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The testis-determining gene, *SRY*, exists in multiple copies in Old World rodents

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Summary

SRY is a unique gene on the Y chromosome in most mammalian species including the laboratory mouse, *Mus musculus*, and the closely related European wild mouse species *M. spicilegus*, *M. macedonicus*, and *M. spretus*. In contrast, *SRY* is present in 2–6 copies in the more distantly related Asian mouse species *M. caroli*, *M. cervicolor*, and *M. cookii* and in 2–13 copies in the related murid species *Pyromys saxicola*, *Coelomys pahari*, *Nannomys minutoides*, *Mastomys natalensis*, and *Rattus norvegicus*. Copy numbers do not correlate with known phylogenetic relationships suggesting that *SRY* has undergone a rapid and complex evolution in these species. *SRY* was recently proposed as a molecular probe for phylogenetic inferences. The presence of multiple *SRY* genes in a wide range of murid species and genera, and at least one cricetid species, necessitates caution in the use of *SRY* for phylogenetic studies in the Rodentia unless it is ascertained that multiple *SRY* genes do not exist.

1. Introduction

Mammalian sex determination pivots on the absence or presence of a Y chromosome. In the absence of a Y (XX or XO karyotypes), the fetal gonads differentiate into ovaries and a female phenotype is formed. In the presence of a Y (XY karyotype), the fetal gonads develop into testes, which, in turn, secrete hormones, e.g., testosterone and Müllerian inhibiting substance, that give rise to the male phenotype. The locus on the Y that induces testis differentiation is called testis-determining factor (*TDF*) in humans and Y-linked testis determining (*Tdy*) in mouse.

Convincing molecular genetic data suggest that sex determining region on the Y (*SRY/Sry*, human and non-*Mus/Mus* gene symbols) is allelic to *TDF/Tdy* (for review see Goodfellow & Lovell-Badge, 1993). These data include its conservation on the Y of metatherian (marsupials) and eutherian (placental) mammals, identification of *de novo SRY* mutations in approximately 10% of human XY female patients, deletion of *Sry* in a mouse strain that produces XY females, and the generation of transgenic XX male mice by the introduction of a 14·6-kilobase pair (kb) genomic fragment that contains *Sry* (Berta *et al.*, 1990; Foster *et al.*, 1992; Gubbay *et al.*, 1992; Koopman *et al.*, 1991). *SRY* encodes a member of the High Mobility Group-1 and -2 (HMG 1/2) protein family whose signature or characteristic amino acid pattern is a DNA-binding domain of approximately 85 amino acids designated the HMG domain. The DNA sequence encoding the HMG domain is called the HMG box. HMG 1/2 proteins non-specifically bind to bent DNA and DNA four-way junctions. In addition, some induce a bend in target DNA sequences. Interest in HMG 1/2 proteins recently intensified when it was suggested that some members, including SRY, recognize specific nucleotide sequences called response elements and function as transcription factors (Alexander-Bridges *et al.*, 1992; Grosschedl *et al.*, 1994; Haqq *et al.*, 1993; Harley *et al.*, 1992; Landsman & Bustin, 1993).

Zinc finger protein on the Y (*ZFY/Zfy*, human and non-*Mus/Mus* gene symbols) maps close to *SRY/Sry* and was initially described as candidate for *TDF/Tdy* (Page *et al.*, 1987). Although the function of *ZFY* remains elusive, its conservation on the Y of all eutherian mammals studied to date suggests an important male-specific role. A highly homologous and equally well conserved gene, *ZFX/Zfx*, is present on the X chromosome. Although ZFY is a single copy gene in humans and many mammalian species, copy number in the family Muridae, the rodent family which includes the laboratory mouse and rat, varies from 1 to 26 (Nagamine *et al.*, in press; Nagamine *et al.*, 1989). The laboratory mouse, *Mus musculus*,

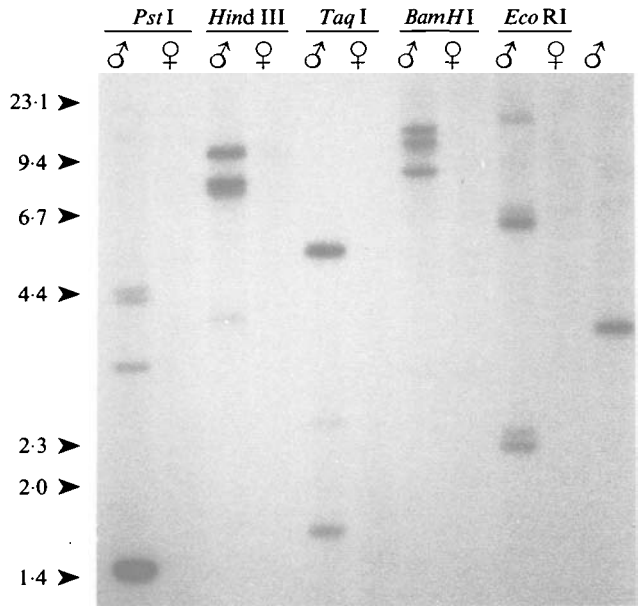

Fig. 1. Autoradiograph of a Southern blot containing male and female rat (*R. norvegicus*) DNA digested with five different restriction enzymes and hybridized with a mouse *Sry* HMG box probe. Multiple *Sry*-homologous bands are observed with male but not female rat samples. Note that a laboratory mouse (C57BL/6) male sample (last lane) gives only a single band, in keeping with *Sry* being a single copy gene in *M. musculus*.

has two copies, *Zfy-1* and *Zfy-2*, both of which have complete open reading frames (Ashworth *et al.*, 1989; Mardon & Page, 1989). Differential expression of *Zfy-1* and *Zfy-2* during fetal development and during spermatogenesis suggests that the genes are not functionally redundant (Nagamine *et al.*, 1990). Whether the additional copies of *ZFY* in other murid species are pseudogenes or, as in *M. musculus*, potentially functional remains to be determined.

SRY is single copy in a variety of eutherian and metatherian mammals including humans, primates, and *M. musculus* (Foster *et al.*, 1992; Gubbay *et al.*, 1990; Sinclair *et al.*, 1990). Therefore it was unexpected when 4–5 male-specific bands were obtained from Southern blots containing male and female DNAs of the laboratory rat (*Rattus norvegicus*) following hybridization with a murine *Sry* HMG box probe (Fig. 1). The data suggested at least 4 *SRY* genes in the laboratory rat. This study addresses whether other murid species have multiple copies of *SRY*. In addition we compared copy numbers of *SRY* and *ZFY* among species to determine if these varied relative to known phylogenetic relationships.

2. Materials and methods

(i) *Nomenclature and species studied*

The nomenclature used follows Auffray *et al.* (1990) and Bonhomme & Guénet (1989). The species were divided into three groups for data interpretation. The first includes *Mus musculus*, which is comprised of four subspecies (*M. m. musculus*, *M. m. domesticus*, *M. m. bactrianus*, *M. m. castaneus*), and three European mouse species that are *M. musculus*' closest relatives: *M. spicilegus*, *M. macedonicus*, and *M. spretus*. The second includes the more distantly related Asian *Mus* species: *M. caroli*, *M. cervicolor*, and *M. cookii*. Last are five non-*Mus* species: *Pyromys saxicola*, *Coelomys pahari*, *Nannomys minutoides*, *Mastomys natalensis* (= *Praomys natalensis*), and *Rattus norvegicus*.

The origins of the samples were: *M. m. musculus*, *M. spretus*, *M. spicilegus*, *M. caroli*, *M. cervicolor* (strain CpTAK), *M. cookii*, *P. saxicola*, *C. pahari*, and

Table 1. *SRY copy numbers based on number of fragments observed on Southern blots and PhosphorImager analysis. PhosphorImager values is the average of two hybridizations of the same blot and are corrected for differences in DNA loading.* * = *copy numbers of SRY calculated relative to M. musculus.* † = *copy number assuming 2 SRY fragments co-migrating as one band in Hind III and Taq I digests.* ‡ = *hybridization intensity of one or more bands is considerably stronger than the M. musculus Sry band. ND = not determined.*

Species	Southern Blots: *Hind* III fragments	Southern Blots: *Taq* I fragments	PhosphorImager counts × 13^3 (Sry copies)*	Copies of SRY
M. musculus	1	1	153 (1·0)	1
M. spicilegus	1	1	ND	1
M. macedonicus	1	1	ND	1
M. spretus	1	1	ND	1
M. caroli	1‡	1‡	290 (1·9)	2
M. cervicolor	5‡	5‡	606 (4·0)	6†
M. cookii	2‡	2‡	282 (1·8)	3†
P. saxicola	4	5	441 (2·9)	3–5
C. pahari	2‡	5	738 (4·8)	2–5
N. minutoides	2‡	> 3‡	1956 (12·8)	13
M. natalensis	1	6	240 (1·6)	2–6
R. norvegicus	4	5	712 (4·7)	4–5

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N. minutoides – Dr M. Potter (National Cancer Institute, Maryland); *M. macedonicus, M. spicilegus*, and *M. cervicolor* (strain CRP) – Drs F. Bonhomme and P. Boursot, Université de Montpellier II, France; *M. spretus* – Dr J.-L. Guénet, Pasteur Institute, France; *M. natalensis* – Dr M. Fahnestock, SRI International, California, and *R. norvegicus* – Dr M. LaVail, University of California, San Francisco.

(ii) *Southern blot analysis*

Ten to 15 μg of genomic DNA were digested with restriction enzymes, size-fractionated on 0.8% agarose gels, then transferred to Hybond-N (Amersham) or MagnaCharge (Micron Separations, Ins.) nylon membranes by capillary blotting. The transferred DNAs were fixed to the blots by UV-cross-linking. After prehybridization for 1–3 h at 65 °C in 0·25 M NaH_2PO_4 (pH 7·2) and 7% sodium dodecyl sulfate (SDS), the blots were hybridized for 14–16 h at 65 °C with the ^{32}P-labelled denatured probe ($2{\cdot}0 \times 10^6$ to $3{\cdot}0 \times 10^6$ cpm/ml) in 0·25 M NaH_2PO_4 (pH 7·2), 7% SDS, and 10% dextran sulfate. The blots were washed twice at low stringency (2X SSC, 0·1% SDS, 55 °C, 30 min/ea; 1X SSC = 0·15 M NaCl, 0·015 M sodium citrate) then once at high stringency (0·1X SSC, 0·1% SDS, 55 °C, 15 min). Autoradiography was performed at −85 °C with Kodak X-OMAT film and an intensifying screen.

A *M. musculus* Sry HMG box probe was generated using the polymerase chain reaction (PCR) (Nagamine *et al.*, 1992). A HMG box probe was chosen since this is the only region evolutionarily conserved among *SRY* genes. Primers flanking the *Sry* HMG box (sense = 5′-GTG ACA ATT GTC TAG AGA GCA TGG A-3′, antisense = 5′-GCA GCT CTA CTC CAG TCT TGC C-3′) were used to amplify a 382-base pair (bp) fragment from C57BL/6 or B6.Y^{Dom} genomic DNA. This fragment served as a template for a second PCR reaction using nested primers (sense = 5′-GTC CCG TGG TGA GAG GCA CAA GT-3′, antisense = 5′-TTT CTC TCT GTG TAA GAT CTT CAA TC-3′) to generate a 160-bp ^{32}P-labeled-*Sry* probe. The probe lies entirely within and represents 66% of the *M. musculus Sry* HMG box.

The *ZFY* probe is a 1·28-kb partial cDNA fragment representing most of the last exon of the *M. musculus Zfy-2* gene (Nagamine *et al.*, 1989; Nagamine *et al.*, 1992). The last exon encodes all of the zinc finger domain, the ZFY domain that is most conserved evolutionarily (Mardon & Page, 1989). The *Zfy* probe was ^{32}P-labeled using a random primer labeling kit (Prime-It II, Stratagene). Hybridization and washes were as above.

(iii) *Quantification of* Sry *and* ZFY *copy numbers*

SRY and *ZFY* copy numbers were estimated using the PhosphorImager (Molecular Dynamics, Inc.). Southern blots were hybridized with mouse *Sry* and *Zfy-2* probes, washed at high stringency, then exposed to phosphor screens for 2–12 days. The sum of the pixels of each band, which is proportional to the strength of the band's ^{32}P signal, was calculated using the ImageQuant 3·3 software program using the settings for volume integration and local background correction. Variations due to differences in DNA loading were corrected using *ZFX*. *ZFX* is a single copy gene on the eutherian X chromosome (Page *et al.*, 1987). All blots were hybridized with the *Zfy-2* probe, which recognizes *ZFX*, and the samples standardized to *M. musculus*. A given species' *ZFX* value was divided by the *Zfx* value of *M. musculus* and the resulting ratio was subsequently multiplied to the species' *SRY* and *ZFY* values to correct for DNA loading.

SRY copy numbers were determined by totaling the values for the *SRY* band(s) of a given species, correcting for DNA-loading, then dividing by the *Sry* value for *M. musculus*. *ZFY* copy numbers were determined similarly but relative to the *M. musculus Zfy* gene.

3. Results

(i) Sry *is a single copy gene in European* Mus

The European *Mus* (*M. spicilegus*, *M. macedonicus*, *M. spretus*) are ancestral to and the closest relatives of

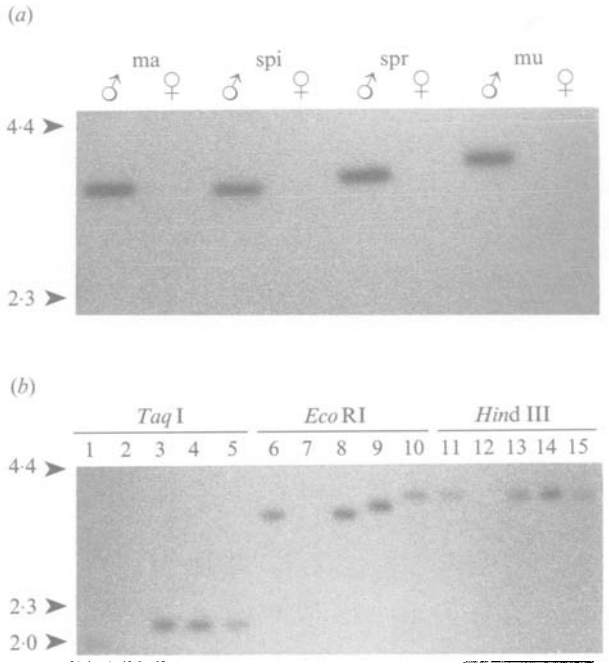

Fig. 2. *Sry* is single copy in European *Mus*. 2*a*. *Eco* RI digest. Male and female *M. macedonicus* (ma), *M. spicilegus* (spi), *M. spretus* (spr), and *M. m. musculus* (mu). 2*b*. *Taq* I, *Eco* RI, *Hind* III digests. *M. macedonicus* – male (lanes 1, 6, 11) and female (lanes 2, 7, 12), *M. spicilegus* male (lanes 3, 8, 13), *M. spretus* male (lanes 4, 9, 14), and *M. m. musculus* male (lanes 5, 10, 15).

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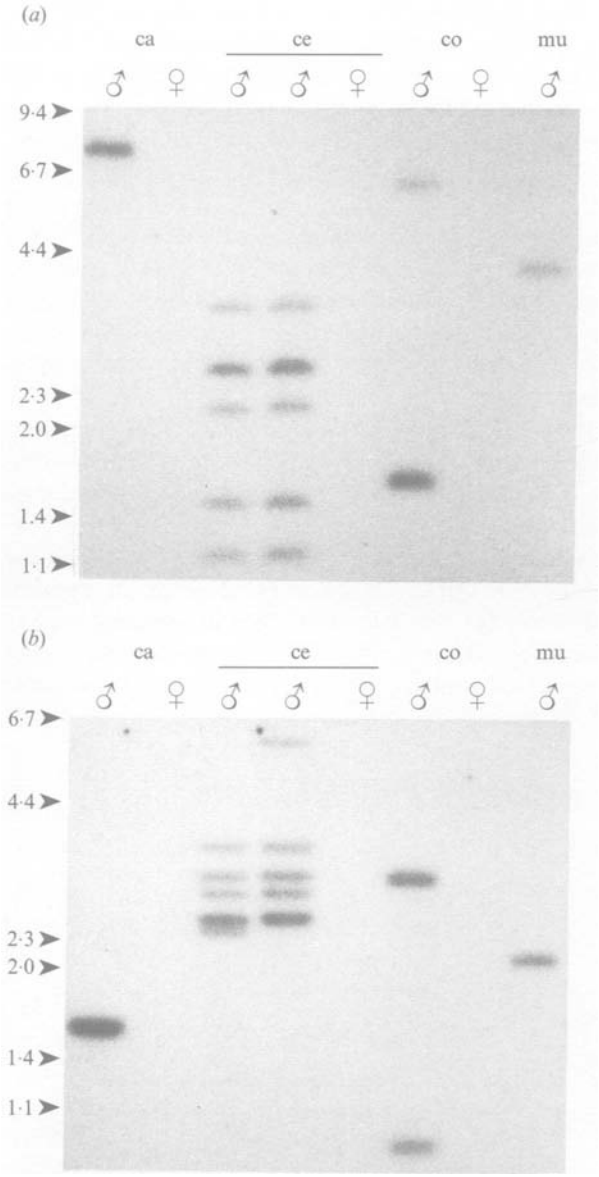

Fig. 3. *Sry* in Asian *Mus*. 3*a*. *Hin*d III digest. Male and female *M. caroli* (ca), *M. cervicolor* (ce), *M. cookii* (co), and *M. m. musculus* (mu). 3*b*. *Taq* I digest. An RFLP is present between *M. cervicolor* males from strain CRP (lane 3) and CpTak (lane 4). Progenitors of these strains were trapped in Thailand. Note that for both restriction enzyme digests the *Sry* band from *M. caroli* and one from *M. cookii* and *M. cervicolor* are darker relative to the *Sry* band from *M. musculus*. This is not due to underloading of the *M. musculus* sample. Hybridization for *Zfy* indicates approximately equal loading of DNA in each lane (Fig. 5*b*).

M. musculus. *M. musculus* harbors a single *Sry* gene on its Y (Gubbay *et al.*, 1990). Southern blots containing male and female DNAs of the European *Mus* gave a single male-specific fragment when hybridized with the 160-bp *Sry* HMG box probe (Fig. 2*a*, *b*; Table 1). No bands were obtained in female lanes demonstrating the specificity of the hybridization (Fig. 2*a*). The bands hybridized to a level equivalent to the single copy *M. musculus Sry* band. The data suggest that like *M. musculus*, *Sry* is single copy in *M. spicilegus*, *M. macedonicus*, and *M. spretus*.

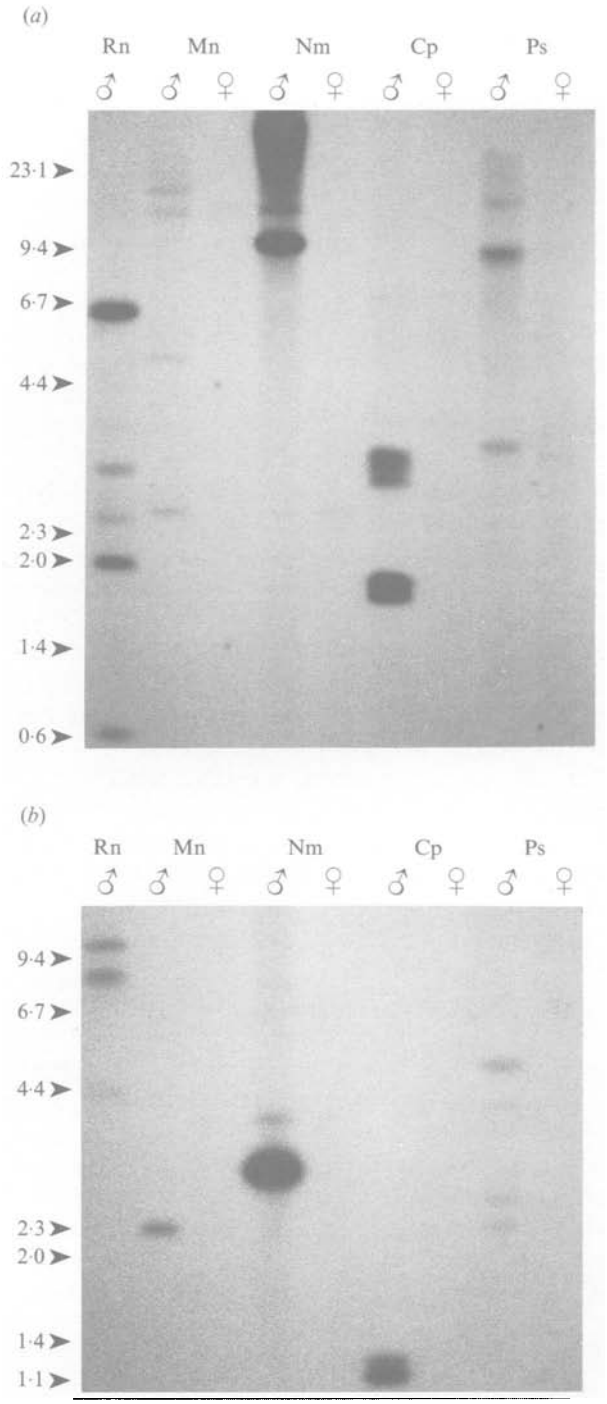

Fig. 4. *SRY* in non-*Mus* murids. *Rattus norvegicus* (Rn) *Mastomys natalensis* (Mn), *Nannomys minutoides* (Nm), *Coelomys pahari* (Cp), and *Pyromys saxicola* (Ps). 4*a*. *Taq* I digest. Weak male–female common bands represent *SOX* genes. Two *Taq* I fragments > 23 kb are just visible in *M. natalensis*. 4*b*. *Hin*d III digest.

(ii) *Amplification and polymorphism of* Sry *in Asian* Mus

M. caroli, *M. cervicolor*, and *M. cookii* are ancestral to the European *Mus*. *M. caroli* gave a single male-specific fragment with *Hin*d III and *Taq* I (Fig. 3*a*, *b*; Table 1). However, the fragment consistently hybridized with a greater intensity relative to the *M. musculus Sry* band despite approximately equal amounts of

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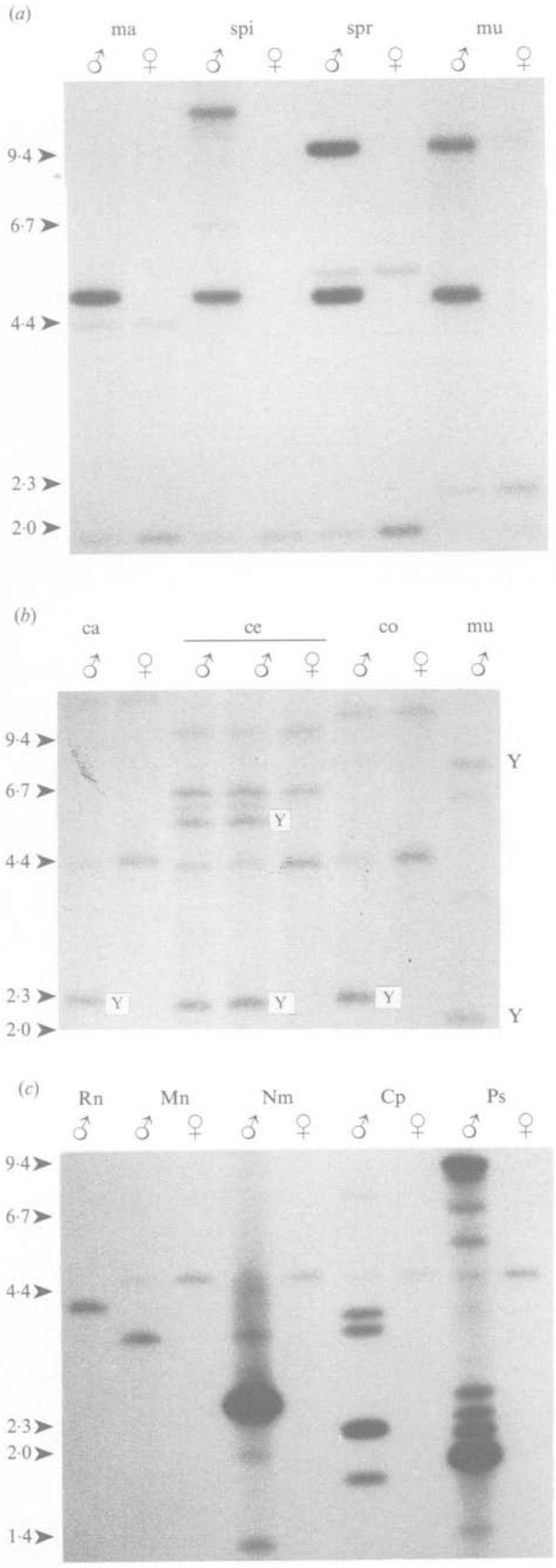

Fig. 5. Duplication and amplification of *ZFY*. 5*a*. European *Mus*. Same blot as Fig. 2*a* (*Eco* RI digest) but hybridized for *Zfy*. Weaker, male–female common bands are homologous *Zfx* or *Zfa* bands. *Zfx* bands are differentiated from *Zfa* bands by their hybridizing with twice the intensity for females relative to males and by the tendency for *Zfx* fragments of different species to co-migrate due to *ZFX* being highly conserved evolutionarily. The 6·7 kb *Zfa* band in the female *M. spicilegus* sample is weak. 5*b*. Asian *Mus*. Same blot as Fig. 3*b* (*Taq* I digest). Y = *Zfy* fragments. Male–female common bands represent *Zfx* or *Zfa* fragments. Note that *M. cervicolor* has two *Zfy* genes. What appears to be a third *Zfy* fragment at about 6·0 kb is a *Zfa* RFLP. In the *M. cervicolor* female sample, two *Zfa* fragments are co-migrating at about 9·4 kb (Nagamine *et al.*, in press). For *M. musculus*, *Zfx* = 3·6 kb and *Zfa* = 6·0 kb (Nagamine *et al.*, 1989). 5*c*. Non-*Mus* murids. Same blot as Fig. 4*b* (*Hind* III digest). All co-migrating male–female common bands are derived from *ZFX*; *ZFA* does not exist in these species (Nagamaine *et al.*, in press). For *N. minutoides*, the strongly hybridizing *ZFY* band is not identical to the strongly hybridizing *SRY* band in Fig. 4b.

DNA being loaded per sample (e.g. see Fig. 5*b* which is the same blot as Fig. 3*b* but hybridized for *Zfy*). Intensity of hybridization is a function of copy number and degree of homology to the *Sry* probe. The stronger *Sry* signal for *M. caroli* relative to *M. musculus* suggests that *M. caroli* has at least 2 *Sry* genes and that the fragments obtained from these genes are identical or close to identical in size following *Hind* III and *Taq* I digests. This interpretation was confirmed by PhosphorImager analysis (see below).

M. cookii gave 2 and *M. cervicolor* gave five bands with *Hind* III and *Taq* I digests (Fig. 3*a*, *b*; Table 1). All bands were male-specific suggesting that the fragments were derived from Y chromosomal sequences and were not due to homologous X or autosomal *Sry*-related HMG-box (*Sox*) genes (Gubbay *et al.*, 1990). In both species and with both digests, one band consistently hybridized stronger than the *M. musculus* *Sry* band suggesting that it represented the co-migration of at least 2 fragments. In addition, a *Taq* I RFLP was identified in *M. cervicolor* male samples from two different strains (CpTak, CRP) (Fig. 3*b*).

It should be reiterated that the size of the *Sry* HMG probe is 160-bp. If the multiple bands are due to *Hind* III and *Taq* I sites being present in the HMG box, only two fragments can be > 160 bp. In fact, the *M. cookii* and *M. cervicolor* *Hind* III fragments and the *M. cervicolor* *Taq* I fragments were considerably larger suggesting that this cannot be the case. Furthermore, given that all data to date suggest that the SRY HMG domain is encoded by a single exon (Graves & Erickson, 1992; Gubbay *et al.*, 1990; Su & Lau, 1993), it is unlikely that the multiple bands are due to an intron in the HMG box. The simplest explanation is that these species have more than one *Sry* gene on their Y. The Southern data are interpreted as *M. caroli* having 2, *M. cookii* having 3, and *M. cervicolor* having 6 copies of *Sry* (Table 1).

(iii) Sry *in non*-Mus *murids*

P. saxicola, *C. pahari*, *N. minutoides*, *M. natalensis*, and *R. norvegicus* are distant relatives of the genus

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Mus (Bonhomme & Guénet, 1989; She *et al.*, 1990). For these species, 3–6 male-specific bands were observed with *Taq*I (Fig. 4*a*, Table 1). For *N. minutoides*, the number of *Taq*I bands could not be determined with certainty due to the presence of a strongly hybridizing, high molecular weight band that resulted in smearing. For *Hind* III digests, *P. saxicola* and *R. norvegicus* gave four male-specific bands while *C. pahari*, *N. minutoides*, and *M. natalensis* gave either 1 or 2 male-specific bands (Fig. 4*b*, Table 1). For *C. pahari* and *N. minutoides*, the intensity of hybridization of certain bands suggested two or more co-migrating fragments. The Southern data suggest multiple *SRY* genes exist in these species. However, the complex hybridization patterns made it difficult to estimate the exact copy number.

(iv) *PhosphorImager analysis*

The number of copies of *SRY/Sry* as determined by the PhosphorImager and Southern blot analyses are listed in Table 1. The copies estimated using the PhosphorImager were generally less than that estimated from the number and hybridization intensity of fragments observed on Southern blots. This is attributed to a combination of differences in homology between the *M. musculus Sry* probe and the *SRY/Sry* genes in other species resulting in an underestimation of the values of all non-*M. musculus* bands and to the inefficiency of high molecular weight fragments to transfer during Southern blotting resulting in an under representation of the values for these fragments. Despite these technical limitations, the PhosphorImager data confirmed that two or more *Sry/SRY* genes are on the Y in Asian *Mus* and non-*Mus* species.

(v) SRY *and* ZFY *copy numbers do not correlate with known phylogenetic relationships*

ZFY is either a unique, duplicated, or amplified gene in murid species (Fig. 5*a–c*) (Nagamine *et al.*, in press). ZFY's hybridization patterns are more difficult to interpret due to the presence of *ZFX/Zfx* bands in all species and *Zfa* bands in all *Mus* species (Ashworth *et al.*, 1990; Nagamine *et al.*, in press). *ZFX/Zfx* and *Zfa* fragments can be identified by their being present in male and female samples and by their hybridizing with stronger intensity relative to the *ZFY/Zfy* bands when blots are probed with a human *ZFY* zinc finger probe (Nagamine *et al.*, 1989). The estimated copy numbers of *SRY* and *ZFY* relative to the known phylogenetic relationships of these species are shown in Fig. 6. Three observations can be made. First, the copy numbers of either *SRY* or *ZFY* do not follow known phylogenetic relationships. For example, given that *Sry* is a single copy gene in *M. musculus*, multiple copies of *Sry* would not have been predicted for the more ancestral Asian *Mus* (*M. caroli*, *M. cervicolor*, *M. cookii*). Similarly, the presence of a single *Zfy* gene

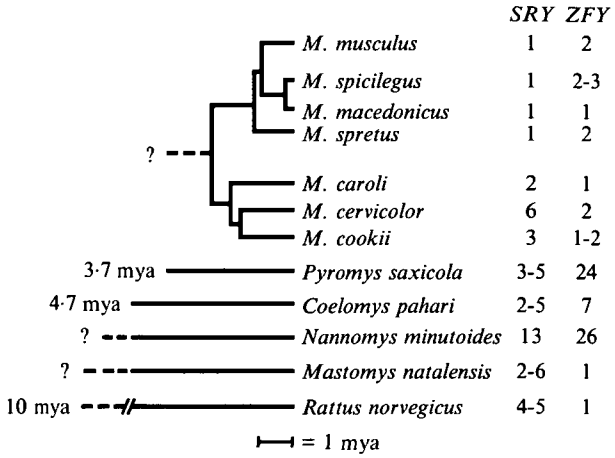

Fig. 6. Phylogenetic relationship of murid species based on Bonhomme, 1986; Boyer *et al.*, 1991; Morita *et al.*, 1992, and She *et al.*, 1990 and shown relative to the estimated copy numbers of *SRY* and *ZFY* as determined by PhosphorImager and/or Southern blot analyses (Nagamine *et al.*, in press; this report). A range of values is given when a specific copy number could not be determined with accuracy. mya = millions of years ago.

in *M. macedonicus* suggests that either a *Zfy* gene was lost or independent duplications of *Zfy* occurred in *M. spretus* and *M. musculus*. Second, despite *SRY* and *ZFY* being neighboring genes, they do not correlate with regard to copy number. *M. natalensis* and *R. norvegicus* both have multiple copies of *SRY* but a single copy of *ZFY*. The reverse is seen in *M. musculus* which has a single *Sry* but two *Zfy* genes. Last, in *P. saxicola*, *C. pahari*, and *N. minutoides*, both *SRY* and *ZFY* exist as multiple copies although not to the same amount.

4. Discussion

All data to date support the hypothesis that *SRY* triggers testis determination in mammals and, by definition, is the testis-determining gene on the Y. The presence of multiple *SRY* genes, especially if they are functional, complicates attempts to understand how SRY induces testis determination. The Southern blot and PhosphorImager analyses reveal 2–6 copies of *Sry* for *M. caroli*, *M. cervicolor*, and *M. cookii* and 2–13 copies of *SRY* for *P. saxicola*, *C. pahari*, *N. minutoides*, *M. natalensis*, and *R. norvegicus*. Recently, 2–6 copies of *SRY* were reported to be present in five species of the South American field mouse *Akodon* (Cricetidae) (Bianchi *et al.*, 1993). The *Akodon SRY* fragments co-migrated, being revealed by an increase in hybridization intensity of the *SRY*-specific band, similar to that observed for *M. caroli*. The Cricetidae is distantly related to the Muridae. It is of interest to determine if multiple copies of *SRY* occur in other mammalian orders or is restricted to the Rodentia.

In four species of *Akodon*, 15–40 % of fertile females have an XY karyotype and thus illustrate XY sex

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reversal. The presence of multiple *SRY* genes does not correlate with XY sex reversal (Bianchi *et al.*, 1993). Similarly, in the present study, we know of no reports of abnormal sexual differentiation for the species with multiple *SRY* genes.

SRY in primates and rodents have relatively high numbers of non-synonymous amino acid substitutions in the regions flanking the HMG domain suggesting that the *SRY* locus is rapidly evolving (Tucker & Lundrigan, 1993; Whitfield *et al.*, 1993). It has been suggested that SRY variation reflects positive adaptation fitness and is instrumental for reproductive isolation and speciation (Whitfield *et al.*, 1993). In *M. musculus*, although *Sry* exists as a single copy gene, it is polymorphic within and among subspecies (Coward *et al.*, 1994; Nagamine *et al.*, 1992). Molecular genetic data suggest that the polymorphisms may correlate with differences in SRY's ability to induce testes on certain genetic backgrounds (Coward *et al.*, 1994). The present data suggest an additional type of *SRY* variability, an increase in copy number.

Whether the additional *SRY* loci in murid or akodontine species are pseudogenes or encode functional proteins can be determined by the cloning and sequencing of the *SRY/Sry* cDNAs. However, in the absence of these data insight can be obtained from the neighboring gene, *ZFY*. Like *SRY*, *ZFY* is a single copy gene in most mammalian species (Page *et al.*, 1987). However, in several species in the Muridae (Nagamine *et al.*, in press) and Cricetidae (Bianchi *et al.*, 1992; Lau *et al.*, 1992) *ZFY* is present as two or more copies. For *M. musculus*, molecular data suggest that a tandem duplication gave rise to two genes, *Zfy-1* and *Zfy-2*, both of which have complete open reading frames and therefore have the potential for generating functional proteins (Ashworth *et al.*, 1989; Mardon & Page, 1989; Simpson & Page, 1991). *Zfy-1* and *Zfy-2* may be functionally distinct since RT-PCR studies show that they are differentially expressed during fetal development and during spermatogenesis (Nagamine *et al.*, 1990). By analogy to the *M. musculus Zfy-1/Zfy-2* model, it is probable that more than one copy of *SRY/Sry* may be functional in some species.

Multiple *SRY* genes may not be deleterious to normal sex determination. Human XYY patients are normal in sexual phenotype and fertility (Gorlin, 1977). In laboratory mice, males carrying the sex reversed mutation, *Sxr*, have two *Sry* genes due to a duplication of the short arm of the Y (McLaren *et al.*, 1988; Roberts *et al.*, 1988). Carrier males (XYSxr) are fertile but have testes that are smaller relative to those from XY siblings (Lyon *et al.*, 1981). However, the smaller testes are due to a higher incidence of X–Y univalence during meiosis and not to *Sry* itself (Cattanach *et al.*, 1990). Analysis of fetal mice at 13 days post coitus has not revealed any obvious differences with regard to testicular differentiation between XY and XYSxr male siblings (Nagamine, unpublished observations).

Exactly how the *SRY* and *ZFY* loci undergo amplification is unclear. Unlike autosomal or X chromosomal genes, unequal crossing over during meiosis cannot be proposed since these genes are present on the region of the Y that does not pair and recombine with the X during meiosis. Alternative explanations include unequal sister chromatid exchange and/or amplification through overreplication as suggested for the dihydrofolate reductase gene (Schimke *et al.*, 1986).

Partial *SRY/Sry* sequences of *M. caroli* and *C. pahari* (= *M. pahari*) have been published, the data having been obtained by directly sequencing PCR amplified *SRY/Sry* fragments (Graves & Erickson, 1992; Lundrigan & Tucker, 1994; Tucker & Lundrigan, 1993). The present data suggest that *SRY* occurs in 2 or more copies in these species. Since no ambiguities in the *SRY/Sry* sequences were reported either the multiple *SRY/Sry* genes are identical over the region sequenced or the PCR or sequencing primers were specific for only one of the copies.

SRY has been proposed as a molecular probe for phylogenetic inferences in the rodents (Lundrigan & Tucker, 1994). The presence of multiple *SRY* genes in a wide range of murid species and genera, and at least one cricetid species, necessitates caution in the use of *SRY* for phylogenetic studies unless it is ascertained that multiple *SRY* genes do not exist.

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