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The evolution of burrowing behaviour in deer mice (genus *Peromyscus*)

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The evolutionary history of most behaviours remains unknown. Here, we assay burrowing behaviour of seven species of deer mice in standardized environments to determine how burrowing evolved in this genus (*Peromyscus*). We found that several, but not all, species burrow even after many generations of captive breeding. Specifically, there were significant and repeatable differences in both the frequency of burrowing and burrow shape between species. Moreover, these observed species-specific behaviours resemble those reported in wild mice. These results suggest that there is probably a strong genetic component to burrowing in deer mice. We also generated a phylogeny for these seven species using characters from four mtDNA and two autosomal loci. Mapping burrowing behaviour onto this phylogeny suggests a sequence for how complex burrowing evolves: from small, simple burrows to long, multi-tunnel burrows with defined entrance and escape tunnels. In particular, the most 'complex' burrows of *P. polionotus* appear to be derived. These behavioural data, when examined in a phylogenetic context, show that even closely related species differ in their burrowing behaviours and that the most complex burrows probably evolved by the gradual accumulation of genetic change over time.

A major goal of evolutionary biology is to explain the appearance of species-specific behaviours. Specifically, do behaviours evolve through a few large steps or many small changes over time? Darwin (1859, 210) himself proposed a comparative approach to study behavioural evolution: if instincts evolve through intermediate forms, then, as he said, 'we ought to find in the collateral lines of descent some evidence of such gradations'. We investigated burrowing behaviour in several closely related species of deer mice to understand how burrowing behaviour evolved in the genus *Peromyscus*.

Differences in species-specific behaviours may reflect innate genetic differences, environmental influences, or often a combination of these factors. Characterizing genetic effects on behaviour is challenging for two reasons. First, many behaviours lack discrete, quantifiable features (Skinner 1966), which make them difficult to assess quantitatively and can hamper genetic and evolutionary analyses. Second, environmental factors that increase variation in behaviour can make behaviours difficult to characterize and, similarly, phenotypic plasticity can obscure evolutionarily interesting differences in behaviour (Clutton-Brock & Harvey 1984).

Burrow construction, however, provides an excellent model to study the origin and evolution of complex behaviours. Burrows serve a number of purposes, including predator avoidance, thermoregulation, food storage and facilitating social interaction and mating (Fleming & Brown 1975; Wolfe & Esher 1977; Ellison 1995; Ebensperger & Blumstein 2006). Differences between burrows may thus reflect an adaptive response to local environments. Moreover, although burrows are not bodily features, they can be viewed as traits subject to natural selection, or as 'extended phenotypes' (Dawkins 1999). Changes in burrow shape may also alter the organism's environment and thereby change the selective forces acting on a variety of traits. Under this scenario, burrowing presents a clear case of niche construction (Laland & Sterelny 2006). Finally, burrowing behaviour is amenable to comparative evolutionary analysis. While many behaviours leave no permanent record, burrowing, in contrast, leaves a physical trace that is easy to measure (Dawkins 1976; King 1980). In at least a few species, burrowing has been successfully studied under standardized laboratory conditions (Layne & Ehrhart 1970; Dawson et al. 1988; Bouchard & Lynch 1989). Together these features allow us to examine variation in burrow morphology in a common environment.

Fossorial and semifossorial lifestyles have originated independently in many animal groups, but we know almost nothing about how burrowing evolves within a clade. Molecular phylogenetics provides a powerful tool for testing hypotheses about the evolution of complex behaviours. For example, phylogenetic analyses of nest

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building (Winkler & Sheldon 1993), sociality (West-Eberhard 1996) and species-specific bird song (Price & Lanyon 2002) show that complex behaviours can evolve from simple behavioural precursors. Conversely, the loss of complex behaviours also has been demonstrated (e.g. Ryan & Rand 1995; Sturmbauer et al. 1996). Thus, studying variation in behaviour among closely related species in a phylogentic context can inform our understanding of how behaviours evolve.

Deer mice (genus *Peromyscus*) are a valuable group for studying the evolution of behaviour. This genus contains many recently diverged species that inhabit a wide range of environments and that show a number of habitat-specific behaviours, one of which is complex burrow-building. In particular, burrows of the oldfield mouse, *Peromyscus polionotus*, have been thoroughly described in a series of natural history studies (Sumner & Karol 1929; Hayne 1936; Rand & Host 1942; Ivey 1949). Oldfield mice build stereotyped burrows, complete with an entrance tunnel, nest chamber and an escape tunnel that extends to just below the soil surface (Sumner & Karol 1929). This specific burrow architecture is thought to minimize snake predation and provide refuge from avian predators in exposed habitats (Blair 1951; Wolfe & Esher 1977). Moreover, *P. polionotus* will produce these 'complex' burrows when placed in seminatural environments (Dawson et al. 1988). Thus, combining laboratory-based behavioural assays with a molecular phylogeny for *Peromyscus* species enables us to examine how this complex burrowing of *P. polionotus* may have evolved.

Here we examine seven captive-bred *Peromyscus* species in a standardized environment. We address two main questions. (1) How does burrowing vary within and among species? (2) How have species-specific differences in burrowing evolved when examined in a phylogenetic context? Overall, we document significant behavioural differences between *Peromyscus* species and find that 'complex' burrowing behaviour may evolve through the gradual accumulation of genetic change through time.

METHODS

Mouse Acquisition and Care

We tested mice from seven *Peromyscus* species (eight subspecies), all acquired from the *Peromyscus* Genetic Stock Center (University of South Carolina, Columbia, SC, U.S.A.). Each strain was descended from wild-caught ancestors that were bred in the laboratory for 13–58 years (depending on strain) without exposure to natural environments (Table 1). Before testing, we housed mice in 17.78 × 25.4 cm plastic cages containing wood shavings and nesting material, with no more than five mice of the same sex per cage. We housed animals at 22 °C with a 16:8 h light:dark cycle and provided standard rodent food and water ad libitum. We made behavioural observations on sexually mature animals that were between 90 and 350 days old. We weighed each individual after testing. The Institutional Animal Care and Use Committee of the University of California-San Diego approved our animal care standards and experiments, Protocol No. S0-310-7.

Standardized Environments

We assayed behaviour in four identical chambers (1.22 × 1.52 × 1.07 m) constructed from 0.5-inch (1.27 cm) PVC sheets; lids consisted of 0.25-inch (0.635 cm) metal grating attached to PVC frames. We filled the enclosures to two-thirds capacity with sandy loam soil and contoured the soil in each enclosure to form two surfaces: one at 0.85 m high and the other at 0.40 m high, connected by a slope of approximately 60° (Fig. 1; following Dawson et al. 1988). This contouring simulated natural variation in terrain. We attached water bottles to one chamber wall and placed approximately 10 g of food (sunflower seeds) in 2.0-inch^2 (5.08 cm^2) plastic trays on the lowest level of each enclosure. We also scattered seeds sparsely throughout the enclosure to encourage exploration. Finally, we provided one square of cotton nesting material (Ancare Corp., Bellmore, NY, U.S.A.) per trial.

Burrowing chambers were kept in a large room where the temperature was maintained between 15 °C and 30 °C (fluctuations were caused by seasonal changes in ambient temperatures). The chambers were exposed to ambient light with one exception; we covered enclosures at dusk to eliminate exposure to moonlight, which may affect activity level. We removed all faeces and nesting debris between trials, and then thoroughly rinsed and mixed the soil to maintain consistent moisture levels and reduce the influence of scent marking. We also scrubbed the enclosure walls with ethanol to minimize residual scents. By examining *Peromyscus* in a controlled environment, we aimed to minimize environmentally induced variation in behaviour.

Table 1
Geographical origin, years of captive breeding and natural burrowing behaviours (as reported in the literature) of the seven *Peromyscus* species tested in this study

Species (common name)	Original collecting site	Years in captivity	Burrowing behaviour in nature	Source
P. californicus (Californa mouse)	Santa Monica Mts, CA, U.S.A.	19	Inept at digging; nests under logs or in rock crevices; often inhabits abandoned woodrat dens	Merritt 1978
P. eremicus (cactus mouse)	Pima Co., AZ, U.S.A.	13	Poor digger; nests in rock heaps, stone walls and near mesquites; occasionally inhabits abandoned burrows	Lewis 1972; Veal & Caire 1979
P. melanophrys (plateau mouse)	Zacatecas, Mexico	28	No information	—
P. aztecus (Aztec mouse)	Michoacan, Mexico	20	Poorly described; nests in rock crevices and burrows at the base of trees	Baker 1968; Vasquez et al. 2001
P. leucopus (white-footed mouse)	Avery Co., NC, U.S.A.	21	Usually nests above ground in rock piles, logs and stumps, but also in excavated or abandoned underground burrows	Baker 1968; Lackey et al. 1985
P. maniculatus bairdii (prairie deer mouse)	Washtenaw Co., MI, U.S.A.	58	Constructs small burrows in soil but will also inhabit abandoned burrows	Eisenberg 1993
P. maniculatus sonoriensis (Sonoran deer mouse)	White Mtn., CA, U.S.A.	11	Poorly described; suggested to be similar to *P. m. bairdii*	Cahalane 1939
P. polionotus subgriseus (oldfield mouse)	Ocala National Forest, FL, U.S.A.	54	Skilled burrower; burrows usually found on sandy slopes; normally 4–6 burrows per home range; complex burrow design	Sumner & Karol 1929; Hayne 1936; Rand & Host 1942; Ivey 1949
*P. polionotus leucocephalus** (beach mouse)	Santa Rosa Island, FL, U.S.A.	18	Identical to *P. polionotus subgriseus*	Sumner & Karol 1929; Hayne 1936; Rand & Host 1942; Ivey 1949

* We report only anecdotal data for *P. p. leucocephalus*.

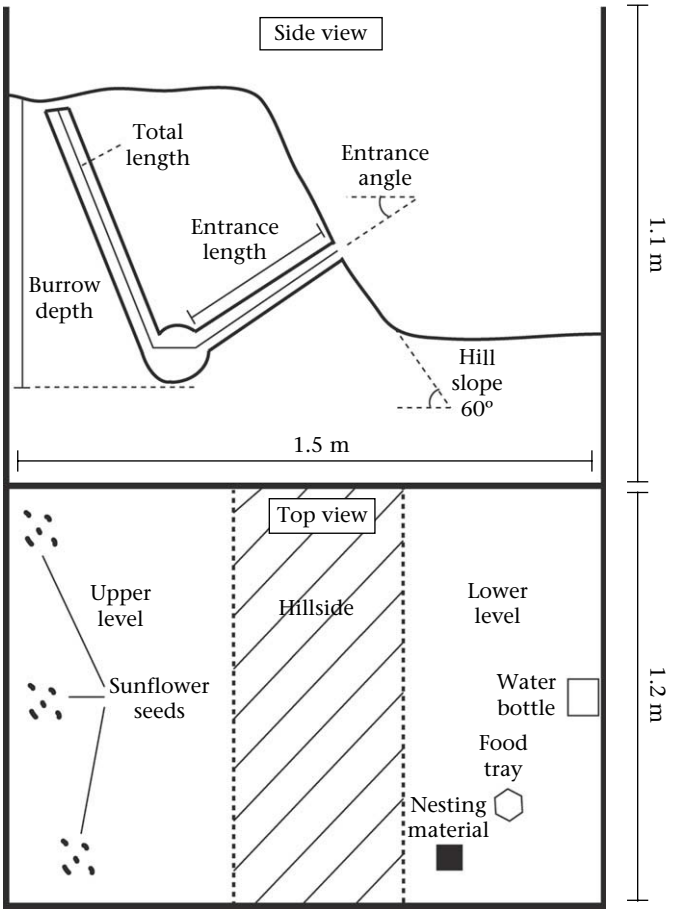

Figure 1. Diagram of standardized laboratory burrowing enclosure. We contoured the soil in each enclosure to create two levels, connected by a slope. Linear dimensions of the enclosure are provided. All quantitative measures of burrow shape are shown.

Behavioural Trials

Each trial consisted of three steps: (1) placing a single mouse in an enclosure between 1300 and 1700 hours Pacific Standard Time, (2) removing the animal after 48 h (giving each individual two full nights of activity) and (3) recording all burrowing-related alterations to the enclosure. To determine the repeatability of behaviours within individuals, each mouse underwent three consecutive trials. Unless otherwise noted, burrow measurements represent the average of all three trials. After each trial, we transferred mice to a new enclosure, allowing us to test for the effects of different enclosures on behaviour. Each round of trials included four trials conducted in parallel: one mouse in each of the four burrow chambers. We tested mice of only a single species and sex at any time.

Measuring Phenotypes

We inspected burrowing chambers after each 48 h trial. First, we made qualitative observations on whether there was no digging, some digging (e.g. shallow excavations or divots) or discrete burrows. We defined 'discrete burrows' as any excavation including both an entrance tunnel and a defined nest chamber. We calculated frequency of burrowing by dividing the number of trials in which discrete burrows were constructed by the total number of trials. Second, we measured the burrow itself. In trials with no discrete burrows, we measured the largest excavation (i.e. tunnel or divot). When burrows were constructed, we injected them with polyurethane filling foam (Hilti Corp., Schaan, Liechtenstein) that expands to fill the burrow cavity and then solidifies to create a permanent cast (Felthauser & McInroy 1983). Thus, each cast was a physical representation of an individual's burrowing behaviour. We measured the following burrow traits from each cast: depth (distance from surface to nest chamber), length of entrance tunnel, total length and angle of entrance (Fig. 1).

Phylogenetic Analysis

Because of conflicting hypotheses about the phylogenetic relationships of *Peromyscus* species (e.g. Carleton 1989; Bradley et al. 2007), we constructed a molecular phylogeny among the seven *Peromyscus* species whose burrowing we studied (Fig. 2). The phylogeny was based on sequences from four mitochondrial (*ND3*, *CO-III*, *ATPG* and *TRNG*) and two nuclear (*Mc1r* and *LCAT*) loci (2414 base pairs total) in three individuals from each species. We performed PCRs under standard conditions (Table 2) using previously published primer sequences (Robinson et al. 1997; Hoekstra et al. 2004). We edited and aligned gene sequences using the software Sequencher v4.2.2 (Gene Codes Corp., Ann Arbor, MI, U.S.A.). We observed congruence in substitution rates among genes using the partition homogeneity test implemented in PAUP* (Swofford 1999) and therefore concatenated all sequences. Topologies were generated in PAUP* (Swofford 1999) and MrBayes (Huelsenbeck & Ronquist 2001) using maximum likelihood and Bayesian algorithms, respectively, with a GTR + gamma model for substitution rates (determined with FINDMODEL, Los Alamos National Laboratories, Los Alamos, NM, U.S.A.). For the Bayesian analyses, we ran simulations for 3 million generations (burn-in of 750 000), after which the average standard deviation of split frequencies was less than 0.01. We also calculated bootstrapping support (1000 replicates of branch swapping) and posterior probabilities to assess confidence in the topology. We rooted the tree following previous studies (Carleton 1989; Turner & Hoekstra 2006).

Statistical Analyses

We tested the effect of body mass, age, temperature and enclosure on burrowing behaviour as well as differences in burrowing at the intra-individual, intraspecific and interspecific levels. To normalize the distribution of data points, burrow measurements were $\log(x+1)$ transformed and frequencies were arcsine transformed. We performed principal component (PC) analysis on burrow measurements, in which the longest burrow from each individual was considered. We then calculated all pairwise correlations between environmental factors (mass, animal age and temperature) and behaviour (frequency of burrowing, PC1, or angle of entrance) within each species. To account for the possibility that closely related species have similar traits that were not independently evolved, we also calculated interspecific correlations between mass, frequency of burrowing and PC1 using the independent contrast method implemented in the program CONTRAST (Felsenstein 2005), with branch lengths estimated from the phylogeny. We also used ANOVA to test for behavioural differences between species, and within species for enclosure and sex effects. Using Friedman's test for related samples, we compared an individual's behaviour across multiple trials. We then calculated repeatability of total burrow length across all three trials by dividing the among-individual variance by the sum of among- and within-individual variances (also known as the coefficient of intraclass correlation). Finally, we calculated the percentage of PC1 variation that segregates among species. All correlations, ANOVAs, related-sample tests and PCAs were performed in the SPSS v.11 statistics package (SPSS, Chicago, IL, U.S.A.).

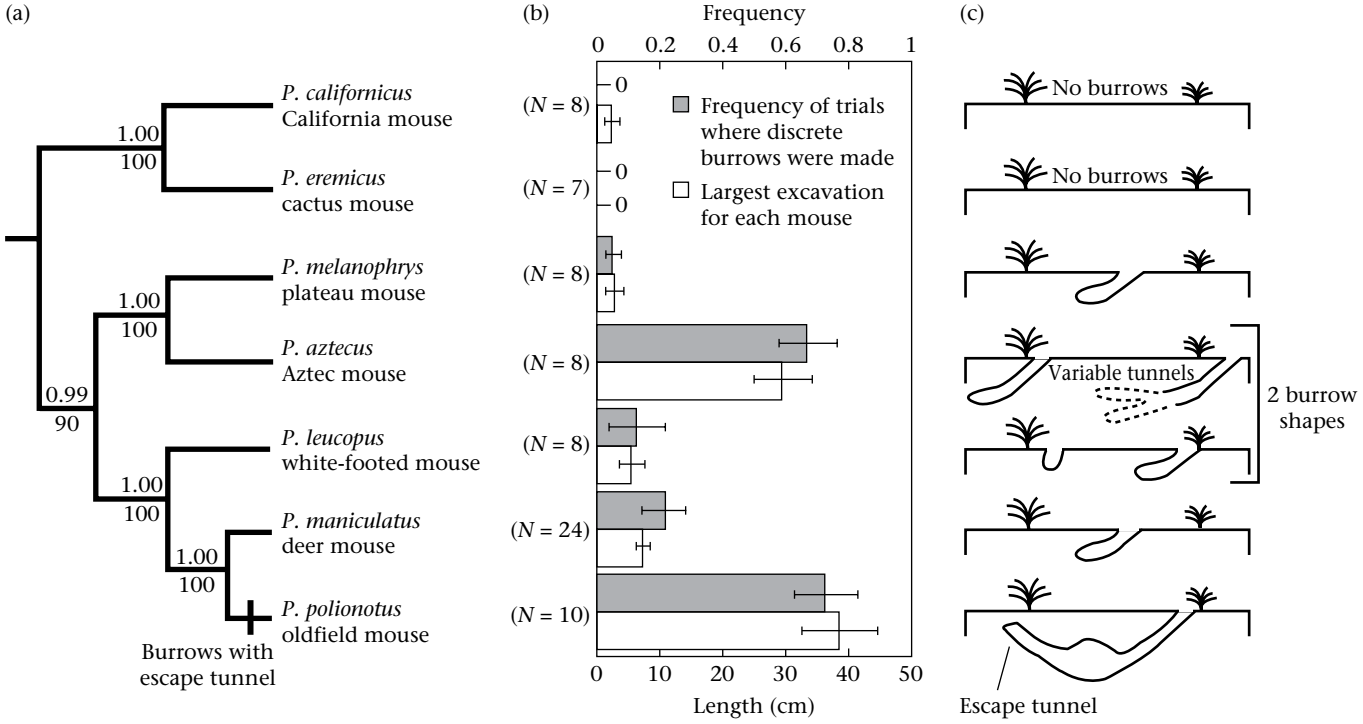

Figure 2. Phylogenetic analysis of burrowing behaviour in the genus *Peromyscus*. (a) Molecular phylogeny of seven species, representing eight taxa, in which three individuals were used for each taxon. Numbers above and below the nodes represent posterior probabilities and maximum likelihood bootstrap values (1000 replicates), respectively. Precise branch lengths are not shown. The origin of escape tunnels is indicated. (b) Quantitative measures of burrowing behaviour in each species. Bars represent species means (±SE) for: (1) frequency of trials (total of three trials per mouse) in which a discrete burrow was constructed and (2) largest burrow observed for each mouse. (c) The most commonly observed burrow shape of each species. *Peromyscus aztecus* and *P. leucopus* regularly produced two burrow shapes and the different burrows are shown.

RESULTS

Analysis of Behavioural Variation

We collected data from 73 mice (including males and females from all seven species) for five behavioural measures associated with burrowing: entrance length, depth, total length, entrance angle and frequency of burrowing (Table 3). Principal component analysis of burrow shape (entrance length, depth of burrow and total burrow length) collapsed these three variables into a single component (PC1) that explained 97% of the total variation. The PC1 weightings were: entrance length = 0.988, burrow depth = 0.987, and total burrow length = 0.990. Neither body mass, individual age, nor temperature were significantly correlated with PC1, frequency of burrowing or angle of entrance within species (correlations were not performed in species with fewer than two individuals burrowing), with two exceptions: in *P. maniculatus*, entrance angle was positively correlated with individual age (Pearson correlation: $r_{22} = 0.49$, $P = 0.02$) and PC1 was negatively correlated with temperature ($r_{20} = -0.50$, $P = 0.02$). We also found no significant behavioural differences between mice tested in the different standardized enclosures for any species.

Table 2
PCR conditions (extension time, annealing temperature and annealing time) used to amplify nuclear and mtDNA genes

Gene	Size (base pairs)	Extension time (s)	Annealing temperature (°C)	Annealing time (s)
Mc1r	772	120	56.0	60
Lcat	455	30	56.5	10
Nd3/Co3/Atpg/Trng	1184	60	42.0	45

The expected sizes of PCR products are listed.

Intra-individual Variation

Because each mouse was tested in three consecutive trials, we were able to compare burrowing behaviour across trials for each individual. We calculated the repeatability of total burrow length, as it was the only continuous variable measured in every trial. The repeatability of total burrow length varied among species (Table 3). While one species consistently did not burrow (*P. eremicus*: $r = 1.0$), the repeatability of burrow length ranged between $r = 0.14$ and 0.54 for the other six species. Only one species burrowed differently between trials (Friedman test for three related samples: $\chi^2_2 = 6.615$, $P = 0.037$): *P. polionotus* consistently produced significantly longer burrows in their final trial than in the first two trials.

Intraspecific Variation

To test for burrowing differences within species, we performed ANOVA between the two subspecies of *P. maniculatus*: *bairdii* and *sonoriensis*. We found no significant differences in any burrow traits between these two taxa. Within the subspecies *P. maniculatus sonoriensis*, however, we found a difference between the sexes: males sometimes built burrows with entrance tunnels and nest chambers (3 of 8 males), whereas females only built shallow divots, never burrows. This resulted in a significant difference between the sexes for PC1 (two-tailed *t* test: $t_{10} = 2.607$, $P = 0.026$). Sex-specific differences were not seen in any other species.

Interspecific Variation

Most of the variation in burrowing behaviour was between rather than within species. Statistical analyses showed significant differences between species for PC1 (ANOVA: $F_{6,72} = 30.7$,

Table 3
Summary of intraspecific data from burrowing trials

Peromyscus species	Sex	Sample size	Mass (g)	Age range (days)	Burrowing frequency (per 3 trials)	Total length (cm)*	Total length repeatability (per 3 trials)	Entrance length (cm)*	Burrow depth (cm)*	Entrance angle (degrees)*
P. californicus	Male	4	32.2 (1.4)	114–147	—	2.9 (1.9)	0.19	—	—	—
	Female	4	34.5 (1.9)		—	1.0 (0.0)		—	—	—
P. eremicus	Male	3	20.8 (0.4)	168–349	—	0.0 (0.0)	1.00	—	—	—
	Female	4	17.8 (0.4)		—	0.0 (0.0)		—	—	—
P. melanophrys	Male	4	36.0 (3.9)	110–173	—	0.3 (0.3)	0.25	—	—	—
	Female	4	32.9 (2.8)		0.08 (0.08)	4.9 (2.5)		5.5 (—)	14.5 (—)	66.0 (—)
P. aztecus	Male	4	35.1 (2.2)	99–139	0.75 (0.16)	32.0 (7.0)	0.14	22.1 (7.9)	17.9 (3.2)	28.0 (7)
	Female	4	43.6 (3.8)		0.58 (0.08)	26.9 (13.7)		16.0 (7.1)	17.9 (4.4)	26.0 (11)
P. leucopus	Male	4	16.8 (0.4)	76–108	0.17 (0.17)	5.4 (2.6)	0.54	4.0 (—)	10.5 (—)	35.0 (—)
	Female	4	17.4 (1.0)		0.08 (0.08)	5.5 (3.1)		9.5 (—)	8 (—)	15.0 (—)
P. maniculatus sonoriensis	Male	8	23.2 (1.1)	70–250	0.29 (0.13)	9.3 (2.4)	0.49	8.7 (1.0)	8.8 (1.8)	32.0 (3)
	Female	4	20.5 (2.0)		—	2.3 (1.1)		—	—	—
P. maniculatus bairdii	Male	7	17.3 (1.3)	70–165	0.29 (0.13)	9.6 (1.7)	0.24	7.6 (0.8)	9.1 (0.5)	60.0 (7)
	Female	5	17.5 (0.7)		0.13 (0.13)	6.9 (2.3)		9.0 (—)	14.0 (—)	40.0 (—)
P. polionotus subgriseus	Male	6	13.6 (0.3)	73–319	0.67 (0.17)	40.1 (9.3)	0.28	16.6 (2.7)	16.7 (3.0)	29.8 (5.7)
	Female	4	13.2 (0.4)		0.83 (0.10)	37.0 (7.0)		16.6 (2.8)	11.6 (1.2)	46.3 (3.8)

Data for each species are grouped by sex, and all values, except those for age, represent means (±SE). Dash (—) indicates that a group did not show the behaviour.
* Mean values when only the largest burrow was considered. Individuals that did not build a discrete burrow were excluded from calculations of entrance length, burrow depth and entrance angle.

$P < 0.001$), with 75% of the total variation in PC1 segregating among species. In post hoc comparisons between all species pairs, the mean PC1 for *P. polionotus* and *P. aztecus* differed significantly from all other species (Dunnett's T3, assumes unequal variance: $P < 0.01$). *Peromyscus polionotus* and *P. aztecus* burrows were longer than those of all other species; mean burrow length (based on each individual's largest burrow) was 41.9 cm for *P. polionotus* and 29.4 cm for *P. aztecus*. Moreover, *P. polionotus* was the only species that built 'complex burrows' (i.e. those having entrance tunnels, nest chambers (including nesting material) and escape tunnels; Fig. 2), with the exception of two individuals that produced burrows without escape tunnels. Some *P. aztecus* burrows had secondary tunnels radiating out from the nest (4 burrows out of 24 trials), but these were fundamentally different from *P. polionotus* escape tunnels, which always radiated towards the surface. Only three other species, *P. maniculatus*, *P. leucopus* and *P. melanophrys*, produced discrete burrows. However, these burrows were always small; the mean burrow length for each species (based on the largest burrow from only those individuals that produced burrows) ranged from 12.0 cm to 14.1 cm. *Peromyscus californicus* and *P. eremicus* never produced a discrete burrow, although they sometimes made shallow divots. There were no significant differences in angle of burrow entrance between species.

The mean frequency of burrow construction also varied significantly among species (ANOVA: $F_{6,72} = 10.4$, $P < 0.001$), ranging from 0 (neither *P. californicus* nor *P. eremicus* burrowed) to 0.73 in *P. polionotus* (Fig. 2). *Peromyscus polionotus* burrowed significantly more often than all species except *P. aztecus* (burrowing frequency = 0.67). However, two *P. polionotus* individuals burrowed in only one of the three trials (the same two mice that failed to produced a complex burrow). Two species, *P. melanophrys* and *P. leucopus*, rarely burrowed: in a total of 24 trials for each species, *P. melanophrys* built only one burrow and *P. leucopus* built only four burrows (two individuals each produced burrows in two of three trials).

Phylogenetic Patterns

The maximum-likelihood algorithm produced a single, best tree and the Bayesian analysis produced a high consensus tree; both had identical topologies with strong support (Fig. 2) that are consistent with the traditional morphology-based topology (Carleton 1989). Using independent contrasts and this topology, we found a significant correlation between burrowing frequency and burrow shape (Pearson correlation: $r_5 = 0.87$, $P = 0.01$; Fig. 3). We found no significant correlations between species means for mass and PC1, or between mass and burrowing frequency.

When we superimposed behavioural variables onto the molecular phylogeny, several patterns emerged (Fig. 2). The only two species that never burrowed, *P. californicus* and *eremicus*, clustered together on the phylogeny. However, most often closely related species differ in their behaviours. For example, species that burrow frequently and build large burrows have relatives that burrow less frequently and construct only small burrows: *P. polionotus* and *P. maniculatus* are sister species, but consistently produce distinct

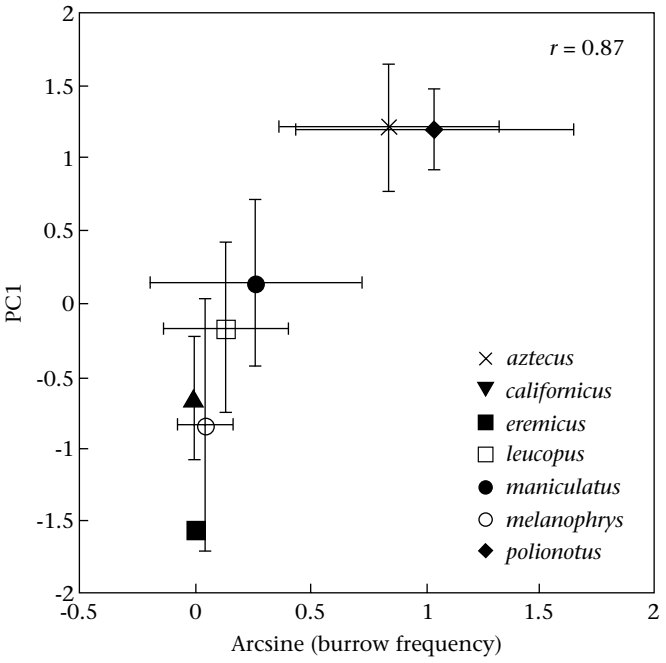

Figure 3. Interspecific correlation between burrowing frequency and burrow shape. All points represent species means (±SD). Positive correlation was significant at the $P = 0.05$ level.

burrows. Moreover, both *P. aztecus* and *P. polionotus* frequently build long burrows but are not each other's closest relatives. Finally, using this phylogeny, we find that the complex burrowing shown by *P. polionotus* is a derived trait.

DISCUSSION

While it is clear that burrowing has evolved independently in many groups of animals, it is not clear how burrowing behaviour evolves among closely related species. Our comparative study shows that burrowing behaviours vary considerably in *Peromyscus*. Although this represents only a partial sample of all the species in the genus and our evolutionary conclusions may change with additional sampling, the variation we observed in complexity suggests how burrowing may have evolved. Based on the phylogenetic pattern with large and complex burrows being derived, we suggest the following evolutionary sequence: there was a propensity to dig, existing tunnels were elongated, additional tunnels were added and then the orientation of tunnels was fixed (e.g. angles of entrance and escape tunnels). This progression suggests that complex burrowing probably evolved through the gradual accumulation of genetic changes, some of which were probably driven by natural selection.

After more than 20 generations of captive breeding, without the opportunity to burrow, most species still show distinctive burrowing behaviours, suggesting that burrowing may be an innate behaviour (Table 2). We cannot comment on whether mice behave differently in other environments, but we intend to test the effect of different substrates on burrowing in future experiments. Despite the large differences in size between species, we did not find an association between species size and either burrow shape or burrowing frequency. Moreover, we did not find an association between either age or temperature and burrowing within or between species (with the exception of within *P. maniculatus*). Thus, differences in size, age and temperature alone do not explain the behavioural variation we observed among species (but we cannot rule out the influences of other unexamined variables). Although captive breeding may affect *Peromyscus* behaviour (McPhee 2003), the behaviours of our seven species seen in the laboratory closely match behaviours described in the wild. The lack of evidence for learning and the close match between burrows in our enclosures and in nature imply a genetic contribution to *Peromyscus* burrowing.

Although burrowing behaviour was highly repeatable within individuals (our estimates of repeatability were conservative because we included all trials in our calculations, regardless of whether a mouse burrowed), some mice showed variation in their burrows across trials. However, only one species showed consistent differences across the three test trials: *P. polionotus* constructed significantly larger burrows in their final trial than in either of the first two. In this species it appears that either burrow length increases through experiential learning or mice produce longer burrows after they have acclimated to the testing environment. Future studies can further examine this hypothesis in two ways: (1) by increasing the number of trials per individual and (2) by rearing mice in an environment where they can burrow.

Within species, we found only minimal variation among individuals (either between sexes or subspecies). For example, we identified only a single sex-specific burrowing difference: in *P. m. sonoriensis*, females never burrowed, whereas males occasionally burrowed. However, the small sample size of females ($N = 4$) may mean that the behaviour, rather than being absent in females, is rare and was simply not observed in this study. Assaying a larger sample of *P. sonoriensis* females should clarify whether a true sex difference exists in this subspecies. In preliminary experiments, we also found that two subspecies of *P. polionotus* constructed burrows that were statistically indistinguishable. These observations support the idea that most variation in burrowing accrues between species.

Although some behavioural variation occurs within species, between-species differences explained most of the total variation in burrow shape and burrowing frequency. This species-specific variation has several implications. First, since some species that consistently construct long burrows have close relatives that construct short, simple burrows or sometimes do not burrow at all, it appears that even large burrowing differences can evolve over short periods. Second, we found a strong correlation between the frequency of burrowing and the size of the burrows, suggesting a common mechanism linking the propensity to burrow and burrow size. Moreover, although we were unable to sample every species, the observed burrowing variation can be reduced to four general states: no burrowing, infrequent construction of small burrows, frequent construction of large burrows with irregular shapes, and frequent construction of large, regularly shaped burrows. The most complex evolutionary state that we observed was the derived behaviour (i.e. long and deep burrows with escape tunnels) of *P. polionotus*. Based on our data, the construction of escape tunnels is unique to *polionotus* and probably evolved from a shallow burrow similar to those made by its closest relatives, *P. maniculatus* and *P. leucopus*. The uniformity and complexity of *P. polionotus* burrow shape implies that it may be the product of natural selection, perhaps an adaptive response to predation in a habitat with sparse cover (Blair 1951; Wolfe & Esher 1977). *Peromyscus aztecus* appears to have independently evolved long burrows with secondary tunnels, and it lives in environments with sparse cover, providing anecdotal evidence that exposed habitats may drive the evolution, or maintenance, of long burrows.

Overall, our results show that *Peromyscus* burrowing can be easily quantified, that there is probably a genetic component to interspecific burrowing differences, and that burrowing behaviour varies among even closely related species. Most notably, two sister species, *P. maniculatus* and *P. polionotus*, show distinct and repeatable differences in their burrow construction. Because the unique burrow of *P. polionotus* appears derived, this suggests a gain in behavioural complexity caused by novel genetic change(s). Moreover, although allopatric in the wild, in laboratory environments, *P. maniculatus* and *P. polionotus* are interfertile (Watson 1942). A small-scale backcross experiment suggests that this interspecific behavioural difference may be controlled by a few loci (Dawson et al. 1988; J. N. Weber & H. E. Hoekstra, unpublished data). These results, combined with the recent publication of a linkage map for *Peromyscus* (Steiner et al. 2007), give us a unique opportunity to identify the genetic changes underlying the evolution of this mammalian behaviour.

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