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Evolution of the immunoglobulin heavy chain variable region (*Igh-V*) locus in the genus *Mus*

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Abstract. The evolution of the mouse immunoglobulin heavy chain variable region (*Igh-V*) locus was investigated by the comprehensive analysis of variable region (*Vh*) gene family content and restriction fragment polymorphism in the genus *Mus*. The examination of natural *Mus domesticus* populations suggests an important role for recombination in the generation of the considerable restriction fragment polymorphism found at the *Igh-V* locus. Although the sizes of individual *Vh* gene families vary widely both within and between different *Mus* species, evolutionary trends of *Vh* gene family copy number are revealed by the analysis of homologues of mouse *Vh* gene families in *Rattus* and *Peromyscus*. Processes of duplication, deletion, and sequence divergence all contribute to the evolution of *Vh* gene copy number. Certain *Vh* gene families have expanded or contracted differently in the various muroid lineages examined. Collectively, these findings suggest that the evolution of individual *Vh* family size is not driven by strong selective pressure but is relatively neutral, and that gene flow, rather than selection, serves to maintain the high level of restriction fragment polymorphism seen in *M. domesticus*.

Introduction

In the mouse, the immunoglobulin heavy chain variable region (*Igh-V*) locus encodes 100–200 variable region (*Vh*) genes, which are assembled into complete heavy chain variable region genes by recombination with members of the *Dh* (diversity) and *Jh* (joining) region gene families (reviewed by Alt et al. 1987). *Vh* genes are grouped into at least 12 multigene families whose members cross-hybridize and share greater than 80% identity at the nucleotide level (Brodeur and Riblet 1984, Brodeur et al. 1985, Dildrop 1984, Winter et al. 1985, Kofler 1988, Reininger et al. 1988, Pennell et al. 1989). *Vh* gene families are particularly remarkable for their polymorphism, which includes both variation in restriction fragment number and length, and differences in coding and flanking region sequences (Siekevitz et al. 1982, Clarke et al. 1983, Loh et al. 1983, Brodeur and Riblet 1984, Near et al. 1984, Perlmutter et al. 1985, Kaartinen et al. 1986, Riblet et al. 1986). The *Igh-V* locus has been extensively examined in common inbred strains of mice, but the uncertain origin and possible shared ancestry of these strains makes it difficult to interpret how they represent variation within *Mus domesticus* or other *Mus* species from which they may be derived (Morse 1978, Potter 1978, Ferris et al. 1982). Preliminary investigations into the extent and distribution of polymorphism at the *Igh-V* locus in natural populations of *M. domesticus* and other species referable to the complex genus *Mus* have been made (Hartman et al. 1986, Hilbert and Cancro 1986, Riblet et al. 1986, Blankenstein et al. 1987). However, the nature of the evolutionary mechanisms and forces responsible for the generation and maintenance of *Igh-V* polymorphism in *Mus* are poorly understood.

Our previous analysis of the *Igh-V* locus in inbred strains of mice revealed that characteristics changes in *Vh* gene family copy number are small, restricted to a single *Vh* gene family, and apparently randomly distributed; recombination was identified as a mechanism generating *Vh* gene family polymorphisms (Tutter and Riblet 1988a). Here, we continue our study of the evolution of the mouse *Igh-V* locus with the characterization of *Vh* gene families in various wild populations of *M. domesticus*, other *Mus* species, and the muroid rodents *Rattus norvegicus* and *Peromyscus maniculatus*, using blot hybridization techniques and *Vh* gene family-specific probes.

Abbreviations used in this paper: Igh-V, immunoglobulin heavy chain variable region locus; *Vh,* immunoglobulin heavy chain variable region gene; V_κ, immunoglobulin light chain (kappa type) variable region gene.

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Materials and methods

Animals. Fresh or frozen tissue samples from various *Mus* species were obtained from several different sources, as listed in Table 1. Tissue and/or DNA samples from inbred strains of *R. norvegicus* (listed in the text) were generously provided by Peter Wettstein, Darcy Wilson, Daniel Gold, and Philip Tsichlis. Specimens of *P. maniculatus* (deer mouse) were collected in Jenkintown, Pennsylvania, by Furball Esther, a domestic cat.

Genomic DNA. DNA was prepared from fresh tissue (liver, spleen, or kidney) as described (Tutter and Riblet 1988a); we also used the following method, which gives a good yield of high relative mass DNA from fresh or frozen spleen or liver. Samples were broken into small pieces, Dounce homogenized in 5 ml of saline, and mixed with 15 ml of 50 m*M* Tris, 100 m*M* ethylenediaminetetraacetate (EDTA), 100 m*M* NaCl, 1% sodium dodecyl sulfate (SDS), pH 8. Protease was added to 50 μg/ml followed by overnight incubation at 37 °C. The mixture was extracted once or twice with buffer-saturated phenol, incubated with 20 μg/ml RNAse at 37 °C for 1 h, and extracted once with phenol and once with chloroform. DNA was precipitated by the addition of 2 volumes of 95% ethanol, spooled onto glass rods, air dried, resuspended in 1–2 ml 10 m*M* Tris, 10 m*M* NaCl, 1 m*M* EDTA, pH 7.5, and rocked overnight.

Southern blotting. All methods have been described in detail (Tutter and Riblet 1988a). Briefly, 7 μg of genomic DNA were digested with *Eco* RI, electrophoresed through 0.7% agarose gels, transferred to nitrocellulose or nylon membranes in 20 × standard sodium citrate (SSC), and hybridized in the presence of 10% dextran sulfate, 10 × Denhardt's, 0.1% SDS, and 10^6 cpm/ml radiolabeled probe. Cloned *Vh* gene family-specific DNA probes were as described (Tutter and Riblet 1988a). All blots were washed at a final stringency of 0.2 × SSC, 65 °C. Size markers were *Hind* III digests of lambda phage DNA.

Results

The biological classification, geographical origin, source, and breeding status of all *Mus* isolates analyzed are presented in Table 1, following the classification of Marshall and Sage (1981), Marshall (1986), and Bonhomme (1986). Phylogenetic relationships between the represented *Mus* species are diagrammed in Figure 1. *Eco* RI-digested, Southern-blotted genomic DNA from multiple individuals of each species was hybridized to probes specific for 7 of the 12 identified *Vh* gene families. The two-membered *VhX24* family (Hartman and Rudikoff 1984) has been characterized in the genus *Mus* (Hartman et al. 1986) and was therefore excluded from our analysis; other small *Vh* gene families (*Vgam3-8, Vh10, Vh11, Vh12*) also were not examined. The inbred strain BALB/c was used as a standard *M. domesticus* reference (Potter 1978, Yonekawa et al. 1982) on all blots. Throughout the remainder of this paper, "polymorphism" will refer to restriction fragment length polymorphism, i. e., variation in number and/or length of restriction fragments, unless specifically noted otherwise.

Generation and distribution of Igh-V polymorphism in natural populations of M. domesticus. Eight wild *M. domesticus* mice were trapped during a 2-week period from a house in the Manayunk neighborhood of Philadelphia, Pennsylvania, in order to assess the *Igh-V*

Table 1. Classification, geographic origin, and source of species referable to the genus *Mus*.

Subgenus	Species	Geographic origin	Source*	Status
Mus	*M. caroli*	Chonburi province, Thailand	1	outbred
	M. castaneus	Thailand	2, 3	outbred
	M. cookii	Tak province, Thailand	1	outbred
	M. domesticus	Philadelphia, Pennsylvania, USA	4	wild-trapped
		Philadelphia, Pennsylvania, USA	5	inbred strains
		California, USA	2	outbred
		Antioch, California, USA	6	outbred
		Eastern Shore, Maryland, USA	1	outbred
		Oberlin/Bowling Green, Ohio, USA	5	outbred
	(M. brevirostris)†	Azrou, Morocco	1, 2	outbred
	(M. praetextus)†	Erfoud, Morocco	1	outbred
		Jerusalem, Israel	2	outbred
		Egypt	2	outbred
	M. molossinus	Kyushu, Japan	1	outbred
Mus	*M. musculus*	Brno, Czechoslovakia	2, 3, 6	outbred
		Belgrade, Yugoslavia	2	outbred
		Northern Jutland, Denmark	2	outbred
		Grimso/Tovetorp/Tyresta, Denmark	6	outbred
	M. spicilegus	Debljica/Pancevo, Yugoslavia	2	outbred
	M. spretus	Spain	2	outbred
Coelomys	*M. pahari*	Tak province, Thailand	1	outbred
Pyromys	*M. saxicola*	Mysore, India	1	outbred
Nannomys	*M. minutoides*	Nairobi, Kenya	1	outbred

* Tissues from *Mus* species were provided by: 1, M. Potter; 2, V. Chapman; 3, R. Sage; 4, this laboratory; 5, N. Henderson; 6, A. Wilson.
† *M. brevirostris* and *M. praetextus*, formerly considered distinct subspecies of *M. domesticus*, are now included within *M. domesticus* (see text).

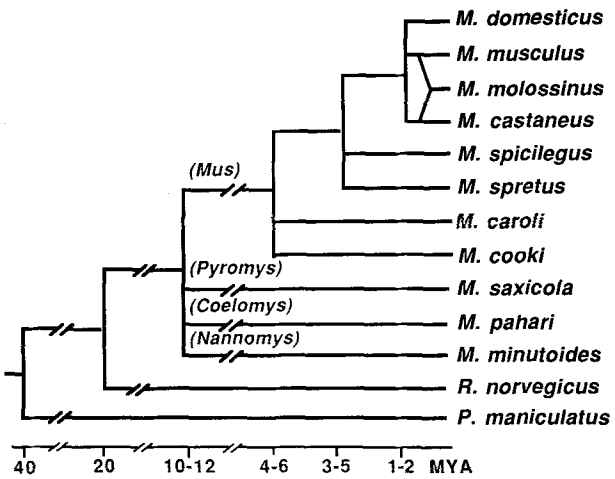


Fig. 1. Phylogenetic tree of the *Mus*, *Rattus*, and *Peromyscus* species included in this study. The four *Mus* subgenera are indicated with *parentheses*. Phylogenetic relationships and divergence times are taken from Yonekawa et al. 1981, 1986; Brownell 1983; Ferris et al. 1983a; Bonhomme et al. 1984; Sarich 1985; E. Prager and V. Sarich, personal communication. *M. molossinus* is diagrammed as a hybrid between *M. musculus* and *M. castaneus* (Yonekawa et al. 1986, 1988). Divergence times are tentative; the *Mus/Rattus* divergence is particularly problematic and is discussed by Sarich (1985). Alternative branching orders are possible at branch points involving more than two taxa. MYA, millions of years ago.

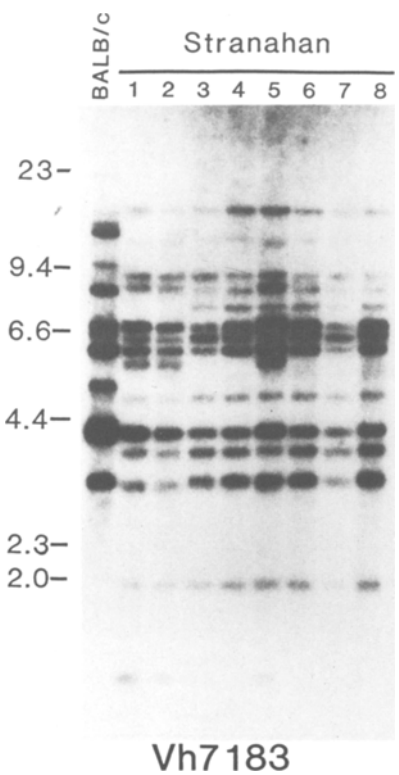


Fig. 2. *Eco* RI Southern blot of the *Vh7183* gene family in eight individual *M. domesticus* ("Stranahan"), trapped from a single house in the Manayunk neigborhood of Philadelphia, Pennsylvania, USA. The inbred strain BALB/c is included for comparison. Size markers in this and in all figures are in kilobases.

polymorphism among the individuals at a single location. These individuals are denoted Stranahan 1–8, and the restriction fragment patterns of their *Vh7183* gene families are shown in Figure 2. At least three distinct *Vh7183* patterns are present in these mice (the pattern of Stranahan 3,7 can be distinguished from that of Stranahan 4, 6, 8 by differences in relative intensity of the upper and lower of the three restriction fragments in the 7–9 kb range, possibly reflecting copy number variation). One individual (Stranahan 5) appears to be a heterozygote, summing the patterns of Stranahan 1, 2 and Stranahan 4, 6, 8. Thus, in a population of eight individuals, only one obvious heterozygote at the *Igh-V* locus is seen; although the sample is small, this contrast with the extremely high level of heterozygosity observed at the *H-2* locus in wild *M. domesticus* (Duncan et al. 1979, Nadeau et al. 1981).

While distinct, the different Stranahan *Vh7183* patterns show only small differences; this is also observed when other *Vh* gene families are examined in these mice (data not shown). The lack of unrelated haplotypes suggests that this population is derived from a small number of individuals. The pattern of slight differences resembles changes seen in inbred strains of mice, which were interpreted to reflect unequal recombination between identical *Vh* gene arrays, (intrahaplotype recombination, Tutter and Riblet 1988a), and suggests that the variation present at the *Igh-V* locus within the sampled Stranahan population resulted from a series of such events.

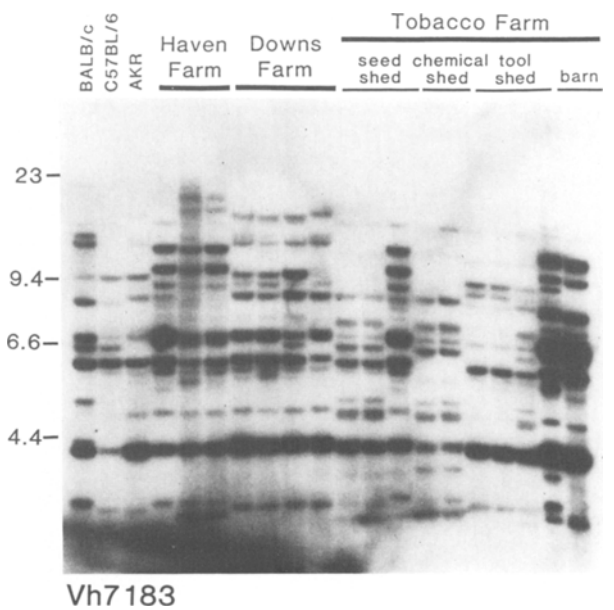
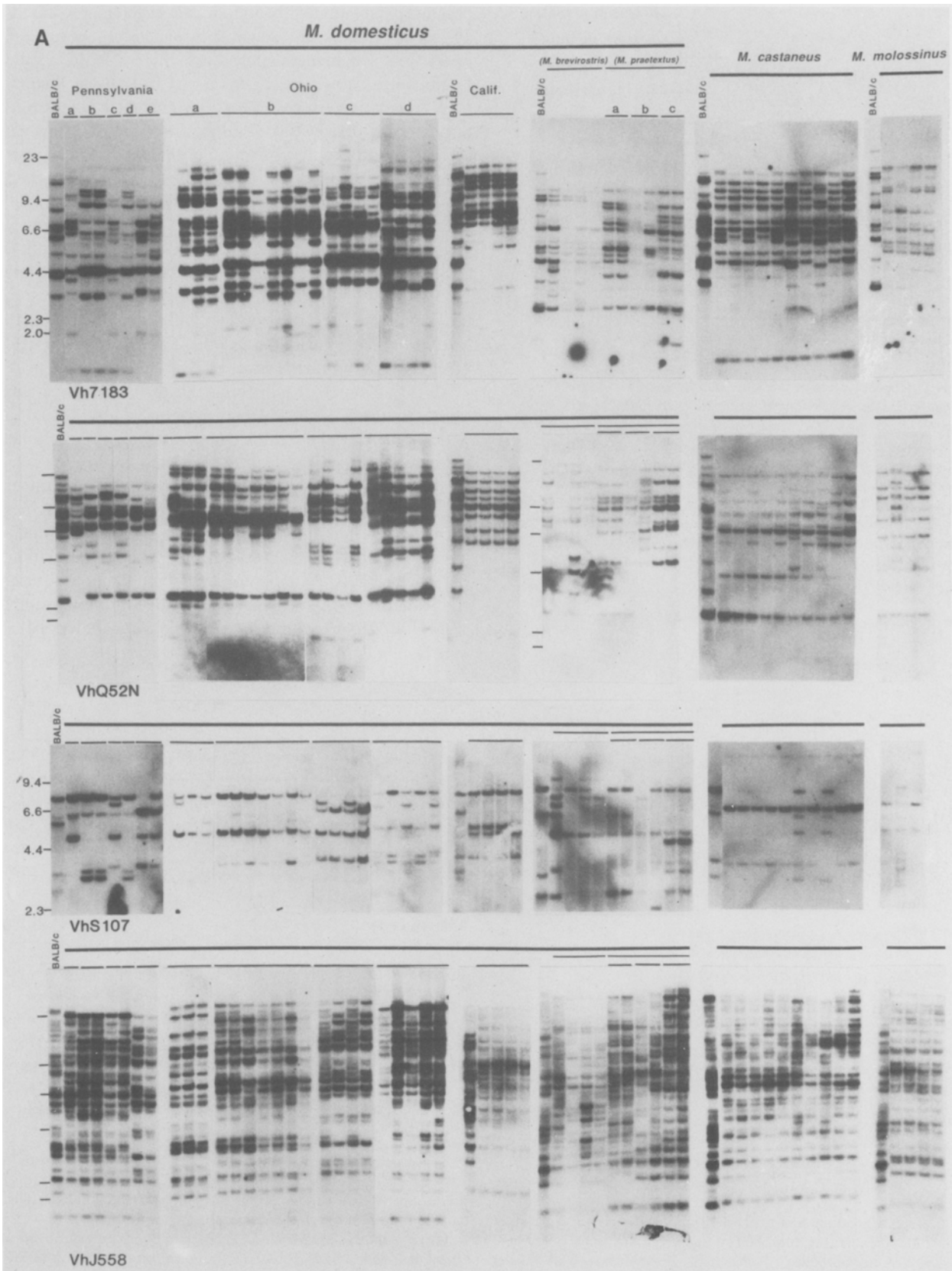
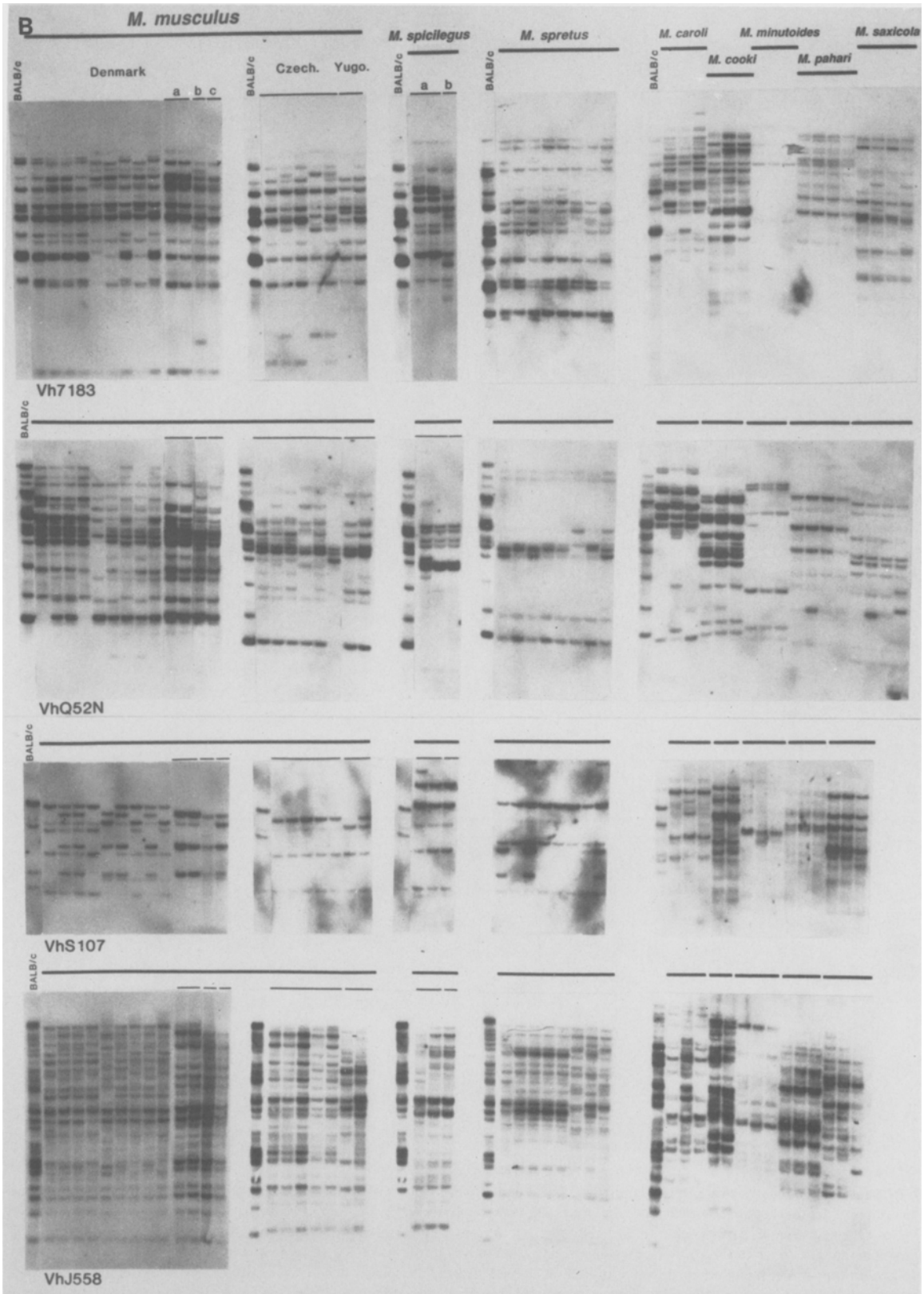


Fig. 3. *Eco* RI Southern blot of the *Vh7183* gene family in individuals outbred from *M. domesticus* trapped from three farms, located within a 100 km radius in Maryland, USA (Eastern Shore mice, maintained by M. Potter; see text). Tobacco Farm mice were trapped from four separate locations, as indicated. The inbred strains BALB/c, C57BL/6, and AKR are included for comparison.


A
M. domesticus
Pennsylvania
Ohio
Calif.
(M. brevirostris)
(M. praetextus)
M. castaneus
M. molossinus
BALB/c
a b c d e
a b c d
a b c
23
9.4
6.6
4.4
2.3
2.0
Vh7183
VhQ52N
9.4
6.6
4.4
2.3
VhS107
VhJ558


B
M. musculus
M. spicilegus
M. spretus
M. caroli
M. minutoides
M. saxicola
M. cooki
M. pahari
Denmark
Czech.
Yugo.
a b c
a b
BALB/c
Vh7183
VhQ52N
VhS107
VhJ558

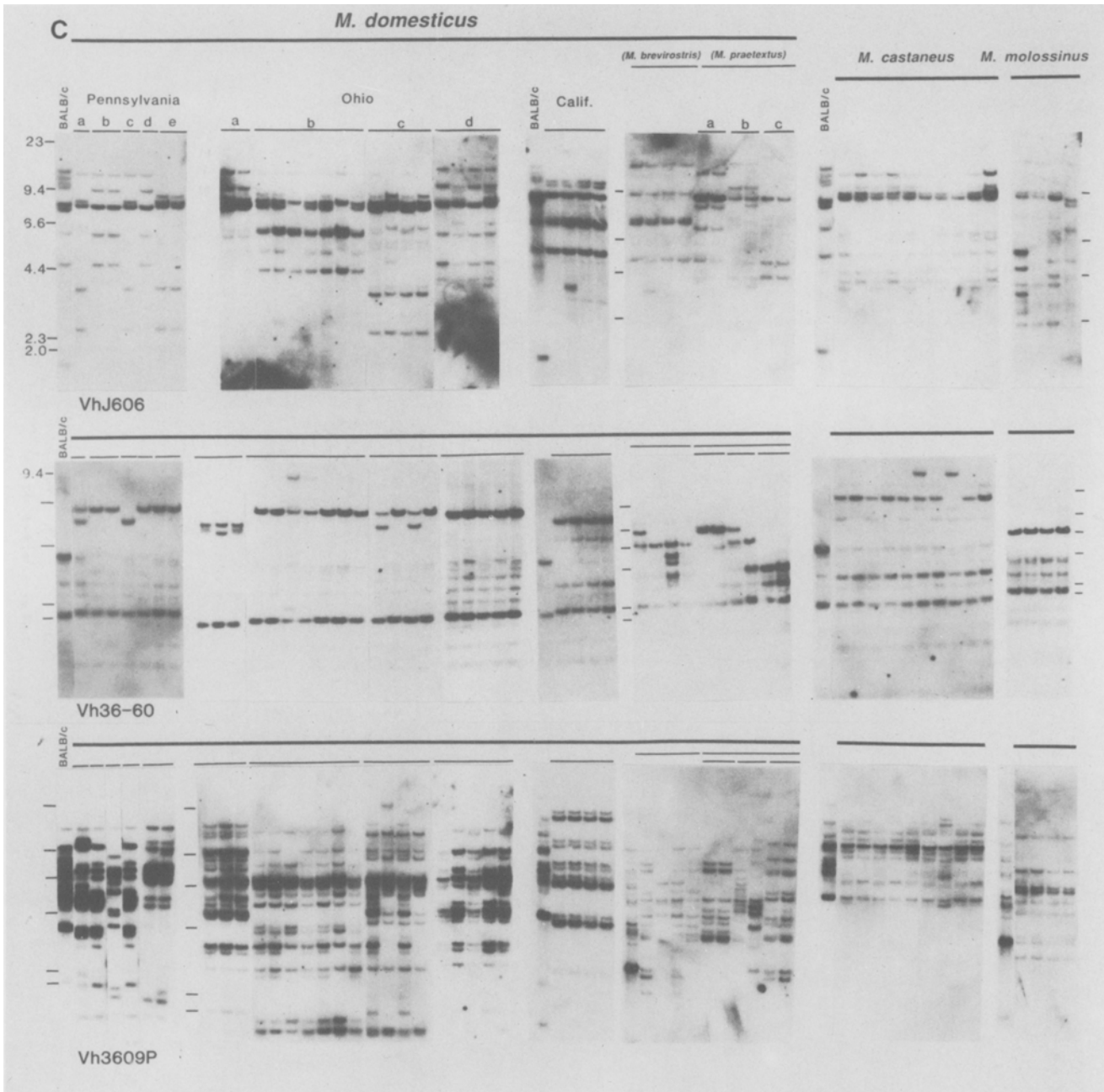
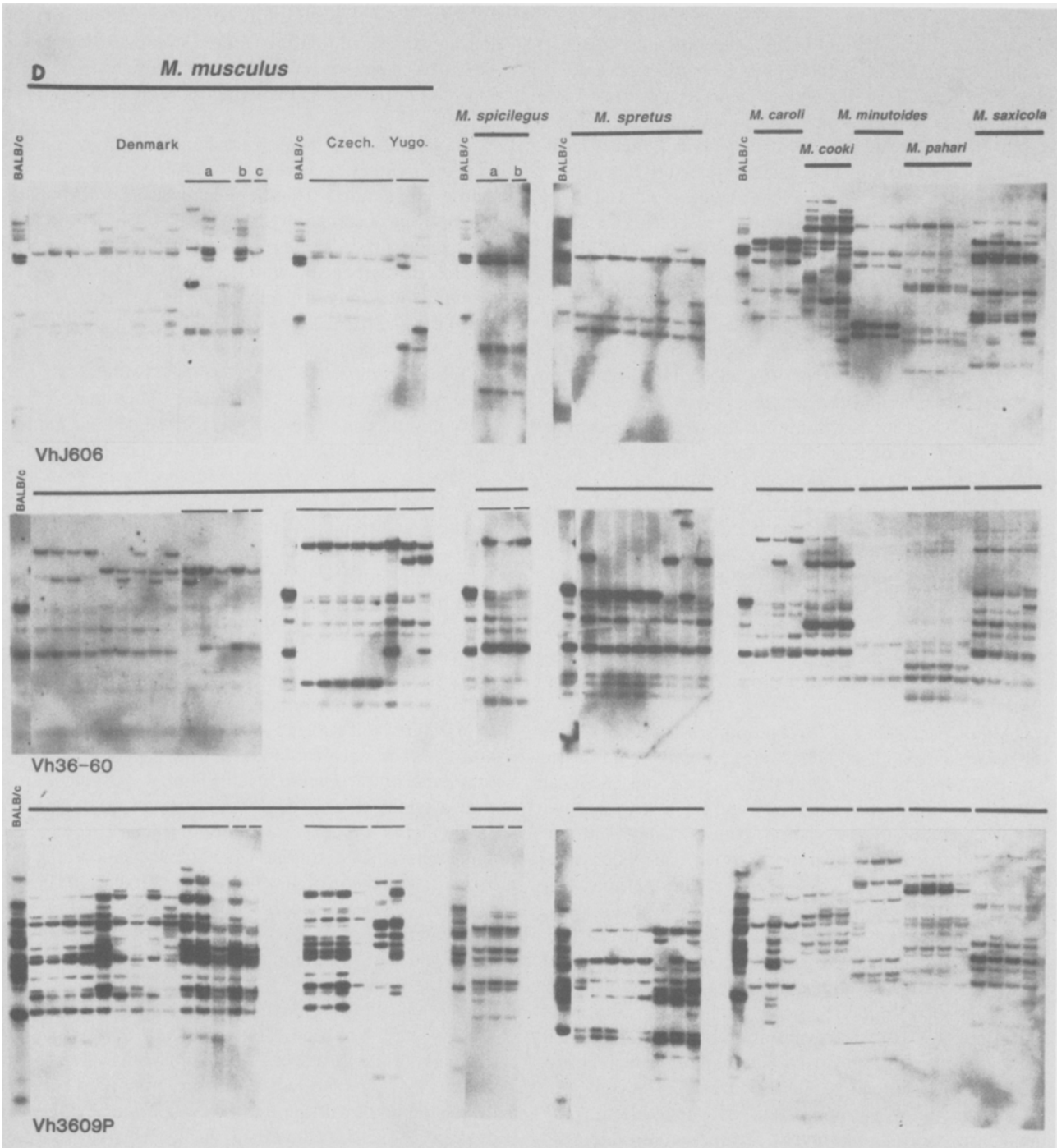


Fig. 4 A–D. *Eco* RI Southern blot analysis of *Vh* gene family content in multiple individuals of species referable to the genus *Mus*. Each row shows a different *Vh* gene family, as indicated *below* each row: the preceding two pages, *Vh7183, VhQ52N, VhS107,* and *VhJ558;* on this and the facing page, *VhJ606, Vh36-60,* and *Vh3609P*. *Mus* species are ordered from *left* to *right* with increasing phylogenetic distance to *M. domesticus* (see Fig. 1). Species designations appear *above* the *top row* in each figure and carry down to the rows *below,* as indicated by the *thick solid lines*. In the same manner, *thin solid lines* group members of different inbred or outbred strains, or isolates of the same species having different geographical origins. The inbred strain BALB/c is included in most blots as an internal reference, also shown *above* the *top row* only. Size markers are generally shown only for the first blot from the *left* in each row; where a blot does not include a BALB/c lane, the size markers of the blot to its *left* apply, unless size markers are shown for that blot. *M. domesticus,* Pennsylvania: inbred strains derived from mice trapped in a spaghetti factory in Philadelphia, Pennsylvania, USA (Connor 1975). a, b, c, d: inbred "Connor" strains PAA, PAB, PAC, and PAE, respectively, maintained by N. Henderson;



c: partly inbred strain (gen. 6) PAF. The PAB and PAD strains share identical patterns in all *Vh* gene families. *M. domesticus*, Ohio: outbred lines derived from mice trapped in Ohio, USA. a: line BG, derived from mice trapped in Bowling Green; b, c, d: lines OW, PW, and WRL, respectively, derived from mice trapped near Oberlin College. Included under *M. domesticus* are several outbred *M. brevirostris* and *M. praetextus* individuals, once considered subspecies of *M. domesticus* (see text), also indicated with *thin solid lines*. a, b, c: *M. praetextus* bred from isolates trapped in Jerusalem, Egypt, and Morocco, respectively. *M. musculus*, Denmark: outbred from isolates trapped in Northern Jutland, except for a, b, c, which are derived from *M. musculus* isolates with introgressions of *M. domesticus*-type mitochondrial DNA (Ferris et al. 1983b), trapped in Grismo, Tovetorp, and Tyresta, respectively. *M. spicilegus:* mice outbred from isolates trapped in Yugoslavia. a, b: Deblijca and Pancevo, respectively.

The extent and distribution of *Igh-V* polymorphism in a small geographic area was investigated with a panel of *M. domesticus* mice bred from individuals trapped from a variety of locations and breeding populations (demes) within a large tobacco farm (TF) near the Chesapeake Bay, Maryland, from the Haven farm (HF) 20 km away, and from the Downs farm (DF), located across the bay, about 100 km away (D'Hoostelaere and Potter 1986). These populations are collectively referred to here as the Eastern Shore mice. These mice have been outbred in an attempt to preserve polymorphisms present in the original isolate but can yield only a minimum estimate of the diversity present in the sampled population.

The *Vh7183* patterns of the Eastern Shore mice are shown in Figure 3, along with those of several inbred strains. Again, these data are representative of results obtained with probes for other *Vh* gene families (data not shown). It is clear that considerable polymorphism is present at the *Igh-V* locus within the sampled area, and even within the limits of the tobacco farm. Polymorphism is evident within all but one deme (TF chemical shed): three patterns are observed in three individuals from HF, four in the four DF individuals, two in the three TF seed shed individuals, two in the three TF tool shed individuals, and two in the two TF barn individuals. In fact, the variation among the TF demes is comparable to that observed between the three different farms, in keeping with studies showing that biochemical variation within farms approaches that found between farms (reviewed by Sage 1981).

Although certain *Vh7183* patterns present in neighboring demes appear closely related (e. g., note the similarity between those in mice from the TF seed and chemical sheds) and the TF seed shed mice and HF mice are found to have one pattern in common, patterns found within a deme almost always appear more similar than patterns between demes (Fig. 3). These observations are consistent not only with those of Hartman and co-workers (1986), who found different *VhX24* patterns between the various tobacco farm demes, but also with studies of biochemical variation and histocompatibility, in which similar genotypes were found in mice within demes (Duncan et al. 1979, Sage 1981). The closely related patterns found within demes are suggestive of intrahaplotype recombination, as discussed above for the Stranahan mice.

Evidence for recombination between different *Vh* gene arrays (interhaplotype recombination) in wild populations of *M. domesticus* can be found in inbred strains derived from mice trapped in a spaghetti factory in Pennsylvania (Fig. 4: *M. domesticus,* Pennsylvania). For example, in the *VhJ606* family, inbred strains *a* and *c* exhibit a pattern that appears recombinant between that of the *e* strain and that of the *b* and *d* strains. In the *Vh36-60* family, the *a* strain pattern appears recombinant between the pattern of the *c* strain, and either that of the *e* strain or that of the *b* and *d* strains. It is difficult to derive gene orders from the recombinant patterns in different *Vh* gene families, as we cannot identify parental-recombinant relationships in the wild-derived inbred strains. Furthermore, the observed patterns may well result from the accumulation of multiple events involving more than two original haplotypes.

In summary, both intra- and interhaplotype recombination appear to generate new *Igh-V* polymorphism in natural populations. These mechanisms may in part account for the observation that many different inbred, outbred, and wild *M. domesticus Vh* patterns have several restriction fragments in common (Figs. 2–4). For example, note the number of restriction fragments shared between the *Vh7183* patterns of the DF mice and the inbred strain AKR (Fig. 3).

When comparing the *Vh7183* patterns of *M. domesticus* trapped in California, Maryland, Pennsylvania, and Ohio (Figs. 2–4), the greatest differences between patterns present in a restricted locality appear as great as those seen between patterns present in isolates from distant sites. This, as well as the above-mentioned finding that variation at the *Igh-V* locus between demes within a farm approximates that between farms, indicates that there is little or no micro-geographic structuring of *Igh-V* polymorphism in *M. domesticus.*

Generally, the complexities (number of restriction fragments) of individual *Vh* gene families observed in the various geographical isolates of *M. domesticus* are similar to those observed in laboratory strains (Brodeur and Riblet 1984, Tutter and Riblet 1988a). The *Vh* gene families of several *M. brevirostris* and *M. praetextus* are included with those of *M. domesticus* in Figure 4. Although these were originally classified as subspecies of *M. domesticus* on the basis of coat color, more recent protein and mitochondrial DNA studies have failed to support the taxonomic subdivision of *M. domesticus* (Ferris et al. 1983a, Marshall 1986). In keeping with these reports, all *Vh* gene family patterns of *M. brevirostris* and *M. praetextus* fall within the range of complexities seen in *M. domesticus.*

Content and polymorphism of Vh gene families in other Mus species. *Vh* gene family patterns of multiple individuals of the *Mus* species listed in Table 1 are shown in Figure 4. The various species are ordered in columns from left to right with approximate respect to phylogenetic distance from *M. domesticus* (Fig. 1). All analyzed species contain a complement of each *Vh* gene family examined, hybridizing with intensities comparable to that of a BALB/c control.

When comparing the *Vh* gene family patterns of different *Mus* species, polymorphism appears more limited in the smaller *Vh* gene families. For example, restriction fragments or combinations of restriction fragments in the *VhS107, Vh36-60,* and *VhJ606* gene families are frequent-

ly shared between members of different species (Fig. 4); note particularly the similarity of *Vh36-60* patterns, both within and between species of the *Mus* subgenus (Table 1, Fig. 4). Likewise, Hartman and co-workers (1986) report identical patterns in the small *VhX24* gene family in certain *M. musculus* and *M. spretus* individuals. Varying levels of polymorphism in *Vh* gene families of different sizes might be expected if there has been no selection for restriction fragment polymorphism, since a smaller *Vh* gene family would have presented fewer opportunities for recombination or mutational events generating new polymorphisms. Consistent with this is the lack of observed duplications or deletions in the smaller *Vh* gene families in inbred strains of mice (Tutter and Riblet 1988a). However, even in the larger *Vh* gene families in which many different and unique patterns are observed, certain common bands are also seen. For example, a similar low relative mass *Vh7183* band is frequently seen in individual *M. domesticus, M. musculus,* and *M. castaneus* (Fig. 4). Also, certain V_κ families share many comigrating bands in individuals of different *Mus* species (Huppi et al. 1985).

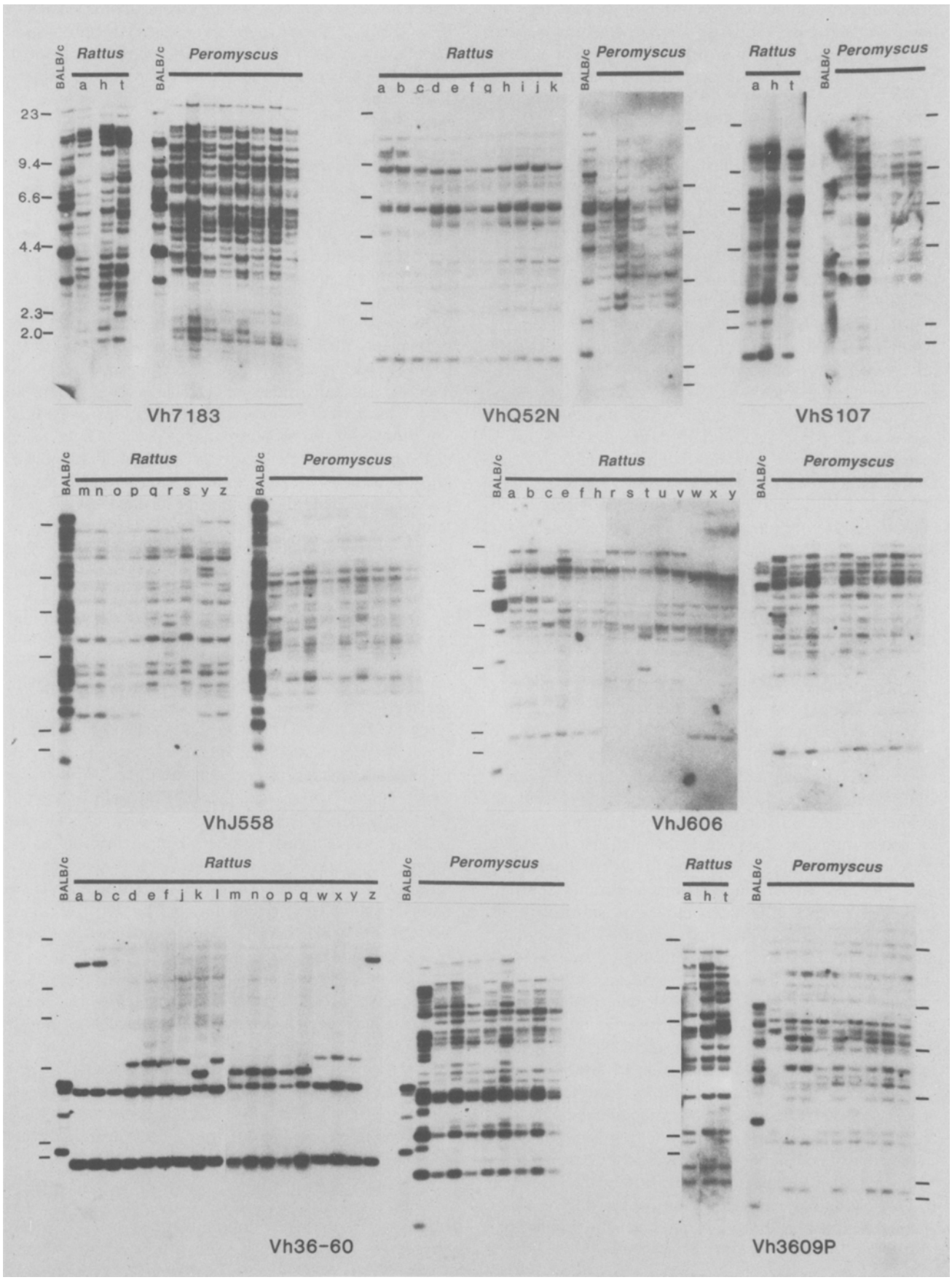
Because only few individuals from relatively small outbred colonies of most *Mus* species were available for analysis, we cannot assess the extent and distribution of *Igh-V* polymorphism within these species; however, in all species examined, polymorphism is observed in almost every *Vh* gene family (Fig. 4). Considerable *Igh-V* variation is present within the sampled *M. musculus* (Fig. 4), yet although *M. musculus* and *M. domesticus* have been separated for over 1 million years (Sage et al. 1986), they do not exhibit "species-specific" *Vh* gene family patterns. In fact, patterns found in isolates of these two species are sometimes quite similar, as illustrated by the comparison of the *Vh7183* patterns of BALB/c and certain individual Danish *M. musculus* (Fig. 4). In a more striking example of interspecific similarity, the *Vh36-60* pattern of several *M. spretus* individuals appears identical to that of BALB/c (Fig. 4). The occasional sharing of *Igh-V* haplotypes across species barriers suggests that a portion of current *Igh-V* polymorphism predates the speciation of *Mus,* as Klein and Figueroa (1986) have suggested for polymorphism at the *H-2* complex of the mouse. However, it should be remembered that while sizes and intensities of restriction fragments in Southern blot patterns may appear identical, coding and flanking sequences of individual *Vh* genes present on these restriction fragments are expected to have diverged after the separation of these species.

The analysis of *Vh* gene families in different *Mus* species reveals a large degree of variation in both the size and number of hybridizing restriction fragments, and thus in the relative contribution of individual *Vh* gene families to the *Igh-V* locus (Figs. 1–4). Interestingly, the difference in *Vh* gene family complexity observed in different *Mus* species is often correlated with phylogenetic distance between these species. For example, compare the small size of the *VhQ52N* family in *M. spicilegus* and the increased complexity of the *VhS107* family in *M. cookii,* relative to *M. domesticus,* with the similar sizes of these *Vh* gene families in the more closely related *M. domesticus, M. musculus,* and *M. castaneus* (Fig. 4).

Content of mouse Vh gene family homologous in P. maniculatus and R. norvegicus. In order to determine whether further variation in *Vh* gene family content exists in other muroid rodents, inbred strains of *R. norvegicus* (family Muridae) and individual wild-caught *P. maniculatus* (family Cricetidae) were surveyed with the panel of mouse *Vh* gene family probes used in our analysis of the genus *Mus.* These species diverged from *Mus* approximately 20 and 40 million years ago, respectively (Fig. 1). As shown in Figure 5, both species retain sequences which hybridize under stringent conditions to probes for each of the mouse *Vh* gene families examined. The number of restriction fragments detected by each *Vh* gene family probe often varies greatly from that found in *Mus,* while other *Vh* gene families remain similar in size. For example, note the complexity of the *Vh36-60* gene family in *P. maniculatus* and the *Vh7183* gene family in both *R. norvegicus* and *P. maniculatus,* which makes clear that individual *Vh* gene families have expanded and contracted differently in the three muroid lineages examined.

While probes for most mouse *Vh* gene families hybridize to *R. norvegicus* and *P. maniculatus* with intensities comparable to that of the BALB/c control, a probe for the *VhJ558* family yields weaker hybridization in both species (Fig. 5; note the overexposure of the BALB/c lane in the *VhJ558* blots). This indicates that the *VhJ558* family may be less conserved than others in muroid lineages. It is unclear whether probes for *Rattus* or *Peromyscus VhJ558* homologues would detect additional members of this family in these species; however, essentially the same patterns are seen when Southern blots hybridized to the mouse *VhJ558* probe are washed under less stringent conditions (3 × SSC; data not shown). Also of interest is the complex, faint background detected by *Vh36-60* in *R. norvegicus,* which may represent sequences that have diverged from this *Vh* gene family (Fig. 5).

In summary, the analysis of mouse *Vh* gene family homologues in *R. norvegicus* and *P. maniculatus* indicates that the complement of these homologues is highly variable between these species and *Mus,* exceeding the variation seen within *Mus.* In addition, most *Vh* gene families appear well conserved across these lineages, while the *VhJ558* family, and likely some *Vh36-60* members in *R. norvegicus,* have undergone significant sequence divergence.


BALB/c
Rattus
a h t
BALB/c
Peromyscus
23
9.4
6.6
4.4
2.3
2.0
Vh7183
Rattus
a b c d e f g h i j k
BALB/c
Peromyscus
VhQ52N
Rattus
a h t
BALB/c
Peromyscus
VhS107
BALB/c
Rattus
m n o p q r s y z
BALB/c
Peromyscus
VhJ558
BALB/c
Rattus
a b c e f h r s t u v w x y
BALB/c
Peromyscus
VhJ606
BALB/c
Rattus
a b c d e f j k l m n o p q w x y z
BALB/c
Peromyscus
Vh36-60
Rattus
a h t
BALB/c
Peromyscus
Vh3609P

Discussion

Evolution of Vh gene family restriction fragment polymorphism. The analysis of the multigene families comprising the *Igh-V* locus in the genus *Mus* has revealed a strikingly large degree of restriction fragment polymorphism. In several cases, putative *Vh* alleles from different mouse strains were shown to have sequence differences in the *Vh* coding regions, as well as the difference in flanking regions creating the restriction fragment length polymorphism (Clarke et al. 1983, Loh et al. 1983, Near et al. 1984, Perlmutter et al. 1985, Kaartinen et al. 1986). Thus, restriction fragment polymorphism most likely reflects sequence divergence throughout the fragments.

We have shown that recombination appears to be an important source of new *Igh-V* polymorphisms in natural populations of *M. domesticus*. The potential role of recombination in the generation of the high restriction fragment length polymorphism at the *Igh-V* locus is underscored by the lack of observed mutations changing restriction fragment length in inbred strains of mice (Tutter and Riblet 1988a). As shown in Figures 2–5, when a *Vh* gene family is examined in different *Mus* individuals and species, very different patterns of restriction fragments are observed; yet at the same time, many shared fragments are evident. Although the identity of such bands is not proven, their frequency argues against chance coincidence. In general, we presume that fragments of identical length and similar intensity in different haplotypes are more closely related than fragments of different length. The sharing of similar restriction fragments between different wild and inbred *M. domesticus Igh-V* haplotypes, and between individuals of different *Mus* species, further supports the view that recombinational processes serve to shuffle and reassort a discrete number of restriction fragments into a greater number of haplotypes.

Another important issue concerns the forces maintaining polymorphism at the *Igh-V* locus. The wealth of local variation at this locus [e. g., at least six distinct patterns in the TF mice (Fig. 3), and at least five distinct patterns in the Pennsylvania factory mice (Fig. 4)] could result from one of several mechanisms serving to counter the tendency of local inbreeding to reduce levels of polymorphism. Strong selection for polymorphism at the *Igh-V* locus is appealing; extensive diversity of antibody specificities is of obvious importance for survival. However, the diversity seen in *Mus* is not universal. Little polymorphism has been observed in those *Vh* gene families examined in man and in the grey squirrel, *Sciurus carolinensis* (unpublished observations; see also Johnson et al. 1984, Van Dijk et al. 1989). In contrast, evidence for selection for polymorphism at the *H-2* locus in the mouse has been paralleled by similar evidence in man (discussed in Nadeau et al. 1988). This complex locus is also of vital importance in immune recognition of foreign antigens; a crucial difference may be in the low degree of functional redundancy at *H-2* , as compared to *Igh-V* (see below).

High levels of *Igh-V* polymorphism could also result from the rapid generation of new haplotypes by frequent recombination. Recombination in the *Igh-V* locus in *Mus* is readily observable, both in laboratory crosses and in the wild, but the frequency (approximately 1%) is consistent with the physical size of the locus (1–2 million base pairs) and not with enhanced recombination (Brodeur et al. 1985, unpublished observations). This frequency does not appear large enough to affect the maintenance of polymorphism and, indeed, has not done so in the human *Igh-V* locus which is of similar genetic size (Johnson et al. 1984).

A third and more likely possibility is that in *Mus*, migration between populations is responsible for the maintenance of *Igh-V* polymorphism. The gene flow attending migration appears to be increased in commensal species, in which movement of individuals with humans is common; dispersal between populations of *M. domesticus* may yield a very large effective population, which would help maintain diversity by retarding genetic drift. Berry (1986) has stressed the importance of dispersal in the population dynamics of *M. domesticus*. Studies of biochemical variation at many different loci have revealed less variability in aboriginal *Mus* species (which are not commonly dispersed by interaction with human society); estimates of individual heterozygosity are less than half that found in commensal species (reviewed by Sage 1981). Consistent with these observations, in the small sample of aboriginal *M. spicilegus* analyzed here, identical patterns are shared in most *Vh* gene families in isolates trapped near two different Yugoslavian towns (Fig. 4). The analysis of a larger sample of wild trapped commensal and aboriginal *Mus* species will be necessary to determine the degree to which gene flow may contribute

◀ **Fig. 5.** *Eco* RI Southern blot analysis of the content of *Vh* gene family homologues in inbred strains of *R. norvegicus* and wild-caught *P. maniculatus*. BALB/c lanes are included within most blots as an internal reference; size markers are shown when a blot lacks a BALB/c lane. Other experiments show that probes for *VhS107, VhQ52N,* and *Vh3609P* hybridize to *R. norvegicus* with intensity comparable to BALB/c (not shown). Not all inbred strains of *R. norvegicus* were examined with every *Vh* gene family. Blots were washed at high stringency (0.2 × SSC, 65 °C). Inbred strains of *R. norvegicus* are as follows: a, SHR/Dw; b, OKA/Dw; c, MR/N; d, ALB/N; e, RCS/N; f, SD; g, BUF/Wi; h, LEW/Wi; i, M520/N; j, F344/Wi; k, YOS/Dw; I, PETH/N; m, CAR/N; n, AUG/Dw; o, A28807/N; p, ACP; q, WF/Wi; r, Black hooded (BH/Dw); s, NSD/N; t, DA/Wi; u, ACI/N; v, BN/Wi; w, Osborne Mendel (OM) #4; x, OM#6; y, OM#8; z, WKA/Dw. A compilation of *Igh-V* haplotypes defined by strains of *R. norvegicus* will be provided elsewhere.

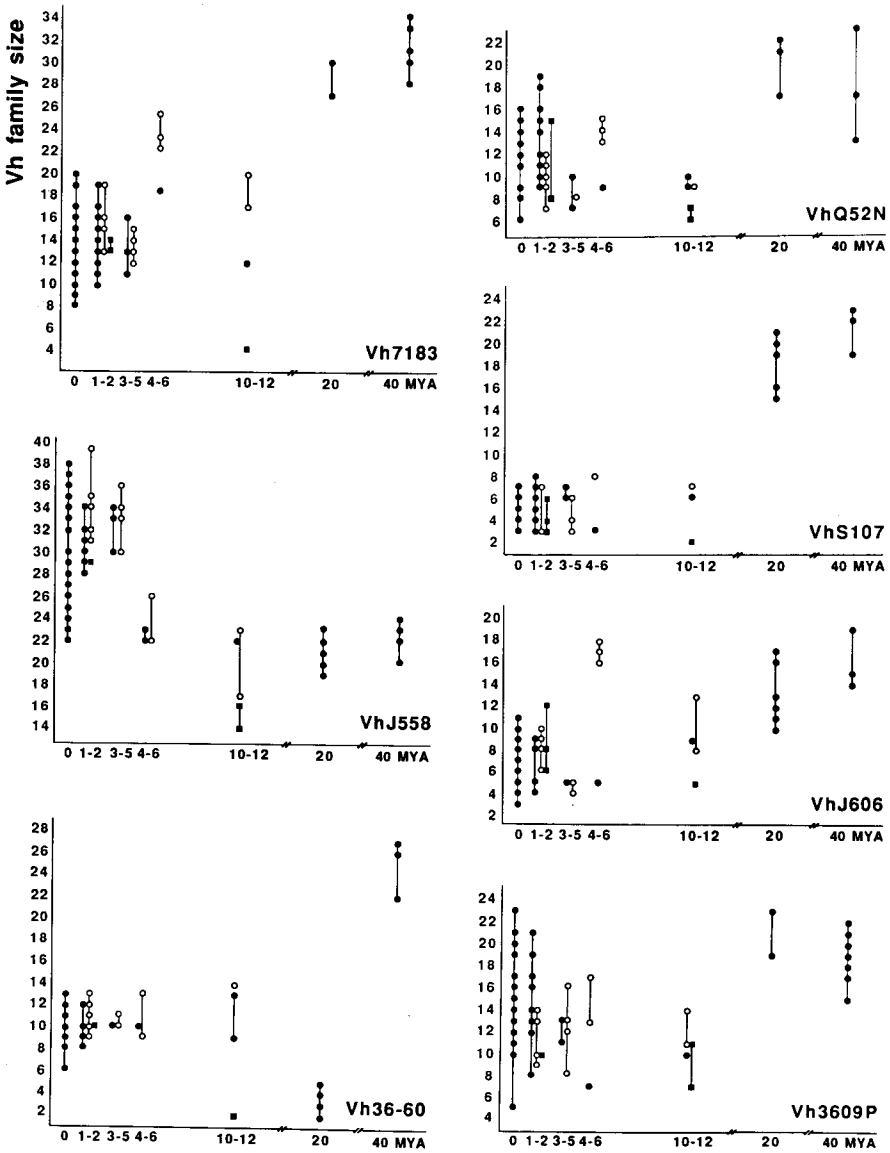


Fig. 6. Association of the range of individual *Vh* gene family complexity in *Mus* species, *R. norvegicus*, and *P. maniculatus* (y-axis), with phylogenetic distance of these species from *M. domesticus* (x-axis). MYA, millions of years ago. 0 MYA: *M. domesticus*. 1–2 MYA: *M. musculus (closed circles), M. castaneus (open circles), M. molossinus (closed squares);* 3–5 MYA: *M. spicilegus (closed circles), M. spretus (open circles);* 4–6 MYA: *M. caroli (closed circles), M. cookii (open circles);* 10–12 MYA: *M. pahari (closed circles), M. saxicola (open circles), M. minutoides (closed squares);* 20 MYA: *R. norvegicus;* 40 MYA: *P. maniculatus*. References for divergence times are given in the legend to Figure 1. The ranges of *Vh* gene family complexity were determined by scoring the number of hybridizing restriction fragments in each *Vh* gene family of every individual shown in Figure 4 and 5, excepting several apparent heterozygotes. For *M. domesticus*, this analysis also includes restriction fragment patterns of inbred strains (Brodeur and Riblet 1984, Tutter and Riblet 1988a) as well as other wild and outbred individuals (A. Tutter, unpublished observations). In cases such as the *Vh36-60* family *R. norvegicus*, where no continuum exists between strongly and weakly hybridizing bands, the latter were excluded. Note that to avoid weighting of the data by the sampling of primarily related individuals from small colonies, we do not distinguish between single and multiple observations of the same complexity; thus, some points represent more than one individual. Replicate experiments and multiple exposures (not shown) were often used to ensure the inclusion of as many separate restriction fragments as possible.

to the maintenance of *Igh-V* polymorphism in commensal *Mus* species.

Evolutionary trends of Vh gene family copy number. In order to identify evolutionary trends of *Vh* gene family size in *Mus, Rattus,* and *Peromyscus,* we determined the size of each *Vh* gene family, as reflected by the number of hybridizing restriction fragments (Brodeur and Riblet 1984), in each isolate of each species shown in Figures 2–5. Implicit in this analysis is the assumption that for each *Vh* gene family, the number of hybridizing restriction fragments is similarly correlated to *Vh* copy number in all species examined. In general, restriction fragments carry only a single *Vh* gene (Crews et al. 1981, Near et al. 1984, unpublished observations); a few instances have been observed where a single band reflects the superposition of several hybridizing fragments of the same size (Siekevitz et al. 1983) or two *Vh* genes on a single fragment (Yancopolous et al. 1984, Wang and Calame 1985). Short of extensive cloning analysis in each species, the Southern blot approach offers the best way to compare *Vh* gene complements in multiple individuals from multiple species. Where *Vh* genes have been cloned from several *Mus* species, copy number per restriction fragment has not varied (A. Hartman, personal communication). Therefore, while some variation is anticipated, we do not believe this potential source of error has obscured any significant evolutionary trends of copy number.

For each *Vh* gene family examined, the range of size observed in a given species was plotted with respect to the time of the divergence of that species from *M. domesticus* (Fig. 6). These data represent the sizes of *Vh*

gene families in contemporary species, the termini of the phylogenetic tree in Figure 1. If one assumes that the most likely evolutionary pathway involves the smallest amount of change, then the sizes of the different *Vh* gene families in the ancestral species at the branchpoints of the phylogenetic tree can be inferred. Although this simple analysis cannot identify a uniform trend of expansion or contraction in all contemporary species, a change local to one segment of the tree should be evident. For example, the *Vh7183* and *VhS107* gene families of most *Mus* species appear to have contracted since the divergence of *Mus* and *R. norvegicus,* about 20 million years ago. The *VhJ606* plot may reflect a slight contraction in the *Mus* lineages, with independent expansion in the *M. spretus* lineage. On the other hand, the simplest interpretation of the *VhJ558* plot is that net expansion, possibly a large duplication, of this family has occurred in the lineages comprising the *Mus* subgenus (Fig. 1), within 10 to 12 million years ago. This last result differs from the conclusions of Blankenstein and co-workers (1987), who propose independent expansions of the *VhJ558* gene family in *M. domesticus* and *M. spretus,* the former a two- to fourfold expansion within the past million years. Their conclusions were based on the size of the *VhJ558* gene family in one or a few individuals of the species included in their study and thus did not take into account the variation in *VhJ558* size observable within species.

In the *Vh3609P* and *VhQ52N* gene families, no copy number trend is evident; these *Vh* gene families appear to have diverged randomly in each lineage from an ancestral size range (Fig. 6). The interpretation of the evolutionary history of the *Vh36-60* gene family is more difficult; this *Vh* gene family may have undergone contraction in the *Mus* and *Rattus* lineages, or alternatively, unique expansion in the *Peromyscus* lineage. If the faint bands seen in the *Vh36-60* family in *R. norvegicus* (Fig. 5) are indicative of sequences which have diverged from a larger, ancestral *Vh36-60* family, then the former history is likely. This is supported by the complex pattern of bands detected by *Vh36-60* in *M. domesticus* under relaxed stringency; these bands do not appear to result from cross-hybridization with characterized *Vh* gene families (data not shown). Thus, contraction of a *Vh* gene family may result from the divergence of a subset of its members, as well as from their deletion. Although not obvious from Figure 5, approximately one third of the restriction fragments detected by probes for the *Vh7183, VhJ606,* and *VhS107* gene families in *Rattus* actually cross-hybridizes with all three families, indicating that divergence, rather than deletion, may account for most of the apparent contraction experienced by these families in *Mus* (Tutter and Riblet 1988b). Therefore, the total number of unique restriction fragments comprising the *Vh7183, VhJ606,* and *VhS107* gene families in *Mus, Rattus*, and *Peromyscus* is actually more similar than would appear from the plots of these families in Figure 6, and the largest differences in *Vh* gene content in these genera are due to variation in the *VhJ558* and *Vh36-60* gene families.

The wide range of *Vh* gene family complexity within *M. domesticus,* as much as three- to fourfold in certain *Vh* gene families (e. g., *Vh3609P* and *Vh7183,* Fig. 6), is more easily reconciled with a model of *Vh* gene family copy number evolution which is primarily stochastic, rather than selective. This is consistent with the apparently random distribution of *Vh* copy number variation in inbred strains (Tutter and Riblet 1988a). If *Vh* gene family size were selectively maintained, less variation would be expected within a species; yet as shown in Figure 6, as more individuals are sampled, the observed range of *Vh* family size is broadened, suggesting that in *Mus, Vh* family size is even more variable than is evident from this study. A primarily stochastic model of *Vh* gene family copy number evolution is also consistent with the finding that individual *Vh* gene families have expanded or contracted differently in *Mus, Rattus,* and *Peromyscus.* An important extension of such a model is that particularly large *Vh* gene families, such as *VhJ558* in *M. domesticus* or *Vh36-60* in *P. maniculatus,* are not the result of positively selected expansion. Huppi and co-workers (1985) found the $V_\kappa 19$ family to be expanded in *R. norvegicus* with respect to *Mus* and the $V_\kappa 21$ family contracted, suggesting that the evolution of copy number in V_κ families may proceed in a fashion similar to that in *Vh* gene families.

Although the size of individual *Vh* gene families varies between species, the total number of *Vh* genes in *Mus, Rattus,* and *Peromyscus* appears similar. Indeed, since the germline *Vh* genes comprising the *Igh-V* locus contribute considerable diversity to the immunoglobulin variable region (antibody-combining site) repertoire, there should be selection for at least a minimum necessary *Vh* gene pool, encoding a minimum level of *Vh* gene diversity (including essential antigen binding specificities), above which the evolution of *Vh* gene family size might be comparatively neutral. This threshold may be similar to the complement of *Vh* genes in *M. minutoides,* which apparently numbers less than 50 (Figs. 4 and 6). The threshold value could be even lower with an adequately diverse repertoire created by compensatory expansion of *Dh* or *Jh* gene families and/or light chain variable region genes, which also contribute to combining site diversity. Another possibility is that a loss of diversity does not necessarily accompany a smaller *Vh* gene pool. The functional redundancy of the *Igh-V* locus has been established by the demonstration that multiple *Vh* genes, often belonging to different *Vh* gene families, are usually used in response to immunization with even a simple antigen [e. g., the antigens ARS (Margolies et al. 1981), NP (Boersch-Supan et al. 1985), HA (Clarke et al. 1985), OX (Kaartinen et al. 1986), TNP (Riley et al. 1986), dextran

(Alkokar et al. 1987), and TGAL (Busto et al. 1987); see also Riblet et al. 1987]. Conversely, a particular *Vh* (or *V*) gene may give rise to antibodies directed against distinct and apparently unrelated antigens (e. g., Weiss et al. 1984, Schiff et al. 1986, Naparstek et al. 1986). Above a certain *Vh* gene pool size, additional functional diversity may not be gained with an increase in pool size; rather, beyond this point, the pool becomes progressively more redundant. At a level of high redundancy, a sizable contraction of the *Vh* gene pool may have little effect on the level of encoded diversity. The analysis of the various heavy and light chain variable region elements in *M. minutoides* and other *Mus* species should help clarify these issues.

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