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MICROSATELLITES CAN BE MISLEADING: AN EMPIRICAL AND SIMULATION STUDY

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Abstract.—It has been long recognized that highly polymorphic genetic markers can lead to underestimation of divergence between populations when migration is low. Microsatellite loci, which are characterized by extremely high mutation rates, are particularly likely to be affected. Here, we report genetic differentiation estimates in a contact zone between two chromosome races of the common shrew (*Sorex araneus*), based on 10 autosomal microsatellites, a newly developed Y-chromosome microsatellite, and mitochondrial DNA. These results are compared to previous data on proteins and karyotypes. Estimates of genetic differentiation based on *F*- and *R*-statistics are much lower for autosomal microsatellites than for all other genetic markers. We show by simulations that this discrepancy stems mainly from the high mutation rate of microsatellite markers for *F*-statistics and from deviations from a single-step mutation model for *R*-statistics. The sex-linked genetic markers show that all gene exchange between races is mediated by females. The absence of male-mediated gene flow most likely results from male hybrid sterility.

Key words.—Common shrew, *F*-statistics, mitochondrial DNA, *R*-statistics, *Sorex araneus*, Y chromosome.

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Studies of genetic differentiation usually rely on estimates based on loci within one family of genetic markers. But within the same class of markers, the loci are likely to be affected by the same systematic bias. For instance, a recent article by Hedrick (1999) shows that microsatellites are likely to underestimate genetic divergence between populations due to their high polymorphism.

Among studies comparing genetic differentiation estimated from different classes of markers, those comparing microsatellites and allozymes are particularly relevant, because both are codominant, biparentally inherited, nuclear markers. Although a number of such studies found no significant differences in differentiation estimates between these two classes of markers (Barker et al. 1997; Estoup et al. 1998; Lynch et al. 1999; Ross et al. 1999), one study (Lehman et al. 1996) found lower differentiation for microsatellites than for allozymes. It is noteworthy that this latter investigation is the only one in which migration between populations was thought to be very low, because high mutation rates of microsatellites are expected to produce most serious underestimation of genetic structure with *F*-statistics when mutation is higher or of the same order of magnitude as migration. This situation can be expected in populations that are strongly differentiated. One such case is hybrid zones, which are characterized by partial reproductive isolation that arises when two adjacent populations are genetically too distinct to interbreed freely but have not acquired full reproductive isolation.

In this study, we analyze the population structure across a hybrid zone between two chromosome races of the common shrew (*Sorex araneus*). Three previous studies, one with karyotypes and allozymes (Brünner and Hausser 1996) and two with microsatellites (Lugon-Moulin et al. 1996, 1999), have shown gene exchange to be strongly reduced between these two races. Furthermore, when analyzed with microsatellites, chromosomal hybrids were genetically indistinguishable from karyotypically pure individuals sampled on the same side of the hybrid zone, thus suggesting that the sampled hybrids were backcrossed individuals of ancient hybrid ancestry (Lugon-Moulin et al. 1999). This indicates that interracial gene exchange is very rare. However, whereas karyotypes and allozymes were characterized by extremely steep clines and suggested very low genetic exchange ($F_{ST} \simeq 0.7$; Brünner and Hausser 1996), estimates based on microsatellites revealed much less differentiation ($F_{ST} \simeq 0.1$; Lugon-Moulin et al. 1996, 1999) with the presence of few race-specific alleles. *R*-statistics (Slatkin 1995), devised for microsatellite loci evolving under a single-step mutation model (SSM), were also much lower than *F*-statistic estimates inferred from chromosomes and allozymes ($R_{ST} \simeq 0.2$; Lugon-Moulin et al. 1999).

There are two possible explanations for this strong discrepancy. One is selection against introgressed chromosomes and allozymes. The other is underestimation of the overall nuclear genetic structure as inferred from microsatellite markers. Strong selection against introgressed chromosomes cannot be neglected because karyotypic hybrids could experience lower fertility through the formation of chain elements at meiosis (Brünner and Hausser 1996). Systematic selection against introgressed allozymes is less likely, although these results were based on only two loci. As for the second explanation, the high mutation rate of microsatellites can lead to underestimates of genetic differentiation by means of *F*-statistics when migration is low (e.g., Hedrick 1999), and violations of a strict SSM process could modify the func-

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tional relation between gene-flow and *R*-statistics (Slatkin 1995).

To clarify the cause of the discrepancy between these estimates of genetic differentiation, we use both empirical data and simulations. First, we expand the number of autosomal microsatellites to 10 and analyze two additional classes of genetic markers, mitochondrial DNA (mtDNA) and a microsatellite on a Y chromosome. Results from these two sex-specific markers are compared to the results obtained from chromosomes, allozymes, and microsatellites. Second, we explore through simulations how high polymorphism and the particular mutation models of microsatellites could lead to underestimates of the degree of genetic structure between populations.

Materials and Methods

Study Area and Contact Zone

The hybrid zone is located in the Arve Valley between Les Houches and Chamonix at the foot of the northern slopes of the Mont Blanc massif (western France). At this site the Cordon and Valais races meet at La Griaz stream. Individuals from both races can be distinguished on the basis of their karyotypes, morphology (Hausser et al. 1991), diagnostic allozymes (Neet and Hausser 1991), and by their mtDNA (Taberlet et al. 1994). For this study, we used individuals sampled by Brünner and Hausser (1996). These authors sampled shrew populations on a 13-km transect that runs across the contact zone. The sample is biased toward the Valais race because the Cordon populations are replaced after about 2 km from the contact zone by a sister species, the Millet's shrew (*Sorex coronatus*). A total sample of 260 individuals has been analyzed, 75 on the Cordon side (populations 1–5) and 185 on the Valais side (populations 6–17). All individuals were live caught and released after tissue samples were taken. A total of 195 individuals was karyotyped; only a single Valais individual was found on the Cordon side of the zone, no Cordon individual was found on the Valais side and no F_1 karyotype was detected (for details, see Brünner and Hausser 1996).

Genetic Data

All individuals were genotyped for the following 10 autosomal microsatellite loci: L9, L14, L16, L33, L45, L57, L62, L67, L68, L69 (Wyttenbach et al. 1997; Balloux et al. 1998). Polymerase chain reaction (PCR) conditions are given elsewhere (Lugon-Moulin et al. 1996; Balloux et al. 1998). End products were run on polyacrylamide sequencing gels (6%, 8 M urea), together with a sequencing reaction and the amplified clone as a size marker. An additional microsatellite locus L8Y (Genbank accession number AF175743) was also amplified. This locus is located either on the male Y1 or Y2 chromosome and was previously tested on individuals for which karyotypes were available ($N = 195$). L8Y was amplified to yield a unique end product in 117 males, whereas amplification never succeeded in 78 females. L8Y was amplified together with locus L14 in the same PCR reaction. This allowed the identification of females, which show an amplification pattern for the autosomal locus, but not for locus L8Y, versus PCR reactions which failed completely.

For the mtDNA, a fragment of 307 bp located in the cytochrome *b* gene was amplified with primers L14841 and H15149 (Kocher et al. 1989; Taberlet et al. 1994). PCR was performed in 25-μl volumes overlaid with mineral oil. The reaction mixture contained 125–250 ng DNA template, 1 μM primer, 4 mM $MgCl_2$, 200 μM dNTP, 1 × Taq polymerase buffer, and 0.5 U Extrapol II Taq polymerase (Chemie Brunschwig, Amsterdam, The Netherlands). Amplification lasted for 35 cycles with denaturation for 45 sec at 93°C, annealing for 45 sec at 50°C, and polymerization for 1 min at 72°C. Amplified DNA (8 μl) was double digested with the restriction enzymes *Asn*I (AT//TAAT) and *Dde*I (C//TNAG). *Asn*I and *Dde*I were chosen by comparing the sequences of the cytochrome *b* haplotypes of the two races (Taberlet et al. 1994). *Asn*I cleaves the amplified part of the cytochrome *b* haplotype Valais at position 14978 into two fragments of 137 bp and 170 bp, and *Dde*I cleaves at position 15073 of the Cordon haplotype into two fragments of 232 bp and 75 bp (positions according to the standard human mtDNA numbering from Anderson et al. 1981). Restriction profiles were read after migration on a 1.6% agarose gel stained with ethidium bromide.

Population Structure Analysis

For all loci, hierarchical estimates of *F*-statistics (Weir 1996) were obtained with the computer package S-PLUS (Statistical Sciences 1995). Four levels are defined; I, individuals; S, site (populations); R, race; and T, total. The heterozygote deficiency within populations is estimated as F_{IS}, between populations within races as F_{SR}, and between races as F_{RT}. For haploid markers (mtDNA and the microsatellite on the Y chromosome), F_{IS} and F_{IT} are meaningless. However, F_{SR} and F_{RT} are appropriate measures of the Wahlund effect. Samples with fewer than three males (sites 2 and 12) were not included in the analysis of the Y-chromosome microsatellite.

Fixation indices provide the correct criterion for differentiation if and only if diversity is low (Wright 1978; Charlesworth 1998; Nagylaki 1998; Hedrick 1999). In an infinite-island model and assuming mutation follows the infinite-allele model (IAM), F_{ST} is a decreasing function of $N(m + \mu)$, the product of local population size and the sum of migration and mutation (Rousset 1996). When mutation is negligible compared to migration, F_{ST} becomes a simple function of the number of migrants. But this assumption is unlikely to hold for microsatellites, especially when migration rates are low. An additional difficulty arises when the mutation model cannot be assumed to be an IAM. Indeed, under IAM, *F*-statistics are ratios of identities by descent (IBD; e.g., Rousset 1996). Any mutation model that introduces homoplasy will change IBD to identity in state (IIS). Under mutation models generating homoplasy, such as the SSM that has been proposed to be characteristic of microsatellites (e.g., Weber and Wong 1993; Di Rienzo et al. 1998), the relation between F_{ST} and the number of migrants plus mutants no longer holds (Rousset 1996). To account for these factors in the estimation of population differentiation with microsat-

ellites, we also computed *R*-statistics, which were specifically defined for loci undergoing stepwise mutations and take into account the variance in size of alleles (Slatkin 1995). *R*-statistics are independent of the mutation rate under an SSM. Under this model or a mixed model with both stepwise mutations and larger deletions or insertions, Slatkin (1995) showed that *R*-estimates are expected to be higher than *F*-estimates. Because our sampling scheme is hierarchical, we computed hierarchical *R*-estimates (R_{SR}, R_{RT}) through an analysis of variance on allele size (Rousset 1996; Michalakis and Excoffier 1996) using the statistical package S-PLUS (Statistical Sciences 1995). We also tested whether R_{RT}-estimates were significantly higher than their F_{RT} counterparts on the 10 autosomal microsatellites with a bootstrap procedure described in Lugon-Moulin et al (1999).

Simulations

The F_{ST}- and R_{ST}-estimates obtained from microsatellites were much lower than for other markers (chromosomes and allozymes). To explore the possible causes of this discrepancy, we performed simulations with various migration rates under a pure SSM, and a mixed model of SSM with a proportion of random mutations, which have the same probability to end up as any of the *K* possible allelic states. The model underlying these random mutations is called the *K*-allele model (KAM). The simulations were performed with a slightly modified version of EASYPOP, an individual based model of population structure (Balloux 1999).

For each replicate, two populations of 1000 individuals with 10 loci were simulated. We used the mutation rates (10^{-2}, 10^{-3}) corresponding to $N\mu = 10$ and $N\mu = 1$, and migration rates (0, 10^{-3}, 10^{-4}) corresponding respectively to no migration, one migrant every generation, and one migrant every 10th generation. The simulations were run under pure SSM models and models of mainly stepwise mutations but with a small proportion (5% and 10%) of KAM, while the remainining mutations are following a strict SSM model. When the number of possible allelic states under KAM is very large, the variance of allele size becomes unrealistically large. To keep this variance at a biologically realistic level, a model allowing for KAM mutations must have a finite possible range of allelic states. Therefore, we also explore the effect of a constraint on allele sizes (Nauta and Weissing 1996). We set the number of allelic states in our simulations to either 30 or 1000. Thirty allelic states mimics high constraint on allele size, whereas 1000 corresponds to no constraint. To check whether the constraint on the size of alleles was effective, we reasoned as follows: Because each replicate was followed over 100,000 generations, an allele in our simulations will undergo on average $10^2 \pm 10$ or $10^3 \pm 32$ mutations, for mutation rates respectively of 10^{-3} and 10^{-2}, because the number of mutations follows a Poisson distribution. We estimated the number of mutations necessary for an allele to reach the size limit for both 30 and 1000 allelic states by simulations. For 50,000 simulations, the lowest number of mutations before reaching the limit in size was 15 and 12,574 with 30 and 1000 allelic states, respectively. Even with the higher mutation rate (10^{-2}), the probability of reaching a size limit with 1000 possible allelic states can be neglected (it would require a deviation from the mean number of mutations of more than 361 standard deviations). Simulations with 1000 allelic states will therefore be considered as unconstrained. In contrast, a strong constraint will act on simulations performed with 30 allelic states, where most alleles will reach the limit at least once.

The simulations were run for 100,000 generations and replicated 20 times. Each 1000th generation, gene diversity and variance in allele size within population, F_{ST} and R_{ST}, were estimated. Multilocus F_{ST} and R_{ST} were computed as the ratio of the averaged variances following Weir and Cockerham (1984) and Goldstein et al. (1995).

Expectations of F_{ST}, R_{ST}, Heterozygosity (H_s) and Variance in Allele Size (V_s) under Single-Step Mutation

Expectations for *R*- and *F*-statistics, as well as that of the variance in allele sizes and gene diversity, were derived by Rousset (1996) for the stepwise mutation process. He showed that one way to obtain these expectations is to consider the generating function $\Psi(z)$ of the probabilities that a randomly chosen gene differs by *k* steps from another randomly chosen gene. The simulations were checked by comparing the expected values of the different parameters under a strict SSM with the results of simulations. In our model, individuals rather than gametes migrate. We therefore used Nagylaki's (1983) transition equation (78) instead of Rousset's (1996) equation (9) to account for diploid migration. This leads us to the following system of equations for the generating functions of identity in state at equilibrium:

$$\psi_1(z) = \{cr(z)[1 + (b - 1)r(z)]\} \div \{1 + [b - 1 - c + a(2c - 1)]r(z) + [a - b + c - 2ac + bc]r(z)^2\}, \quad (1a)$$

$$\psi_2(z) = \{cr(z)[a + (b - a)r(z)]\} \div \{1 + [b - 1 - c + a(2c - 1)]r(z) + [a - b + c - 2ac + bc]r(z)^2\}, \quad (1b)$$

$$\psi_3(z) = [bcr(z)]/\{1 + [b - 1 - c + a(2c - 1)]r(z) + [a - b + c - 2ac + bc]r(z)^2\}, \quad (1c)$$

where $a = (1 - m)^2 + m^2/(n - 1)$, $b = (1 - a)/(n - 1)$, $c = 1/2N$, $r(z) = [1 - (2 - z - 1/z)u/2]^2$ and *m* is the migration rate, *u* the mutation rate, *N* the population size, and *n* the number of populations; the indices 1, 2, and 3 refer to pairs of genes within individuals, among individuals within populations, and between populations, respectively.

Solutions for expected variances in allele size *Vj* can be readily derived from the properties of generating functions as

$$V_j = E[k_j^2]/2 = \frac{1}{2}(d^2\psi_j(z)/dz^2)\big|_{z=1}$$

(Rousset, 1996), where *k* is the number of steps apart between the alleles in the pair, and $j = 1$, 2, or *3* refers to the level (individuals, subpopulations, or between subpopulations) at which the variance is calculated. R_{ST} expectation was taken as the ratio $(V_3 - V_2)/V_3$. For gene diversities, we first com-

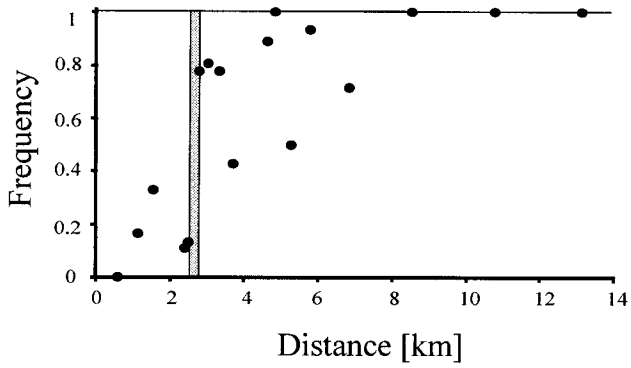

FIG. 1. Cline for the mitochondrial DNA haplotypes on a transect through the hybrid zone. Plotted are the frequencies of the Valais characteristic haplotype. The gray vertical bar indicates the location of the contact zone.

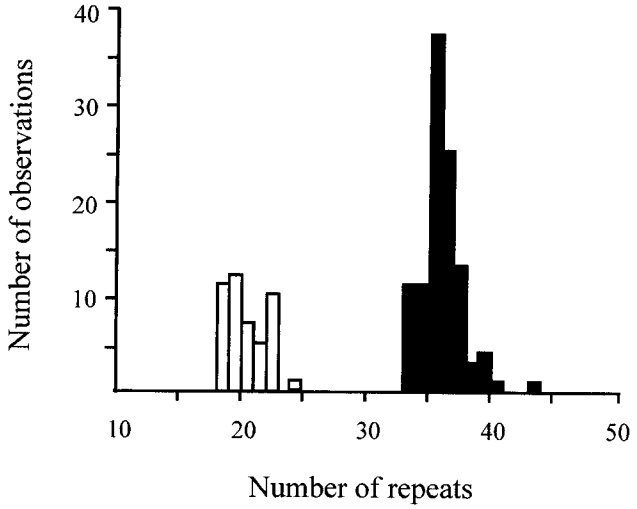

FIG. 2. Allele distribution of the Y-chromosome microsatellite L8Y. Males of the Cordon race are represented by black bars and Valais males by white bars.

puted the probabilities of identity in state Q_1, Q_2, and Q_3 according to

$$Q_j = \frac{1}{\pi}\int_0^{\pi} \psi_j(e^{ix})\, dx \qquad (2)$$

(Rousset 1996), using the computer package Mathematica (Wolfram 1991), and obtained expectations of diversities using the relation $E[H_j] = 1 - Q_j$. The expectation of F_{ST} is $(Q_2 - Q_3)/(1 - Q_3)$.

RESULTS

Uniparentally Inherited Markers

The mtDNA analysis showed that 85% of the individuals on the Cordon side of the contact zone and 79% on the Valais side bear their race-specific haplotype. Introgression of the mtDNA alleles occurs on both sides of the contact zone (Fig. 1). The highest frequencies of haplotype admixtures are recorded close to the contact zone, with samples most distant from it being fixed for the race-specific allele (populations 1, 15, 16, 17). This translates into an F_{SR} of 0.03 and an F_{RT} of 0.56.

We observed 15 alleles at the male-inherited microsatellite L8Y and genetic diversity was estimated to be $H_r = 0.71$. A striking feature appears in the allelic distribution. Males of the Valais race clearly show a very different, disjunct allele distribution compared to males of the Cordon race (Fig. 2). All males sampled on the Cordon side ($n = 46$) bear alleles shorter than 25 repeats, whereas all males sampled on the Valais side ($n = 105$) have alleles longer than 32 repeats (Fig. 2). This indicates a complete absence of Y chromosome exchange between the races. The relative genetic structure for the Y-locus within and between races estimated with *F*-statistics are $F_{SR} = 0.04$ and $F_{RT} = 0.19$, respectively. F_{RT} does not reflect the absence of alleles shared between races. This stems from the high within-race polymorphism, which lowers the estimate of higher-level structure (Wright 1978; Charlesworth 1998; Nagylaki 1998; Hedrick 1999). *R*-statistics for this Y-chromosome microsatellite show a completely different picture. No within-race structuring is detected. However, differentiation is nearly complete between the races ($R_{RT} = 0.98$). As *R*-statistics account for the relative size of alleles, R_{RT} is much higher than F_{RT} due to the nonoverlapping allele distributions of L8Y between the races (Fig. 2). The high between-race structure estimated with the two sex-linked genetic markers are in general agreement with the protein and karyotype data reported previously (Brünner and Hausser 1996), and indicate very restricted genetic exchange.

Autosomal Microsatellites

Autosomal microsatellite analysis revealed a large amount of polymorphism, with a mean genetic diversity of $H_r = 0.77$. An important part of the overall variance is due to the difference between races with both *F*- and *R*-statistics ($F_{RT} = 0.10$, $R_{RT} = 0.27$). Structure within races lies at about 0.02 with *F*-statistics, but slightly negative estimates are obtained with *R*-statistics with $R_{SR} < 0$. These F_{RT}- and R_{RT}-values are similar to those previously obtained with seven microsatellite loci (Lugon-Moulin et al. 1999). However, they are much lower than the estimates based on other markers (allozymes and chromosomes), including sex-specific markers. The difference between *F*- and *R*-estimates for the autosomal microsatellites is not significant (Bootstrap test, 1000 replicates $P = 0.13$).

Simulations

Single-step mutation with unconstrained number of alleles

Simulations of this ideal case serve two purposes. First, they allow us to verify that our individual-based simulations are correct, by comparison of the simulation results with the theoretical expectations. Second, they allow us to quantify the variance around these theoretical expectations.

Under pure SSM with no constraint on the number of alleles, the fit of the simulations with analytical expectations is excellent, as expected (Table 1, Fig. 3). However, the variations around the mean are smaller for gene diversity H_e and F_{ST} than for variance in allele size (V_s) and R_{ST} (Fig. 3). This is confirmed by looking at the standard deviations (SDs) in Table 1. The SDs for R_{ST} vary between 0.1 and 0.3 and those

TABLE 1. Analytical expectations and simulation results after 100,000 generations under a constrained and unconstrained single-step mutation model (SSM). N, the number of individuals in each population; n, the number of populations; μ, the mutation rate; m, the diploid migration rate. The expectations for gene diversity and variance in allele size within populations, F_{ST} and R_{ST}, are respectively denoted as E(H_s), E(V_s), E(F_{ST}), and E(R_{ST}). Gene diversity (H_s) and variance in allele size (V_s) have been computed as within-population estimates. Simulation results have been averaged over the 20 replicates. Standard deviations (SD) have been computed over all loci of the 20 replicates. The constrained model examined 30 allelic states, the unconstrained model examined 1000.

				Expectations				Constrained							Unconstrained							
N	n	μ	m	E(H_s)	E(F_{ST})	E(V_s)	E(R_{ST})	H_s	SD	F_{ST}	SD	V_s	SD	R_{ST}	SD	H_s	SD	F_{ST}	SD	V_s	SD	R_{ST}	SD
1000	2	0.01	0	0.889	0.111	20.000	1.000	**0.870**	0.030	**0.103**	0.046	**13.1**	8.1	**0.821**	0.306	**0.888**	0.020	**0.103**	0.029	**19.0**	13.4	**0.978**	0.262
1000	2	0.01	0.0001	0.897	0.065	39.990	0.556	**0.881**	0.028	**0.064**	0.031	**21.6**	17.6	**0.478**	0.271	**0.894**	0.019	**0.068**	0.029	**42.4**	62.7	**0.623**	0.283
1000	2	0.01	0.001	0.909	0.031	39.990	0.111	**0.888**	0.031	**0.031**	0.016	**21.9**	18.0	**0.102**	0.095	**0.907**	0.021	**0.039**	0.014	**41.3**	53.9	**0.138**	0.118
1000	2	0.001	0	0.667	0.333	2.000	1.000	**0.653**	0.077	**0.325**	0.098	**1.7**	1.4	**0.972**	0.242	**0.665**	0.069	**0.312**	0.093	**1.8**	1.3	**0.980**	0.255
1000	2	0.001	0.0001	0.693	0.202	3.999	0.556	**0.684**	0.075	**0.216**	0.108	**3.6**	4.1	**0.580**	0.299	**0.693**	0.070	**0.214**	0.100	**4.0**	4.4	**0.597**	0.299
1000	2	0.001	0.001	0.733	0.075	3.999	0.111	**0.719**	0.067	**0.073**	0.046	**3.5**	3.9	**0.120**	0.107	**0.731**	0.071	**0.076**	0.050	**3.4**	2.9	**0.114**	0.113

for V_s between 1.3 and 63, whereas those for F_{ST} and H_s are all less than 0.1. If we take the ratio of SD to the mean, we obtain the coefficient of variation (CV), which is much larger for variance of allele size than for heterozygosity (between seven and 70 times larger in our simulations; Table 1), whereas the CV for R_{ST} is close to that of F_{ST} when there is no migration, and 2.5 as large when migration equals mutation (Table 1). This confirms the results of Slatkin (1995), whose simulations showed that R_{ST} has a larger variance than F_{ST}.

Single-step mutation with constrained number of alleles

The effect of the constrained number of alleles is very slight both on gene diversity (H_s) and F_{ST}. Gene diversity is very slightly decreased compared to the unconstrained case, but F_{ST} is virtually unaffected (Table 1, Fig. 3). Similarly, with the lower mutation rate (10^{-3}, bottom of Table 1), both variance in allele size (V_s) and R_{ST} are nearly unaffected by the constraint on the number of allelic states. In contrast, at the higher mutation rate (10^{-2}), there is a very strong effect of decreasing V_s in the constrained simulations. There is also a weak effect on genetic differentiation estimators with constrained simulations giving slightly smaller R_{ST}-values than under no constraint (Table 1, Fig. 3). Overall, these simulations show that constraining the number of allelic states to 30 has a limited effect on genetic differentiation estimators. It is only at the higher mutation rate (10^{-2}) that V_s and to a much lesser extent R_{ST} estimates are lower than the situation with no constraint (Table 1, Fig. 3). Because R-statistics are defined as ratios of variance, they are only slightly affected by the reduction of the variance in allele size.

Mixed mutation model

Results when the mutation model includes a small proportion of KAM are presented in Table 2. When there are 1000 possible allelic states, V_s within populations is unrealistically large (Table 2, upper part). The effect on R_{ST} depends on the mutation rate. With a low mutation rate, there is virtually no effect, whereas R_{ST} is nearly halved with the high mutation rate. At both mutation rates, F_{ST} is very similar to what is expected under pure SSM and H_s is slightly increased as is seen from a comparison of the expectations in Table 1 with the upper part of Table 2. Under the constrained mutation model (30 allelic states), variance in allele size is much smaller. The maximum possible variance is the variance of a rectangular distribution with 30 states, namely 70. This would be the variance under pure KAM. The lower part of Table 2 shows that the higher mutation rates give estimates of variance in allele size close to this maximum. A portion of random mutation slightly increases H_s, thereby decreasing F_{ST} slightly (Fig. 3). The effect is more pronounced at the high mutation rate (Table 2), but the most dramatic effect is on R_{ST}. Even with as little as 5% KAM, R_{ST}-values are much lower than what is expected under pure SSM (Table 2, Fig. 3). Not only is R_{ST} much lower, but, because its SD does not change much, its CV is increased drastically compared to the simulations under pure SSM, whereas the CV of F_{ST} diminishes only slightly (Tables 1, 2).

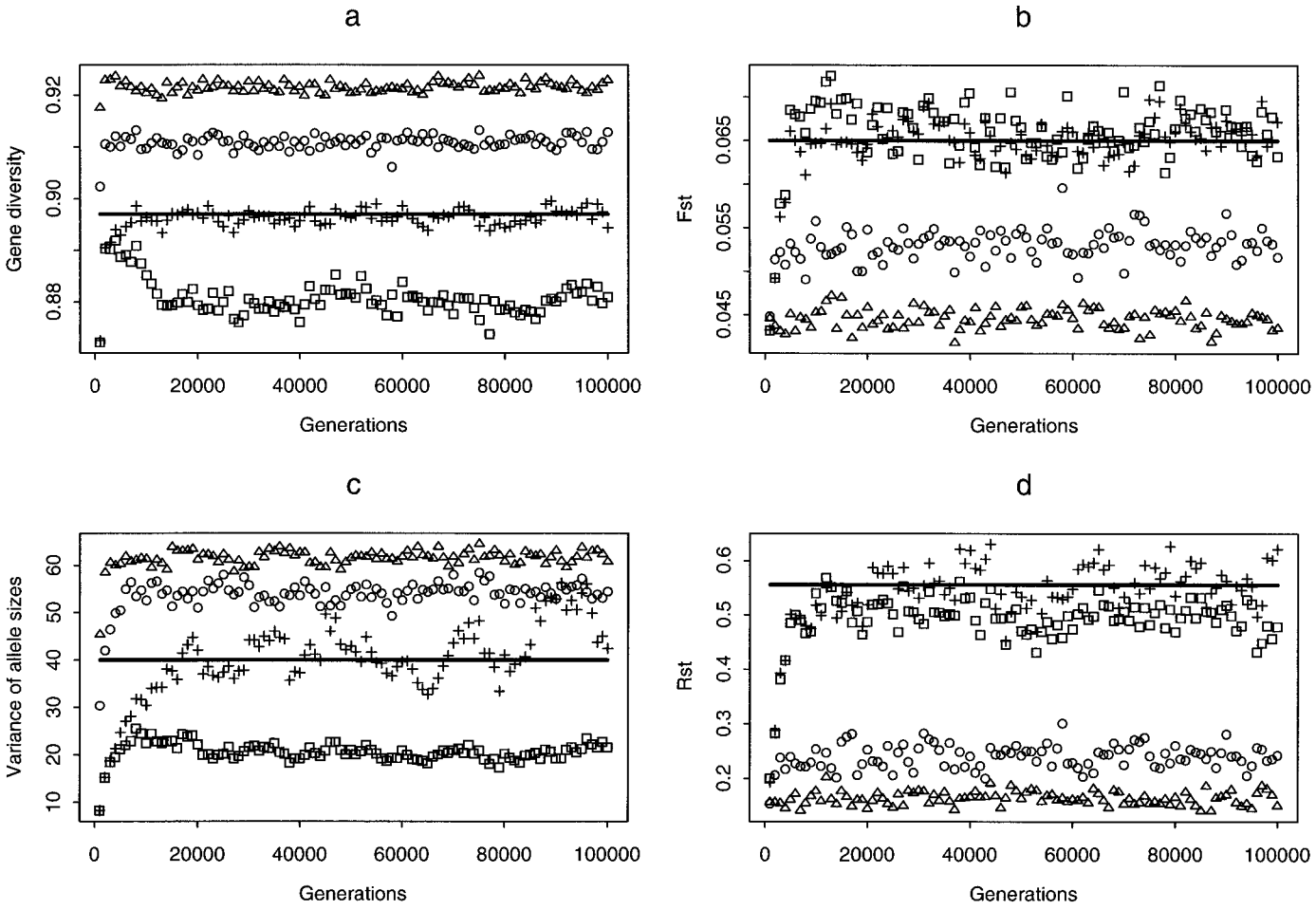

FIG. 3. Results of simulations. For all four panels, the simulations consisted of two populations with $N = 1000$ individuals each. Mutation rate was set to 10^{-2} and migration rate to 10^{-4}. Crosses, simulations under pure single-step mutation (SSM) with 1000 allelic states; squares, pure SSM, 30 allelic states; circles, 5% KAM, 30 allelic states; triangles, 10% KAM, 30 allelic states. (A) Gene diversities (H_e) within populations; (B) F_{ST}; (C) variance of allele sizes within populations; and (D) R_{ST}. The solid line on each panel corresponds to the expectation of the relevant statistics under a strict SSM model, according to the equations of Rousset (1996) modified for diploid migration according to Nagylaki (1983).

DISCUSSION

Before discussing the biases affecting differentiation estimators, we discuss the biological interpretation of the striking allele distribution of the Y-chromosome microsatellite. The lack of introgression at the Y microsatellite locus indicates complete absence of male-mediated gene flow, whereas mtDNA shows some limited interracial gene exchange. This discrepancy cannot be explained simply by female-biased dispersal, because both sexes undergo juvenile dispersal in *S. araneus* (Hanski et al. 1991). Furthermore, males are much more vagile than females during the reproductive season (Stockley et al. 1994). Because interracial matings in the laboratory are fertile and produce offspring of both sexes (Castagné et al. 1994), the absence of male-mediated gene flow implies either extremely strong intraracial assortative mating with choosy females and/or male hybrid sterility. Admixture of autosomes and mtDNA, but not of Y markers, could theoretically be the outcome of female-controlled assortative mating. However, it is rather unlikely that this prezygotic barrier would completely impede male gene flow. This suggests that male F_1 hybrids could be sterile in the Les Houches hybrid zone. Because chromosomal incompatibilities generally act on both males and females (Coyne and Orr 1998), the most likely explanation for the male hybrid hypothesis would be classical postzygotic isolation. It is well known that inviability or sterility is acquired first by the heterogamic sex in animals with sex-determining chromosomes (Haldane 1922; Coyne and Orr 1998).

The very low introgression revealed by the sex-specific markers is congruent with the previous results based on chromosomes and allozymes (Brünner and Hausser 1996). Therefore, the most likely cause of the discrepancies between the autosomal microsatellites and all other markers is systematic underestimation of differentiation with the microsatellites. However, the explanation for this underestimation is different for *F*- and *R*-statistics. If the reasons for underestimation are well known for *F*-statistics, the problem is more subtle for *R*-statistics. Indeed, it has been documented several times in the literature that *F*-statistics provide the correct criterion for differentiation if and only if diversity is low (Wright 1978;

TABLE 2. Simulation results after 100,000 generations under a single-step mutation model (SMM) with 5% and 10% random mutations. N, the number of individuals in each population; n, the number of populations; μ, the mutation rate; and m, the diploid migration rate. Gene diversity (H_s) and variance in allele size (V_s) have been computed as within population estimates. Simulation results have been averaged over the 20 replicates. Standard deviations (SD) have been computed over loci and replicates.

				5% random mutations							10% random mutations						
N	n	μ	m	Hs	SD	F_{ST}	SD	V_s	SD	R_{ST}	SD	H_s	SD	F_{ST}	SD	V_s	SD	R_{ST}	SD
Unconstrained number of alleles																			
1000	2	0.01	0.0001	**0.930**	0.013	**0.058**	0.014	**58147**	25587	**0.278**	0.210	—	—	—	—	—	—	—	—
1000	2	0.001	0.0001	**0.725**	0.067	**0.206**	0.090	**20972**	25180	**0.540**	0.290	—	—	—	—	—	—	—	—
Constrained number of alleles																			
1000	2	0.01	0	**0.907**	0.019	**0.058**	0.025	**53.7**	20.2	**0.288**	0.221	**0.921**	0.012	**0.046**	0.017	**59.8**	18.0	**0.162**	0.147
1000	2	0.01	0.0001	**0.913**	0.015	**0.052**	0.023	**54.4**	20.2	**0.243**	0.201	**0.923**	0.012	**0.044**	0.016	**60.8**	16.8	**0.151**	0.148
1000	2	0.01	0.001	**0.919**	0.017	**0.030**	0.012	**60.0**	22.3	**0.080**	0.088	**0.928**	0.012	**0.028**	0.012	**64.9**	18.5	**0.075**	0.080
1000	2	0.001	0	**0.664**	0.091	**0.310**	0.113	**13.5**	18.1	**0.821**	0.325	**0.696**	0.079	**0.282**	0.092	**21.3**	18.5	**0.716**	0.312
1000	2	0.001	0.0001	**0.710**	0.071	**0.196**	0.095	**24.8**	27.5	**0.463**	0.272	**0.714**	0.081	**0.210**	0.101	**29.3**	25.0	**0.474**	0.280
1000	2	0.001	0.001	**0.745**	0.089	**0.075**	0.046	**23.2**	25.7	**0.088**	0.103	**0.771**	0.074	**0.076**	0.043	**32.9**	27.9	**0.088**	0.096

Charlesworth 1998; Nagylaki 1998; Hedrick 1999). This effect is clearly illustrated by the male-inherited microsatellite locus. Even if no allele is shared between both races, the male microsatellite F_{RT}-estimate ($F_{RT} = 0.19$) is three times lower than its biallelic mtDNA counterpart ($F_{RT} = 0.56$), where the two alleles are present in both races.

R-statistics have been developed to take into account the high homoplasy inherent to microsatellite markers (Slatkin 1995). *R*-statistics perform better than *F*-statistics as long as there is some memory in the mutation process (i.e., a new allele generated by mutation is more similar to its previous state than to a randomly selected allele). Slatkin (1995) has shown that *R*-statistics are less biased than *F*-statistics under a two-phase model, but this latter model produces new alleles that are more similar to their previous state than random. In contrast, even a small proportion of random mutation events (KAM) tends to erase an important part of the memory of the mutation process, thereby strongly reducing R_{ST}.

The effect of a constrained number of alleles was rather weak in our simulations. We used 30 possible allelic states, whereas Nauta and Weissing (1996) used 10 allelic states for most of their simulations. The latter would be an underestimate in our case because we observed a difference between the smallest and the largest allele of 20.1 ± 13.1 repeats. The number of possible allelic states could also be quite variable between loci and go well beyond 30 for some microsatellites. For instance, we observed for locus 57 a range of 48 repeats. As previously shown by Nauta and Weissing (1996), the effect of a constrained range of alleles has a major effect on the variance of allele size, and we find the same results in our simulations. However, with 30 allelic states, this has only minor effects on R_{ST}-estimates, which are defined as ratios of variances.

The effects of constraint, proportion of random mutations, and mutation rate are not independent. There are nontrivial positive interactions among these effects. Thus, there is a need for analytical work to disentangle these effects on gene diversity, variance in allele size, F_{ST}, and R_{ST}. The important result of the simulations of this study is that even a small proportion of random mutations can shift R_{ST}-estimates downward.

In our empirical data, the R_{RT} based on the autosomal microsatellites is much lower than the estimates of structure obtained from other genetic markers (sex-specific markers, chromosomes, and allozymes). The autosomal R_{RT}-estimate is larger than its F_{RT} counterpart, although not significantly so ($P = 0.13$). This result is consistent with the simulations, and it strongly suggests that the mutation process of our autosomal microsatellite loci is different enough from an SSM to lose most memory of allele size. This latter result is also confirmed by a study by Wyttenbach et al. (1999), who tested six of the microsatellites used in the present study to determine whether their variation was better explained by a stepwise or an infinite allele model. These authors could not detect any trend in favor of either model. Empirical work on diploid microsatellite mutations has shown that they generally mutate under complex models, with frequent addition or deletion of several repeat units (Fitzsimmons 1998; Primmer et al. 1998; Schlötterer et al. 1998). Several other analyses of population structure have reached the conclusion that many

microsatellite loci do not fit an SSM process (Estoup et al. 1995; Goodman 1998; Ross et al. 1997).

However, *R*-statistics give a credible result for the Y microsatellite with almost complete genetic divergence (R_{RT} = 0.98). The disjunct allele distribution at this locus suggests that it could mutate under a fairly strict SSM model. Because locus L8Y is haploid, this raises the question of whether microsatellite mutations on haploid and diploid genes could be driven by different mechanisms. Adding or deleting one or a few repeats by slippage during replication would be the only cause of mutation at haploid loci, whereas in diploids other mechanisms like unequal crossing over could generate large insertions or deletions. It should be noted, however, that both mutation rate and polymorphism of microsatellites on the Y chromosome do not seem to differ from their autosomal counterparts (Roewer et al. 1992; Heyer et al. 1997).

To our knowledge, this study constitutes the first report for extreme underestimation of population genetic structure inferred from microsatellites. Previous studies that compared microsatellites to other classes of genetic markers (Lehman et al. 1996; Barker et al. 1997; Estoup et al. 1998; Lynch et al. 1999; Ross et al. 1999) did not detect such strong discrepancies. The obvious reason is that gene flow across the hybrid zone we studied is probably much lower than in all other studies. Although this is an extreme example of very restricted gene exchange, this kind of situation might be common outside hybrid zones in at least two fields of research where populations are often isolated: conservation biology and biogeography. Generally, particular caution should be taken when interpreting population structure based on very polymorphic loci when gene flow is reduced. For instance, in this study, population structure estimates (*F*- and *R*-statistics) based solely on autosomal microsatellites would have lead to a strong overestimate of the extent of gene flow between the two races.

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