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Multiple origins of XY female mice (genus *Akodon*): phylogenetic and chromosomal evidence

Hopi E. Hoekstra* **and Scott V. Edwards**

Department of Zoology and Burke Museum, University of Washington, Seattle, WA 98195, USA

Despite the diversity in sex determination across organisms, theory predicts that the evolution of XY females is rare in mammals due to fitness consequences associated with infertility or the loss of YY zygotes. We investigated this hypothesis from a phylogenetic perspective by examining the inter- and intraspecific distribution of Y chromosomes in males and females (XY females) in South American field mice (*Akodon*). We found that XY females occurred at appreciable frequencies (10–66%) in at least eight *Akodon* species, raising the possibility that this system of sex determination has arisen multiple times independently. To determine the number of origins of XY females in *Akodon*, we constructed a molecular phylogeny of 16 species of *Akodon* based on mitochondrial DNA control region sequences. Both parsimony and maximum-likelihood reconstruction of ancestral states suggest that multiple steps (gains or losses of XY females) best explain the evolution of XY females, but do not clearly differentiate between single and multiple origins. We then directly compared functional and non-functional Y chromosomes in six species by Southern blot analysis. We found that male and female Y chromosome restriction fragment length polymorphism patterns were identical within species, but always differed between species, providing evidence that XY females arose at least six times within the *Akodon* lineage. To our knowledge, this pattern in *Akodon* is the first documentation of a novel sex-determining system arising multiple times within a tight clade of mammals. In addition, this system provides a clear test of the accuracy of phylogenetic methods to reconstruct ancestral states.

Keywords: sex determination; Y chromosome; sex chromosomes; XY females; ancestral states; *Akodon*

1. INTRODUCTION

Unlike many basic developmental processes, the mechanisms employed for sex determination are highly diverse across organisms (Ohno 1967; Bull 1985; Wibbels *et al*. 1994). How such different sex-determining mechanisms arose and diversified has long puzzled biologists (Bull 1985). Clear answers to account for this diversity have eluded scientists for three primary reasons: first, the deep divergence between taxa with different sex-determining mechanisms complicates comparative studies; second, observing a sex-determining system early in its evolution is difficult; and finally, the mechanisms of sex determination for many clades are incompletely described, for example reptiles, where fewer than 10% of all species have been characterized (Viets *et al*. 1994). Mammals provide an excellent system in which to study sex determination because mechanisms are so well described across a variety of species (Vorontsov *et al*. 1980; Fredga 1983). However, the diversity of sex determination is greatly reduced within mammals, which generally employ a system of heterogametic (XY) males and homogametic (XX) females (Ohno 1967; Bull 1983). Exceptions to this pattern generally appear to have evolved once within a genus. For example, the mole (*Talpa*; Sanchez *et al*. 1996), which has hermaphroditic females; the wood lemming (*Myopus*; Fredga *et al*. 1976), which has XY females caused by a mutation on the X chromosome; and finally, a single species of microtine vole (*Microtus*; Burgos *et al*. 1988), which also has XY females (for a review, see Fredga 1983).

To explain the lack of diversity of sex-determining schemes in mammals, Bull (1983) suggested that novel sex-determining mechanisms do not often evolve in species with heteromorphic sex chromosomes like mammals because (i) a male-determining mutation (formation of XX males) faces the lack of necessary Y-chromosome genes for male function, and (ii) a female-determining mutation (formation of XY females) faces the production of one-quarter YY offspring and the potential adverse effects of Y-chromosome genes on the feminizing process (Marin & Baker 1998). This hypothesis predicts an evolutionarily conserved mechanism of mammalian sex determination and very infrequent origins of novel sex-determining systems, specifically XX males or XY females.

South American field mice (*Akodon*) present a unique opportunity to study the evolution of sex determination because the aforementioned difficulties are overcome by the presence of several closely related species in which XY females occur (see, for example, Bianchi *et al*. 1971). In species with XY females, the presence or absence of the Y chromosome no longer plays the decisive role in sex determination. In *Akodon* XY females, the Y chromosome fails to trigger the male pathway and is hence referred to as Y* (Lizarralde *et al*. 1982). Evidence that the cause of sex-reversal lies on the Y* chromosome includes cytogenetic, molecular, breeding and phylogenetic studies, and no individuals of XYY* karyotype have been reported within this clade of *Akodon* (Bianchi & Contreras 1967; Lizarralde *et al*. 1982; Vitullo *et al*. 1986; Bianchi *et al*. 1993; Espinosa & Vitullo 1996; H. E. Hoekstra, unpublished data).

In several species of *Akodon*, XY* females persist at high frequency along with normal XX females in natural populations. Because XY* females occur only in some species, the *Akodon* clade allows us to address an

*Author for correspondence (hopi@u.washington.edu).

important question regarding the evolution of novel sex-determining systems that cannot be addressed in other mammalian clades, namely, how many times did XY* females evolve in *Akodon*? Bull's (1983) hypothesis predicts a single ancient origin of XY* females because the mode of sex determination is a conserved trait, whereas if sex determination in *Akodon* is more labile, XY* females may have evolved independently in several species. In order to determine the number of origins of the Y* chromosome, we tested a series of predictions specifying single and multiple origins of the Y* chromosome at both the species and chromosomal levels (figure 1). We address these predictions through a combination of phylogenetic analysis of *Akodon* species lineages using mitochondrial DNA (mtDNA) control region sequences, as well as an examination of the ancestry of the Y* chromosome itself.

2. METHODS

(a) *Tissue samples*

Samples of *Akodon azarae* and *Akodon molinae* were collected in Argentina during March–April 1998. Remaining tissue samples for detection of XY* females and phylogenetic reconstruction from 14 species were obtained from the Museum of Vertebrate Zoology, Berkeley, CA; Field Museum of Natural History, Chicago, IL; National Museum of Natural History, Washington, DC; and Museum of Southwestern Biology, Albuquerque, NM. Although there are *ca*. 25 species of *Akodon* currently described from across South America (Smith & Patton 1991), the majority of specimens are inaccessible and not represented in museum collections. However, we have tissues from a geographically diverse sample of well-described species from all of the major clades of *Akodon* (see Appendix A).

(b) *Screening for XY* females*

To detect the presence of XY* females in *Akodon* species, polymerase chain reaction (PCR) genotyping was performed on 179 phenotypic females from 16 species of *Akodon*. PCR primers designed to amplify an intron of the *Smcy* gene on the Y chromosome of mammals (Agulnik *et al*. 1994) were used to determine the presence or absence of the Y* chromosome in phenotypically female *Akodon*. Male DNA of the same species was used as a positive control in every case. A second PCR reaction, using the single copy autosomal gene *tubulin* (Palumbi 1996), was used as a control to ensure that the absence of a band in a PCR reaction was not due solely to poor DNA template quality. PCR results from ten females from five species (a putative XX and XY* female from each species) were confirmed by Southern blots. We used a second Y-chromosome gene, the sex-determining gene, *Sry* as a probe for the Y and Y* chromosomes (following the protocol described in §2(f)) and as an independent locus on the Y chromosome. The Southern blot restriction patterns from *Sry* matched our PCR results exactly, confirming our assumption that the *Smcy* gene is, in fact, Y-chromosome specific and that our PCR screening method is accurate.

(c) *Sequencing of mtDNA control region and phylogenetic reconstruction*

To reconstruct species relationships, we sequenced the entire mtDNA control region of 20 individuals representing 16 species of *Akodon*. Amplification primers were designed from flanking conserved regions in sigmodontine rodents: 12S rRNA (Sullivan *et al*. 1995) and the cytochrome *b* gene (Smith & Patton 1993). The primer sequences are 12Sl: 5′-CGTTCATTGCTTAATTTTTAT CACTGC-3′ and C2: 5′-TGGTCTTGTAAATCAGTAATG-3′. PCR products were cleaned using a purification kit (Quiagen, Valencia, CA, USA). Cycle sequencing using fluorescently labelled dye terminators of both strands was carried out on an ABI 373A automated sequencer. Sequences were aligned manually using the program Sequencher (GeneCodes) and adjusted by eye as necessary. Sequences were deposited in GenBank (accession numbers AF296258–AF296278).

Topologies were generated using neighbour-joining (NJ) and maximum-likelihood (ML) methods. Trees were rooted by a designated outgroup, *Auliscomys pictus*, another akodontine rodent (Smith & Patton 1993). To develop an empirical model of sequence evolution, ML construction was based on an initial NJ tree from which ML estimates of α (0.377; among-site rate variation), κ (3.901; transition bias) and the proportion of invariant sites (0.507) were generated. These values were then used in combination with an HKY85 model of sequence evolution, which was chosen on the basis of hierarchical hypothesis testing (Huelsenbeck & Rannala 1997), to generate an ML tree (Voelker & Edwards 1998). Tree topologies and bootstrap support were generated using PAUP 4.0 (Swofford 1999).

(d) *Reconstruction of XY* evolution*

The presence (1) or absence (0) of XY* females within a species was coded as a binary character (Wiens 1999) and mapped on to the ML topology using MacClade (Maddison & Maddison 1992). Maximum-parsimony (MP) algorithms were used to determine ancestral states. Ambiguous lineages were resolved using the ACCTRAN (accelerated transitions) and DELTRAN (delayed transitions) options of MacClade. ML estimations of ancestral states were calculated using DISCRETE 1.01b (Pagel 1994, 1997). Rates of character change and ML estimates of ancestral character states were calculated following Mooers & Schulters (1999) using branch lengths estimated from the NJ tree. Because DISCRETE does not handle unknown states, the unknown tips were either assigned state by MP, or alternatively all possible permutations were averaged.

(f) *Southern blot analysis*

High molecular weight DNA was isolated from an XX female, XY* female and XY male for each of six *Akodon* species for which high quality tissues were available. XX female DNA served as a control to ensure that bands were Y-chromosome specific. Approximately 10 µg of DNA from each individual was cut with *Eco*R1, *Hin*dIII, *Pvu*II and *Pst*I restriction enzymes. Standard Southern hybridization methods and solutions were used; hybridizations were conducted at 60° for 24 h, and blots were exposed for four to six days. The probe was PCR amplified from total genomic *Akodon boliviensis* DNA using primers designed from the conserved HMG box region of the sex-determining gene, *Sry*, in *Mus musculus* (SRY1 5′-AGCGCCCATGAATGCATT-3′ and SRY4 5′-GTTTGGGTATTTCTCTCTG-3′). The 197 base pair (bp) product was then cloned and sequenced for verification. The probe did not contain any relevant restriction sites.

3. RESULTS

(a) *Frequency of XY* females across species*

Results of the PCR survey show that XY* females are present in eight out of 16 species tested (table 1). Three species (*Akodon mimus*, *Akodon orophilus* and *Akodon juninensis*) did not have large enough samples to determine the status

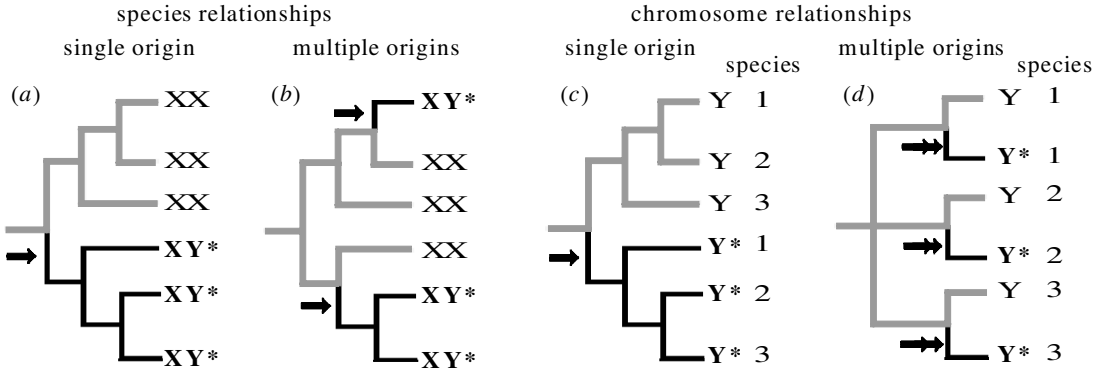

Figure 1. Phylogenetic hypotheses of the evolutionary origin(s) of XY* females. Scenarios (*a*) and (*b*) depict hypothetical phylogenetic relationships of species that either have XY* females or do not, represented by XY* and XX, respectively. A single origin of XY* females predicts that those species with XY* females are each others closest relatives (a monophyletic origin) as in (*a*). Multiple origins of XY* females may produce a pattern where those species with XY* females are scattered throughout the tree as in (*b*). A pattern similar to that shown in (*a*) could still be reconstructed by parsimony if there were multiple origins of XY* females if the parsimony assumptions were violated. Scenarios (*c*) and (*d*) show phylogenetic relationships of the Y and Y* chromosomes from three hypothetical species. Species are represented by a number. A single origin of Y* chromosome would produce a pattern of clustering of Y* chromosomes independent of species. Multiple origins of the Y* chromosome produces a pattern where Y and Y* chromosomes from a single species cluster together. Arrows indicate origins of XY* females.

of XY* females, and are thus considered unknown in the MP reconstructions. We discovered XY* females in two species in which they had not yet been reported: *Akodon torques* and *Akodon kofordi*. Overall, our results extended but did not contradict any previously reported data on the taxonomic distribution of XY* females in *Akodon*. The frequency of XY* females among all females in each species tested ranged from 0 to 66% (table 1), although for some species (*A. kofordi* and *Akodon puer*), sample sizes were too low to calculate a reliable frequency.

(b) *Phylogenetic analysis*

Currently there are no complete *Akodon* phylogenies, but incomplete trees based on allozymes (Barrantes *et al*. 1993; Rieger *et al*. 1995) or cytochrome *b* gene sequences (Patton & Smith 1992; Smith & Patton 1993) are available. None of these phylogenies, however, are strongly resolved or include all the species of *Akodon* that contain XY* females. Our phylogeny, based on the mtDNA control region for the 16 species of *Akodon*, included the eight species that have XY* females (figure 2). In several species, two individuals were sequenced. In all cases, mtDNAs from the same species clustered together with greater than 95% bootstrap support. The topology upholds previously described affinities, i.e. between *puer*, *subfuscus*, and *juninensis* (Myers *et al*. 1990), as well as *torques*, *mollis* and *orophilus* (Smith & Patton 1993). The two major clades of *Akodon* shown in figure 2 are very similar to the clades generated by the cytochrome *b* gene (Smith & Patton 1991), although differing in the species included. The basal position of *Akodon urichi* is consistent with the hypothesis that *A. urichi* may not belong in the genus *Akodon* (Smith & Patton 1993). Weakly supported nodes are due primarily to the uncertain position of *A. juniensis* and to the 'flip-flop' of *A. mimus*, which also falls basal to *Akodon cursor* in some trees. The uncertainty in *A. mimus*'s position is also evidenced by its previous assignment to the *Microxus* genus (Smith & Patton 1993). Thus, despite low bootstrap values in some nodes, the topology is well supported by other phylogenetic studies using independent loci.

Table 1. *Frequency of XY* females among and within species of* Akodon *resulting from our PCR genotyping* survey

(Our results confirm those of previous studies, which demonstrated the presence of XY* females in *A. varius* and *A. mollis* and the absence of XY* females in *A. molinae* and *A. urichi* for which large samples in this study were unavailable. For some species, samples sizes are small and resulting frequencies may be inflated.)

species	*n*	no. XY*	%XY*
A. azarae	39	4[a,b]	10
A. bolviensis	23	8[c]	35
A. subfuscus	13	5	38
A. torques	11	4	36
A. kofordi	3	2	66
A. puer	2	1[d]	50
A. varius	—	—[c]	—
A. mollis	—	—[e]	—
A. aerosus	10	0	0
A. a. baliolus	10	0	0
A. cursor	10	0	0
A. molinae	4	0[f]	0
A. urichi	1	0[c]	0
A. orophilus	3	0	?
A. mimus	2	0	?
A. juninensis	1	0	?

[a] Bianchi & Contreras 1967; [b] Bianchi *et al*. 1968; [c] Bianchi *et al*. 1971; [d] Vitullo *et al*. 1986; [e] Lobato *et al*. 1982; [f] Bianchi & Merani 1984.

(c) *Reconstruction of XY* origins*

The phylogenetic tree shows that those species with XY* females do not form a monophyletic clade. A likelihood-ratio test of monophyly suggests that a topology which forces those species with XY* females to be monophyletic is

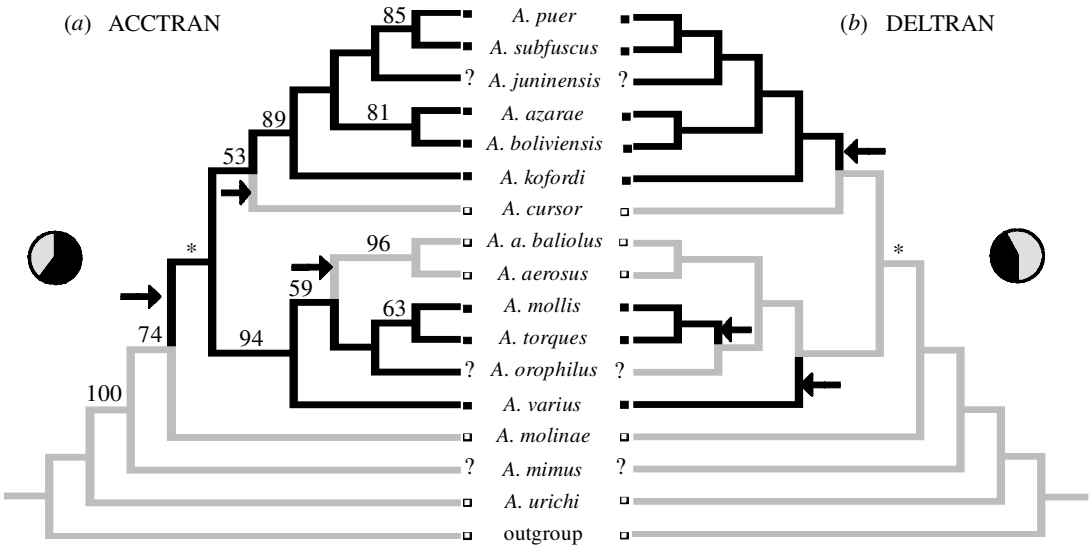

Figure 2. Phylogenetic pattern of XY* female evolution. The presence of XY* females was mapped onto the ML topology of *Akodon* species (based on 1085 bp of mtDNA control region) using equal-weighted parsimony reconstruction. Solid lines indicate presence of XY* females. Boxes at tips indicated absence (empty box), presence (filled box) or unknown state (?) of XY* females in the species. Gain or loss of XY* females (steps) are indicated by arrows. Numbers above the lineages represent bootstrap values (1000 replicates). (*a*) Reconstruction using ACCTRAN (accelerated transitions) to resolve branch assignments. (*b*) Reconstruction using DELTRAN (delayed transitions). The relative areas in the pies represent the relative ML support for the presence (black) or absence (grey) of XY* females at the starred node (*) using the one-rate model.

significantly worse than the 'best' phylogeny ($p < 0.01$) (Huelsenbeck *et al.* 1996).

The presence or absence of XY* females was then mapped onto this topology, using ACCTRAN (accelerated) and DELTRAN (delayed) options to resolve unresolved branches (figure 2). To evaluate the phylogenetic lability of the XY* system across species, the number of character state changes (steps, as shown by arrows in figure 2) was calculated on the ML topology and compared to the number of steps required by 1000 reconstructions after randomly shuffling the characters at the tips (Maddison & Slatkin 1991). The number of steps on the best topology ($x = 3$) is not significantly different to the average number of steps on trees with random character distributions ($x = 4.227$; $p > 0.05$), implying that the observed distribution of XY* females is not different from a random scattering of XY* females. This result also suggests that XY* females are gained and lost with little phylogenetic inertia or historical contingency.

Thus, instead of a single event, the phylogeny implies multiple gains or losses of XY* females (figure 2). The accelerated (ACCTRAN) reconstruction suggests a single ancient origin of XY* females with loss of XY* females in *A. cursor* and the *Akodon aerosus–Akodon aerosus balious* lineage. On the other hand, the delayed (DELTRAN) reconstruction shows three independent origins of XY* females and no losses. These two reconstructions differ in assigning one unknown state in *A. orophilus*. The uncertainty in phylogenetic position of *A. juniensis* and *A. mimus* does not affect the character reconstruction because both species are of unknown state and are assigned the same state in both reconstructions.

When all possible assignments of the three unknown states were tried ($n = 8$), multiple origins were supported in four cases, and in the remaining four cases multiple origins were as likely as a single origin reconstruction.

ML analysis estimated the rate of gain and loss of XY* females as approximately equal, thus a two-rate model that allows rates of gain and loss to differ was not statistically better than a one-rate model (Mooers & Schulter 1999). Over all eight trees, the ML estimate of rate varied from 0.0056 to 0.0137. ML was also used to estimate the ancestral character state at the node that is expected to have XY* females in the single origin scenario and no XY* females in the multiple origin scenario (the asterisk in figure 2). When unknown tips were reconstructed using ACCTRAN, ML estimated the relative support for XY* females present as 0.67. However, when *A. orophilus* was coded as having no XY* females, the ML support for XY* females at the ancestral node dropped to 0.42, and the multiple origin hypothesis was favoured. When all the possible permutations of the unknown tips were averaged (using a one-rate model), the ML estimate was 0.70 for having XY* females and 0.30 for the absence of XY* females at the node of interest.

(d) *Y and Y* chromosome relationships across species*

To determine relationships between Y and Y* chromosomes within and between species, restriction pattern variation in XY males and XY* females was examined using a Y-chromosome-specific probe consisting of a portion of the *Akodon* sex-determining gene, *Sry*. Southern blots reveal that Y and Y* chromosome patterns are always similar within a species, but always differ between species, for all six species tested (figure 3). Restriction patterns differed in both band number (range 1–3) and band size between species. This pattern holds when additional restriction enzymes (*Pst*I and *Pvu*II) are used (data not shown). DNA from XY* females of *Akodon mollis* and *Akodon varius* was unavailable, but XY male restriction

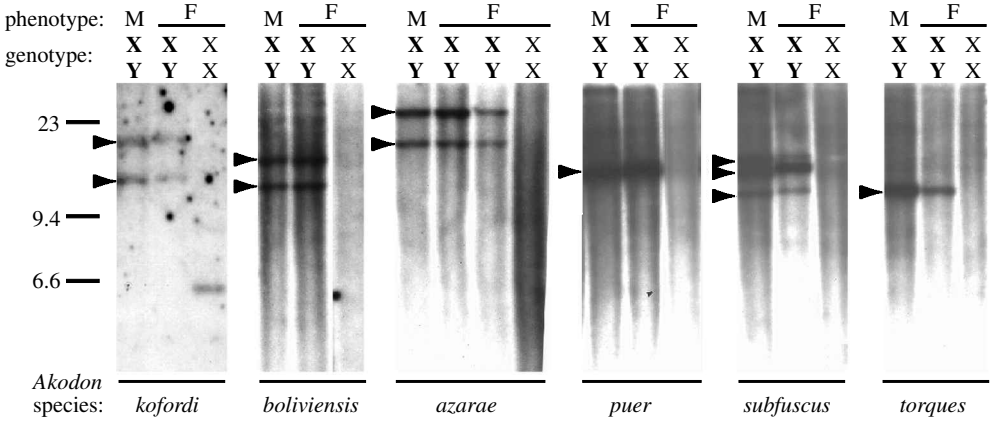

Figure 3. Molecular comparison of the Y and Y* chromosomes. For each of six *Akodon* species, DNA from an XY male, XY* female and XX female was probed with a Y-chromosome fragment. Phenotype, male (M) or female (F). Genotype, XY (XY male or XY* female) or XX (XX female). Arrows highlight strongly hybridizing fragments shared between XY males and XY* females. Scale in kilobases is indicated at the left.

patterns from these species are also unique (data not shown).

4. DISCUSSION

The phylogenetic distribution of XY* females across *Akodon* species is itself unable to distinguish between single or multiple origins of XY* females. While the parsimony results suggest that single and multiple evolutions of XY* females are equally likely, these analyses rely on the traditional assumptions of parsimony reconstruction, specifically equal weighting of character loss versus gain (Ree & Donoghue 1998). ML reconstructions rely on estimates of rate that are based on the distribution of characters on the tips, rather than independent biological data (Schultz & Churchill 1999). Relative rates of gain and loss can dramatically influence results. For example, if we weight character gain (the appearance of XY* females in a species) as more likely than character loss (the extinction of XY* females from a species), a scenario with multiple origins of XY* females in several species becomes more likely. Additionally, if rates of character change are rapid, this can also complicate ancestral state reconstruction (Cunningham 1999). Ultimately then, a species tree approach, although useful, is not definitive without *a priori* information about the relative probability of loss versus gain of XY* females in a species.

To complement the phylogenetic reconstruction of XY* females, we further tested the hypotheses by direct examination of the genealogical relationships of Y and Y* haplotypes. The predicted patterns in figure 1 (clustering by species or clustering by sex) stem from the fact that Y chromosomes are inherited paternally and Y* chromosomes are inherited maternally. Thus, Y and Y* chromosomes are never found in a single individual and therefore do not have an opportunity to recombine, thereby avoiding possible Y–Y* conversion or recombination within or between species. Unlike the phylogenetic mapping, direct analysis of relationships between Y and Y* using Southern blots can differentiate between the single and multiple origin hypotheses and suggests that XY* females arose multiple times. In every case, restriction patterns are identical within each species tested but always differ between species, suggesting a new origin of Y* chromosomes within each species. This analysis suggests that there are at least six independent origins of XY* females, rather than three as predicted by equally weighted parsimony reconstruction using DELTRAN or one origin as predicted by ACCTRAN. These restriction pattern data are particularly relevant because they provide independent evidence that does not rely as strongly on the assumptions associated with character mapping. Additionally, our particular results are robust to discordances between gene and species trees (e.g. hybridization and incomplete lineage sorting) or uncertainties in character scoring. These restriction fragment length polymorphism (RFLP) data also show that it would be incorrect to suggest the presence of XY* females in common ancestors of even closely related pairs of *Akodon* species that both possess XY* females, since RFLP patterns in such pairs (e.g. *azarae* and *boliviensis*) are different. The presence of different patterns in both Y and Y* chromosomes in these species suggests independent origins even among close relatives, although this does not exclude the possibility of XY* female extinctions in species or common ancestors. These six origins of XY* females triples the number of stable XY female systems described in mammals and is the first time multiple species within a genus have been shown to share this trait. The estimate of six independent origins of XY* females is a minimum estimate, as there are still many species of *Akodon* to survey. Additional detailed sampling may also be able to detect temporal and spatial variation in XY* female frequencies.

Why can XY* females evolve repeatedly in *Akodon*? While XY females occur in several mammalian species, including humans and house mice (*M. musculus*), they experience dramatic declines in fitness. Human XY females are infertile, suffering from 46,XY gonadal dysgenesis (Kent & Watchel 1989) and house mice are sub-fertile, having significantly smaller litters if any (Eicher *et al*. 1982). First, unlike humans and house mice, *Akodon* XY* females are both viable and fertile. The mechanisms involved in *Akodon* XY* female fertility are currently being explored (A. Vitullo, personal communication). Second, XY* female *Akodon* produce more eggs than can implant and therefore do not suffer from a

decrease in litter size due to the loss of YY* zygotes (Lizarralde *et al*. 1982; Espinosa & Vitullo 1996). Finally, XY* females produce more offspring than *Akodon* XX females over equal lifetimes (Hoekstra & Hoekstra 2000). These unique characteristics give *Akodon* the potential to maintain XY* females once they have evolved.

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APPENDIX A

List of species, field numbers, museum sources and general location of individuals used in reconstructing the *Akodon* phylogeny. Abbreviations for sources: University of Washington Burke Museum (UWBM), National Museum of Natural History (USNM), Museum of Southwestern Biology (MSB) and Museum of Vertebrate Zoology (MVZ).

species	number	source	locality
A. azarae	HEH373	UWBM	Argentina, P. Buenos Aires
	HEH391	UWBM	Argentina, P. Buenos Aires
A. molinae	HEH236	UWBM	Argentina, P. Mendoza
	HEH214	UWBM	Argentina, P. Mendoza
A. urichi	USNM560865	USNM	Venezuela, Amazonas
A. mollis	NK27682	MSB	Ecuador, Rio Tatahuazo
A. varius	NK21722	MSB	Bolivia, Rio Limon
A. bolviensis	JLP12055	MVZ	Peru, D. Puno
	JLP13063	MVZ	Peru, D. Puno
A. subfuscus	JLP13654	MVZ	Peru, D. Arequipa
	JLP14004	MVZ	Peru, D. Arequipa
A. torques	JLP11771	MVZ	Peru, D. Cusco
A. aerosus	JLP13444	MVZ	Peru, D. Cusco
A. a. baliolus	JLP13040	MVZ	Peru, D. Puno
A. kofordi	JLP12205	MVZ	Peru, D. Puno
A. puer	JLP12081	MVZ	Peru, D. Puno
A. juninensis	JLP13385	MVZ	Peru, D. Junin
A. mimus	JLP12264	MVZ	Peru, D. Puno
A. orophilus	JLP13433	MVZ	Peru, D. Junin.
A. cursor	JLP16278	MVZ	Paraguay, D. Caaguaza
Auliscomys pictus	JLP12929	MVZ	Peru, D. Puno

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