and *Pithecellobium elegans* (Chase

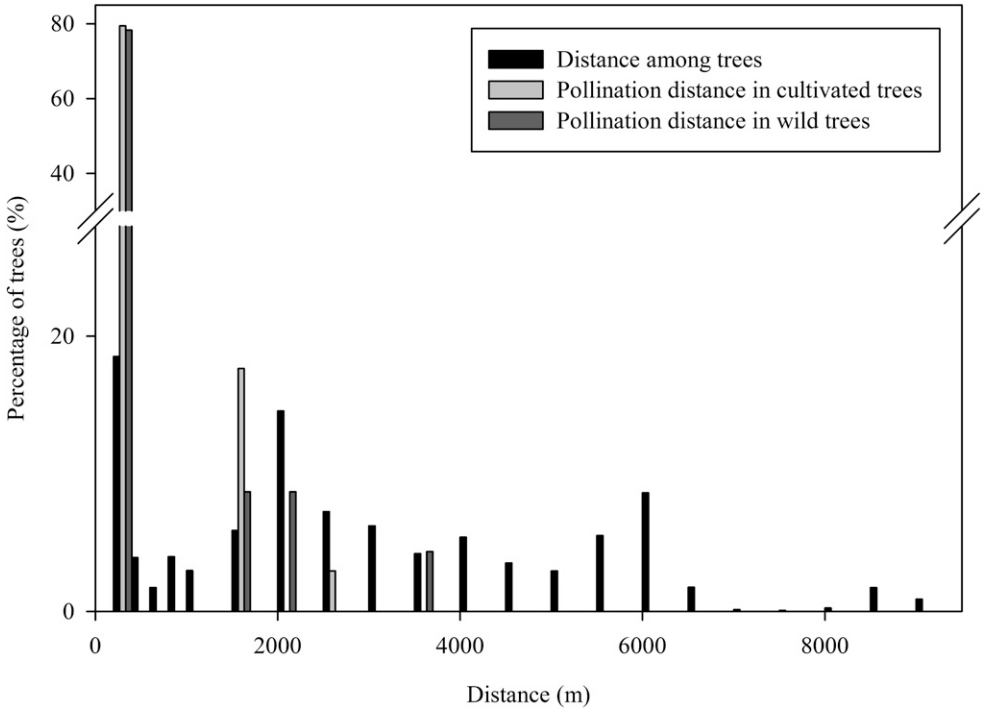

Fig. 4. Spatial separation of trees and distributions of pollen dispersal distance in wild and cultivated trees of *Theobroma cacao*. Note that self-pollinated individuals are included.

et al., 1996). Furthermore, we documented pollen dispersal distances up to 3 km in single individuals. Such pollen dispersal distances are unusual for an insect-pollinated tropical understory tree species, although distances >2 km have been found in *Symphonia globulifera* (Carneiro et al., 2009). In canopy trees, pollen dispersal distances tend to be larger, as exemplified by distances of 5.8–14.2 km in seven species of the genus *Ficus*, which are pollinated by small wasps (Nason et al., 1998).

The large pollen dispersal distances in *T. cacao* are surprising, considering the minute sizes of the pollinating ceratopogonid midges (0.5–2 mm). Although dispersal over long distances is poorly understood in insects, individuals of small species of Diptera, Homoptera, and Hymenoptera are known to be wind dispersed (Nason et al., 1998). However, such wind dispersal is unlikely in the interior of the forest understory and suggests either that the insects disperse actively or that they emerge over the forest canopy to be transported in the aerial plankton, as also assumed for fig-pollinating wasps (Nason et al., 1998).

***Crop–wild gene flow*—**Our results revealed bidirectional pollen flow between wild and cultivated cacao. This was evident both for seeds from the paternity analysis and for adult plants, indicating that hybrids can establish under natural conditions. Pollen flow from crop to wild relatives carries the risk of genetic contamination of wild populations (Ellstrand et al., 1999; Fénart et al., 2007). According to Arias and Rieseberg (1994) and Fénart et al. (2007), in general, spatial separation among wild and cultivated forms alone is rarely enough to prevent gene flow. Indeed, considering the large pollen dispersal distances we found in cacao, physical distances of a few hundred meters as in our study cannot totally prevent pollen flow between the cacao forms. Wild populations are considered reservoirs of genetic diversity, but the studied wild populations showed lower genetic diversity than the crops. In this case, gene flow actually could increase the genetic diversity of wild cacao, although long-term consequences are currently unknown and need more study. In Bolivia, most wild populations of *Theobroma cacao* occur in the low-lying Amazonian plain, whereas cacao cultivation mainly takes place on the Andean foothills. Our study region was located right at the boundary of these two zones, indicating that crop-to-wild gene flow is most relevant in areas were both wild and cultivated gene pools are common.

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APPENDIX 1. Allele frequency analysis of wild and cultivated *Theobroma cacao*.

Locus	*k*	*n*	H_O	H_E	PIC	NE-1P	NE-2P	NE-PP	NE-I	NE-SI	HW	*F* (null)
Cir6	6	562	0.247	0.331	0.318	0.941	0.811	0.669	0.46	0.7	***	+0.1751
Cir25	14	571	0.578	0.779	0.747	0.6	0.422	0.234	0.081	0.381	ND	+0.1479
Cir11	10	561	0.353	0.592	0.565	0.791	0.609	0.404	0.193	0.503	ND	+0.2535
Cir12	17	571	0.708	0.835	0.814	0.494	0.326	0.151	0.048	0.345	***	+0.0832
Cir7	8	561	0.449	0.646	0.597	0.768	0.599	0.418	0.174	0.471	ND	+0.1728
Cir18	9	571	0.469	0.521	0.496	0.844	0.673	0.48	0.255	0.553	***	+0.0497
Cir1	10	571	0.536	0.619	0.554	0.8	0.649	0.487	0.21	0.494	***	+0.0656
Cir15	14	569	0.343	0.447	0.423	0.889	0.734	0.561	0.33	0.609	***	+0.1477
Cir21	11	568	0.41	0.623	0.603	0.756	0.565	0.348	0.162	0.479	***	+0.2378

Note: 572 samples of adults and offspring. *k* = number of alleles; *n* = number of individuals; H_O = observed heterozygosity; H_E = expected heterozygosity; PIC = polymorphic information content; NE-1P = combined nonexclusion probability (first parent); NE-2P = combined nonexclusion probability (second parent); NE-PP = combined nonexclusion probability (parent pair); NE-I = combined nonexclusion probability (identity); HW = Hardy-Weinberg equilibrium; *F* (null) = frequency of null allele.

APPENDIX 2. Paternity assignment in *Theobroma cacao* (mean observed error rate across loci = 0.1166; dispersal distance range: 10–3007 m).

			Assignments		Assignment rate	
Level	Confidence (%)	Critical Delta	Observed	Expected	Observed	Expected
Strict	95	5.59	44	73	13%	21%
Relaxed	80	2.13	57	107	17%	31%

APPENDIX 3. Summary of private alleles in parental trees and offspring of *Theobroma cacao.*

Type	Locus	Allele	Frequency
Parental tree	mTcCir25	132	0.007
Parental tree	mTcCir25	142	0.024
Parental tree	mTcCir25	160	0.007
Parental tree	mTcCir25	176	0.002
Parental tree	mTcCir11	300	0.002
Parental tree	mTcCir12	184	0.002
Parental tree	mTcCir12	192	0.002
Parental tree	mTcCir12	210	0.002
Parental tree	mTcCir1	136	0.002
Parental tree	mTcCir1	140	0.002
Parental tree	mTcCir1	148	0.002
Parental tree	mTcCir1	152	0.002
Parental tree	mTcCir15	244	0.009
Parental tree	mTcCir15	246	0.013
Parental tree	mTcCir15	248	0.002
Parental tree	mTcCir15	250	0.004
Parental tree	mTcCir21	184	0.004
Offspring	mTcCir11	296	0.003
Offspring	mTcCir12	194	0.001
Offspring	mTcCir12	198	0.001
Offspring	mTcCir12	200	0.001
Offspring	mTcCir7	148	0.005
Offspring	mTcCir7	152	0.002
Offspring	mTcCir7	164	0.002
Offspring	mTcCir7	166	0.003
Offspring	mTcCir18	330	0.001
Offspring	mTcCir18	350	0.006
Offspring	mTcCir1	144	0.003