Locus-Specific Genetic Differentiation at *Rw* Among Warfarin-Resistant Rat (*Rattus norvegicus*) Populations

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ABSTRACT

Populations may diverge at fitness-related genes as a result of adaptation to local conditions. The ability to detect this divergence by marker-based genomic scans depends on the relative magnitudes of selection, recombination, and migration. We survey rat (*Rattus norvegicus*) populations to assess the effect that local selection with anticoagulant rodenticides has had on microsatellite marker variation and differentiation at the warfarin resistance gene (*Rw*) relative to the effect on the genomic background. Initially, using a small sample of 16 rats, we demonstrate tight linkage of microsatellite *D1Rat219* to *Rw* by association mapping of genotypes expressing an anticoagulant-rodenticide-insensitive vitamin K 2,3-epoxide reductase (VKOR). Then, using allele frequencies at *D1Rat219*, we show that predicted and observed resistance levels in 27 populations correspond, suggesting intense and recent selection for resistance. A contrast of F_{ST} values between *D1Rat219* and the genomic background revealed that rodenticide selection has overwhelmed drift-mediated population structure only at *Rw*. A case-controlled design distinguished these locus-specific effects of selection at *Rw* from background levels of differentiation more effectively than a population-controlled approach. Our results support the notion that an analysis of locus-specific population genetic structure may assist the discovery and mapping of novel candidate loci that are the object of selection or may provide supporting evidence for previously identified loci.

THE genetic structure of natural populations can potentially be utilized to test the fitness relevance of previously identified candidate genes underlying adaptation or to identify novel genes under selection (Lewontin and Krakauer 1975; Taylor *et al.* 1995). Specifically, alleles should be distributed among populations according to their selective values and levels of drift and migration (*e.g.*, Wright 1951; Slatkin 1993a). Following periods of local selection, allele frequencies of fitness-related genes should be dominated by selection. Therefore, population pairs experiencing divergent selection at fitness-related genes are expected to exhibit high levels of differentiation (*e.g.*, Lewontin and Krakauer 1975; Robertson 1975; McDonald 1994) as measured by F_{ST} (Wright 1951) or one of its analogs. Similarly, allele frequencies at loci linked to the genes under selection will be altered as a function of selection intensity and recombination rates (see Barton 2000 and references therein), a scenario that has been extended to subdivided populations (*e.g.*, Stephan 1994; Charlesworth *et al.* 1997; Slatkin and Wiehe 1998). Low migration rates are expected to reduce the opportunity for recombination between divergent haplotypes (*e.g.*, Slatkin and Wiehe 1998). Therefore, as predicted by theory that specifically dealt with microsatellite evolution (Slatkin 1995; Slatkin and Wiehe 1998; Wiehe 1998), sometimes it may be possible to detect the effects of natural selection on fitness-related genes by studying linked microsatellites (*e.g.*, Paterson 1998; Kohn *et al.* 2000; Harr *et al.* 2002).

A problem with this approach is that stochastic processes may cause populations to diverge in their allele frequencies as well, thereby leading to potentially large variances of F_{ST}-based estimates of population differentiation (*e.g.*, Nei and Chakravarti 1977; Wang *et al.* 2001). Therefore, inferences concerning selection that are based on F_{ST} may have high uncertainty (*e.g.*, Tsakas and Krimbas 1976). Patterns of variation averaged over many unlinked loci should reflect such stochastic genome-wide historical demographic effects, including founder events, dispersal, and inbreeding (*e.g.*, Pritchard *et al.* 2000). To evaluate the degree to which divergence at candidate genes and the regions flanking them is caused by stochastic processes and sampling effects, genetic variation at neutral unlinked loci needs to be surveyed as well (Schlötterer *et al.* 1997; Pritchard and Rosenberg 1999).

We examine populations of the brown rat (*Rattus norvegicus*) that vary dramatically in resistance levels to anticoagulant rodenticide poisons (Figure 1). Anticoagulant rodenticides remain one of the main tools avail-

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able to control rodent populations worldwide yet their effectiveness is jeopardized by the evolution of resistance (Jackson 1986; Hadler and Buckle 1992). Warfarin has been the most widely used rodenticide in the past but has now been largely replaced by alternative anticoagulants such as coumatetralyl or second-generation, more potent anticoagulants such as bromadiolone and difenacoum (Greaves 1986; Hadler and Buckle 1992). Fieldwork has documented intense selection in anticoagulant-exposed rat populations (Greaves and Rennison 1973; Bishop *et al.* 1977; Partridge 1979). Progress has been made toward the elucidation of the biochemical mechanism of resistance (*e.g.*, Hildebrandt and Suttie 1982; Thijssen 1995). Further, the physiological response characteristic of resistant rats (see below) now can be measured, thereby allowing for the more routine detection of resistant rats in the field (Martin *et al.* 1979; Gill *et al.* 1994; Pelz and Endepols 1999). The approximate genomic location of the warfarin resistance locus *Rw* has been derived from linkage mapping with phenotypic markers during laboratory crosses (Greaves and Ayres 1969, 1982; Wallace and MacSwiney 1976) and in a congenic resistant strain of rats with microsatellite markers (Kohn and Pelz 1999, 2000). A screen for localized patterns of linkage disequilibrium on rat chromosome 1 allowed the assignment of *Rw* to an ~2.2-cM interval that contains the microsatellite marker *D1Rat219* (Kohn *et al.* 2000).

The anticoagulant resistance phenotype is manifest as prolonged prothrombin times [or percentage of clotting activities (PCA)] after a diagnostic dose of anticoagulant has been administered. PCA is estimated with a blood clotting response (BCR) test the values of which are then used to separate resistant from nonresistant phenotypes (Martin *et al.* 1979; Gill *et al.* 1994). Biochemical analyses show that the resistance mechanism involves an enzyme complex that has vitamin K 2,3-epoxide reductase (VKOR) activity (*e.g.*, Thijssen and Janssen 1994; Cain *et al.* 1998; Guenthner *et al.* 1998). For small sample sizes, resistance phenotypes and genotypes of rats now can be determined with an *in vitro* VKOR activity assay (Thijssen and Pelz 2001). Resistance to various anticoagulants may be due to either different alleles at *Rw* or additional loci closely linked to it (*e.g.*, Greaves and Ayres 1982). Strain-specific modifier loci, some of which are sex linked, likely affect the resistance phenotype (MacNicoll 1986, 1995; Kerins and MacNicoll 1999; Sugano *et al.* 2001).

Here we utilize new mapping resources, and resistance phenotyping and genotyping technology, to design a study that examines the joint effect of selection, migration, and drift on marker variation and differentiation in resistant rat populations. First, we present further evidence for the tight linkage between *Rw* and microsatellite *D1Rat219* by association mapping, using wild-caught rats for which resistance genotypes are now available (Thijssen and Pelz 2001). We also examine the involvement of the *Rw* locus in resistance to other anticoagulant rodenticides and describe aspects of its quantitative genetics. Second, we examine the association between *D1Rat219* and resistance phenotype frequencies in a large sample of rats. Third, we contrast variation and differentiation at *D1Rat219* with presumably neutral loci. And fourth, we compare these results obtained using the population-controlled approach to those obtained using a case-controlled design.

MATERIALS AND METHODS

Notation: Capital letters denote the phenotypes that are resistant to warfarin, bromadiolone, coumatetralyl, and difenacoum (RW, RB, RC, and RD, respectively). Resistance loci and alleles are denoted in italic as *Rw*, *Rb*, and so on, the distinction between the locus and allele symbols being evident from the context. Susceptible phenotypes and alleles are denoted by a plus symbol (+); thus, a heterozygous warfarin-resistant rat would be designated as RW for its phenotype and *+/Rw* for its genotype. A warfarin susceptible rat would be designated as RW+ for its phenotype and +/+ for its genotype.

Sample populations: Study farms and townships are located in the Münsterland area of northwestern Germany where warfarin has been used since the early 1950s (Pelz *et al.* 1995; Pelz 2001). In 1990, rodent control problems were reported and a survey using the BCR method revealed a resistance area, ML, of >8000 km^2 (Figure 1A, top right inset). The focus of the survey was on rat-infested townships and farms (Pelz *et al.* 1995). Resistance may dramatically differ between adjacent farms as is depicted in Figure 1A (*e.g.*, populations 9 and 28, 3 and surrounding farms). Resistance frequencies are given in Table 1. Other anticoagulants now have largely replaced warfarin in our study area. Resistance to these agents also has evolved (Figure 1; *cf.* Pelz *et al.* 1995; Pelz 2001). This progression toward the use of alternative anticoagulants in response to the evolution of resistance in Germany parallels that observed in the United Kingdom and in many other localities around the world (Greaves 1986; Hadler and Buckle 1992). In our study area warfarin resistance has expanded in range and prevalence over the past decade, and resistance to bromadiolone, coumatetralyl, and difenacoum has established itself at localities where it was previously undetectable (Pelz 2001).

Figure 1 depicts the 27 localities from which 727 rats were collected (*cf.* Table 1). Samples were obtained on several occasions between 1995 and 1999 and thus are unlikely to represent family groups. Of these, 677 rats were tested for warfarin resistance with the BCR method (*cf.* OEPP/EPPO 1995) as applied previously (Pelz *et al.* 1995; Kohn and Pelz 1999; Pelz 2001), 482 for bromadiolone resistance, 364 for coumatetralyl resistance, and 369 for difenacoum resistance. Initially, difficulties were encountered with tests for coumatetralyl resistance that we were able to address during later stages of the project (Pelz and Endepols 1999). Our analyses with respect to the RC phenotype thus should be considered as preliminary.

Warfarin resistance generally occurs in conjunction with resistance to the other anticoagulants in our study area (Table 1). With the exception of RD, the frequency of resistance to one anticoagulant was significantly correlated with the frequency of resistance to another anticoagulant (not shown). Such cross-resistance appears to be a general feature of resistant rodent populations (MacNicoll 1986, 1995; Hadler and Buckle 1992; Pelz *et al.* 1995; Thijssen 1995). Resistance groups RW, RC, RB, and possibly RD therefore should not be considered as independent samples during further analyses.

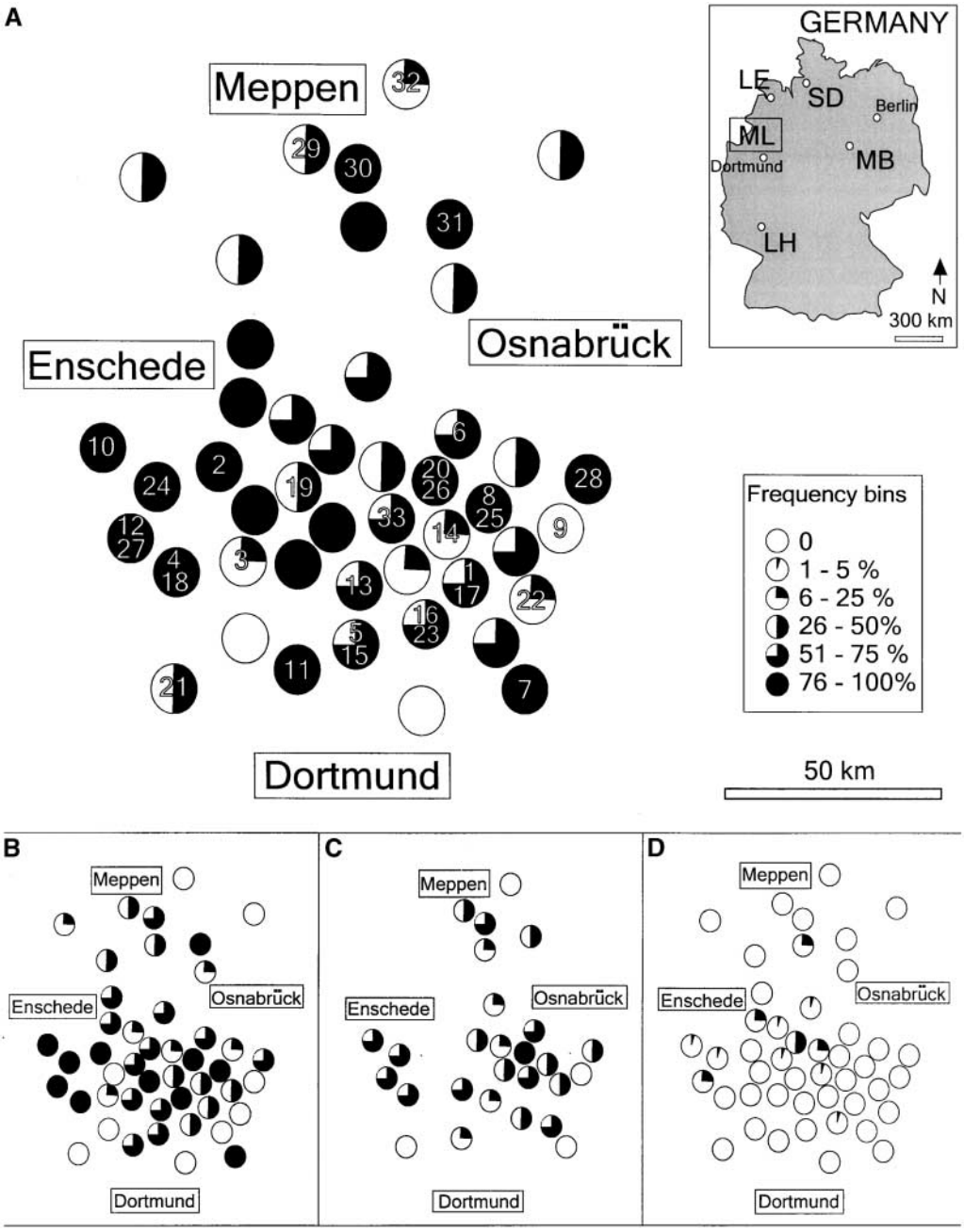

FIGURE 1.—Distribution of resistance to warfarin, RW (A), bromadiolone, RB (B), coumatetralyl, RC (C), and difenacoum, RD (D) in the Münsterland area (inset, ML) of Germany (summary of data published in PELZ 2001). Distribution map based on 1168 rats trapped live and tested for resistance with the BCR method between 1990 and 1999 (*cf.* PELZ *et al.* 1995; PELZ 2001). Of the 46 localities depicted, those 25 localities for which tissue samples for genetic typing were available show a population identification number. Samples from farms belonging to the same township were pooled to generate this figure (yielding average resistance frequencies for the locality) and each population identification number is shown. LE, SD, MB, and LH denote populations outside the resistance area tested with the BCR method; only samples from MB and LH were available for the genetic study. Populations numbered 1, 7, 8, 9, 22, 27, and 31 had $N < 3$ and were excluded from genetic data analyses. Population resistance frequencies given in Table 1 were used for all analyses. Farm names cannot be provided by prior agreement, but details on sampling locations will be provided by request to M. H. Kohn or H.-J. Pelz.

Conversely, warfarin-susceptible rats almost always were susceptible to the other anticoagulants. Only 3 of 101 (~3%) RW+ rats were RB (1), RC (2), or RD (0). For our analyses we considered only one group (RW+) of susceptible rats.

Microsatellite typing and analysis: DNA from 727 rats was extracted and the microsatellite loci *D1Rat219*, *D2Rat31*, *D10Rat6*, *D13Rat18*, *D14Rat15*, and *D17Rat38* were assayed following standard procedures (KOHN and PELZ 1999). The latter five loci are located on rat chromosomes 2, 10, 13, 14, and 17 and were typed as an indicator of background levels of variation. *D1Rat219* was chosen to represent *Rw* on rat chromosome 1 (see RESULTS; *cf.* KOHN *et al.* 2000). For map position data of loci, see The Rat Genome Database at the Medical College of Wisconsin, Milwaukee, Wisconsin (http://rgd.mcw.edu/; April 2002).

Each of the presented analyses used a subset of the total sample. First, 16 rats from population 24 were used for association mapping of *Rw* (Table 2). Their resistance phenotypes and genotypes were previously determined using the *in vitro* assay of VKOR activity (THIJSSEN and PELZ 2001). Resources were insufficient to apply the VKOR assay to a more extensive sample. In addition to the five unlinked microsatellite loci *D2Rat31*, *D10Rat6*, *D13Rat18*, *D14Rat15*, and *D17Rat3*, these 16 rats were typed for four loci that mapped within the ~2.2-cM interval on chromosome 1 containing *Rw* (Table 2; *D1Rat67*, *D1Rat364*, *D1Rat219*, and *D1Rat288*; KOHN *et al.* 2000).

Second, the entire sample of 727 rats from 27 populations was analyzed (Table 1). Rats for which no BCR testing was done but that were obtained from populations with known resistance phenotype frequencies were included in this population-controlled analysis. Unless stated otherwise, we focused on the analysis of population genetic data with respect to RW. Allele frequencies underlying the population-controlled design are given in supplement 1 at http://www.genetics.org/supplemental/.

TABLE 1

Sample resistance frequencies

Population	*N*	RW (*N*)	RB (*N*)	RC (*N*)	RD (*N*)
2	3	1.00 (3)	0.67 (3)	ND	— (1)
3	5	0.25 (4)	0.33 (3)	ND	— (1)
4	52	0.93 (46)	0.92 (37)	0.71 (21)	— (31)
5	20	0.74 (19)	0.87 (15)	ND	— (13)
6	24	0.96 (24)	0.90 (20)	0.55 (11)	— (16)
10	42	0.95 (38)	0.88 (32)	0.70 (23)	0.04 (28)
11	70	0.83 (65)	0.84 (51)	0.47 (30)	— (39)
12	25	0.95 (21)	1.00 (17)	0.63 (8)	0.06 (17)
13	78	0.64 (60)	0.76 (45)	0.52 (38)	— (39)
14	19	0.44 (16)	0.50 (12)	0.67 (3)	— (4)
15	10	0.17 (6)	— (3)	ND	ND
16	19	— (18)	ND	— (8)	ND
17	53	0.86 (51)	0.95 (42)	0.50 (44)	— (37)
18	29	0.91 (23)	0.89 (19)	0.63 (8)	— (15)
19	56	0.65 (49)	0.76 (33)	0.46 (28)	0.04 (23)
20	27	0.62 (26)	1.00 (15)	0.71 (14)	— (14)
21	23	0.33 (21)	— (14)	— (3)	ND
23	37	0.11 (37)	0.29 (7)	— (9)	— (2)
24	73	0.93 (55)	0.90 (51)	0.68 (56)	— (43)
25	20	0.89 (19)	0.93 (15)	0.38 (13)	— (12)
26	6	1.00 (6)	1.00 (6)	1.00 (2)	— (6)
28	37	0.86 (35)	0.76 (29)	0.50 (20)	— (21)
29	6	0.67 (6)	0.50 (4)	0.40 (5)	— (2)
30	9	0.78 (9)	0.71 (7)	0.89 (9)	— (5)
32	4	0.25 (4)	— (2)	— (2)	ND
LH	13	— (13)	ND	— (9)	ND
MB	3	0.33 (3)	ND	ND	ND
Mean	29.9	0.63 (25.1)	1 (17.9)	1 (13.5)	1 (13.7)
Total	**727**	**677**	**482**	**364**	**369**

N, number of sampled individuals tested with the BCR method for each rodenticide (in parentheses); —, no resistance found in *N* individuals tested. ND, not determined; RW, warfarin resistance; RB, bromadiolone resistance; RC, coumatetralyl resistance; RD, difenacoum resistance.

Third, only rats of known BCR phenotype were analyzed using a case-controlled design. Warfarin-resistant rats formed the case group, RW, to be compared to the control group, RW+, composed of warfarin-susceptible rats. This analysis ignored the population origin. The groups RB, RC, and RD should not be considered as independent from the RW group (see above) and only brief mention of results will be made. Allele frequencies underlying the case-controlled design are given in supplement 2 at http://www.genetics.org/supplemental/.

Analyses and computations were done as implemented in the Genetic Data Analysis (GDA) software (Lewis and Zaykin 2002). The software implements analytical and randomization procedures outlined in Weir (1996; and references given therein). Descriptive statistics computed included the number of chromosomes sampled ($2N$), the number of alleles per locus (k), expected and observed heterozygosity (H_e and H_o), and fixation indices estimated with respect to sample configuration (f). The coefficient D_A was computed and χ^2 analysis was used to test for Hardy-Weinberg equilibrium (HWE). In addition, HWE exact tests were done using the shuffling method for 3200 runs followed by Fisher's exact tests. Loci with $P < 0.05$ were considered in HW disequilibrium. Composite gametic phase disequilibrium D_{AB} (*i.e.*, not assuming HWE) between the most frequent alleles at the loci was estimated in the same fashion.

For each locus separately and across loci, Wright's (1951) *F*-statistic analogs θ (F_{ST}), F (F_{IT}), and f (F_{IS}) were estimated and analyzed within an ANOVA framework using GDA software following Weir (1996). If applicable, to estimate significance of *F*-statistics, the 95% confidence interval (CI) about each measure was computed using 1000 bootstrap replicates over loci. Similarly, jackknifing over populations was used to compute mean values and a 1 SD interval.

Spearman's correlation coefficients among the variables θ, geographical distance, and ΔRW were computed. Here, ΔRW was used as a surrogate measure for divergent selection with warfarin and was computed from Table 1 as the difference in warfarin resistance frequency between population pairs (supplement 3 at http://www.genetics.org/supplemental/). Geographic isolation was measured in kilometers as a straight line connecting any two sampling sites (supplement 3 http://www.genetics.org/supplemental/). Significance of correlation coefficients was assessed within the framework of a two-tailed Mantel's (1967) test (Slatkin 1993b; Raymond and Rousset 1995).

RESULTS

Choice of the genetic marker for resistance: Microsatellite genotyping results for 16 rats were compared

TABLE 2

Association of warfarin resistance (VKOR) genotypes with microsatellite genotypes in 16 rats

ID[a]	VKOR[b]	*D1Rat288* (chromosome 1, 98.09 cM)	*D1Rat364* (chromosome 1, 98.74 cM)	*D1Rat219* (chromosome 1, 98.82 cM)	*D1Rat67* (chromosome 1, 99.87 cM)	*D2Rat31* (chromosome 2)	*D10Rat6* (chromosome 10)	*D13Rat18* (chromosome 13)	*D14Rat15* (chromosome 14)	*D17Rat38* (chromosome 17)
4083	+/+	139/151	152/152	250/250	170/172	150/154	144/144	136/136	152/152	176/176
4100	*Rw/Rw*	139/139	152/156	250/254	170/174	154/154	144/146	136/136	152/156	176/176
4130	*Rw/Rw*	139/151	152/156	254/254	174/174	150/154	144/144	136/136	152/156	172/176
4131	+/*Rw*	139/151	152/156	242/254	158/174	150/150	144/146	136/136	152/156	172/176
4133	*Rw/Rw*	139/151	152/156	254/254	174/174	152/154	144/146	136/136	152/156	172/176
4134	+/*Rw*	139/151	152/152	242/254	158/170	150/154	146/148	140/152	152/152	176/176
4135	*Rw/Rw*	151/151	152/152	254/254	170/174	150/154	144/146	136/152	152/152	172/172
4136	*Rw/Rw*	139/139	156/156	254/254	174/174	150/154	144/154	136/136	152/152	172/172
4137	*Rw/Rw*	139/139	156/156	254/254	174/174	154/154	144/144	136/136	148/152	176/176
4138	+/*Rw*	139/151	156/156	242/254	152/174	150/154	144/144	136/136	152/152	172/172
4139	*Rw/Rw*	139/139	156/156	254/254	174/174	150/154	144/146	136/136	152/152	172/176
4140	*Rw/Rw*	139/151	152/156	254/254	174/174	154/154	146/146	136/150	152/156	172/176
4142	*Rw/Rw*	139/139	156/156	254/254	174/174	152/154	144/144	136/150	152/152	176/176
4143	*Rw/Rw*	139/139	156/156	254/254	174/174	150/154	144/148	140/152	152/156	176/176
4144	*Rw/Rw*	139/139	156/156	254/254	174/174	152/154	144/154	136/144	156/156	176/176
4145	*Rw/Rw*	139/151	156/156	254/254	174/174	150/154	144/148	140/152	152/152	172/176
	P_{HWE}	0.64	0.28	0.19	0.36	0.23	0.93	0.02	1	0.59
	D_{AB}	0.06	0.11	0.16	0.16	0.05	−0.03	−0.04	−0.06	−0.02
	(±1SD)	(0.029)	(0.074)	(0.080)	(0.074)	(0.026)	(0.056)	(0.054)	(0.039)	(0.056)
	χ^2	1.8	4.0	13.4	10	2.1	0.5	0.4	1.6	0.2
	$P(D_{AB})$	0.1600	0.0344	<0.0001	0.0066	0.2818	0.5800	0.3234	0.7591	0.3425

Microsatellite alleles are designated according to their size.

[a] Rats from population 24 and their identification numbers (ID) are as in Thijssen and Pelz (2001).

[b] Warfarin resistance genotype determined with an *in vitro* VKOR activity assay in 2 μM warfarin (from Thijssen and Pelz 2001).

to warfarin resistance phenotypes determined with the VKOR method (Table 2). At each marker we designated the allele most commonly associated with warfarin resistance as the *Rw* allele and the remaining ones as + alleles. On the basis of the presence or absence of the assigned *Rw* allele, and assuming that the resistance allele was dominant (see below), *D1Rat219* correctly classified all 16 rats as either resistant or susceptible according to VKOR testing results (Table 2). *D1Rat67* correctly classified 15 (94%) rats, whereas *D1Rat288* and *D1Rat364* each correctly classified 14 (86%) rats. Loci on the other chromosomes classified between 12 (75%) and 14 (86%) rats correctly by chance.

Microsatellite genotypes at *D1Rat364*, *D1Rat219*, and *D1Rat67* corresponded to warfarin resistance genotypes determined with the VKOR method in 9 (56%), 15 (94%), and 13 (81%) of 16 cases, respectively (Table 2). Genotypes at *D1Rat288* and at the five unlinked loci corresponded to the VKOR genotyping results in five or fewer cases. The single case of inconsistency between *D1Rat219* with a VKOR genotype was due to the heterozygous resistant rat 4100 whose VKOR activity was between that of heterozygous and homozygous resistant rats and thus was ambiguous (Thijssen and Pelz 2001). Overall, only 1 of 18 VKOR genotypes determined in the previous study by Thijssen and Pelz (2001) was ambiguous.

Larger values of D_{AB} are expected between markers closely linked to the trait locus than between more distant markers. The highest D_{AB} coefficient of 0.16 was found between *Rw* and *D1Rat67* and between *Rw* and *D1Rat219*. A Fisher's exact test on the permutated contingency tables yielded the strongest support ($\alpha = 0.0001$) for tight linkage between *Rw* and *D1Rat219* (Table 2). D_{AB} coefficients and associated statistics for the loci situated on other chromosomes were not significant. Analysis of D_{AB} assumed the preservation of haplotypes (*i.e.*, no double crossovers) and thus represented a best-case scenario that was partly supported by significant levels of higher-order composite disequilibria coefficients (D_{ABC}) among *D1Rat364*, *D1Rat219*, and *D1Rat67* (not shown).

This analysis further implicated the locus group *D1Rat364-D1Rat219-D1Rat67* in the expression of a warfarin-insensitive VKOR and suggested that *D1Rat219* is the most suitable marker for resistance of those surveyed (*cf.* Kohn *et al.* 2000). We chose *D1Rat219* as the marker to represent *Rw* for two additional reasons. First, *D1Rat219* is the only framework marker, implying the lowest degree of uncertainty concerning its map location. Second, *D1Rat219* has been placed in the newest available gene maps of the rat [*e.g.*, Steen *et al.* (1999); a high-density integrated genetic linkage and radiation hybrid map of the laboratory rat, The Rat Genome Database: ftp://rgd.mcw.edu/pub/publications/1999/steen_genome_research/].

Quantitative genetics: We analyzed *D1Rat219* genotypes and VKOR activities of the 16 rats from Table 2 within a coarse quantitative genetic framework (Falconer and Mackay 1996). Genotypic values derived from the VKOR activity assay of the 254/254 and +/+ rats were set as a and $-a$, respectively, and that of the +/254 genotype was set as d (Figure 2). The point of zero was set midway between the genotypic values of the two homozygotes. The value of d depends on the degree of dominance (d/a), zero being the expectation for codominant alleles. We found that genotypes at *D1Rat219* had significant effects on VKOR activities in the presence of warfarin (ANOVA; $P < 0.001$, F_{Ratio} =

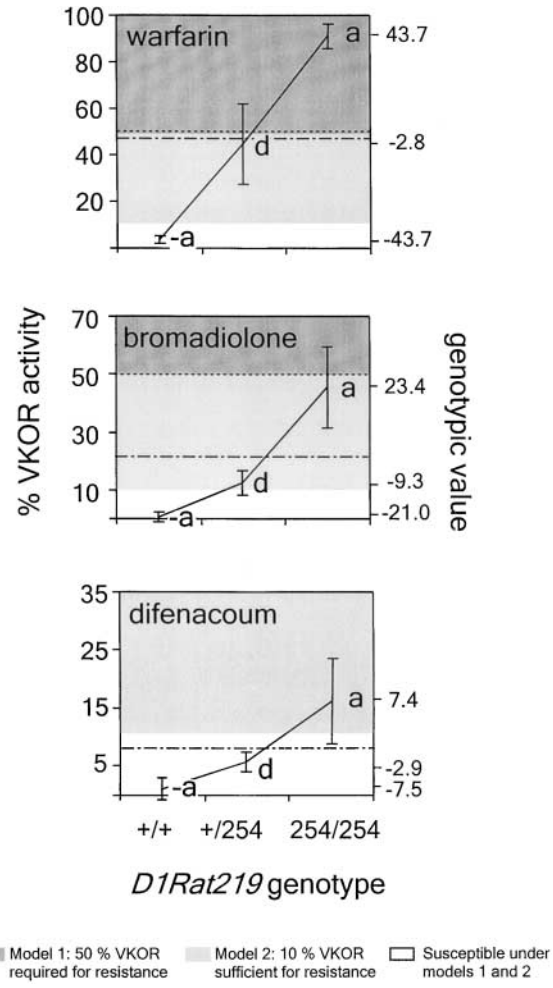

Figure 2.—Association between *in vitro* VKOR activity and *D1Rat219* genotypes deduced from 18 rats (*cf.* Table 2) in the presence of 2 μM warfarin (top), bromadiolone (middle), and difenacoum (bottom). Absolute percentage of VKOR activities are shown on the left axis, and corresponding genotypic values are on the right axis. Assigned genotypic values $-a$, d, and a as defined in Falconer and MacKay (1996), and the point of zero (dashed line) is midway between $-a$ and a. The degree of dominance is defined as d/a. The dotted line demarcates 50% VKOR activity. Susceptibility alleles were pooled as + (see text). Two additional susceptible rats (IDs 4038 and 4060 in Thijssen and Pelz 2001) from populations 12 and 28, respectively, with the *D1Rat219* genotypes 250/250 and 248/250 were included in this analysis.

132.1, $R^2 = 0.94$, d.f. 15; Figure 2, top). Genotypes that lacked the 254-bp allele (+/+) had genotypic values of −43.7 ± 2.1 (SD) whereas genotypes containing one and two copies of the 254-bp allele had genotypic values of −2.8 ± 17.8 and 43.7 ± 5.3, respectively. The effect of two essentially codominant alleles was indicated by a d/a of −0.06. The genotypic values of 254/254 genotypes were significantly higher than the genotypic values of +/254 and +/+ genotypes (Tukey-Kramer comparison of means, all $P < 0.01$), and the +/254 genotypes had a higher value than the +/+ genotypes (Tukey-Kramer comparison of means, $P < 0.01$). The mean VKOR activity of 254/254 genotypes was 87% of the expected full (100%) activity, suggesting incomplete penetrance with respect to warfarin.

Similarly, genotypes at *D1Rat219* had significant effects on VKOR activities in the presence of bromadiolone (ANOVA; $P < 0.01$, $F_{ratio} = 22.7$, $r^2 = 0.75$, d.f. 15; Figure 2, middle) and difenacoum (ANOVA; $P < 0.003$, $F_{ratio} = 8.7$, $r^2 = 0.54$, d.f. 15; Figure 2, bottom). The effects of recessive alleles with respect to bromadiolone and difenacoum were indicated by a degree of dominance of −0.4 for both anticoagulants. Low penetrance of *Rw* with respect to bromadiolone and difenacoum exposure, respectively, was suggested by a VKOR activity of the 254/254 genotype that was 50 and 15% of the expected full activity. Finally, in the presence of bromadiolone and difenacoum, the 254/254 genotypic values were significantly higher than the genotypic values of the +/254 and +/+ genotypes (for both, Tukey-Kramer comparison of means was $P < 0.01$). With respect to difenacoum, however, the +/254 genotypic value was not significantly higher than the +/+ genotypic value (Tukey-Kramer comparison of means, not significant (n.s.) at $\alpha = 0.01$).

These data suggest that *D1Rat219* is closely linked to one or several tightly linked loci (*Rw*) that mediate warfarin insensitivity of the VKOR. The incomplete dominance and penetrance inferred from *D1Rat219* either were caused by its incomplete association with *Rw* or reflect real properties of *Rw*. The RB and RD phenotypes either are due to separate resistance loci *Rb* and *Rd* that are less closely linked to *D1Rat219* than *Rw* is or are determined by the *Rw* locus, which differs in its penetrance and dominance with respect to the three anticoagulants examined. Conceivably, the expression of resistance to bromadiolone and difenacoum then requires the action of modifier loci whose relative contribution to resistance depends on assumptions made concerning the required VKOR activity for proper blood coagulation homeostasis. For instance, if we assume that 50% VKOR activity is needed for coagulation homeostasis (model 1, Figure 2), then *Rw/Rw* rats would be considered predominantly RW and RB, *+/Rw* rats likely would be considered RW and RB+, and none would be considered RD. However, lower VKOR activity thresholds needed to maintain coagulation homeostasis have been suggested (Thijssen and Janssen 1994; Thijssen and Pelz 2001). When only genotypes that express a VKOR with <10% activity are considered as susceptible (model 2, Figure 2), then both *+/Rw* and *Rw/Rw* rats would be considered RW and RB, whereas only *Rw/Rw* rats would be considered RD and *+/Rw* rats RD+.

Association between *D1Rat219* and population resistance frequency: We considered the *Rw* allele (254-bp allele) and + alleles (all others) with frequencies p and q, respectively, which were measured in the entire sample of 727 rats (*cf.* supplement 1 at http://www.genetics.org/supplemental/). We assumed that *Rw* was dominant (*i.e.*, both model 1 and model 2 in Figure 2) and fully penetrant with respect to warfarin. At HWE we then expected a total of ~49% *+/Rw* rats and ~18% *Rw/Rw* rats in our sample, corresponding to a predicted RW phenotype frequency of ~67%, which differed by only 4% from the observed RW phenotype frequency of 63% (Table 1). A BCR classification error may explain as much as 2% of this discrepancy (*cf.* Martin *et al.* 1979; Kohn and Pelz 1999).

Similarly, assuming dominance and full penetrance of *Rw* with respect to bromadiolone (model 2 in Figure 2) and using HWE frequencies at *D1Rat219*, we estimated that ~67% of rats were RB, only 1% less than the observed RB sample frequency of 68% (Table 1). In contrast, observed frequencies of RC (47%) and RD (1%) could not be predicted using allele frequencies at *D1Rat219*. The discrepancy with respect to RC likely was related to our initial difficulties in adopting the BCR method for RC resistance testing (Pelz and Endepols 1999). Our ability to predict RD frequencies may have been diminished by small sample size and by the presumably recessive nature and low penetrance of *Rw* with respect to difenacoum (Figure 2, bottom; *cf.* Greaves and Cullen-Ayres 1988).

The sample mean of enzymatic activity of the VKOR (genotypic value *M*) can be predicted on the basis of the underlying HWE genotype frequencies at the trait locus as $M = a(p - q) + 2dpq$ (Falconer and Mackay 1996). First we applied this approach to the case-controlled sample. Allele frequencies were obtained from supplement 2 and a, $-a$, and d were derived from Figure 2. Predicted *M*, given as percentage of VKOR activity in the presence of the respective anticoagulant, was ~57, 24, and 13% for the case groups RW, RB, and RD, respectively (Figure 3A). These percentages exceeded a 10% cutoff value for VKOR activity (model 2) and hence may be considered RW, RB, and RD. Only the RW group exceeded 50% of VKOR activity expected under a single dominant genetic model (*cf.* Figure 2, model 1). Control group RW+ had a predicted VKOR activity of <5% and would be classified as susceptible under both models (*cf.* Figure 2). Thus, allele frequencies at *D1Rat219* measured for the case and control groups enabled predictions to be made concerning VKOR activities (*M*-values) and resistance status.

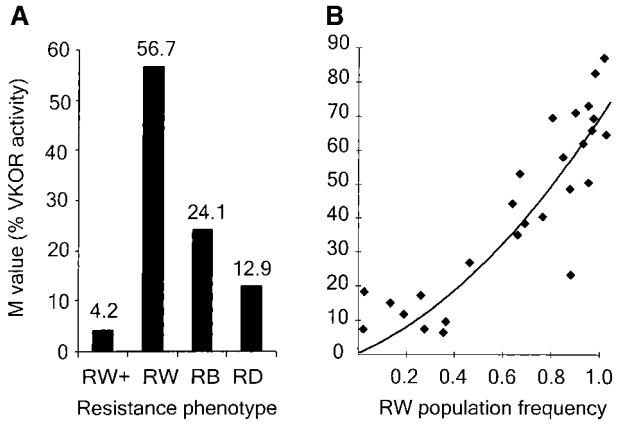

FIGURE 3.—Percentage of VKOR activities (*M*-value) in a case-controlled (A) and population-controlled design (B) predicted on the basis of allele frequencies at *D1Rat219*. In A, we considered the phenotypes warfarin resistance RW, bromadiolone resistance RB, and difenacoum resistance RD. In B, only the RW phenotype and its frequency in populations were considered (see text).

Similarly, we predicted the *M*-value for each population listed in Table 1 on the basis of *D1Rat219* allele frequencies (supplement 1 at http://www.genetics.org/supplemental/). We found a significant association between predicted *M*-values and RW frequencies (ANOVA, $F_{\text{Ratio}} = 50.3$, $R^2 = 0.79$, d.f. 26, $P < 0.0001$; Figure 3B) and between predicted *M*-values and RB frequencies (ANOVA, $F_{\text{Ratio}} = 18.5$, $R^2 = 0.58$, d.f. 25, $P < 0.0001$; not shown), but not between predicted *M*-values and RD frequencies ($P = 0.27$, not shown). To conduct a corresponding analysis for RC, VKOR activities measured with respect to warfarin were used (Figure 2, top). Like warfarin, coumatetralyl is a first-generation, nonacute-acting anticoagulant. The *M*-value that was obtained was associated with RC frequencies (ANOVA, $F_{\text{Ratio}} = 13.8$, $R^2 = 0.57$, d.f. 19, $P = 0.003$, not shown). Overall, this coarse regression-based approach (*cf.* MANTEL 1967) allowed for a prediction to be made regarding the mean VKOR activity of populations (*M*-value) underlying resistance to warfarin, bromadiolone, and coumatetralyl, but not difenacoum.

Population-controlled analysis of variation and differentiation: We compared variation and differentiation at *D1Rat219* and the five neutral loci across 27 rat populations. An average of 2.9 (2.5–3.2) alleles per population occurred at *D1Rat219* and 4.3 (3.8–4.7) alleles per locus and population at the neutral loci (Table 3). The mean H_e at *D1Rat219* was 0.43, whereas H_e at the neutral loci was 0.60. The 95% confidence intervals of the estimates overlapped. Similarly, the confidence intervals about the mean H_o values of *D1Rat219* (0.43) and the neutral loci (0.54) overlapped.

Four populations (23, 24, 28, and 29) deviated from HWE at *D1Rat219*. Only one of the four deviations (28) was in the direction of heterozygote excess. Sixteen populations displayed deviations from HWE at neutral loci (populations given in parentheses): *D2Rat31* (6, 10, 14, 18, 25, 26, LH), *D10Rat6* (11, 12, 17, 20, 24, 28, 29), *D13Rat18* (6, 12, 26, 28), *D14Rat15* (LH), and *D17Rat38* (11, 12, 17–20, 23–25). Of the 28 observed deviations from HWE at the five neutral loci, all were in the direction of heterozygote deficiency. Population substructure within farms and townships presumably has caused Wahlund's effect (*cf.* SELANDER and YANG 1969).

Descriptive population genetics statistics from Table 3 yielded no significant relationships between them and resistance frequencies given in Table 1 (ANOVA; $P > 0.05$). This was valid for the presumably neutral loci as well as for *D1Rat219*. However, the mean of f at *D1Rat219* calculated over all populations (0.01) was one order of magnitude lower than the mean of f at the neutral loci (0.10; Table 3), but since the 95% CIs overlapped, this observation remained statistically inconclusive.

Analysis of population structure at the neutral loci revealed high *F*-statistics that were significantly different from zero ($P < 0.05$) at each hierarchical level f, F, and θ (Table 4A). F (0.226) was most pronounced, followed by θ (0.133) and f (0.107). Computed standard deviations for each individual neutral locus suggested that with the exception of some f values, *F*-statistics were significantly different from zero throughout (Table 4A). In contrast, f (−0.024) at *D1Rat219* was not significantly different from zero, whereas F (0.214) and θ (0.232) were significantly different from zero. Hence, overall, *D1Rat219* differed from the genomic background by more pronounced levels of outbreeding (f) and population subdivision (θ). The locus-specific patterns of genetic subdivision measured as θ at *D1Rat219* were in agreement with expectations for loci that are the object of selection. Following POGSON *et al.* (1995), we compared θ values of *D1Rat219* with the genomic background using the expression $\chi^2_{(n-1)} = (n-1)\,[\theta_{(D1Rat219)}/\theta_{(\text{genomic background})}]$, where n is the number of populations examined and θ is computed over the five neutral loci (*cf.* Table 4A). Using this approach, we found that the difference between *D1Rat219* and the genomic background was significant ($\chi^2_{(26)} = 45.4$; $P < 0.01$). None of the permutations that placed a neutral locus in the nominator were significant at $\alpha = 0.05$.

We tabulated θ at *D1Rat219* and at the neutral loci to test their interrelationship with geographical distance and divergent selection with warfarin (ΔRW; supplement 3 at http://www.genetics.org/supplemental/). The θ-value at the five neutral loci was significantly related to distance (Figure 4A; Mantel's test; $P = 0.02$; $a = 0.136$, $b < 0.001$), where a and b represent the interception and slope of the linear regression fitted to the points, respectively. Testing the relationship $\theta/(1-\theta)$ *vs.* ln(distance) (SLATKIN 1993b) was also significant ($P = 0.02$). Similarly, at locus *D1Rat219* distance was significantly correlated with θ (Figure 4B; $P < 0.0001$;

TABLE 3

Variation data for each population at *D1Rat219* (first number) and across the genomic background (second number)

ID	$2N^a$	k	H_e	H_o	f
2	6/6	2.0/2.8	0.60/0.65	0.3/0.60	0.50/0.10
3	6/8	2.0/2.6	0.33/0.46	0.33/0.45	0.00/0.04
4	86/92	4.0/4.8	0.53/0.59	0.51/0.54	0.03/0.07
5	38/40	2.0/4.0	0.49/0.58	0.68/0.54	−0.41/0.06
6	46/44	2.0/4.2	0.23/0.63	0.17/0.58	0.25/0.08
10	76/76	3.0/3.0	0.47/0.43	0.53/0.40	−0.12/0.09
11	136/120	2.0/7.0	0.49/0.70	0.54/0.64	−0.12/0.08
12	50/40	3.0/4.6	0.44/0.73	0.48/0.61	−0.10/0.17
13	78/64	3.0/5.4	0.47/0.58	0.46/0.51	0.01/0.13
14	30/28	4.0/4.4	0.47/0.60	0.53/0.51	−0.14/0.16
15	18/12	2.0/3.5	0.11/0.54	0.11/0.64	0.00/−0.22
16	36/32	3.0/4.4	0.42/0.56	0.50/0.57	−0.20/−0.03
17	104/88	3.0/4.6	0.61/0.50	0.67/0.44	−0.11/0.11
18	58/40	3.0/5.0	0.48/0.68	0.38/0.60	0.22/0.12
19	94/86	5.0/5.8	0.59/0.66	0.51/0.57	0.14/0.14
20	44/50	2.0/4.6	0.50/0.70	0.59/0.66	−0.18/0.06
21	44/38	3.0/4.8	0.53/0.64	0.64/0.59	−0.20/0.09
23	66/64	4.0/4.8	0.25/0.60	0.15/0.48	0.39/0.21
24	144/136	4.0/4.4	0.40/0.46	0.33/0.43	0.17/0.07
25	28/36	3.0/3.8	0.42/0.60	0.50/0.46	−0.21/0.24
26	12/12	2.0/3.0	0.17/0.54	0.17/0.52	0.00/0.05
28	62/62	3.0/5.2	0.62/0.60	0.81/0.53	−0.31/0.11
29	8/10	4.0/4.4	0.82/0.73	0.25/0.68	0.73/0.08
30	18/16	2.0/3.4	0.42/0.60	0.33/0.62	0.23/−0.04
32	8/6	3.0/2.5	0.61/0.56	0.75/0.60	−0.29/−0.12
LH	26/24	2.0/5.6	0.21/0.65	0.23/0.55	−0.09/0.17
MB	6/6	1.0/2.4	0.00/0.57	0.00/0.33	0.00/0.47
Mean	49.2/45.6	2.9/4.3	0.43/0.60	0.43/0.54	0.01/0.10
95% CI		2.5–3.2/3.8–4.7	0.36–0.57/0.56–0.63	0.34–0.51/0.51–0.57	−0.09–0.11/0.04–0.14

Sample size ($2N$), number of alleles (k), expected and observed heterozygosity (H_e and H_o), inbreeding coefficient (f).
[a] For neutral loci, rounded mean sample size is given; see supplement 1 at http://www.genetics.org/supplemental/ for N.

$a = 0.192$, $b < 0.001$) and with $\theta/(1 - \theta)$ ($P < 0.0001$). In sharp contrast, θ at *D1Rat219* was significantly related to ΔRW (Figure 4B; $P < 0.0001$; $a = 0.053$, $b = 0.510$) whereas no such relationship was supported for the neutral loci (Figure 4A; $P = 0.10$; $a = 0.138$, $b = 0.020$). ΔRW had no systematic relationship with geographic distance separating localities ($P = 0.11$; $a = 0.317$, $b = 0.001$; *cf.* Figure 1). Hence, both the neutral alleles and alleles at *D1Rat219* were distributed according to geographic distance ($R^2 \sim 40$ and 21%, respectively). However, while differentiation at *D1Rat219* clearly was dominated by ΔRW ($R^2 \sim 67\%$), the effect that ΔRW had on differentiation over the genomic background was negligible ($R^2 < 1\%$).

Case-controlled analysis of variation and differentiation: Rats were grouped by their warfarin resistance phenotype and analyzed within the framework of a case-controlled design. Descriptive statistics k, H_e and H_o, and f at the five neutral loci did not differ between the RW+ and RW groups (paired t-tests, all n.s. at $\alpha = 0.05$; Table 5). Sample size ($2N$) of the RW+ group (319.2) was lower than that of the RW group (825.6). However, standard deviations associated with statistics were similar between both groups, suggesting sample size had little effect (Table 5). With one exception (*D13Rat18*, RW+) neutral loci generally departed significantly from HWE ($P < 0.001$) and exhibited heterozygote deficiency in both groups (not shown), as was expected for intentionally pooled samples derived from subdivided populations.

D1Rat219 was set apart from the genomic background in both the case and control group in that it differed in the magnitude and equity of H_e and H_o values. Specifically, we observed a high level of inbreeding at *D1Rat219* (0.38) in the RW+ group that was similar to that observed over the genomic background (0.23 ± 0.16). In contrast, we found no evidence for inbreeding (−0.08) at *D1Rat219* in the RW group even though the genomic background in the RW group displayed levels of inbreeding (0.21 ± 0.10) equal to those of the RW+ group (Table 5). Genotype frequencies at *D1Rat219* in the RW category were marginally compatible with HWE expectations ($P = 0.058$) but HWE at *D1Rat219* was rejected in the RW+ group ($P < 0.01$). Results for the

TABLE 4

Analysis of *f*, *F*, and θ at *D1Rat219* and five neutral loci computed for the population-controlled (A) and the case-controlled design (B)

Analysis	Locus (2*N*)	*f*	*F*	θ
A. Population controlled	*D1Rat219* (1328)	−0.024 (0.040)	0.214 (0.066)	0.232 (0.046)
	D2Rat31 (1300)	0.019 (0.031)	0.141 (0.034)	0.125 (0.020)
	D10Rat6 (1140)	0.108 (0.028)	0.212 (0.030)	0.116 (0.020)
	D13Rat18 (1312)	0.048 (0.031)	0.192 (0.039)	0.151 (0.026)
	D14Rat15 (1298)	0.049 (0.025)	0.170 (0.044)	0.126 (0.033)
	D17Rat38 (1118)	0.311 (0.044)	0.413 (0.043)	0.148 (0.037)
	Mean	0.107	0.226	0.133
	(95% CI)	(0.037–0.203)	(0.164–0.320)	(0.122–0.147)
B. Case controlled	*D1Rat219* (1228)	0.037	0.337	0.311
	D2Rat31 (1192)	0.132	0.143	0.013
	D10Rat6 (1062)	0.217	0.222	0.010
	D13Rat18 (1222)	0.174	0.187	0.016
	D14Rat15 (1204)	0.158	0.157	−0.002
	D17Rat38 (1044)	0.406	0.421	0.025
	Mean	0.214	0.224	0.012
	(95% CI)	(0.151-0.309)	(0.160-0.321)	(0.005-0.018)

Mean and 95% CI computed for neutral loci by 1000 bootstrap replicates over loci, 1 SD (in parentheses following *F*-statistic) computed by jackknifing over populations. 2*N*, sampled chromosomes. Sample size smaller for A than for B because only rats for which the BCR phenotype was known were included in analysis.

RB and RC groups were similar to those presented for the RW group (not shown), and sample size for RD was too low for analysis.

Genetic subdivision between case and control groups at the five presumably neutral loci was low (θ = 0.012) yet significantly different from zero at α = 0.05 (Table 4B). Moreover, *f* and *F* were pronounced (0.214 and 0.224, respectively) and significant at α = 0.05 each (Table 4B). In contrast, resistant and nonresistant rats were highly differentiated with regard to *D1Rat219*, a locus closely linked to *Rw*. θ between case and control groups was 0.311 or ~30 times more pronounced than that of the genomic background. Although *f* was small at *D1Rat219* (0.037), *F* was pronounced (0.337). Statistics at *D1Rat219* could not be tested for significance using the bootstrap or jackknife procedures. To obtain

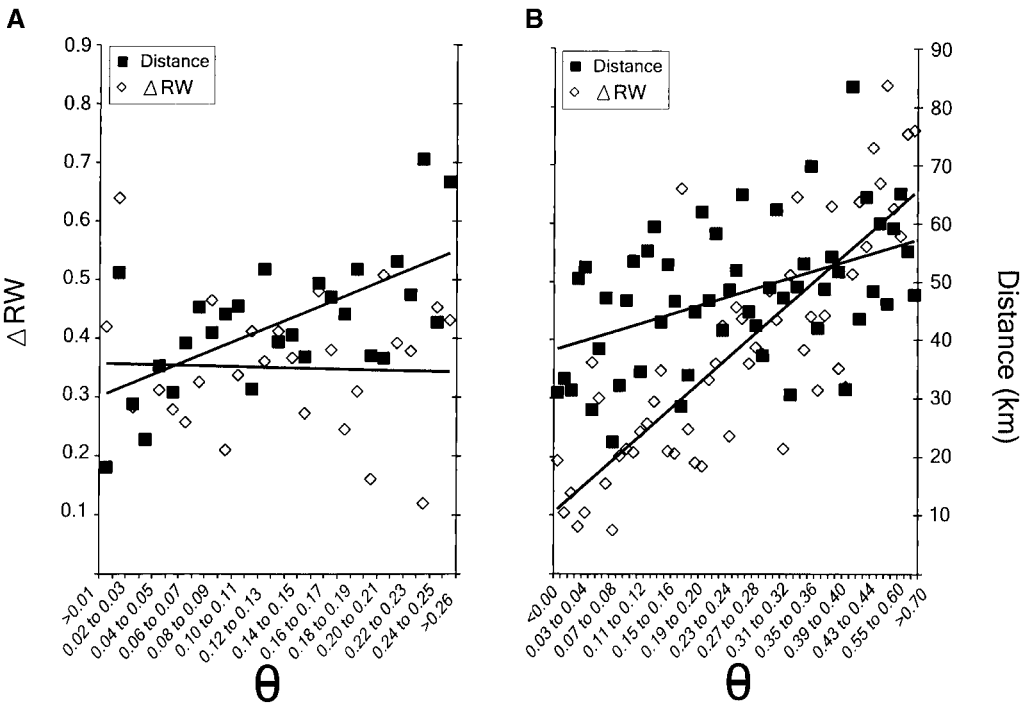

FIGURE 4.—Isolation-by-distance and isolation-by-divergent selection (ΔRW) at neutral loci (A) and at *D1Rat219* (B). For clarity of presentation, θ-values were grouped in bins and distances >100 km were omitted. Statistical analysis used unmodified data given in supplement 3 at http://www.genetics.org/supplemental/ (see text).

TABLE 5

Case-controlled comparison of descriptive statistics of resistance groups RW+ and RW

Group	Locus	$2N$	k	H_e	H_o	f
RW+	*D1Rat219*	352	6	0.43	0.28	0.38
	D2Rat31	346	10	0.77	0.64	0.17
	D10Rat6	288	11	0.66	0.55	0.17
	D13Rat18	330	14	0.84	0.77	0.08
	D14Rat15	334	4	0.56	0.42	0.24
	D17Rat38	298	10	0.68	0.33	0.51
Mean ± 1 SD[a]		319.2 ± 24.9	9.8 ± 3.6	0.70 ± 0.11	0.54 ± 0.17	0.23 ± 0.16
RW	*D1Rat219*	876	5	0.54	0.58	−0.08
	D2Rat31	846	13	0.78	0.69	0.12
	D10Rat6	774	9	0.65	0.49	0.23
	D13Rat18	892	15	0.76	0.60	0.21
	D14Rat15	870	6	0.55	0.48	0.13
	D17Rat38	746	9	0.63	0.40	0.36
Mean ± 1 SD[a]		825.6 ± 62.8	10.4 ± 3.6	0.67 ± 0.10	0.53 ± 0.11	0.21 ± 0.10

Sample size ($2N$), number of alleles (k), expected and observed heterozygosity (H_e and H_o), inbreeding coefficient (f).

[a] Mean and SD computed for the five presumably neutral loci. SD computed as the deviation from the mean, with exception of f, for which SD was computed using 1000 bootstrap replicates over loci.

a measure for the robustness of these estimates, random assignment of 6150 *D1Rat219* genotypes (sampled with replacement) to the RW and RW+ groups was done, yielding a nonsignificant θ (95% CI: −0.001–0.091) and significant ($\alpha = 0.05$) f (95% CI: 0.129–0.181) and F (95% CI: 0.154–0.225). We predicted that *D1Rat219* should be highly differentiated between the case group RW and the control group RW+. For this to be informative, we further predicted that the genetic differentiation across the genomic background should be negligible. We found that when $\chi^2_{(n-1)} = (n - 1)\ [\theta_{(D1Rat219)}/\theta_{(\text{genomic background})}]$ (Pogson *et al.* 1995) was computed, θ values at *D1Rat219* (0.311) and for the genomic background (0.012) were significantly ($\chi^2_{(1)} = 25.9$; $P < 0.001$) different. None of the permutations that placed any of the putatively neutral loci in the nominator was significant at $\alpha = 0.05$.

DISCUSSION

***D1Rat219* as a marker for resistance:** Our previous research established an association between warfarin resistance as measured by the BCR method and allele frequencies at *D1Rat288*, *D1Rat364*, *D1Rat219*, and *D1Rat67* contained in an ~2.2-cM interval of rat chromosome 1 (Kohn *et al.* 2000). In accord with previous linkage mapping data, we hypothesized that this interval corresponds to the warfarin resistance locus *Rw* (Kohn *et al.* 2000). Here, we determined VKOR activity for a small sample of rats (Table 2, Figure 2). VKOR measurement should be linked to the resistance mechanism more directly than prothrombin time (BCR) measurements previously taken (*e.g.*, Hildebrandt and Suttie 1982; Thijssen and Janssen 1994; Cain *et al.* 1998; Guenthner *et al.* 1998; Thijssen and Pelz 2001). Correspondingly, our genotyping data (Table 2) now suggest that the interval defined by markers *D1Rat364*, *D1Rat219*, and *D1Rat67* is tightly linked to a locus *Rw* that mediates warfarin insensitivity via a pathway that involves an anticoagulant-insensitive VKOR (Figure 2) and that promotes normal prothrombin times despite exposure to anticoagulant poison. Our data support earlier suggestions that as little as 10% VKOR activity (model 2 in Figure 2) may be sufficient to maintain coagulation homeostasis, *i.e.*, to express the resistance phenotype (*cf.* Figure 3A). Finally, our data are compatible with *Rw* being a component of the VKOR complex, but we cannot exclude the possibility that the *Rw* locus represents a gene that acts farther upstream of the VKOR (*cf.* Wallin *et al.* 2001).

Our results prompt the working hypothesis that *Rw* also corresponds to *Rb*, *Rc*, and *Rd* and thus is a major locus or cluster of loci underlying resistance to warfarin, bromadiolone, coumatetralyl, and difenacoum (Figures 2 and 3). At least four previous observations support this hypothesis. First, all of these anticoagulant compounds are derivatives of coumarin. Second, VKOR activities measured in the presence of all four anticoagulants are correlated (Thijssen and Pelz 2001). Third, with the exception of RD, resistance segregated as a major and dominant locus in laboratory crosses (Greaves and Ayres 1969, 1982; Wallace and MacSwiney 1976; Kohn and Pelz 1999). RD mapped to the same genomic interval but was found to be recessive (Greaves and

Cullen-Ayres 1988). Finally, cross-resistance to several different anticoagulants occurs in our rat populations (Figure 1 and Table 1; *cf.* Pelz *et al.* 1995; Pelz 2001; Thijssen and Pelz 2001) and elsewhere in the world (Hadler and Buckle 1992).

Ecological genetics: The nearly ubiquitous presence of the 254-bp allele at *D1Rat219* in resistant rats (Figures 2 and 3) suggests a single and recent origin of resistance, followed by a rapid spread throughout our study area (Figure 1). The origin of the resistance allele could be due to *de novo* mutations resulting from the introduction of the resistance allele from elsewhere. The *Rw* allele likely became common and spread rapidly in the early 1990s, given the increasing control problems with anticoagulants and high frequencies of resistance on farms where it was not detected less than a decade ago (Pelz *et al.* 1995; Pelz 2001). Assuming dominance, a net selection coefficient of as little as 0.05 would be sufficient to attain the observed average *Rw* allele frequency (~26%) within a decade (*cf.* Hartl and Clark 1988, p. 156). Mortality rates between 60 and 100% (Lund 1985) suggest more intense selection (*e.g.*, $s > 0.4$) may occur in exposed rat populations. Hence, intense selection may have led to high levels of resistance within even shorter time periods (*e.g.*, <2 years) at sites (*e.g.*, 7 and 28) located at the eastern border and that until recently had undetectable resistance levels. Overall, given the widespread use of anticoagulants in the ML area, resistance to warfarin, bromadiolone, and coumatetralyl could have developed and easily spread since their initial discovery about a decade ago. The slow increase in difenacoum resistance frequency is compatible with its recessive genetic underpinnings (Greaves and Cullen-Ayres 1988; *cf.* Hartl and Clark 1988, p. 156).

Owing to the recent and intense selection that has dominated our study system, we were able to estimate warfarin and bromadiolone resistance frequencies within 4% or less of the BCR-deduced value simply by using allele frequencies at *D1Rat219*. Moreover, using allele frequencies at *D1Rat219*, we deduced the *in vitro* VKOR activity of case and control groups (Figure 3A) and of field populations of varying resistance levels (Figure 3B). However, in some populations the association between the 254-bp allele and *Rw* was weak (*e.g.*, in populations 21 and 29–32). Moreover, we were unable to determine coumatetralyl and difenacoum resistance frequencies. Therefore, PCR-based diagnosis of resistance in the field merits further development and our approach should be adopted only with caution.

Knowledge of the mode of selection at *Rw* provides insight into the ecological genetics and management of resistant rodent populations (Greaves 1986; Hadler and Buckle 1992). Warfarin resistance has been adopted as a textbook example of overdominant selection (Hartl and Clark 1988; Falconer and Mackay 1996). A high nutritional need for vitamin K constitutes the presumed pleiotropic cost associated with the resistant *Rw/Rw* genotype. In contrast, due to the dominant nature of warfarin resistance, the *+/Rw* genotype is protected against poisoning but may not suffer measurable vitamin K deficiency. Hence, a balanced polymorphism may be maintained. However, while most previously described resistant rat strains suffered from vitamin K deficiency, other strains did not (Smith *et al.* 1993; Thijssen 1995). Hence, an overdominant selection mode at *Rw* cannot be simply assumed and merits investigation within a strain-specific context.

Overdominance, as narrowly defined, exists when the heterozygote has a higher fitness than both homozygotes at all times and across niches (*e.g.*, Hartl and Clark 1988). This narrow definition may not hold for our rat populations. First, we previously found that variation at microsatellite loci linked to *Rw* was drastically reduced (Kohn *et al.* 2000). This observation is difficult to reconcile with theoretical expectations for microsatellites linked to genes under balancing selection (Slatkin 1995; Wiehe 1998). Second, balancing selection should reduce F_{ST} values between populations (Lewontin and Krakauer 1975; Karl and Avise 1992; McDonald 1994). Here we found, to the contrary, that F_{ST} at *D1Rat219* was more pronounced relative to F_{ST} values distributed on other chromosomes (Table 4A). Fourth, we found no consistent support for heterozygote excess at *D1Rat219* within populations (Table 3). However, tests for HWE are not an especially powerful method to detect selection (Hartl and Clark 1988).

Other observations are compatible with overdominant selection models at *Rw*. First, expected heterozygosity (0.43) equaled the observed heterozygosity (0.43) at *D1Rat219*, whereas at the neutral loci we found that the expected heterozygosity (0.60) exceeded the observed heterozygosity (0.54; Table 3). Population substructure ($\theta = 0.133$; Table 4A) conceivably has led to heterozygote deficiency and Wahlund's effect over the genomic background (*i.e.*, Hartl and Clark 1988). Selection could have counteracted this pattern for microsatellite loci linked to *Rw* (Slatkin 1995). Second, within populations we find 13 negative *f* values at *D1Rat219* and only 4 at the genomic background (Table 3), an observation that is compatible with a viability of the *+/Rw* genotype that exceeds the geometric mean of the *Rw/Rw* and +/+ genotypes (Weir 1996). In all, evidence for overdominant selection at *Rw* remains ambiguous in our populations and the mode of selection may depend on the poison used. Alternative models that employ a less narrow definition of overdominance (Dempster 1955) that may explain facets of our variation data should be explored. These may include those that assume a heterogeneous selection regime over space and time and low migration rates (*cf.* Levene 1953; Slatkin and Wiehe 1998; Schmidt and Rand 2001).

Locus-specific population structure at *D1Rat219*: Lev-

els of genetic differentiation at *D1Rat219* should exceed those observed over the genomic background. Whereas the former should be dominated by selection, the latter should be influenced predominantly by drift. We found significant differences in F_{ST} between neutral loci and those linked to *Rw* (Table 4A). Specifically, the mean value of θ for *D1Rat219* was ~1.7 times that for neutral loci (0.232 *vs.* 0.133; Table 4A). Individual θs between populations reached even higher values at *D1Rat219*, up to ~0.8 for populations separated by a mere 37 kilometers (populations 15 and 26; *cf.* supplement 3 at http://www.genetics.org/supplemental/).

To quantify the relative influence that selection had on the distribution of resistance alleles over the spatial scale represented by our study (Figure 1), we assessed patterns of differentiation with distance. For the neutral loci, the amount of variation in θ that was explained by variation in geographical distance was ~40% (Figure 4A), whereas the contribution of ΔRW to values of θ was nonsignificant ($R^2 < 1\%$; Figure 4A). The average value of θ for the neutral loci was 0.133, corresponding to ~1.6 genetically effective migration events per generation under an island model. In contrast, at the resistance marker *D1Rat219*, 67% of variation in θ was explained by variation in our surrogate measure of selection ΔRW (Figure 4B) and only ~21% was explained by variation in geographical distance. Hence, net rates of migration and fixation at *Rw* are determined by the scope and intensity of warfarin application, resulting in substantial population differentiation at *D1Rat219*, a locus linked to *Rw*. Accordingly, our results support the previously formulated notion (Lewontin and Krakauer 1975; Taylor *et al.* 1995) that a comparison of θ between a candidate locus and the genomic background is a valid method for detecting fitness-related genes.

In contrast to the warfarin resistance allele, most genetic polymorphisms in nature appear to be weakly selected (Endler 1986; Conner 2001). Structured populations provide more favorable conditions for polymorphism maintenance (*e.g.*, Karlin 1982; Nagylaki 1992; Slatkin and Wiehe 1998; Nagylaki and Lou 2001). However, the ability to detect loci under selection may be limited if pronounced population structure has caused the genomic background to be highly differentiated (*e.g.*, Rousset 1999). Therefore, to explore a strategy that might reduce the noise caused by within-deme events, we pooled our samples into the resistant and nonresistant categories RW and RW+. When genetic differentiation was analyzed within this case-controlled framework, the signal-to-noise ratio was increased compared to a population-controlled design (Table 4). Specifically, while we found that differentiation over the genomic background between the RW and RW+ groups was low (θ = 0.012), the value of θ at *D1Rat219* was 0.311, which was 26 times higher than that for the neutral loci (Table 4B). Overall, differentiation at *Rw* in the case-controlled design was ~15 (26/1.7) times more pronounced than that obtained from the population-controlled analysis (Table 4).

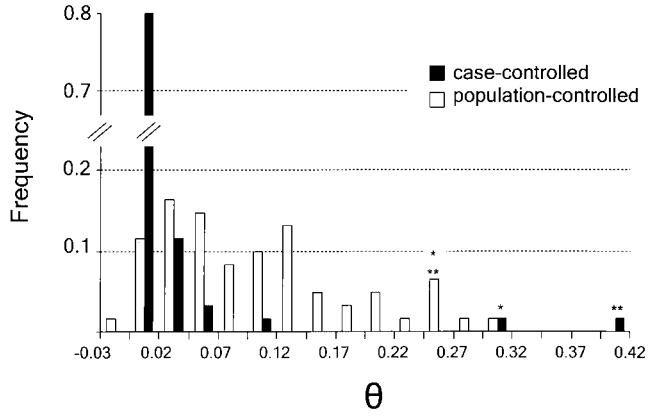

Figure 5.—Distribution of allele-specific θ-values computed for a population-controlled design (open bars) and for a case-controlled design (solid bars). Positions of the 250-bp and 254-bp alleles at *D1Rat219* in the distribution are indicated by * and **, respectively.

Tabulation of all 61 possible allele-specific θ values obtained from the case-controlled and population-controlled analyses further showed that a case-controlled analysis has more effectively reduced background levels of F_{ST} (Figure 5). Specifically, in the case-controlled analysis, only 3 of 61 (4.9%) alleles had θ values >0.1, including the 254-bp allele (θ = 0.404) and the 250-bp allele (0.313) at *D1Rat219* and one allele at *D2Rat31* (0.101). None of the neutral alleles exceeded a θ value of 0.2 (*i.e.*, $N_m \sim 1$). In contrast, in the population-controlled analysis, 29 (47.5%) alleles had θ values >0.1, and 8 (13.1%) alleles exceeded 0.2. Moreover, θ values of the 254-bp allele and the 250-bp allele at *D1Rat219* were lower than those during the case-controlled analysis (θ = 0.243 and 0.246, respectively) and equal to or lower than those of four alleles at the neutral loci *D17Rat38*, *D10Rat6*, and *D2Rat31*. Case-controlled designs may generally assist the mapping of adaptive trait loci, and we suggest that theoretical models analogous to those now used in human disease association studies should be explored (*e.g.*, Pritchard and Donnelly 2001).

Extensive genetic hitchhiking also presents difficulties for gene localization. For some of our anticoagulant selected rat populations, intense selection has resulted in genetic hitchhiking over an extended genomic interval (Kohn *et al.* 2000). We computed the F_{ST} analog θ for the five populations numbered 11, 21, 23, 24, and LH for which we have typed 26 microsatellite loci spanning a 32-cM genomic interval on rat chromosome 1 (Kohn *et al.* 2000). We found a systematic relationship between θ and our surrogate measure for divergent selection ΔRW (Figure 6; Mantel's test; $P < 0.001$; $R^2 = 69\%$). Thus, genetic hitchhiking has attenuated genetic differences between populations far beyond the *Rw* locus,

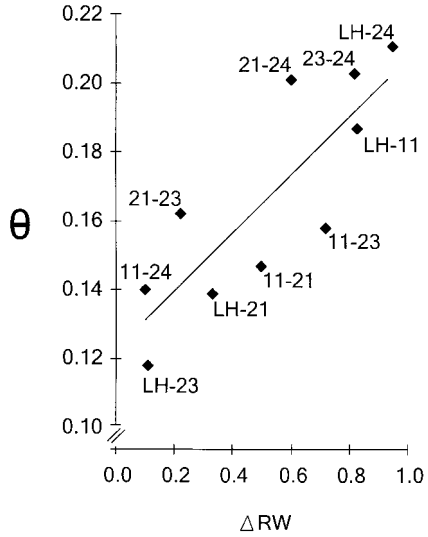

FIGURE 6.—Chromosomal effects of selection on *Rw*. The differences in warfarin resistance frequency (ΔRW) was plotted *vs.* θ computed for a 32-cM interval on rat chromosome 1 between population pairs (24, 23, 21, 11, and LH) having warfarin resistance levels between 0 and 93%. No isolation-by-distance relationship was supported (not shown).

impairing our ability to narrow the genomic location of the gene in strongly selected populations. A range of parameter values—notably low migration and recombination compared to selection, epistasis, and the difference in the timing of fixation between adjacent and divergently selected demes—affects the time window for which such isolation-by-linkage disequilibrium may persist (FELSENSTEIN 1981; SLATKIN and WIEHE 1998). For example, in populations having low and moderate levels of resistance, the genomic interval in linkage disequilibrium was much less than that in highly resistant populations (KOHN *et al.* 2000).

The existence of genetic differentiation over large genomic intervals as a result of selection at a nearby locus suggests that populations may diverge in characters that initially were not a direct target of natural selection. This mechanism might account for components of the phenotypic divergence observed between some populations (RICE and HOSTERT 1993; but see FELSENSTEIN 1981). In fact, such hitchhiking effects led to the initial discovery of linkage between *Rw* and the coat color mutation. In a free-living mouse population from Cambridge, United Kingdom, there was an extraordinarily high frequency of this coat color variant where warfarin selection on the mouse ortholog of *Rw* (*war*) was intense and resistance levels were high (WALLACE and MACSWINEY 1976). We have found previously that as much as ~32 cM (14%) of rat chromosome 1 surrounding the *Rw* locus is in linkage disequilibrium (KOHN *et al.* 2000). Presumably, other traits influenced by genes contained in this region may have diverged as a result of intense selection at *Rw* as well. Inspection of recombination maps and gene annotation data from mouse chromosome 7 (the ortholog to rat chromosome 1) suggested that up to 240 genes and 1360 single nucleotide polymorphic sites (http://www.ensembl.org/Mus_musculus/) potentially might be affected by hitchhiking over this distance.

Conclusions: Our study of the comparative population genetics of neutral and fitness-related markers in rat populations under varying degrees of selection for anticoagulant resistance has led to several consequential findings. First, a small genome interval defined by our study was implicated in the expression of a warfarin-insensitve vitamin K 2,3-epoxide reductase. Second, quantitative genetic analyses were compatible with a model that invokes *Rw* as a major locus that mediates resistance to several anticoagulant poisons, but that varies in penetrance and dominance with respect to the poison used. Third, we documented that allele frequencies at *D1Rat219* were highly predictive for population resistance levels. Fourth, the strong association of *D1Rat219* with resistance was reflected by patterns of variation and differentiation that were clearly dominated by selection. In contrast, background levels of differentiation were dominated by population structure. A case-controlled design reduced background levels of F_{ST}.

Thus far, relatively few studies such as TAKAHASHI *et al.* (2001) have been able to relate population genetic data to a defined and naturally occurring phenotype (LYNCH and WALSH 1998; MCKAY and LOTTA 2002). More commonly, this has been accomplished in systems where selection with pesticides has taken place (CHEVILLON *et al.* 1995; TAYLOR *et al.* 1995; LENORMAND *et al.* 1998). Our study lends further justification to an increasing number of studies that search for beneficial mutations solely on the basis of the statistical signature of selection (see SCHLÖTTERER 2002 and references therein). Moreover, our ongoing study demonstrates that free-living rodent populations, the phenotype for which has been characterized, may provide the raw material for high-resolution gene mapping (JACOB and KWITEK 2002). Specifically, the genetic characterization of *Rw* has important implications for vitamin-K-related medical research and therapy (FEDERMAN *et al.* 2001; LINDER 2001), rodent control (HADLER and BUCKLE 1992), and a fuller characterization of the vitamin K cycle (HILDEBRANDT and SUTTIE 1982).

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