Molecular Phylogenetics and Evolution
Vol. 11, No. 2, March, pp. 213–221, 1999
Article ID mpev.1998.0581, available online at http://www.idealibrary.com on IDEAL®

Cytochrome *b* Phylogeny of North American Hares and Jackrabbits (*Lepus,* Lagomorpha) and the Effects of Saturation in Outgroup Taxa

Kenneth M. Halanych,*,[1,2] John R. Demboski,†,‡,[2] Bettine Jansen van Vuuren,*
David R. Klein,§ and Joseph A. Cook†,‡

**Department of Zoology and Entomology, University of Pretoria, Pretoria 0002, South Africa; †Biochemistry and Molecular Biology Program and §Alaska Cooperative Fish & Wildlife Research Unit, University of Alaska Fairbanks, Fairbanks, Alaska 99775-7020; and ‡University of Alaska Museum, 907 Yukon Drive, Fairbanks, Alaska 99775-6960*

Received August 25, 1997; revised July 17, 1998

Jackrabbits and hares, members of the genus *Lepus,* comprise over half of the species within the family Leporidae (Lagomorpha). Despite their ecological importance, potential economic impact, and worldwide distribution, the evolution of hares and jackrabbits has been poorly studied. We provide an initial phylogenetic framework for jackrabbits and hares so that explicit hypotheses about their evolution can be developed and tested. To this end, we have collected DNA sequence data from a 702-bp region of the mitochondrial cytochrome *b* gene and reconstructed the evolutionary history (via parsimony, neighbor joining, and maximum likelihood) of 11 species of *Lepus,* focusing on North American taxa. Due to problems of saturation, induced by multiple substitutions, at synonymous coding positions between the ingroup taxa and the outgroups (*Oryctolagus* and *Sylvilagus*), both rooted and unrooted trees were examined. Variation in tree topologies generated by different reconstruction methods was observed in analyses including the outgroups, but not in the analyses of unrooted ingroup networks. Apparently, substitutional saturation hindered the analyses when outgroups were considered. The trees based on the cytochrome *b* data indicate that the taxonomic status of some species needs to be reassessed and that species of *Lepus* within North America do not form a monophyletic entity. © 1999 Academic Press

***Key Words: Lepus;* hare; jackrabbit; cytochrome *b;* Lagomorpha; phylogeny; saturation; rooting.**

INTRODUCTION

In comparison to other mammalian orders, the Lagomorpha (rabbits, hares, and pikas) is not diverse and contains numerous monotypic genera. One exception to this generalization is the genus *Lepus,* which has a worldwide distribution and comprises 24 to 30 species of jackrabbits and hares (Corbet and Hill, 1980; Flux and Angermann, 1990; Hoffmann, 1993). The long historical association of several species of *Lepus* with humans has fostered their economic and ecological importance. A lack of morphological variation noted between species compared to within species (Angermann, 1983; Flux and Flux, 1983; Corbet, 1986; Flux and Angermann, 1990) has resulted in numerous taxonomic revisions throughout the last two centuries (reviewed by Flux, 1983). Recent biochemical and molecular analyses have begun to address the evolution and historical biogeography of *Lepus* but have been limited to analyses of only a few species (Robinson and Osterhoff, 1983; Pérez-Suárez *et al.*, 1994).

We examine the evolutionary history of 11 currently recognized species of *Lepus,* with an emphasis on North American taxa. Using sequence data from the mitochondrial cytochrome *b* gene, we provide a phylogenetic framework for hares that can be expanded and tested by subsequent studies. The data indicate that the three arctic species of hares (*L. arcticus, L. timidus,* and *L. othus*) are probably a single species and that North American *Lepus* is not monophyletic.

Based on previous molecular analyses of leporids (Halanych and Robinson, in press) and paleontological data (Dawson, 1981), *Oryctolagus cuniculus* (the European rabbit) and *Sylvilagus* (cottontails) are the most appropriate outgroups to *Lepus.* However, because of the problems of substitutional saturation, the inclusion of these taxa caused inconsistencies to arise between different phylogenetic reconstruction methods (i.e., parsimony, neighbor joining, and maximum likelihood). When the ingroup was examined as an unrooted network (i.e., with no outgroups in the analysis), these three methods of phylogenetic analysis were congruent, suggesting that the resultant phylogeny is robust under a variety of assumptions.

[1] Present address: Biology Department, MS 33, Woods Hole Oceanographic Institute, Woods Hole, MA 02543.

[2] Both J.R.D. and K.M.H. contributed equally to this work.

1055-7903/99 $30.00

MATERIALS AND METHODS

Data Collection

Nineteen specimens, including the two outgroups (*Oryctolagus cuniculus* and *Sylvilagus audubonii*), were analyzed (Table 1). The available karyotypic (Robinson *et al.*, 1983; for *L. callotis* see González and Cervantes, 1996) and fossil dentition (C. Ramos, pers. com.) data support *Lepus* monophyly. Because some species of *Lepus* have large geographic ranges (Flux and Angermann, 1990) and due to the current state of *Lepus* taxonomy, more than one representative per species was employed in several cases.

Total genomic DNA was extracted from fibroblast cells, museum skins, blood, or tissue. Extractions for tissue and blood samples followed a NaCl extraction protocol modified from Miller *et al.* (1988), fibroblast samples employed a standard phenol–chloroform extraction procedure (Maniatis *et al.*, 1982), and DNA from the museum specimens was isolated with a Chelex 100 protocol (Walsh *et al.*, 1991). In all cases, the PCR amplification followed standard protocols (Palumbi, 1996) and cycling profiles were an initial denaturation at 94°C for 1–2 min, 30–40 cycles of amplification (denaturation at 94°C for 15–30 s, oligonucleotide annealing at 50–53°C for 15–30 s, and extension at 72°C for 45–60 s), followed by a final extension at 72°C for 3–5 min. The following oligonucleotide primers were used in amplification and sequencing: H15149, H15494, and L14841 (Kocher *et al.*, 1989); H14724 and L151162 (Pääbo and Wilson, 1988); and MVZ04, MVZ05, MVZ14, and MVZ23 (Smith and Patton, 1993). *Lepus* specific primers, LEPUS 16 (5′-AAATAGRAARTACCATTCAGGYTTRAT-3′) and LEPUS 37 (5′-TATACTTAYYTAGAAACTTGRA-3′), were modified by J.R.D. from Smith and Patton (1993).

PCR products were purified with either a 30% PEG 3350/1.5M NaCl protocol modified from Kusukawa *et al.* (1990; by J.R.D.) or a gel purification using the Cleanmix system from Talent, Inc. (by K.M.H.). PCR products were cycle sequenced by J.R.D. and sequences were collected on an ABI 373 autosequencer using protocols given in Lessa and Cook (1998). For K.M.H., the use of biotinylated primers during PCR and streptavidin-coated beads (Dynal Company) provided single-stranded DNA for sequencing (Sequenase v2.0; US Biochemical). Fragments were visualized using acrylamide gel electrophoresis and standard audioradiographic techniques (Hillis *et al.*, 1990). For all taxa, sequences were verified by sequencing in both directions for multiple PCR products.

Phylogenetic Analyses

An alignment of the cytochrome *b* data was produced with the program Clustal W (Thompson *et al.*, 1994)

TABLE 1

Taxa Used in Phylogenetic Analysis

Species	Common name	Collection locality	DNA source	Tissue no.	GenBank access. no.
Lepus alleni	Antelope jackrabbit	Navojoa, Sonora, Mexico—subspecies: *alleni*	Tissue	NK 6589[a]	AF010156
		Isla Tiburéon, Sonora, Mexico—subspecies: *tiburonensis*	Tissue	NK 4504	AF010157
L. americanus	Snowshoe or varying hare	Fairbanks, Alaska, USA	Tissue	UAM 20297[b]	AF010152
		Booth Bay, Maine, USA	Fibroblast	TJR[c]	U58932[d]
L. arcticus	Arctic hare	Nansens Land, N.E. Greenland	Blood	AF 20299	AF010153
L. californicus	Black-tailed jackrabbit	Bernalillo County, New Mexico, USA	Tissue	NK 21994	AF010160
		Lubbock, Texas, USA	Fibroblast	TJR	U58933[d]
L. callotis (1)	White-sided jackrabbit	Hildago County, New Mexico, USA	Tissue	NK 3800	AF010158
(2)		Hildago County, New Mexico, USA	Tissue	NK 5014	AF010159
L. capensis	Cape hare	Cape Province, South Africa	Fibroblast	TJR	U58934[d]
L. europaeus	European or brown hare	Ven, Sweden	Tissue	AF 21115	AF010161
		Sibbarp, Sweden	Tissue	AF 21116	AF010162
L. othus	Alaskan hare	Chevak, Alaska, USA	Museum skin	UAM 10870	AF010154
L. saxatilis	Scrub hare	Kimberly, South Africa	Tissue	—	AF009731
L. timidus	Mountain, blue, or snow hare	Chukotsk Peninsula, Russia	Museum skin	UAM 23260	AF010155
		Aberdeen, Scotland, U.K.	Fibroblast	TJR	AF009732
L. townsendii	White-tailed jackrabbit	Cache Valley, Utah, USA	Fibroblast	TJR	AF009733
Oryctolagus cuniculus	European rabbit		—	—	U07566[e]
Sylvilagus audubonii	Desert cottontail	Carbon County, Wyoming, USA	Fibroblast	TJR	U58938[f]

[a] NK—Museum of Southwestern Biology, University of New Mexico.
[b] UAM or AF—University of Alaska Museum.
[c] TJR—Fibroblast cultures housed by T. J. Robinson.
[d] Halanych and Robinson, 1999.
[e] Irwin and Árnason, 1994.
[f] Halanych and Robinson, 1997.

and verified based on amino acid sequence. No insertions or deletions were present in the alignment. The aligned data set has been submitted to TREEBASE (http://phylogeny.harvard.edu/treebase).

The PAUP software package (version 3.1.1; Swofford, 1993) was used for parsimony analyses. Additionally, we used PHYLIP version 3.5 (Felsenstein, 1993) for neighbor joining (DNAdist and Neighbor programs) and MacClade version 3.0 (Maddison and Maddison, 1992) to determine various character statistics and tree lengths. Maximum-likelihood estimates were carried out with both the DNAml program of PHYLIP and fastDNAml (Olsen *et al.*, 1994). All parsimony and neighbor-joining bootstrap values reported here were based on 1000 iterations and, in the case of parsimony bootstraps, the general heuristic search algorithm was employed. For neighbor joining, a Kimura 2-parameter model using the empirical Ti/Tv ratio and a γ correction (Jin and Nei, 1991) was employed. The γ correction ($\alpha = 0.5$) was empirically determined following the method of Sullivan *et al.* (1995). Neighbor-joining bootstraps were carried out with the Seqboot and Consense programs of PHYLIP. Due to computation time, maximum-likelihood bootstraps consisted of 100 iterations using fastDNAml.

Although our analyses were based on the empirically derived Ti/Tv ratio, we also employed ratios of 1:1 and 10:1 to determine the robustness of our results. These two particular values were arbitrarily chosen. The empirical ratio (Ti/Tv = 5.67:1) was determined by using the "state changes and stasis" option of MacClade to count the average number of Ti and Tv events on 1000 equiprobable random trees (Halanych, 1996; Halanych and Robinson, 1997). For ease of computation, the value 5.6:1 was used in subsequent analyses. This method of estimation is easily biased by saturation effects, and when the estimation procedure included the two outgroup taxa, the ratio fell to 3.55:1. Thus, to more accurately approximate the model of evolution for the ingroup, the outgroups (which exhibit saturation, see below) were excluded from this calculation.

Oryctolagus cuniculus is the sister taxon to the *Lepus* clade and thus the most appropriate outgroup (Halanych and Robinson, 1999; see also Dawson, 1981). However, *Sylvilagus audubonii* was also used to avoid possible biases of using a single outgroup.

Several workers (Irwin *et al.*, 1991; Graybeal, 1993; Meyer, 1994) have noted the pronounced difference in evolutionary rates between synonymous and nonsynonymous coding positions within the cytochrome *b* gene. In this data set, 93% of the variable characters were synonymous coding positions (i.e., third codon positions and first-position leucines). Due to the small number of nonsynonymous sites that were parsimony informative (eight) within the ingroup, we did not analyze these two classes of data separately.

RESULTS

The cytochrome *b* data set consisted of 702 aligned positions of which 219 (31.2%) were variable and 153 (21.8%) were parsimony informative. When only the ingroup was considered, there were 168 (23.9%) variable sites and 134 (19.1%) informative positions. The g_1 statistic indicated that the data contained significantly more signal than expected at random ($g_1 = -0.897$ with outgroups and -0.745 excluding outgroups when using the empirical Ti/Tv ratio; Hillis and Huelsenbeck, 1992). Calculated g_1 values were relatively robust even when various combinations of taxa, including taxa at the well-supported tips, were excluded.

Analysis of nucleotide composition revealed fewer guanines than expected at random: 23.5% guanine in the first position, 14.5% in the second, and 3.2% in the third. Similar values have been reported for other mammals (Irwin *et al.*, 1991; Matthee and Robinson, 1997). Base composition biases (Irwin *et al.*, 1991) for the codon positions were 0.058, 0.195, and 0.293, respectively.

Saturation

To assess the degree of saturation due to multiple substitutions in the data, the Ti/Tv ratio was plotted against the Jukes–Cantor distance for all pairs of taxa (Fig. 1). The Jukes–Cantor distance was used to facilitate comparison with previously published data (e.g., Adkins and Honeycutt, 1994). The most striking feature in Fig. 1 is that the pairwise comparisons fall into three main clusters. The cluster on the right, $d > 0.14$, corresponds to all of the intergeneric comparisons. All of these comparisons had a Ti/Tv ratio near 1:1 and are approaching a Jukes–Cantor distance of 0.2. For intergeneric comparisons of leporids, such values are indicative of saturation at synonymous positions (Halanych and Robinson, 1999). The second cluster between distances of 0.07 and 0.13 represented most, but not all, interspecific comparisons within the ingroup. The cluster on the left ($d < 0.05$) represented the intraspecific comparisons and comparisons within either the arctic clade or the western American clade (see below). Similar results were obtained under a variety of models that correct for multiple nucleotide substitutions.

Phylogenetic Reconstructions

The branch and bound search program of PAUP with the empirical Ti/Tv ratio of 5.6:1 found 2 most parsimonious trees (Fig. 2). While the 10:1 weighting found the same 2 trees, equal weighting (1:1) found 20 equally parsimonious trees, including the 2 from the empirical weighting. The equally weighted trees had a length of 421 steps and a C.I. of 0.644 (C.I. = 0.568 when excluding uninformative characters). Two geographic clades in particular are evident in the reconstructed topolo-

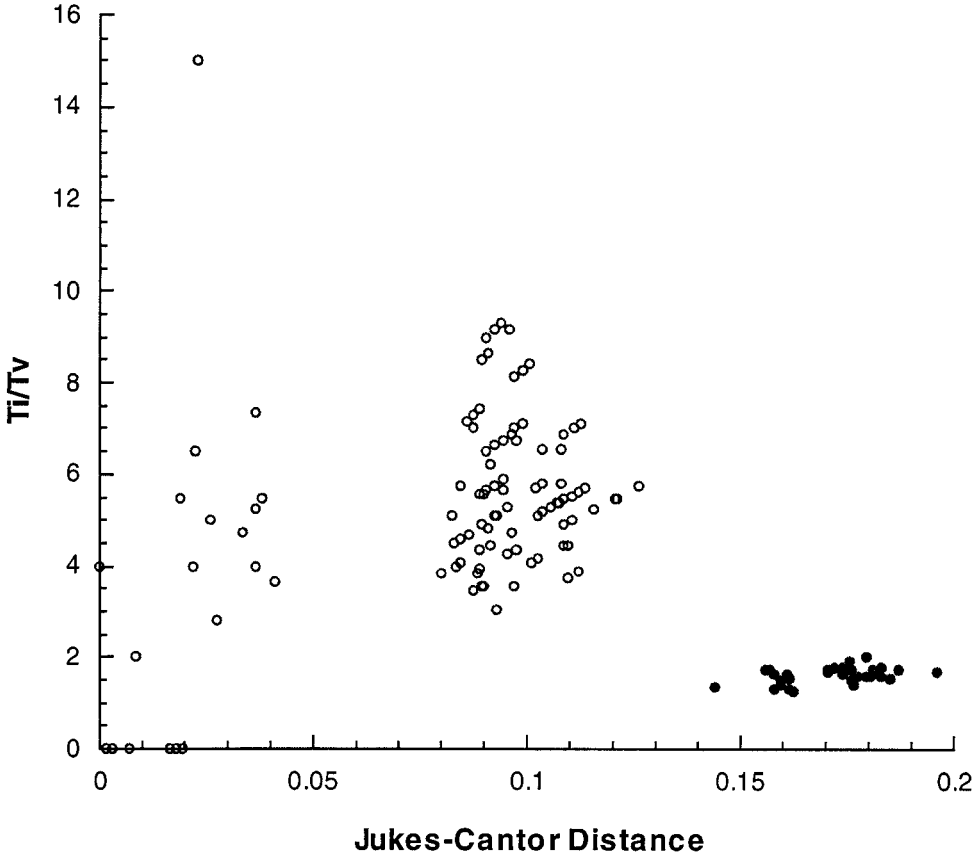

FIG. 1. Substitutional saturation plot. The Ti/Tv ratio was plotted against the Jukes–Cantor distance of all pairwise comparisons of taxa. Empty symbols are pairwise comparisons within the ingroup whereas filled symbols are ingroup to outgroup comparisons. The positions of the ingroup to outgroup comparisons on the plot are suggestive of saturation (see Halanych and Robinson, 1999).

gies: a clade from the high northern latitudes that included *L. townsendii* (named the arctic clade) and a clade from middle and western North America (named the western American clade).

Neighbor joining found a slightly different tree (Fig. 3a; compare deep branches) than the parsimony analyses. A Kimura 2-parameter model with the empirically derived γ correction ($\alpha = 0.5$) and Ti/Tv value of 5.6:1 was used for the NJ analysis. The absolute and corrected distances are shown in Table 2. The deeper relationships suggested by maximum likelihood (Fig. 3b) were different from either the NJ or the parsimony trees. The ML reconstructions employed fastDNAml with the empirical Ti/Tv ratio and 10 iterations of the jumble option. According to the Kishino–Hasegawa (1989) test, there is no significant difference between the empirical parsimony trees, NJ tree, or ML tree when the outgroups were included. Due to software limitations, all possible combinations of the unresolved polytomy in the parsimony tree were explored. Bootstrap values are shown in the relevant figures.

Effects of Distant Outgroups

Several features in the phylogenetic analyses of *Lepus* suggested that the outgroups used are sufficiently distant from the ingroup taxa to be a source of considerable noise in the data. The most obvious of these features was the relatively long terminal branch lengths of the outgroup compared to the ingroup branches. Additionally, the assessment of substitutional saturation revealed that intergeneric comparisons have apparently suffered multiple hits at synonymous positions. Lastly, bootstrap values at the deeper nodes of the trees (i.e., close to the root) were very low, indicating that levels of homoplasy may be high. Admittedly, the outgroups also seem to add phylogenetic signal, probably at the node defining the ingroup; this addition of signal presumably accounts for the more negative g_1 when the outgroups are included. The outgroup problem is exacerbated by the fact that there are no extant taxa which are more closely related to *Lepus*, yet unambiguously outside of the ingroup (see Dawson, 1958, 1981; Hibbard, 1963; Chapman and Flux, 1990; Halanych and Robinson, 1999).

Due to the lack of more appropriate extant outgroups, we explored unrooted networks of our ingroup taxa to determine if the deep internal branches were potentially biased by the saturation effects of the outgroups. When the outgroups were excluded, all

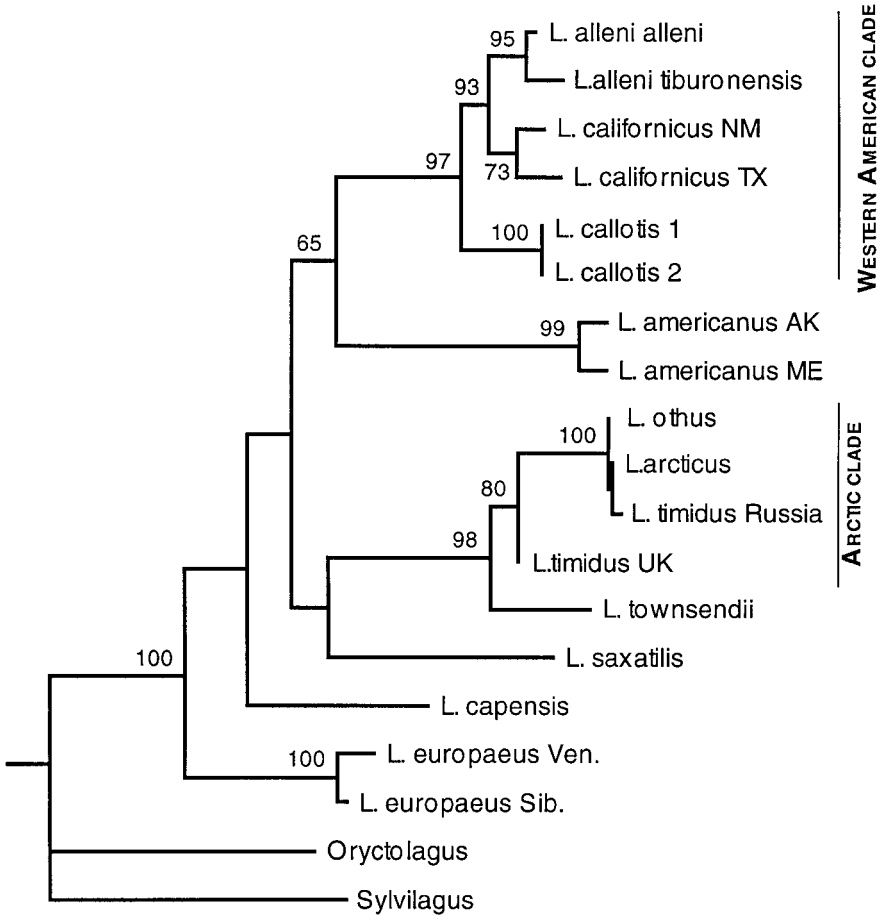

FIG. 2. Results of the parsimony analyses based on the cytochrome *b* sequences. One of two most parsimonious trees obtained, using the branch and bound algorithm of PAUP and the empirical Ti/Tv ratio of 5.6:1, is shown. The other tree differed in that the *L. othus–L. arcticus–L. timidus* (Russia) clade formed an unresolved polytomy. The branch lengths shown are drawn proportional to the amount of change along the branch (assuming Ti and Tv are weighted equally to accurately represent the number of changes along the branch). Bootstrap values obtained from 1000 iterations using a heuristic search (empirical weighting) are shown next to the relevant nodes. If no value is shown, the node was supported in <50% of the iterations.

three reconstruction methods (MP, NJ, and ML) found the same tree (Fig. 4) with the exception of the branching order within the *L. othus–L. arcticus–L. timidus* (Russia) clade. A branch and bound search using the empirical Ti/Tv ratio recovered two most parsimonious trees; one clustered *L. arcticus–L. timidus* (Russia) and the other was an unresolved polytomy for these taxa plus *L. othus*. Both NJ and ML analyses grouped *L. arcticus–L. timidus* (Russia). However, the NJ bootstrap weakly supported a *L. othus–L. arcticus* association. For all three methods, branch lengths between *L. othus*, *L. arcticus*, and *L. timidus* (Russia) were very short (supported by ≤2 nucleotide substitutions), and the association of *L. americanus* with the western American clade was well supported. Interestingly, when the outgroups were included, none of the reconstruction methods produced a topology consistent with this unrooted network.

Although the use of an outgroup is standard practicein phylogenetic analysis, we argue that, in this case, the inclusion of the most appropriate outgroups (*Sylvilagus* and *Oryctolagus*) hindered our reconstructions due to branch attraction (e.g., Hendy and Penny, 1989; Miyamoto and Boyle, 1989; Wheeler, 1990; Maddison *et al.*, 1992). We conclude that the tree in Fig. 4 is more probable for two reasons. First, when the outgroups are excluded, all three methods recover the same topology, thereby supporting the suggestion that this result is robust across a variety of assumptions. Second, the outgroups are known to be saturated (a source of noise) when compared to ingroup taxa.

However, exclusion of the outgroups during phylogeny reconstruction raises another problem. Where should the tree be rooted? By assuming constant evolutionary rates, we used midpoint rooting to determine the direction of evolution on the tree (Fig. 4). This assumption was checked using the rate heterogeneity test of Muse and Gaut (1994). No significant differences across lineages were revealed.

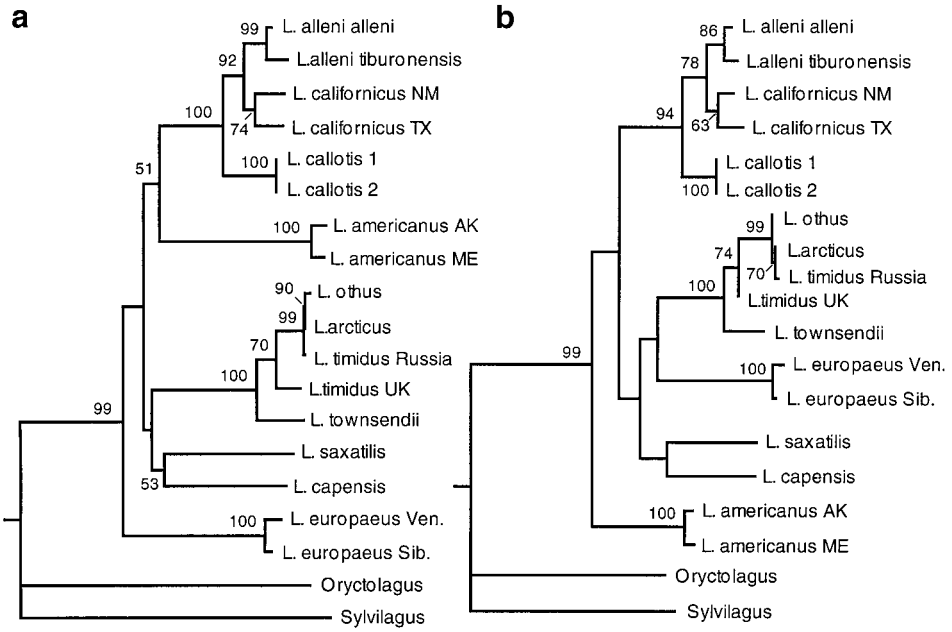

FIG. 3. (a) The neighbor-joining topology produced using a Kimura 2-parameter model with the empirical γ correction (α = 0.5) to account for among-site rate variation (Jin and Nei, 1991) and the empirical Ti/Tv ratio (5.6:1). (b) The maximum likelihood tree reconstructed using fastDNAml (Olsen *et al.*, 1994) with a Ti/Tv ratio of 5.6:1. Neighbor-joining bootstrap values (1000 iterations) and maximum likelihood bootstrap values (100 iterations) are shown on the topologies next to the relevant node, unless the node was supported in <50% of the bootstrap iterations.

DISCUSSION

Lepus Evolution

This study is an initial estimate of evolutionary relationships within *Lepus* which should be expanded and refined by subsequent studies. In all of the analyses herein, bootstrap values tend to be strong for the tips of the tree, whereas the basal nodes are more poorly supported. This lack of support at deeper levels may be due to sampling biases; there is an incomplete representation of *Lepus* species from Asia, Europe, Africa, and Mexico. As these taxa are added, many of the deeper branches may be resolved, but due to the possibility of a rapid radiation within *Lepus*, these

TABLE 2

Distances Based on Cytochrome *b* Data

	Lal-al	*Lal*-ti	*Lam*-AK	*Lam*-ME	*Lar*	*Lca*-NM	*Lca*-TX	*Lcl*-1	*Lcl*-2	*Lcp*	*Leu*-Si	*Leu*-Ve	*Lot*	*Lsa*	*Lti*-UK	*Lti*-Ru	*Lto*	*Ocu*	*Sau*
Lal-a1	—	0.009	0.093	0.093	0.091	0.023	0.022	0.037	0.037	0.087	0.106	0.108	0.092	0.093	0.091	0.089	0.086	0.180	0.201
Lal-ti	6	—	0.102	0.102	0.094	0.026	0.028	0.037	0.037	0.094	0.114	0.116	0.096	0.093	0.098	0.093	0.093	0.183	0.211
Lam-AK	59	64	—	0.009	0.117	0.091	0.097	0.094	0.094	0.115	0.121	0.126	0.119	0.114	0.113	0.119	0.118	0.174	0.204
Lam-ME	59	64	6	—	0.116	0.088	0.094	0.098	0.098	0.115	0.127	0.133	0.117	0.107	0.113	0.117	0.112	0.174	0.205
Lar	58	60	73	72	—	0.094	0.088	0.095	0.095	0.098	0.101	0.102	0.001	0.101	0.018	0.001	0.037	0.205	0.196
Lca-NM	16	18	58	56	60	—	0.019	0.034	0.034	0.097	0.115	0.117	0.096	0.100	0.101	0.096	0.096	0.178	0.208
Lca-TX	15	19	61	59	56	13	—	0.042	0.042	0.092	0.106	0.108	0.090	0.102	0.095	0.090	0.086	0.184	0.202
Lcl-1	25	25	60	62	61	23	28	—	0.000	0.097	0.108	0.113	0.097	0.096	0.099	0.094	0.094	0.160	0.192
Lcl-2	25	25	60	62	61	23	28	0	—	0.097	0.108	0.113	0.097	0.096	0.099	0.094	0.094	0.160	0.192
Lcp	55	59	71	71	62	61	58	61	61	—	0.095	0.101	0.100	0.083	0.095	0.100	0.093	0.196	0.180
Leu-Si	67	71	75	78	64	72	66	68	68	60	—	0.007	0.102	0.112	0.092	0.102	0.108	0.179	0.191
Leu-Ve	68	72	78	81	65	73	67	71	71	63	5	—	0.104	0.114	0.094	0.104	0.110	0.181	0.193
Lot	59	61	74	73	1	61	57	62	62	63	65	66	—	0.103	0.017	0.003	0.039	0.207	0.198
Lsa	59	59	71	67	64	63	64	61	61	53	70	71	65	—	0.087	0.103	0.100	0.195	0.176
Lti-UK	56	60	68	68	12	62	58	61	61	58	57	58	11	54	—	0.020	0.023	0.212	0.196
Lti-Ru	57	59	74	73	1	61	57	60	60	63	65	66	2	65	13	—	0.039	0.205	0.198
Lto	55	59	73	70	25	61	55	60	60	59	68	69	26	63	15	26	—	0.224	0.200
Ocu	101	102	99	99	113	100	102	92	92	109	101	102	114	109	112	113	121	—	0.201
Sau	111	115	112	112	109	114	110	107	107	101	107	108	110	100	105	110	110	112	—

Note. Above diagonal—distances calculated with the Kimura 2-parameter model with an empirical γ correction (α = 0.5) and Ti/Tv ratio of 5.6:1. Below diagonal—absolute distances. Taxa are designated by the first letter of their generic name and the first two letters of their species name, except for *L. callotis* (*Lcl*) and *L. capensis* (*Lcp*).

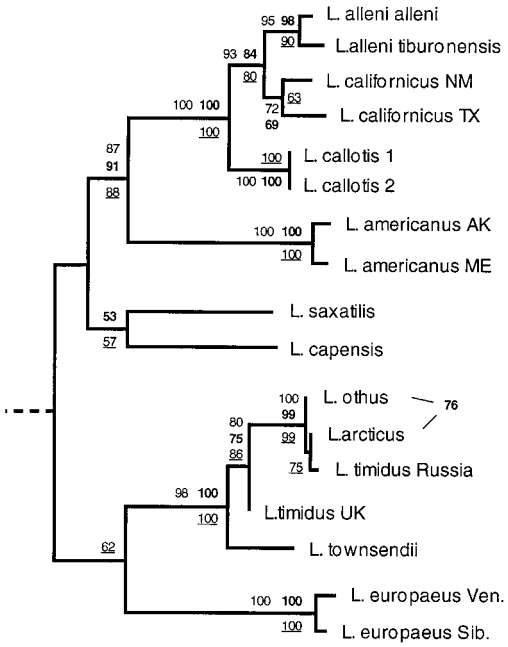

FIG. 4. The unrooted network (i.e., outgroups excluded) produced using a Ti/Tv ratio of 5.6:1 for all methods of phylogeny reconstruction used herein. Parsimony found two best trees which differed in the resolution of the arctic clade. The dashed line at the base of the tree is a tentative midpoint rooting which assumes equal rates of nucleotide change across the tree. Bootstrap values are shown unless the node was supported in <50% of the bootstrap iterations (parsimony, 1000 iterations = light face; neighbor joining, 1000 iterations = bold face; maximum likelihood, 100 iterations = underlined). Although the NJ analysis based on the observed data yielded this tree, a NJ bootstrap supported the tree in which *L. othus* and *L. arcticus* clustered 76% of the time. This is indicated on the tree to the right of the taxon names.

relationships may not be tractable with cytochrome *b*. (Halanych and Robinson, 1999, have shown that cytochrome *b* contained limited phylogenetic signal for intergeneric lagomorph relationships.)

Two species groups were consistently recovered by our analyses, a western American clade and an arctic clade (including *L. townsendii*). Our results support previous groupings based on morphology (Nelson, 1909; Gureev, 1964) and allied *L. townsendii* to recognized arctic species. The distances among the *L. californicus* samples (approximately 0.02) and between *L. alleni* and *L. californicus* (approximately 0.025) suggest that relationships within the western American clade may be more complex than our limited sampling indicates. Greater geographic breadth of sampling for these species, and the inclusion of the other Mexican species (*L. flavigularis* and *L. insularis*), may further refine the evolutionary relationships within the western American clade.

Short branch lengths within the arctic clade were repeatedly recovered with all reconstruction methods employed. Not surprisingly, the taxonomic status of these species (*L. timidus, L. arcticus,* and *L. othus*) has been a long-standing controversy (Baker *et al.,* 1983). Those who recognize three species of arctic hares consider the Eurasian populations to be *L. timidus,* the Alaskan populations to be *L. othus,* and the Greenland and Canada populations to be *L. arcticus* (Flux and Angermann, 1990; Hoffmann, 1993), whereas others suggest a single holarctic population exists that should be recognized as *L. timidus* (see Baker *et al.,* 1983).

Given that no distances greater than 0.02 were observed within the arctic clade (Table 2), the cytochrome *b* data support the interpretation of a single circumpolar species. Within this clade the Scottish representative of *L. timidus* was notably different, confirming Baker *et al.*'s (1983) morphological recognition of that population. Among *L. arcticus* (Greenland), *L. othus* (Alaska), and *L. timidus* (Russia), genetic distances of less than 0.001 were observed. This is an order of magnitude less variability than normally ascribed to distinct mammalian species based on cytochrome *b* data (e.g., Sudman and Hafner, 1992; Dragoo *et al.,* 1993; Groves and Shields, 1996). Admittedly, larger sample sizes are required to understand the variation and genetic structure of these populations. Nelson (1909) and more recently Baker *et al.* (1983) and C. Ramos (pers. com.; morphological data) have noted support for the close relationships within the arctic clade.

Lepus Evolution and Phylogeography

The jackrabbits and hares comprise the most speciose and widespread leporid genus. Based on fossils, Hibbard (1963) proposed that *Lepus* first arose in North America, implying that hares radiated to other continents. Unfortunately, given the biogeographic representation of species in our study, it is not possible to confirm or refute this hypothesis. However, the data provide a clear indication that North American *Lepus* are not a monophyletic clade to the exclusion of other hares; *L. townsendii, L. arcticus,* and *L. othus* are in a clade separate from the remaining North American taxa. The placement of Old World taxa at the base of, and within, the arctic clade suggests that some hares invaded North America secondarily, perhaps via an Asian-American land connection (i.e., Beringia).

In contrast, *L. americanus,* which ranges across the high latitudes of the United States, Canada, and Alaska, is more closely related to taxa from the southwest United States and Mexico (*L. alleni, L. californicus,* and *L. callotis*) than to the arctic clade. While this association was not strongly supported in the analyses which included the outgroups, all three methods found strong bootstrap support in the unrooted network (Fig. 4).

One of the more striking results of our phylogeny is the deep divergence between species pairs that hybridize in the wild (*L. timidus* and *L. europaeus;* Thulin *et al.* 1997; also see Flux, 1983, concerning *L. californicus*

and *L. townsendii*). Hybridization between more distantly related species argues that isolation mechanisms (e.g., geographic, behavioral, or ecological) may be driving speciation within *Lepus.* In fact, some workers (Robinson *et al.*, 1983; Azzaroli Puccetti *et al.*, 1996) have indicated that the lack of chromosomal diversity within *Lepus* points to mechanisms of speciation that do not invoke the chromosomal models suggested for other mammals (Reig, 1989; Dannelid, 1991).

ACKNOWLEDGMENTS

T. J. Robinson provided instrumental help with this manuscript. Our research was supported by an Open Research Programme grant from the South African Foundation for Research Development to T. J. Robinson and a National Biological Service (NBS) grant to D. R. Klein. Anonymous reviewers provided valuable criticism of this manuscript. J.R.D. thanks L. A. Ruedas for insightful discussions. The following people were instrumental in providing specimens: H. Erasmus (*L. capensis*), C.-G. Thulin (*L. europaeus*), and T. L. Yates of the Museum of Southwestern Biology. We also thank C. Ramos for sharing unpublished data. J.R.D. contributed to this work while conducting dissertation research at The University of Alaska Fairbanks and was supported by NBS Research Work Order No. 13, through the Alaska Cooperative Fish and Wildlife Research Unit. K.M.H. contributed to this work while holding a postdoctoral fellowship at the University of Pretoria, and his support from the Foundation for Research Development and the University of Pretoria is gratefully acknowledged.

REFERENCES

Adkins, R. M., and Honeycutt, R. L. (1994). Evolution of the primate cytochrome *c* oxidase subunit II gene. *J. Mol. Evol.* **38:** 215–231.

Angermann, R. (1983). The taxonomy of Old World *Lepus*. *Acta Zool. Fennica* **174:** 17–21.

Azzaroli Puccetti, M. L., Corti, M., Scanzani, A., Civitelli, M. V., and Cappana, E. (1996). Karyotypes of two endemic species of hare from Ethiopia, *Lepus habessinicus* and *L. starcki* (Lagomorpha, Leporidae). A comparison with *L. europaeus*. *Mammalia* **60:** 223–230.

Baker, A. J., Eger, J. L., Peterson, R. L., and Manning, T. H. (1983). Geographic variation and taxonomy of arctic hares. *Acta Zool. Fennica* **174:** 45–48.

Chapman, J. A., and Flux, J. E. C. (1990). Introduction and overview of the Lagomorphs. *In* "Rabbits, Hares and Pikas, Status Survey and Conservation Action Plan" (J. A. Chapman and J. E. C. Flux, Eds.), pp. 1–6. IUCN, Gland, Switzerland.

Corbet, G. B. (1986). Relationships and origins of European lagomorphs. *Mammal Rev.* **16:** 105–110.

Corbet, G. B., and Hill, J. E. (1980). "A World List of Mammalian Species," Comstock, London.

Dannelid, E. (1991). The genus *Sorex* (Mammalia, Soricidae)—distribution and evolutionary aspects of Eurasian shrews. *Mammal Rev.* **21:** 1–20.

Dawson, M. R. (1958). Later Tertiary Leporidae of North America. *Univ. Kans. Paleontol. Contrib. Vertebrata* **(Art. 6):** 1–75.

Dawson, M. R. (1981). Evolution of the modern lagomorphs. *In* "Proceedings of the World Lagomorph Conference" (K. Myers and C. D. MacInnes, Eds.), pp. 1–8. Univ. of Guelph Press, Guelph.

Dragoo, J. W., Bradley, R. D., Honeycutt, R. L., and Templeton, J. W. (1993). Phylogenetic relationships among the skunks: A molecular perspective. *J. Mammal. Evol.* **1:** 255–267.

Felsenstein, J. (1993). PHYLIP (phylogeny inference package), version 3.5c. Department of Genetics, Univ. of Washington, Seattle, Washington.

Flux, J. E. C. (1983). Introduction to taxonomic problems in hares. *Acta Zool. Fennica* **174:** 7–10.

Flux, J. E. C., and Angermann, R. (1990). The hares and jackrabbits. *In* "Rabbits, Hares and Pikas, Status Survey and Conservation Action Plan" (J. A. Chapman and J. E. C. Flux, Eds.), pp. 61–94. IUCN, Gland, Switzerland.

Flux, J. E., and Flux, M. M. (1983). Taxonomy and distribution of East African hares. *Acta Zool. Fennica* **174:** 41–43.

González, F. X., and Cervantes, F. A. (1996). Karyotype of the white-sided jackrabbit (*Lepus callotis*). *Southwest. Nat.* **41:** 93–95.

Graybeal, A. (1993). The phylogenetic utility of cytochrome *b:* Lessons from bufonid frogs. *Mol. Phylogenet. Evol.* **2:** 256–269.

Groves, P., and Shields, G. (1996). Phylogenetics of the Caprinae based on cytochrome *b* sequence. *Mol. Phylogenet. Evol.* **5:** 467–476.

Gureev, A. A. (1964). Zaitzeobrazniye (Lagomorpha). *Fauna SSSR.* **3:** 276 pp.

Halanych, K. M. (1996). Testing hypotheses of chaetognath origins: Long branches revealed by 18S rDNA. *Syst. Biol.* **45:** 223–246.

Halanych, K. M., and Robinson, T. J. (1997). Phylogenetic relationships of cottontails (*Sylvilagus,* Lagomorpha): Congruence of cytogenetic and 12S rDNA Data. *Mol. Phylogenet. Evol.* **7:** 294–302.

Halanych, K. M., and Robinson, T. J. (1999). The utility of cytochrome *b* and 12S rDNA data for phylogeny reconstruction of leporid (Lagomorpha) genera. *J. Mol. Evol.* **48:** 369–379.

Hendy, M. D., and Penny, D. (1989). A framework for the quantitative study of evolutionary trees. *Syst. Zool.* **38:** 297–309.

Hibbard, C. (1963). The origin of the P3 pattern of *Sylvilagus, Caprolagus, Oryctolagus,* and *Lepus. J. Mammal.* **44:** 1–15.

Hillis, D. M., and Huelsenbeck, J. P. (1992). Signal, noise, and reliability in molecular phylogenetic analysis. *J. Hered.* **83:** 189–195.

Hillis, D. M., Larson, A., Davis, S. K., and Zimmer, E. A. (1990). Nucleic Acids III: Sequencing. *In* "Molecular Systematics" (D. M. Hillis and C. Moritz, Eds.), pp. 318–370. Sinauer, Sunderland, MA.

Hoffmann, R. S. (1993). Order Lagomorpha. *In* "Mammal Species of the World: A Taxonomic and Geographic Reference" (D. E. Wilson and D. M. Reeder, Eds.), 2nd ed., pp. 807–827. Smithsonian Inst. Press, Washington, DC.

Irwin, D. M., and Árnason, U. (1994). Cytochrome *b* gene of marine mammals: Phylogeny and evolution. *J. Mammal. Evol.* **2:** 37–55.

Irwin, D. M., Kocher, T. D., and Wilson, A. C. (1991). Evolution of the cytochrome *b* gene of mammals. *J. Mol. Evol.* **32:** 128–144.

Jin, L., and Nei, M. (1991). Relative efficiencies of the maximum-parsimony and distance-matrix methods of phylogeny construction for restriction data. *Mol. Biol. Evol.* **8:** 356–365.

Kishino, H., and Hasegawa, M. (1989). Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *J. Mol. Evol.* **29:** 170–179.

Kocher, T. D., Thomas, W. K., Meyer, W. K., Edwards, S. V., Pääbo, S., Villablanca, F. X., and Wilson, A. C. (1989). Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. USA* **86:** 6196–6200.

Kusukawa, N., Uemori, T., Asada, K., and Kato, I. (1990). Rapid and reliable protocol for direct sequencing of material amplified by the polymerase chain reaction. *Biotechniques* **9:** 66–72.

Lessa, E., and Cook, J. A. (1998). The molecular phylogenetics of tuco-tucos (genus *Ctenomys,* Rodentia; Octodontidae) suggests an early burst of speciation. *Mol. Phylogenet. Evol.* **9:** 88–99.

Maddison, D. R., Ruvolo, M., and Swofford, D. L. (1992). Geographic

origins of human mitochondrial DNA: Phylogenetic evidence from control region sequences. *Syst. Biol.* **41:** 111–124.

Maddison, W. P., and Maddison, D. R. (1992). MacClade: Analysis of phylogeny and character evolution, version 3. Sinauer, Sunderland, Massachusetts.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). "Molecular Cloning. A Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Matthee, C. A., and Robinson, T. J. (1997). Molecular phylogeny of the springhare, *Pedetes capensis*, based on mitochondrial DNA sequences. *Mol. Biol. Evol.* **14:** 20–29.

Meyer, A. (1994). Shortcomings of the cytochrome *b* gene as a molecular marker. *TREE* **9:** 378–380.

Miller, S. A., Dykes, D. D., and Polesky, H. F. (1988). A simple salting procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* **16:** 215.

Miyamoto, M. M., and Boyle, S. M. (1989). The potential importance of mitochondrial DNA sequence data to eutherian mammal phylogeny. *In* "The Hierarchy of Life" (B. Fernholm, K. Bremer, and H. Jörnvall, Eds.), pp. 437–450. Elsevier, Amsterdam.

Muse, S. V., and Gaut, B. S. (1994). A likelihood approach for comparing synonymous and nonsynonymous nucleotide substitution rates, with application to the chloroplast genome. *Mol. Biol. Evol.* **11:** 715–724.

Nelson, E. W. (1909). The rabbits of North America. *North Am. Fauna* **29:** 1–314.

Olsen, G. J., Matsuda, H., Hagstrom, R., and Overbeek, R. (1994). fastDNAml: A tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comput. Appl. Biosci.* **10:** 41–48.

Pääbo, S., and Wilson, A. C. (1988). Polymerase chain reaction reveals cloning artifacts. *Nature* **334:** 387–388.

Palumbi, S. R. (1996). Nucleic acids II: The polymerase chain reaction. *In* "Molecular Systematics" (D. M. Hillis, C. Mortiz, and B. K. Mable, Eds.), 2nd ed., pp. 205–248, Sinauer, Sunderland, MA.

Pérez-Suárez, G., Palacios, F., and Boursot, P. (1994). Speciation and paraphyly in western Mediterranean hares (*Lepus castroviejoi, L. europaeus, L. grantensis,* and *L. capensis*) revealed by mitochondrial DNA phylogeny. *Biochem. Genet.* **32:** 423–436.

Reig, O. A. (1989). Karyotypic repatterning as one triggering factor in cases of explosive speciation. *In* "Evolutionary Biology of Transient Unstable Populations" (A. Fontevilla, Ed.), pp. 246–289. Springer-Verlag, Berlin.

Robinson, T. J., and Osterhoff, D. R. (1983). Protein variation and its systematic implications for the South African Leporidae (Mammalia: Lagomorpha). *Animal Blood Groups Biochem. Genet.* **14:** 139–149.

Robinson, T. J., Elder, F. F. B., and Chapman, J. A. (1983). Karyotype conservatism in the genus *Lepus* (order Lagomorpha). *Can. J. Genet. Cytol.* **25:** 540–544.

Smith, M. F., and Patton, J. L. (1993). The diversification of South American murid rodents: Evidence from mitochondrial DNA sequence data for the akodontine tribe. *Biol. J. Linn. Soc.* **50:** 149–177.

Sudman, P. D., and Hafner, M. S. (1992). Phylogenetic relationships among Middle American Pocket Gophers (Genus *Orthogeomys*) based on mitochondrial DNA sequences. *Mol. Phylogenet. Evol.* **1:** 17–25.

Sullivan, J., Holsinger, K. E., and Simon, C. (1995). Among-site rate variation and phylogenetic analysis of 12S rRNA in Sigmodontine rodents. *Mol. Biol. Evol.* **12:** 988–1001.

Swofford, D. L. (1993). PAUP: Phylogenetic analysis using parsimony, version 3.1.1. Computer program distributed by Illinois Natural History Survey, Champaign, IL.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22:** 4673–4680.

Thulin, C.-G., Jaarola, M., and Tegelström, H. (1997). The occurrence of mountain hare mitochondrial DNA in wild brown hares. *Mol. Ecol.* **6:** 463–467.

Walsh, P. S., Metzger, D. A., and Higuchi, R. (1991). Chelex 100 as a medium for simple extraction of DNA from PCR-based typing from forensic material. *Biotechniques* **19:** 506–513.

Wheeler, W. C. (1990). Nucleic acid sequence phylogeny and random outgroups. *Cladistics* **6:** 363–368.