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SEX-BIASED GENETIC STRUCTURE IN THE VECTOR OF LYME DISEASE, *IXODES RICINUS*

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Abstract.—We analyzed 725 *Ixodes ricinus* ticks (the principal vector of Lyme disease in Europe) collected in Switzerland in 1995 and 1996 (three and eight samples, respectively) and in Tunisia in 1996 (one sample) with five microsatellite markers. We found highly significant genetic differentiation between Swiss and Tunisian samples but detected almost no differentiation within Switzerland, even between those samples separated by the Alps. Interestingly, we found that *I. ricinus* females were more genetically related to one another than were males at a local scale, which would indicate a higher dispersal rate of immature males. Possible explanations for these findings in terms of sex-specific association of ticks with certain hosts (e.g., birds) and their epidemiological consequences are discussed.

Key words.—*Ixodes ricinus*, Lyme disease, population genetics, sex-biased dispersal, ticks.

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Increasing attention is currently devoted to tick-borne pathogens, particularly since the identification of *Borrelia burgdorferi* as the agent of Lyme disease (Parola and Raoult 2001). This disease has major public health and economic impacts (Gubler 1998) and is the most prevalent and widespread vector-borne human infection in the Northern Hemisphere (Smith et al. 2000), where its incidence continues to grow (Gubler 1998).

Lyme disease is transmitted by ticks of the *Ixodes ricinus* complex (e.g., Rich et al. 1995; Gubler 1998; Fukunaga et al. 2000). The species belonging to the *I. ricinus* complex are widely distributed throughout the Northern Hemisphere: Europe, western Turkistan, Turkey, Caucasus, North Africa for *I. ricinus* (Perez and Rohdain 1977), Asia for *I. persulcatus* (Balmelli and Piffaretti 1996), and North America for *I. scapularis* and *I. pacificus* (Rich et al. 1995). These ticks can feed on almost any terrestrial vertebrate, including humans and domestic animals (Hoogstraal and Aeschlimann 1982), and can therefore transmit many different pathogens to a diverse range of hosts (e.g., Bernasconi et al. 1997; Delaye et al. 1997). The distribution of these ticks is spatially discontinuous (Aeschlimann 1981). Moreover, the prevalence of infection by *B. burgdorferi* in ticks has been found to be highly variable (3–58%; Aeschlimann et al. 1986; Hubálek and Halouzka 1998). This patchy distribution of both vector and disease agent suggests that there may be limited dispersal of ticks and independent enzootic cycles occurring within this spatial scale. A study of the population structure and dispersal ability of *I. ricinus* is therefore an essential prerequisite for the understanding of the population biology of this vector and thus of the epidemiology of the diseases transmitted.

Only indirect methods, such as studies of polymorphic codominant genetic markers (Tabachnick and Black 1995), can effectively provide us with ecologically meaningful dispersal data on a tick species. At present, little is known about the genetic structure of populations and dispersal of *Ixodes* species. Earlier studies, based on allozyme data, have provided inconclusive results (Healy 1979a,b; Delaye et al. 1997; Kain et al. 1997). Microsatellite markers appear to be alternative tools for such a purpose (Hughes and Queller 1993), because they are usually highly polymorphic, codominant, abundant throughout the genome, and relatively easy to score (Lehmann et al. 1996). In the seabird tick *I. uriae*, for instance, microsatellites enabled the identification of host races through the comparison of genetic structure of tick populations associated with different bird species and sites (McCoy et al. 2001).

In this paper, we describe results obtained by analyzing five polymorphic microsatellite loci of *I. ricinus* previously described by Delaye et al. (1998). We discuss the role of null alleles, which are suspected to partially explain strong and variable local heterozygote deficits, the genetic differentiation found between years and between the sexes, the weak differentiation between sites in Switzerland (small geographical scale), and the strong differentiation between Switzerland and Tunisia (intercontinental scale). We also discuss the epidemiological relevance of our results and their evolutionary implications.

MATERIALS AND METHODS

Sampling Sites

Ticks were sampled during the springs of 1995 (76 females, 88 males) and 1996 (320 females, 198 males) in Switzerland (Fig. 1) and in the Kroumiry Mountains of northwestern Tunisia (North Africa; 20 females, 23 males). In Switzerland, ticks were located in deciduous forest habitat and collected

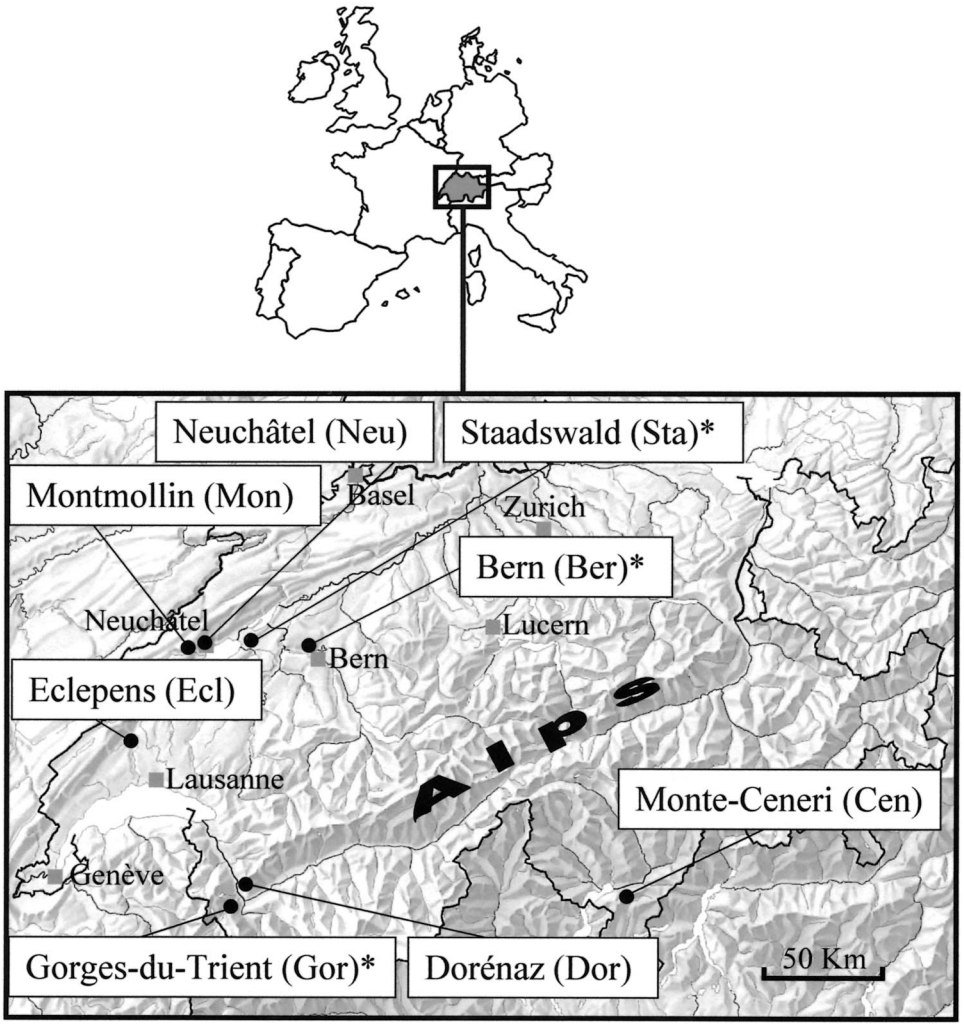

FIG. 1. Sampling locations in Switzerland. Sites sampled both years of the study are marked with an asterisk.

by hand from the understory vegetation. In Tunisia, ticks were sampled from infested cows and by flagging vegetation (e.g., Aeschlimann 1972). All specimens were preserved in 70% ethanol until DNA extraction.

Microsatellite Characterization

We used the five polymorphic microsatellite loci described by Delaye et al. (1998), designated IR8, IR25, IR27, IR32, and IR39. Suspicion for null alleles led us to design an additional pair of primers for the locus IR8 (5′-GCGGTAAATAA-ACAATTCC-3′ and 5′-GGTGTTTGACCAGCACAGTC-3′). This new primer pair was applied in six Swiss samples collected in 1996 (Staadswald, Dorénaz, Monte-Ceneri, Neuchâtel, Eclépens, and Montmollin; Fig. 1).

Data Analyses

Within each sample, the goodness of fit to an equal male: female ratio of sampled ticks was tested by the exact binomial test performed by S-Plus 2000 (MathSoft, Inc., Seattle, WA).

The occurrence of linkage disequilibrium between pairs of loci was tested by the genotypic randomization test for linkage disequilibrium implemented in Genepop version 3.3 (Raymond and Rousset 1995), with the total number of iterations set to 10^7 (lower values did not provide stable *P*-values).

Wright's (1965) *F*-statistics were estimated with the program Fstat version 2.9.1 (Goudet 1995), which calculates unbiased estimates (f for F_{IS} and θ for F_{ST}) according to the method of Weir and Cockerham (1984). Departure of F_{IS} from zero (heterozygote deficiency within samples) was tested by randomly permuting alleles between individuals (15,000 permutations) within each sample and comparing the observed F_{IS}-values to the randomly generated distributions. F_{ST} (between-sample differentiation) was tested by a *G*-based allelic test (Goudet et al. 1996), comparing the observed *G* to the distribution obtained after 15,000 permutations of genotypes between samples.

Highly polymorphic loci reduce the maximum possible value for F_{ST} (Hedrick 1999; Balloux and Lugon-Moulin 2002) because an increase in allele number moves each sample away from fixation. Rousset's (1996) ρ (an estimator of Slatkin's [1995] R_{ST}, designed for microsatellite markers)

was used to provide an alternative to F_{ST} estimates (computed by Fstat ver. 2.9.1).

To test for sex-specific genetic differences between males and females (see Healy 1979a), we used the assignment test described by Favre et al. (1997). The assignment index, *AI*, determines the probability that a genotype originated from the population in which it was sampled. Individuals with a low *AI* have rare genotypes and are, therefore, potential recent immigrants. The assignment index used (AI_c) corrected for population effects (see Favre et al. 1997). The significance of the difference in assignment indices between males and females was tested by a random assignment of sex to individuals in each sample (10^4 permutations) using the program Biasdisp 1.01 provided by J. Goudet (Institut d'Ecologie, Université de Lausanne, Switzerland) and available upon request.

Isolation by distance was assessed by Mantel tests (Manly 1985) using 10^5 permutations and was performed in Genepop (Raymond and Rousset 1995). Because our sampling corresponds to a two-dimensional design, we used Rousset's (1997) method of correlation between ln(geographical distances) and $X/(1 - X)$, where X stands for F_{ST} or R_{ST}.

Null alleles can account for apparent heterozygote deficits, especially when between-locus variance of F_{IS} is strong. The frequency of null alleles was estimated using Brookfield's (1996) method. We tested, by an exact binomial test, the goodness of fit of observed frequencies of blank genotypes (putative null homozygotes) to the expected frequencies under panmixia (unilateral tests).

Multiple testing enhances Type I error; therefore, we applied the sequential Bonferroni procedure when necessary (Holm 1979; see also Rice 1989).

Results

Sex Ratio

After Bonferroni correction, significantly female-biased sex ratios were found in three samples (Bern 1996, $P = 0.0001$; Dorénaz 1996, $P = 0.0004$; Gorges-du-Trient, $P = 0.0011$; Fig. 1).

Polymorphism

Nei's (1978) unbiased heterozygosities were high for loci IR8, IR25, and IR39 (≥ 0.9) but lower for loci IR27 and IR32 (0.52 and 0.72, respectively). Locus IR8 appears to be X-linked, because it never displayed heterozygous phenotypes in males. Therefore, this locus was coded as homozygous in males for statistical methods that are insensitive to correlation between alleles within individuals (i.e., linkage disequilibrium and population differentiation tests). For all other analysis, males were coded as missing data at this locus.

Genetic Homogeneity between Sexes and Years

When genetic differentiation was tested over all loci, male and female allelic frequencies differed significantly in only one sample (Staadswald 1995, $P < 7 \times 10^{-5}$), and differentiation between years was significant only for this same site (Staadswald, $P = 0.0038$). Because variation among the five loci may constitute a confounding factor in global tests, we undertook the same comparisons considering each locus separately for each population. For the comparisons between sexes, 20 of 60 tests were significant, which was more than the 5% expected under the null hypothesis ($P < 10^{-5}$, binomial test). For the comparisons between years, 12 of 30 tests were significant ($P < 10^{-5}$, binomial test). Allelic frequencies thus are sex and year specific.

Table 1. Comparison of assignment probabilities between sexes. See Figure 1 for sample location. *P*-value, probability that assignment probabilities of the two sexes are as or more different than the observed one; 1995, all 1995 samples; 1996, all 1996 samples; with IR8, the X-linked locus IR8 coded homozygous for males; without IR8, locus IR8 removed from the data; CH, Swiss samples only; West, western Swiss samples only (Monte-Ceneri removed, see Fig. 1).

	Mean assignment		
	Females	Males	*P*-value
1995 with IR8	0.31	−0.26	0.2468
1995 without IR8	0.15	−0.12	0.5616
1996 with IR8	0.36	−0.56	0.0006
1996 without IR8	0.23	−0.36	0.0139
CH 1996 with IR8	0.39	−0.63	0.0002
CH 1996 without IR8	0.25	−0.40	0.0120
West CH 1996 with IR8	0.44	−0.75	0.0003
West CH 1996 without IR8	0.30	−0.51	0.0031

To examine the potential proximate cause for the observed differences between sexes, we compared assignment probabilities of males and females in each site. Females were more often correctly assigned than males in all 1996 datasets (Table 1). This did not depend either on the inclusion of distant samples (i.e., Tunisia or Monte-Ceneri) or the inclusion of the X-linked locus (IR8; Table 1).

Years, males, and females thus were considered separately in the following analyses.

Genetic Structure within Samples

For the linkage disequilibrium test, only nine pairs of loci of 223 pairs (4%) gave a significant *P*-value at the 5% level. None of these probabilities were significant after Bonferroni correction. Thus, genotypes at our loci appear statistically independent.

There was a significant overall heterozygote deficiency at every locus ($P < 0.00007$). However, the between-locus variation was high (Fig. 2).

Null Alleles

High levels of local heterozygote deficiency and their variance from one locus to the other suggested the presence of null alleles. We estimated null allele frequencies through Brookfield's (1996) method. Null alleles fully explained the heterozygote deficiency observed for loci IR8, IR25, and IR39 ($P > 0.13$). However, discrepancies between observed and expected cases of nonamplification were significant for locus IR27 ($P = 0.02$, not significant after sequential Bonferroni correction) and highly significant for locus IR32 ($P = 0.0009$). Other factors must be responsible for the observed heterozygote deficiencies.

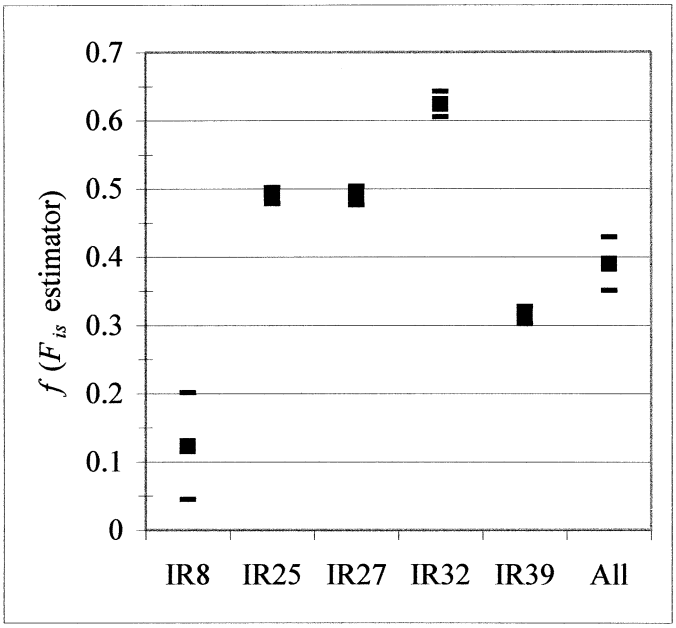

FIG. 2. F_{IS}-estimations per locus and for all loci (All), averaged over all 24 samples (i.e., locations, years, and sexes considered together). Confidence intervals of the means were estimated by jackknifing over populations.

Genetic Differentiation between Swiss Populations

There was no significant differentiation between 1995 samples, for either females ($P = 0.0895$, $\theta = 0.002$, $\rho = 0.015$) or males ($P = 0.4792$, $\theta = -0.001$, $\rho = 0.001$). In 1996, we found a low but significant differentiation between samples for females ($P = 0.030$, $\theta = 0.004$, $\rho = 0.013$), but not for males ($P = 0.281$, $\theta = 0.004$, $\rho = 0.044$). If we excluded the most geographically distant sample, Monte-Ceneri, which is also separated from the other samples by the Alps, differentiation did not remain significant for females ($P = 0.085$, $\theta = 0.003$, $\rho = 0.007$). The differentiation between Monte-Ceneri and other samples was weak ($P = 0.057$, $\theta = 0.01$, $\rho = 0.03$ for females, $P = 0.154$, $\theta = 0.009$, $\rho = 0.021$ for males).

Using the F_{ST}-estimator, geographical distance had no effect on genetic differentiation for females ($P = 0.252$), but it did for males ($P = 0.044$). The opposite pattern was found when using the R_{ST}-estimator ($P = 0.012$ for females, $P = 0.187$ for males).

Genetic Differentiation between Switzerland and Tunisia

There was strong and highly significant differentiation between Tunisia and Switzerland for both females ($P < 0.0001$, $\theta = 0.13$, $\rho = 0.65$) and males ($P < 0.0001$, $\theta = 0.1$, $\rho = 0.75$).

DISCUSSION

We found substantial and highly variable heterozygote deficiencies. Classical population genetics factors such as selfing or Wahlund effects (e.g., Hartl and Clark 1997) cause an excess in homozygotes, but are not relevant in this case because *I. ricinus* is a strictly dioecious species and the F_{IS} variance was high among loci. Null alleles at microsatellite loci are not uncommon and have been invoked in other hard ticks (McCoy et al. 2001). However, the occurrence of null alleles could not explain all heterozygote deficiencies presently found. Molecular imprinting, which would interfere with DNA amplification of some alleles, is another possible explanation (Chakraborty 1989). Asymmetrical amplifications, where one primer is added in excess, led to the identification of further heterozygotes at locus IR39 only (taken into account in our analysis). Parent-offspring studies could likely provide a better understanding of what may be responsible for these heterozygote deficits. Whatever their real cause, they likely decreased the power of the statistical tests. In addition, size homoplasy for microsatellite markers can also lower the power of differentiation tests (Angers et al. 2000). Therefore, nonsignificant results should be considered with caution, in particular the ambiguous results obtained for the weak or null geographical differentiation within Switzerland.

Mitochondrial DNA also suggested weak differentiation at a large scale in a related species, *I. pacificus* (Kain et al. 1999). The development of other microsatellites may be a difficult task because such loci are rare in the *I. ricinus* species complex (Delaye et al. 1998; Fagerberg et al. 2001) and because the primers developed for *I. uriae* did not amplify *I. ricinus* DNA (McCoy and Tirard 2000). Mitochondrial markers could represent an alternative set of markers for *I. ricinus*; however, heterozygosity and sex-biased dispersal, which constitute critical parameters in this species, are not amenable to analysis using mtDNA alone.

Swiss (European) and Tunisian (North African) ticks appear strongly isolated. The role of migrating birds in tick dissemination over long distances, suggested by direct observations (Hoogstraal et al. 1961; Smith et al. 1996), may thus have a weaker impact than expected on effective gene flow and, hence, on micropathogen transport as well.

We found evidence for sex- and year-specific patterns of population genetic structure. Fluctuations of populations and migration events may easily explain the between-year differentiation. However, the differentiation between males and females was unexpected according to previous results (Delaye et al. 1997; but see Healy 1979a). Microsatellites are noncoding markers and we found no evidence for linkage between any locus pair. Selective effects due to hitchhiking are thus unlikely. Size homoplasy also cannot be incriminated here because its existence can only decrease the efficiency of assignment methods (Cornuet et al. 1999).

Male allele frequencies were more similar among samples than female allele frequencies. This suggests that *I. ricinus* males must be more prone to dispersal than females. At the geographic scale considered, this cannot reflect differences in direct mobility. Indeed, all life stages of this tick do not move more than a few meters by themselves (Aeschlimann 1981; Daniels et al. 2000). Ticks are dispersed over greater distances only when passively carried by a host (Aeschlimann 1981). As a result, our findings suggest a sex-associated difference in host choice/compatibility, that is, immature males and females may exhibit different host preferences. For instance, birds might carry larval and/or nymphal males more frequently than females. Such a pattern could have major

epidemiological consequences in terms of pathogen transmission. In Europe, human Lyme disease is caused by different genospecies of *Borrelia* (*B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*, Humair et al. 1995) that are found in specific associations with vertebrate reservoirs (rodents, other mammals, birds; Humair and Gern 1998; Kurtenbach et al. 1998a). It has been shown that the serum-complement of some animals is specifically lethal for some species of *Borrelia* (Kurtenbach et al. 1998b). Moreover, when transmitted to humans, different genospecies are responsible for different clinical manifestations of disease (Hubbard et al. 1998). Male and female *I. ricinus* might thus be involved in different epidemiological routes.

The fact that male and female immature stages of *I. ricinus* may not display the same host specificity has considerable epidemiological implications, and our results highlight the need for further investigations on the distribution of pathogenic agents in males, females, nymphs, and larvae of the *I. ricinus* species complex. This fact also suggests different evolutionary dynamics for sex-specific genes and an increased global heterozygosity of tick populations (Prout 1981).

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