Extreme inbreeding in *Leishmania braziliensis*

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***Leishmania* species of the subgenus *Viannia* and especially *Leishmania braziliensis* are responsible for a large proportion of New World leishmaniasis cases. The reproductive mode of *Leishmania* species has often been assumed to be predominantly clonal, but remains unsettled. We have investigated the genetic polymorphism at 12 microsatellite loci on 124 human strains of *Leishmania braziliensis* from 2 countries, Peru and Bolivia. There is substantial genetic diversity, with an average of 12.4 ± 4.4 alleles per locus. There is linkage disequilibrium at a genome-wide scale, as well as a substantial heterozygote deficit (more than 50% the expected value from Hardy−Weinberg equilibrium), which indicates high levels of inbreeding. These observations are inconsistent with a strictly clonal model of reproduction, which implies excess heterozygosity. Moreover, there is large genetic heterogeneity between populations within countries (Wahlund effect), which evinces a strong population structure at a microgeographic scale. Our findings are compatible with the existence of population foci at a microgeographic scale, where clonality alternates with sexuality of an endogamic nature, with possible occasional recombination events between individuals of different genotypes. These findings provide key clues on the ecology and transmission patterns of *Leishmania* parasites.**

clonality | microsatellites | population genetics | endogamyl | heterozygote defiency

Leishmaniases are worldwide vector-borne diseases of humans and domestic animals, caused by protozoan parasites of the genus *Leishmania*. These parasitoses are a serious public health problem, with about 350 million persons at risk and 2,357,000 new cases per year (1). Leishmaniases occur on all continents except Antarctica. There are more than 20 described species causing human infections (review in ref. 2). Clinical symptoms range from asymptomatic, cutaneous, and mucocutaneous to visceral forms, depending on the *Leishmania* species. Visceral leishmaniasis is mainly caused by species from the *Leishmania donovani* complex; cutaneous and mucocutaneous forms are associated with species from the *Viannia* and *Leishmania* subgenera (3–5). *L. braziliensis* causes cutaneous and mucocutaneous leishmaniases in South America, where these are a severe public health problem.

Despite numerous studies and recent advances in the molecular genetics of these organisms, the reproductive mode of these parasites remains unsettled. Tibayrenc and Ayala (6) proposed that all (or most) *Leishmania* species are clonal. Other authors have challenged this hypothesis, based on pulse field gel electrophoresis (PFGE) data, and argued that some *Leishmania* species are potentially automictic, with frequent genetic exchanges (7). Several studies suggest that recombination may occur in *Leishmania*, and that other complexities may exist (see review in ref. 2). For example, based on evidence from PFGE analyses, Bañuls et al. (8) have proposed the occurrence of pseudorecombination in *Leishmania* populations. Moreover, several genetic studies indicate genetic recombination between *Leishmania* individuals, despite lack of evidence for a sexual stage (9–16). In any case, the molecular data suggest that, after a hybridization event, hybrids propagate clonally in natural populations (9, 12).

The prevailing hypothesis is that *Leishmania* displays a clonal mode of reproduction with occasional pseudorecombination and intragenic recombination, which mimic sexual reproduction processes, and that infrequent genetic exchanges take place in wild populations. Nevertheless, much remains to be elucidated as this interpretation is challenged by certain data, such as the absence of large excess in heterozygosity, as expected in clonal diploids (17, 18), and the lack of a clear structure in individualized lineages at the intraspecific level (2). Indeed, in a clonal model, an excess of heterozygotes and significant linkage disequilibrium are expected. Thus, the known results have failed to resolve the issue of clonality vs. sexuality in these protozoan parasites. Improved knowledge of the population structure and reproductive strategy of *Leishmania* parasites would provide a better understanding of their transmission patterns, as well as useful information for diagnostic purposes, epidemiological surveys, and drug and vaccine development.

Microsatellite loci are highly polymorphic, codominant, abundant throughout the genome, and relatively easy to assay (19, 20). In *Leishmania*, microsatellite studies are relatively recent; a small number of polymorphic microsatellites have been described for *Leishmania* species of the *Viannia* subgenus and especially for *L. braziliensis* (21). We analyze the population structure of *L. braziliensis* in several natural populations from South America (Peru and Bolivia), based on 12 microsatellite loci previously described (22). Peru and Bolivia are 2 of 7 world countries that report 90% of cutaneous leishmaniasis cases. Our population genetics analysis may be the first study of this kind for this *Leishmania* species. It reveals an unexpectedly high level of inbreeding within local samples, a large part of which is explained by local heterogeneity (Wahlund effect), probably due to a microgeographic population substructure, but also to the occurrence of mixed-mating events that include a significant contribution of endogamy (i.e., recombination between 2 genetically identical cells).

Results

We analyzed 124 human strains of *L. braziliensis* from 4 samples: 2 from the Pilcopata department in Peru, isolated in either 1993 or 1994, and 2 from Chapare Natural Park in Bolivia, isolated in either 1994 or 1998 (Tables 1 and 2). Both sites are located in the Amazonian forest and extend over large areas of great faunal and floral diversity.

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Table 1. Genetic diversity at 12 microsatellite loci in 124 strains of *Leishmania braziliensis* strains from 4 populations

Locus	GenBank accession no.	Allele size, bp	*N*	H_s	F_{IS}
AC01	AF139110	198–212	8	0.707	0.576
AC16	AF139112	147–161	11	0.754	0.341
AC52	AF139111	098–126	22	0.914	0.501
ARP	AF045249	121–157	18	0.874	0.441
ITSbraz	AJ300483	100–108	6	0.603	0.923
lbh3	AF044682	116–136	9	0.584	0.599
LRC	BX544585	118–134	15	0.826	0.424
CAK	BX544561	152–170	13	0.743	0.676
EMI	BX541508	183–189	14	0.809	0.645
LBA	BX539885	168–180	14	0.803	0.225
GO9	BX539509	148–168	10	0.673	0.466
E11	BX542509	096–108	9	0.715	0.618
Mean ± SE			12.4 ± 4.4	0.750 ± 0.100	0.537 ± 0.040

N, number of alleles; H_s, Nei's unbiased genetic diversity within subsamples (23); F_{IS}, deviation from panmixia.

Genetic Diversity and Heterozygote Deficiency. We obtained clear electrophoregrams for all genotypes at all 12 loci investigated, with only 1 or 2 alleles per strain at each locus, which excludes events of aneuploidy (for which we would have expected individuals with 3 or 4 alleles). There is considerable genetic diversity, with an average number of alleles per locus of 12.4 ± 4.4, ranging from 6 (*ITSbraz*) to 22 (*AC52*), and a mean genetic diversity $H_s = 0.750 \pm 0.100$ (Table 1).

There is large deficiency of heterozygotes compared with Hardy–Weinberg expectations in each population, for both the multilocus data and each locus separately. The population F_{IS} ranges from $F_{IS} = 0.396$ in Bolivia 1994, to $F_{IS} = 0.687$ in Bolivia 1998 (Fig. 1). For individual loci, the average values range from $F_{IS} = 0.225$ for *LBA* to $F_{IS} = 0.676$ for *CAK* (Fig. 2). The overall mean value is $F_{IS} = 0.504$ (95% CI = 0.427–0.577). All F_{IS} values are significantly different from zero ($P \leq 0.001$).

The selfing rate (s) required to account for the heterozygote deficiency observed over all samples and loci is $s = 0.67$.

Wahlund Effect. The heterozygote deficiency could possibly result from the Wahlund effect—that is, population subdivision within each subsample. This can be investigated with the Bayesian analysis of genetic population structure (BAPS) software. The 2 populations from Bolivia collected in 1994 and 1998 are composed of 15 (with probability $P_{BAPS} = 0.46$) and 13 clusters ($P_{BAPS} = 0.69$), respectively. The other 2 populations from Peru collected in 1993 and 1994 are composed of 18 ($P_{BAPS} = 0.60$) and 11 clusters ($P_{BAPS} = 0.63$), respectively. In each partition identified by BAPS in the 4 subsamples, the heterozygote deficit was calculated again. There was a decrease in F_{IS} with respect to the initial data set. However, $F_{IS.C} = 0.307$ (CI = 0.227–0.584) remains significant ($P \leq 0.001$; Fig. 3). Moreover, analyses with the Wilcoxon test showed a significant decrease ($Z = 1.657, P = 0.0488$). Thus, ≈40% of total F_{IS} can be explained by a Wahlund effect. The selfing rate required to account for this remaining F_{IS} is high, $s_c = 0.47$.

Population Differentiation. The genetic differentiation between Peru and Bolivia for both 1994 collections was small but significant ($F_{ST} = 0.092$, $P < 0.001$). There was also a small temporal differentiation between 1993 and 1994 in Peru ($F_{ST} = 0.004$, $P < 0.001$) and an apparently larger one between 1994 and 1998 in Bolivia ($F_{ST} = 0.114$, $P < 0.001$).

The differentiation between BAPS clusters, using the HIERFSTAT software, was very high ($F_{Cluster/Country} = 0.31$), as expected. The remaining variation between countries was smaller but significant ($F_{Country/Total} = 0.07$, $P < 0.002$).

Linkage Disequilibrium. Linkage disequilibrium for all populations is significant for 46 of the 66 pairs of loci (70%), which is much higher than the 5% (about 3 loci pairs) expected by chance. After sequential Bonferroni correction, 13 pairs remain in significant linkage disequilibrium, so that each of the 12 loci is involved in at least one significant linkage. This cannot be attributed to close physical linkage between loci, as the 12 loci are distributed on different chromosomes (22). These findings indicate strong linkage at a genome-wide scale.

Discussion

Numerous studies published since 1990 suggest that *Leishmania* species may have a predominantly clonal mode of reproduction associated with rare sexual recombination events. The majority of these studies are, however, based on databases that may not be suitable to reach that conclusion. Clonality is mainly inferred from analyses of strong linkage disequilibria observed across loci (24). Yet, computer simulations show that linkage disequilibrium is not a reliable measure of the proportion of clonal versus sexual reproduction in a population (25) because it is too sensitive to population demographic parameters (see also refs. 26–28). Moreover, the genetic markers used (such as multilocus enzyme electrophoresis [MLEE], random amplified polymorphic DNA [RAPD], restriction fragment length polymorphism [RFLP], and pulse field gel electrophoresis [PFGE]) have inherent limitations for inferences on the population genetic structure. These molecular markers have little resolution power (MLEE or RFLP), are dominant (RAPD), or multifactorial (reflecting global genomic organization in PFGE). Thus, even if these approaches can give valuable information on the evolutionary history of *Leishmania* species, they do not allow definitive conclusions about the population structure and the mode of reproduction of such organisms.

Our findings reveal a strong deficiency of heterozygotes (as well as linkage disequilibrium), theoretically incompatible with a strictly clonal reproduction model. Theoretical studies have shown that diploid clones are expected to accumulate heterozygosity at every locus over time (29–33). Clonal diploids should therefore exhibit negative F_{IS} values (17). There are several nonexclusive hypotheses that could account for heterozygote deficiency. They include the presence of null alleles, natural selection, genic conversion, the Wahlund effect, and inbreeding.

Null alleles are often encountered in population genetics studies. They may be frequent in allozymes (34, 35) and in such DNA markers as microsatellites (36–38). In our data, there is relatively little F_{IS} variation across loci, and those loci displaying the strongest F_{IS} variance are not necessarily those with the highest F_{IS} (see Fig. 2), which is what would be expected if null alleles were present. Moreover, no blank has ever been observed in the genotypes (no missing data; i.e., all individuals were amplified at all loci), which, given the high F_{IS}, makes the null allele explanation unlikely.

Table 2. Data set with each sample code, the country, and the year of collection and all genotypes obtained at each locus by PCR

Sample code	Country	Year	Loci											
			AC01	AC16	AC52	ARP	ITSbraz	Ibh3	LRC	CAK	EMI	LBA	G09	E11
LC1568	Peru	1993	202–202	149–161	104–104	139–139	102–102	130–130	124–124	162–162	165–165	180–180	150–152	102–102
LC2231	Peru	1994	202–202	149–155	084–098	139–145	106–106	130–130	132–132	162–162	189–189	178–178	150–154	096–096
LC2280	Peru	1994	198–210	151–161	084–084	137–139	104–106	130–130	132–132	158–158	191–191	176–182	150–162	098–102
LC2282	Peru	1994	206–212	149–149	100–100	151–153	102–102	128–128	120–126	162–162	165–165	176–180	156–156	096–096
LC2291	Peru	1994	202–202	151–151	120–120	133–149	102–106	130–130	128–128	162–162	175–185	174–182	148–148	100–100
LC2292	Peru	1994	204–210	149–149	084–120	139–139	104–104	130–130	132–132	160–160	189–189	174–180	148–152	096–096
LC2293	Peru	1994	202–202	149–151	098–098	133–149	102–106	130–130	128–128	164–168	175–185	174–174	150–152	100–102
LC2308	Peru	1994	204–210	151–169	100–100	149–149	100–102	116–130	120–128	160–160	185–185	174–180	154–154	098–100
LC2310	Peru	1994	204–210	159–159	084–094	137–139	102–102	130–130	132–132	160–160	189–189	176–182	148–156	100–100
LC2315	Peru	1994	202–210	151–161	108–108	141–141	102–102	130–130	130–134	162–162	189–189	176–182	154–154	100–104
LC2316	Peru	1994	200–204	147–161	088–088	135–135	100–100	118–130	130–138	160–160	185–185	176–180	148–154	098–098
LC2318	Peru	1994	202–202	149–167	092–108	139–139	102–102	130–130	130–130	162–162	177–195	174–182	152–152	100–100
LC2319	Peru	1994	200–200	149–157	104–110	141–147	104–108	130–130	124–124	164–164	193–193	174–184	152–152	100–100
LC2320	Peru	1994	202–210	149–155	118–118	155–155	104–104	128–128	132–132	162–162	189–189	174–182	150–154	102–102
LC2321	Peru	1994	208–208	149–149	086–106	149–155	100–100	128–128	122–130	162–162	189–189	176–176	148–156	098–106
LC2352	Peru	1994	202–212	149–161	110–110	143–143	102–104	130–130	126–130	162–162	191–191	178–180	150–154	098–098
LC2353	Peru	1994	202–212	149–161	084–094	129–139	102–102	128–128	132–132	158–162	191–191	176–180	150–152	098–098
LC2355	Peru	1994	210–210	149–159	098–122	129–137	102–102	128–128	132–132	158–160	189–189	176–180	150–150	100–100
LC2367	Peru	1994	202–202	149–161	084–098	131–139	102–106	130–130	132–132	158–164	191–191	174–180	148–148	098–098
LC2368	Peru	1994	202–202	149–161	084–098	135–141	102–104	130–130	132–132	158–162	191–191	174–180	148–148	100–100
LC2369	Peru	1994	200–210	149–161	096–096	135–141	102–106	130–130	126–132	158–164	189–189	174–182	150–150	098–098
LC2371	Peru	1994	200–200	151–161	084–098	135–141	100–106	130–130	132–132	158–164	189–189	174–180	148–148	098–098
LC2373	Peru	1994	202–202	151–161	084–098	133–139	102–104	130–130	132–132	162–162	191–191	174–180	150–150	100–100
LC2284	Peru	1994	210–210	151–161	086–116	139–139	102–102	130–130	130–130	158–160	189–189	174–180	150–152	100–102
LC2322	Peru	1994	202–202	149–161	094–110	149–149	104–104	130–130	130–130	160–160	191–193	174–182	154–154	100–102
CH12B	Bolivia	1994	204–206	149–159	126–126	155–157	102–102	116–130	124–130	168–168	183–183	174–180	154–154	100–100
CH15	Bolivia	1994	206–206	147–157	124–124	155–157	102–102	116–130	122–134	164–164	189–189	168–174	154–154	100–106
CH17	Bolivia	1994	202–202	147–161	096–096	131–131	102–102	130–130	132–132	164–164	185–191	168–168	154–154	098–102
CH25B	Bolivia	1994	202–204	149–149	100–100	121–121	102–102	130–130	124–134	158–164	179–187	168–172	152–168	100–102
CH29B	Bolivia	1994	204–206	149–159	126–126	125–157	102–102	128–128	130–134	158–166	183–185	174–180	154–154	100–100
CUM106	Bolivia	1994	204–206	147–159	100–100	129–131	102–102	128–128	124–128	164–164	185–189	174–174	152–154	100–100
CUM107	Bolivia	1994	202–202	149–149	092–092	121–121	106–106	120–120	118–120	156–156	189–189	170–170	144–144	090–090
CUM31	Bolivia	1994	206–206	149–159	126–126	135–137	102–102	116–128	122134	162–172	187189	168–174	154–154	102–108
CUM38	Bolivia	1994	202–204	149–149	098–104	129–135	102–102	130–130	114134	160–160	185–185	168–168	154–154	102–102
CUM41	Bolivia	1994	202–202	149–149	114–120	131–131	102–102	118–128	126–130	160–160	185187	168–172	154–154	100–102
CUM50	Bolivia	1994	206–206	149–159	100–110	129–147	102–102	116–128	122–124	164–164	185–187	174–174	154–154	100–102
CUM52	Bolivia	1994	206–206	149–149	100–110	129–147	102–102	116–128	122–124	164–164	185–187	174–174	154–154	100–102
CUM53	Bolivia	1994	206–206	149–149	112–112	129–147	104–104	116–116	122–132	164–170	185–189	174–174	154–154	098–104
CUM55	Bolivia	1994	206–206	149–149	126–126	137–137	102–102	128–130	114–130	158–170	187–189	162–168	154–154	098–102
CUM59	Bolivia	1994	206–206	149–159	124–124	129–129	102–102	136–136	122–134	162–174	187–189	168–174	154–154	102–108
CUM65	Bolivia	1994	202–202	149–159	114–114	129–137	102–102	116–128	122–130	164–164	179–185	174–174	154–154	102–102
CUM67	Bolivia	1994	208–208	149–149	088–108	149–155	102–106	128–128	122–130	160–166	187–193	176–178	150–156	100–108
CUM68	Bolivia	1994	202–206	151–151	128–128	129–129	102–108	128–128	122–130	164–168	187–189	168–174	152–154	102–102
CUM82	Bolivia	1994	202–206	149–149	098–112	131–131	100–102	136–136	124–132	164–164	179–189	180–180	154–154	098–100
CUM84	Bolivia	1994	206–206	149–149	096–096	131–131	100–100	116–130	130–130	158–166	181–189	174–174	152–152	100–100
CUM97	Bolivia	1994	206–206	149–159	100–110	129–147	102–102	116–128	122–124	164–164	185–187	174–174	154–154	100–102
CUM96	Bolivia	1994	204–204	149–161	084–098	129–137	102–102	128–128	130–130	162–172	191–193	168–182	154–154	100–102

Selection can strongly affect allele and genotypic frequencies. Underdominance, which decreases the fitness of heterozygous individuals, would result in deficiency of heterozygous genotypes relative to Hardy–Weinberg expectations. Underdominance, however, is not expected to be frequently encountered in nature because it is highly unstable (the rarest allele tends to disappear). We have observed similar F_{IS} patterns across all 12 (dinucleotide, noncoding) microsatellite loci. Widespread, almost genome-wide, underdominance would be required to fit our data, which does not seem reasonable.

Parasites of the Trypanosomatidae family, such as *Leishmania* species, are characterized by genetic plasticity, so that they can use different pathways to generate genetic diversity (e.g., gene conversion; refs. 39 and 40), a process of unidirectional transfer of genetic material between members of a multigenic family (41). Gene conversion generates a transition from the heterozygous stage to the homozygous stage, and thus can result in substantial heterozygote deficiency (40). Given that we obtained similar findings across the 12 independent microsatellite loci, gene conversion could account for our findings only if it occurred among all of the loci studied, across the entire genome, which seems unlikely. If gene conversion significantly affected microsatellite loci heterozygosity, a negative relationship would be expected between differences in allele size in heterozygous individuals and the number of such heterozygous individuals in the data set. This is because heterozygotes recently aroused through mutation have less chance of being immediately converted again compared with older heterozygotes, and with microsatellite loci in clonal organisms, old heterozygotes are expected to carry the most distant alleles in terms of size (30, 31). If gene conversion occurs frequently, microsatellite loci should restore heterozygosity through mutation and thus between alleles that are close in length. We obtained a significant negative relative relationship between the size difference in bases between alleles (Δ) and the number of heterozygous individuals, N_{Hz}, only for locus *E11* (see Fig. S1 and *SI Text*). This locus is located 60 bp before the trifunctional enzyme alpha subunit mitochondrial precursor-like gene, and this observation might reflect frequent conversion in this genomic region. If we exclude *E11* from our data, the overall high F_{IS} values persist.

Clustering within each sample, as performed by BAPS, results in a substantial (40%) decrease in F_{IS} values. This is consistent with the existence of a strong Wahlund effect within each

Table 2. (continued)

Sample code	Country	Year	Loci AC01	AC16	AC52	ARP	ITSbraz	Ibh3	LRC	CAK	EMI	LBA	G09	E11
CUM99	Bolivia	1994	204–204	149–159	088–088	121–121	102–102	122–122	132–132	176–176	185–191	162–162	150–156	098–098
CUM32	Bolivia	1994	204–204	147–161	124–124	129–147	102–102	118–130	120–134	164–172	189–189	168–174	152–152	100–106
CUM46	Bolivia	1994	202–202	149–149	118–118	127–127	102–102	136–136	120–128	152–152	185–187	168–182	154–154	100–100
CUM30	Bolivia	1994	200–200	149–149	090–090	129–131	104–104	122–122	114–130	164–164	179–179	170–170	150–150	096–096
CUM24	Bolivia	1994	204–204	149–149	110–110	129–129	102–102	132–132	124–134	158–164	189–189	174–174	166–166	102–102
CUM42	Bolivia	1994	204–204	149–159	098–110	129–147	100–100	116–128	124–124	164–164	183–183	174–174	152–154	100–100
CUM26	Bolivia	1994	202–204	151–151	118–118	129–137	102–102	116–116	120–132	164–164	183–183	174–180	152–152	102–102
CUM39	Bolivia	1994	204–204	151–151	098–110	135–137	100–100	116–128	120–122	164–164	189–189	172–172	152–152	098–102
CH22B	Bolivia	1994	204–204	149–159	118–118	129–129	102–102	128–128	120–128	162–162	185–187	168–182	154–154	102–102
CUM49	Bolivia	1994	206–206	151–151	122–122	129–129	102–102	130–130	122–124	164–164	185–187	174–174	154–154	100–102
CUM51	Bolivia	1994	202–204	151–161	098–118	135–137	102–102	116–130	132–134	162–162	187–191	168–174	152–154	100–102
CUM251	Bolivia	1998	200–200	149–149	092–092	143–143	102–102	122–122	128–128	166–166	189–189	172–172	152–152	096–096
CUM252	Bolivia	1998	200–200	153–153	116–116	141–141	106–106	122–122	128–128	166–166	183–183	170–172	152–152	096–096
CUM263	Bolivia	1998	204–204	161–161	100–116	131–147	102–102	116–130	122–122	162–162	185–185	176–182	154–154	100–100
CUM266	Bolivia	1998	200–200	149–149	116–116	145–145	106–106	122–122	116–130	164–164	183–189	168–172	152–152	098–098
CUM271	Bolivia	1998	200–200	153–153	098–118	143–143	106–106	122–122	116–128	164–164	181–181	166–170	152–152	096–096
CUM274	Bolivia	1998	200–200	153–157	098–108	139–139	106–106	122–122	116–118	164–164	183–183	170–172	152–152	096–096
CUM275	Bolivia	1998	200–200	151–151	092–092	143–143	106–106	122–122	120–120	164–164	181–181	170–172	152–152	096–096
CUM276	Bolivia	1998	200–200	151–151	092–092	143–143	106–106	122–122	116–130	164–164	183–183	172–172	152–152	096–096
CUM277	Bolivia	1998	204–206	151–151	100–110	131–147	102–102	130–130	116–126	162–162	185–185	168–174	154–154	100–100
CUM279	Bolivia	1998	200–200	151–161	094–094	145–145	100–102	122–122	116–128	164–164	183–183	170–172	152–152	096–096
CUM280	Bolivia	1998	200–200	151–151	092–092	145–145	106–106	122–122	128–128	164–164	183–183	174–174	154–154	096–096
CUM281	Bolivia	1998	200–200	149–149	092–116	145–145	106–106	124–124	132–132	164–164	181–181	187–189	150–150	096–096
CUM282	Bolivia	1998	200–200	149–159	090–090	139–139	106–106	122–122	130–130	164–164	181–181	180–180	152–152	100–100
CUM285	Bolivia	1998	200–200	151–151	092–092	139–149	106–106	122–122	130–130	164–164	183–183	170–170	152–152	096–096
CUM286	Bolivia	1998	200–200	151–151	092–092	141–149	106–106	122–122	130–130	164–164	183–183	170–170	152–152	100–100
CUM288	Bolivia	1998	200–200	149–149	092–092	139–139	106–106	122–122	120–120	164–164	183–183	172–172	152–152	096–096
CUM289	Bolivia	1998	200–200	161–161	092–092	141–149	102–102	122–122	130–130	166–166	183–189	170–170	152–152	098–098
CUM290	Bolivia	1998	200–200	149–159	092–092	145–145	106–106	122–122	118–128	166–166	183–183	172–172	152–152	096–096
CUM291	Bolivia	1998	200–200	149–159	092–092	145–145	106–106	116–130	132–132	164–164	187–187	170–170	152–152	096–096
CUM294	Bolivia	1998	202–202	149–149	088–088	147–147	102–102	122–122	130–130	174–174	189–189	164–164	150–150	094–094
CUM295	Bolivia	1998	202–202	151–151	088–088	131–131	102–102	122–122	132–132	164–164	191–191	164–164	152–152	096–096
CUM310	Bolivia	1998	202–202	151–151	112–112	129–147	102–102	116–130	124–132	162–162	185–189	174–180	154–156	102–102
CUM311	Bolivia	1998	200–200	149–149	100–108	131–131	102–102	116–128	132–132	162–162	189–189	174–174	152–152	098–100
CUM323	Bolivia	1998	206–208	159–159	100–110	131–137	102–102	130–130	124–130	164–164	187–187	174–182	154–154	098–102
CUM324	Bolivia	1998	206–206	161–161	098–098	131–137	100–100	128–128	132–132	166–168	185–185	174–182	154–154	096–096
CUM327	Bolivia	1998	204–204	151–161	104–108	137–137	102–102	128–128	132–132	162–162	181–181	178–180	150–152	102–102
CUM329	Bolivia	1998	204–204	149–149	116–116	145–145	102–102	116–130	124–128	162–162	185–185	174–180	154–154	100–102
CUM331	Bolivia	1998	200–200	149–149	098–112	145–145	106–106	122–122	116–130	164–164	181–189	170–170	152–152	096–096
CUM346	Bolivia	1998	204–204	151–161	104–108	131–137	102–102	130–130	132–132	162–162	185–185	174–180	152–152	098–098
CUM381	Bolivia	1998	200–200	149–153	100–110	145–145	106–106	122–122	116–118	166–166	183–183	168–168	152–152	096–096
CUM384	Bolivia	1998	198–204	149–159	116–116	129–135	102–102	116–130	122–122	162–162	185–185	174–180	154–154	100–102
CUM388	Bolivia	1998	200–200	149–149	098–110	131–137	100–102	116–116	122–132	162–162	183–183	172–180	150–150	098–098
CUM389	Bolivia	1998	204–212	151–151	118–118	131–137	102–102	116–116	122–132	162–162	183–183	172–180	152–152	098–098
CUM393	Bolivia	1998	200–200	153–153	098–098	149–149	106–106	128–128	130–130	164–164	185–185	168–172	148–156	100–100
CUM396	Bolivia	1998	200–200	151–153	116–116	143–143	102–102	122–122	116–118	164–164	183–183	170–170	152–152	096–096

subsample. Considering the large areas investigated (100–200 km^2), it is not unreasonable to expect geographic subdivision within our *Leishmania* samples. In addition to geographic barriers that could influence parasite distribution, the biology of vectors and reservoirs may strongly interfere with the homogeneous spread of genotypes across both regions sampled. We note, for example, that the overall flight distance traveled by a sandfly over its entire lifetime is estimated to be ≈1 km (42); the scale at which our samples were collected is far above this limit. The Wahlund effect we have detected indicates that the Bolivian and Peruvian samples are probably each composed of several strongly differentiated subpopulations. This substructure may result in very large global effective population sizes, as shown by the weak temporal differentiation we observed, and thus may contribute to maintaining the genetic diversity at the scale of each geographic population. This could provide *Leishmania* populations with an advantage in adaptability to environmental differences and changes. The modest but significant differentiation observed between countries (most of the variance is contained within each country) reflects that there is little migration between countries, as well as between subpopulations within each country, where most of the variance occurs (see SI).

Heterozygote deficiency remains high (above 0.307) for every sample and for every locus, even within the clusters defined by BAPS. These findings, together with the high linkage disequilibrium observed, support the idea that these parasites, known to reproduce by clonal fission, also often sexually cross with individuals from the same strain (endogamy), unless our sampling did not allow us to detect a more nested population structure than is apparent. To evaluate this possibility, we studied the distribution of the heterozygous loci in each individual and found a random distribution (adjusted to a Poisson distribution, Kolmogorov–Smirnoff test). A small simulation study undertaken with Easypop v. 2.0.1 (43) suggests that our data are compatible with partially clonal populations. More especially, it seems likely to correspond to very small subpopulations, well structured at a scale much smaller than what can be detected even with clustering procedures (see Figs. S2 and S3 and *SI Text*).

On the basis of the previous considerations, we propose that *Leishmania* parasites use alternative modes of reproduction: clonal-

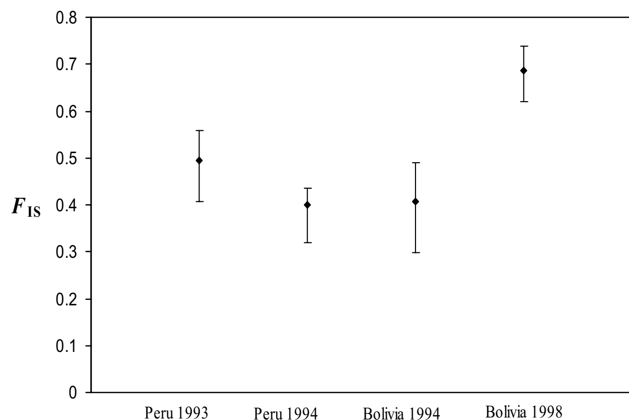

Fig. 1. F_{IS} and 95% confidence intervals obtained by bootstrap over loci, for the 124 human *Leishmania braziliensis* strains collected in 2 different countries in 2 different years. F_{IS} measures the local deficiency of heterozygous genotypes due to nonrandom mating. There is a large heterozygote deficiency in each population, shown because F_{IS} is significantly greater than zero.

ity in both the vertebrate host and the insect vector and occasional sexual fusion in the vector, as has been shown to occur for other kinetoplastid parasites, such as *Trypanosoma brucei* s.l. (44). However, in *Leishmania*, this fusion may frequently involve genetically related parasites or even genetically identical members of the same strain, given the very low incidence of *Leishmania* parasites generally observed in the sandfly vectors (45–47).

We are not aware of any previous evidence of such strong inbreeding in *Leishmania*. This changes the assumption that its mode of reproduction is overwhelmingly clonal. This finding is an important step toward an understanding of leishmaniasis epidemiology. Reproductive mode influences the distribution of alleles within individuals and impacts the rate of selection of recessive or dominant alleles.

An important observation is the high overall genetic diversity observed in each sample. Published studies suggest that there is a link between the genetic polymorphism of circulating strains of *Leishmania* and environmental diversity (48). The large diversity observed in the present samples may be related to the extremely diversified ecosystem (various host and vector species; ref. 49) of the Amazonian forest.

To conclude this detailed population genetics study of *L. braziliensis*, which we believe may be the first of its kind, it seems that these parasites alternate clonal and sexual, although endogamic, reproduction, with infrequent recombination events between different individuals. In addition, our findings show the

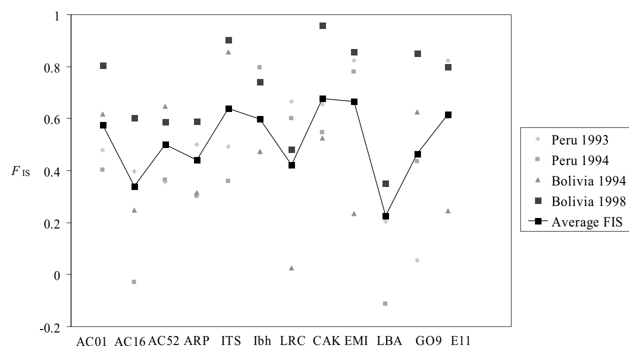

Fig. 2. F_{IS} for each of 12 microsatellite loci in the 4 populations (and their mean) of *Leishmania braziliensis* collected in Peru and Bolivia. There is a large heterozygote deficiency at each locus.

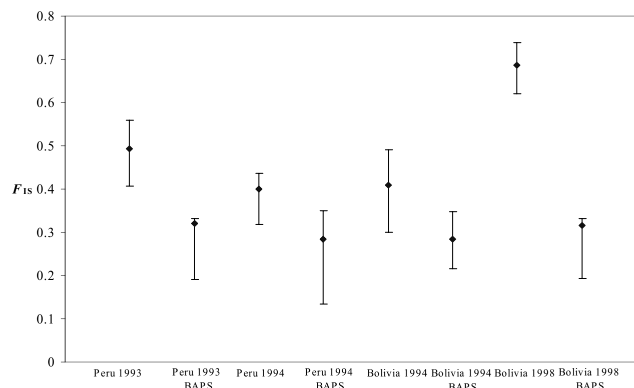

Fig. 3. F_{IS} for *Leishmania braziliensis* strains in each population and within their subdivisions as identified by BAPS. The decrease of F_{IS} in the subdivision suggests a Wahlund effect. However, the residual F_{IS} values are still high, which suggests the persistence of nonrandom mating (due, for example, to selfing).

existence of strong genetic heterogeneity within each country (Wahlund effect), suggesting a substantial population structure at a microgeographic scale. In future studies, it will be important to work at finer geographic scales to detect and delimit this substructuring. The approach used here needs to be applied to other species of *Leishmania* to ascertain the generality of our findings. In vitro experiments could explore whether sexual recombination readily occurs within the sandfly vectors.

Materials and Methods

Parasite Culture and DNA Extraction. One hundred twenty-four human isolates of *Leishmania* (*Viannia*) *braziliensis* were cultured. Promastigote cultures were maintained at 26 °C by weekly subpassages in RPMI1640 medium, buffered with 25 mM Hepes, 2 mM $NaHCO_3$, and supplemented with 20% heat-inactivated FCS, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cultures were harvested by centrifugation and stored at −80 °C until DNA extraction. Fifty-six strains from Peru and 68 from Bolivia were isolated in the Laboratory of Biochemistry, the Alexander Von Humbolt Institute of Tropical Medicine of the University of Lima (Peru), and at the University of Cochabamba Medical Center (Bolivia). We characterized the 124 strains as *L. braziliensis* using the MLEE technique as described in ref. 50. DNA was extracted from parasite mass cultures (promastigotes), using the classical phenol/chloroform extraction technique (51).

Genotyping. The 12 microsatellite loci investigated are listed in Table 1 (see ref. 22). A 30-μL reaction mix was made of 1.2 μL of each primer (10 μM), with the forward primer being labeled with fluorochrome, 100 ng template DNA, 0.9 μL dNTP mix (5 mM), 3 μL buffer 10× and 0.3 μL Taq polymerase (5 UI/μL; Roche Diagnostics). Amplifications were carried out in a thermal cycler: 30 cycles of 94 °C for 30 s, annealing temperature of each locus for 1 min at 72 °C, final extension at 72 °C for 7 min. The reaction products were visualized on a 1.5% agarose gel stained with ethidium bromide. Fluorescence-labeled PCR products were sized on Applied Biosystems Prism 310, with a Genescan 500 LIZ internal size standard. All 124 isolates were genotyped at all 12 loci.

Statistical Analyses. Data were analyzed with the software FSTAT (version 2.9.3.2; ref. 52), which computes estimates and tests the significance of various population genetics parameters. Genetic polymorphism was measured by the number of alleles per locus (*N*) and by Nei's unbiased genetic diversity within subsamples H_s (23). We estimated Wright's *F* statistics (53) with Weir and Cockerham's method (54): F_{IS} measures the relative inbreeding of individuals due to the local nonrandom union of gametes in each subpopulation; F_{IS_C} measures the relative inbreeding of individuals clustered; and F_{ST} measures the relative inbreeding in subpopulations attributable to the subdivision of the total population into subpopulations of limited size. F_{ST} thus also measures genetic differentiation between subpopulations. F_{IS} ranges between −1 and 1. A negative value corresponds to an excess of heterozygotes, a positive value to heterozygote deficiency; 0 is expected under panmixia. F_{ST} varies between 0, when genetic identity between individuals is independent from the subpopulation (no differentiation) and 1, when all individuals of the same subpopulation are homozygous for the same allele but differ from individuals of different subpopulations. The significance of the departure from 0 was

tested by 10,000 randomizations of alleles within subpopulations (for F_{IS}) and of individuals between subpopulations (for F_{ST}). For F_{IS}, the statistic used was Weir and Cockerham's estimator *f*; for F_{ST}, the statistic used was the log-likelihood ratio *G* (55) summed over all loci. Confidence intervals were estimated by bootstrapping over loci or jackknifing over populations with FSTAT. From the F_{IS} parameter, a potential selfing rate *s* was inferred using the formula $s = (2 * F_{IS})/(1 + F_{IS})$ (e.g., ref. 29).

Linkage disequilibrium between pairs of loci (nonrandom association of alleles at different loci) was assessed with a randomization test (genotypes at 2 loci are associated at random a number of times). The statistic used was the log likelihood ratio *G* summed over all subpopulations. Because this procedure was repeated on all pairs of loci, we applied the sequential Bonferroni correction (56) to the *P* values (*P* value × number of tests).

The #3.2 software identifies a hidden structure within populations through a Bayesian analysis. It clusters individuals into genetically distinguishable groups based on allele frequencies. This software was used to detect possible Wahlund effects and has been successfully applied to other parasites (16, 57). The BAPS software used stochastic optimization to infer the posterior mode of the genetic structure. To obtain the best distribution of the 4 populations under study, we ran the program many times to obtain the number of clusters. We also checked that nonstructured populations would not give the same results as ours. This was done by running BAPS on populations simulated with EASYPOP (version 2.0.1). Each of the 4 samples was submitted to a clustering exploration by BAPS with 160 runs with a maximum number of clusters set to 20. F_{IS} was recalculated in each best distribution identified by BAPS and compared F_{IS_C} with the initial F_{IS} using a unilateral Wilcoxon signed-rank test for paired data, the pairing units being the 12 loci. If F_{IS_C} is lower than $F_{IS,}$ it is probable that the initial subsamples were composed of several genetically distinct entities (e.g., geographical microstructure or subpopulations).

To estimate the contribution of macrogeography (between Bolivia and Peru) corrected for the effect of the subpopulation structure (between BAPS clusters), we used HIERFSTAT (version 0.03–2) software (58). This test uses the same statistics as those used for F_{ST} analyses, but the permutation procedure takes into account the hierarchy of the population structure. Differentiation between clusters within countries, $F_{Cluster/Country}$, is tested by randomization of individuals between clusters of the same country. $F_{Country/Total}$, the fixation index due to the distribution of clusters into different countries, is tested by randomizing clusters (including all individuals) between countries.

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