Molecular Systematics of New World Gopher, Bull, and Pinesnakes (Pituophis: Colubridae), a Transcontinental Species Complex

Javier A. Rodríguez-Robles* and José M. De Jesús-Escobar†

*Museum of Vertebrate Zoology and Department of Integrative Biology, University of California, Berkeley, California 94720-3160; and †Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology, University of California, Berkeley, California 94720-3204

Received October 14, 1998; revised June 17, 1999

Pituophis melanoleucus (gopher, bull, and pinesnakes) is among the most widely distributed polytypic species complexes in North America, with most authors recognizing from a single transcontinental species (the melanoleucus complex, composed of 15 subspecies) to four (monotypic and polytypic) species. We used mitochondrial gene sequences from the two middle American species, P. deppei and P. lineaticollis, and from 13 subspecies from most of the range of the melanoleucus complex to test various phylogenetic hypotheses for Pituophis. Maximum parsimony and maximum likelihood methods identified the same major clades within Pituophis and indicated that two segments of the melanoleucus complex, the lodingi-melanoleucus-mugitus eastern pinesnake clade and the affinis-annectens-bimaris-catenifer-deserticola-sayi-ruthveni-vertebralis clade from central and western United States and northern Mexico, represent divergent, allopatric lineages with no known intergradation zone. We recognize each of these two groupings as a different species. Our data also indicate that some ruthveni are more closely related to sayi than to other ruthveni. Nonetheless, ruthveni is an allopatric taxon diagnosable from its closest relatives by a combination of morphometric characters, and because it is likely that at least some of these traits are independent and genetically inherited, we interpret this as evidence that ruthveni has attained the status of independent evolutionary lineage, despite the fact that it retains strong genetic affinities with sayi. The endemic Baja Californian gopher snakes (bimaris and vertebralis) are considered by some taxonomists as a different species, P. vertebralis, but we discovered that these serpents belong to two different clades and hence we do not agree with the recognition of P. vertebralis as presently defined. In summary, we believe that three distinct species are included in the melanoleucus complex, Pituophis melanoleucus (sensu stricto), P. cateni- fer, and P. ruthveni, and that their recognition better represents the evolutionary diversity within this species complex.

Key Words: biogeography; Colubridae; mitochondrial DNA; phylogenetics; Pituophis; snakes; species boundaries; subspecies.

INTRODUCTION

The question as to the number of species included in this genus [Pituophis] is a difficult one to decide. (Cope, 1900, p. 866)

Boundaries between species and subspecies have received considerable attention from systematists (Ball and Avise, 1992; Cracraft, 1983; McKitrick and Zink, 1988; Patton and Smith, 1994; Smith et al., 1997; Wüster and Thorpe, 1992) and have often generated instructive and sometimes intense discussions (Avise and Wollenberg, 1997; Collins, 1992; Dowling, 1993; Edwards, 1954; Frost and Hillis 1990; Frost et al., 1992; Highton, 1998; Inger, 1961; Wake and Schneider, 1998; Wiens, 1982; Wilson and Brown, 1953). During the “molecular systematics revolution” of the last three decades (Avise, 1994; Hillis et al., 1996; Soltis et al., 1998), genetic data have sometimes contradicted previous ideas about species integrity or taxonomic distinctions that were based largely on morphological descriptions and thus led authors to reject named subspecies, recognize new ones, or elevate some to the species level (e.g., Alcobendas et al., 1996; Cicero, 1996; García-Moreno and Fjeldså, 1999; Good and Wake, 1992; Johnson, 1995; Mitthapala et al., 1996; Shaffer and McKnight, 1996; Wüster and Thorpe, 1994; Zamudio and Greene, 1997; Zamudio et al., 1997; Zink et al., 1997). Determining which populations warrant taxonomic recognition contributes to our understanding of the evolution of biodiversity and has significant, practical implications, e.g., for sociology (Keita, 1993) and conservation policies (Avise, 1989; Geist, 1992; Greene, 1994; O’Brien and Mayr, 1991; Sites and Crandall, 1997; Walker et al., 1998; Zhu et al., 1998).

Evolutionary relationships of transcontinental taxa...
have rarely been studied in detail (e.g., Bernatchez and Wilson, 1998). Taxa with such broad geographic ranges provide an excellent opportunity to investigate the practical issues in naming population assemblages. Snakes of the colubrid genus *Pituophis* Holbrook, 1842 are among the most widely distributed polytypic species complexes in North America. Commonly known in English as gopher, bull, and pinesnakes and in Spanish as "alicantes," "cincuates," "coralillos," and "mazacuates," these serpents range from the Pacific to the Atlantic coast of the United States (US) and from southwestern Canada to central Guatemala and the tip of the Baja California peninsula in Mexico (Figs. 1 and 2). Species of *Pituophis* are nonvenomous constrictors; they occur in a wide variety of habitats, including deserts, sandhills, prairies, shrublands, and forests (Degenhardt et al., 1996; Palmer and Braswell, 1995; Sweet and Parker, 1990); they attain body sizes in the wild of up to 2.7 m; they exhibit notable variation in snout morphology (Knight, 1986) and reproductive biology (Fitch, 1985; Palmer and Braswell, 1995; Reichling, 1990; Zuppalorti et al., 1983); and they may be Batesian mimics of rattlesnakes (Kardong, 1980; Sánchez-Herrera et al., 1981; but see Sweet, 1985).

Despite the fact that *Pituophis* has figured prominently in the anatomical, ecological, ethological, and herpetoculturist literature, the genus has had a contentious taxonomic history, which we briefly review as background for our systematic study of these snakes.

The monophyly of *Pituophis* is strongly supported by at least two unique morphological characters, an enlarged epiglottal keel and a laryngeal septum (Cope, 1891; White, 1884; Young et al., 1995), which together produce the unusual defensive hissing made by *Pituophis* (Martin and Huy, 1971; Young et al., 1995), as well as by phylogenetic analyses of mtDNA sequences of the genus and its closest relatives (i.e., *Arizona*, *Bogertophis*, *Cemophora*, *Elaphe*, *Lampropeltis*, *Rhinechilus*, and *Senticolis*; Rodríguez-Robles and De Jesús-Escobar, 1999). In these analyses the monophyly of *Pituophis* was supported by very high (>96%) bootstrap values in maximum parsimony trees.

Beyond the issue of the monophyly of the genus, there has been little agreement among different authors about the number of species and subspecies recognized within *Pituophis*. The most common arguments presented can be summarized in five main theses.

1. The most taxonomically conservative view regarding the number of species in the *Pituophis melanoleu-
cus species complex (hereafter, the melanoleucus complex) in southwestern Canada, the US, and northern Mexico recognizes a single, polytypic species, *P. melanoleucus*, composed of 15 subspecies, including four taxa endemic to islands off the west coast of California and Baja California (Conant, 1956; Dowling, 1958; Smith and Kennedy, 1951; Fig. 1). This taxonomic arrangement is followed in three widely used field guides to amphibians and reptiles from the US (Behler, 1995; Conant and Collins, 1991; Stebbins, 1985), as well as in the Catalogue of American Amphibians and Reptiles (Sweet and Parker, 1990).

(2) Some authors (e.g., Fugler, 1955; Holman, 1996; Klauber, 1947; Wright and Wright, 1957) have at least suggested that the melanoleucus complex is a composite taxonomic unit consisting of two distinct species, *Pituophis melanoleucus* (sensu stricto, including the subspecies *lodingi*, *melanoleucus*, and *mugitus*) in the eastern US and *P. catenifer* (including the subspecies *affinis*, *annectens*, *bimaris*, *catenifer*, *coronalis*, *deserti cola*, *fuliginatus*, *insulanus*, *pumilus*, *sayi*, and *vertebralis*) in the central and western US, northern Mexico, and offshore islands. The Louisiana pinesnake, *ruthveni*, has been assigned to either *P. melanoleucus* (sensu stricto; Stull, 1940; Wright and Wright, 1957) or *P. catenifer* (Fugler, 1955).

(3) The Cape gopher snake, *vertebralis*, has sometimes been claimed to be a distinct species (*Pituophis vertebraalis*; e.g., Cope, 1900; Grismer, 1994; Stull, 1940; Sweet, 1984).

(4) Based on a phenetic analysis of 14 morphometric characters and on its geographic distribution, Reichling (1995; see also Collins, 1991) argued for recognizing *ruthveni* as a distinct species, *Pituophis ruthveni*, therefore effectively implying the recognition of *P. melanoleucus* (sensu stricto) and *P. catenifer* as well.

(5) The taxonomic status of the middle American gopher snakes (Fig. 2), *Pituophis deppei* and *P. lineaticollis*, each with two named subspecies, has been fairly stable since Duellman’s (1960) review. Nevertheless, Dixon et al. (1962) questioned the validity of the two subspecies of *P. deppei* (*P. d. deppei* and *P. d. jani*), whereas Conant (1965) suggested that intergradation or introgression occurred between *P. deppei* and *P. m. affinis* in northern Mexico, perhaps implying that *P. deppei* was not a valid species, a suggestion with which Morafka (1977) agreed.

The taxonomic status of the various forms of *Pituophis* thus has provided systematists with an unresolved controversy, particularly in the melanoleucus complex, within which different authors have recognized from a single transcontinental polytypic species to four (monotypic and polytypic) species. However, with the exception of Cope’s (1900) and Stull’s (1940) studies, all these taxonomic arrangements were proposed after examining only a subset of the described forms of this species complex, or were suggested based on the geographic distribution and presumed diagnosability of the named subspecies. Our study tests the taxonomic hypotheses presented above with phylogenetic trees inferred from mitochondrial DNA (mtDNA) sequences from 16 of the 19 described forms of *Pituophis*, the greatest number of described forms included in any previous taxonomic study of the genus. Our main objective is to assess evolutionary relationships.
among the currently recognized species and subspecies of \textit{Pituophis}, assuming that our gene genealogy accurately reflects these relationships (see Brower \textit{et al.}, 1996; Moore, 1995, 1997). We also compare our findings to previous phylogenetic hypotheses for \textit{Pituophis} (Fig. 3) and discuss some of the biogeographical implications of our results.

\textbf{MATERIALS AND METHODS}

\textit{Taxon Sampling, DNA Isolation, and Sequencing}

We obtained tissue samples from one to four individuals of \textit{Arizona elegans} (glossy snake), \textit{Bogertophis subocularis} (Trans Pecos ratsnake), \textit{Lampropeltis getula} (common kingsnake), \textit{Pituophis d. deppei} (Mexican bullsnake), \textit{P. deppei jani} (northern Mexican bullsnake), \textit{P. lineaticollis gibsoni} (Guatemala gopher snake), and within the \textit{melanoleucus} complex, from \textit{affinis} (Sonoran desert gopher snake), \textit{annectens} (San Diego gopher snake), \textit{bimaris} (northern Baja California gopher snake), \textit{catenifer} (Pacific gopher snake), \textit{desertica} (Great Basin gopher snake), \textit{fuliginatus} (San Martín Island gopher snake), \textit{lodgingi} (black pine-snake), \textit{pumilus} (Santa Cruz Island gopher snake), \textit{ruthveni}, \textit{sayi} (bullsnake), and \textit{vertebralis} (Table 1; Fig. 1). We could not secure samples of \textit{coronalis} (South Coronado Island gopher snake) or \textit{insulanus} (Cedros Island gopher snake) but we believe that the omission of these two taxa does not alter the major findings of our study.

We extracted total genomic DNA from ventral scale clips preserved in 95\% ethanol or from tissue samples (blood, liver, muscle) stored frozen at $-74^\circ$C using the sodium dodecyl sulphate–proteinase K/phenol/RNase method (Sambrook \textit{et al.}, 1989). Using total cellular DNA as template, we amplified (with the polymerase chain reaction, PCR [Saiki \textit{et al.}, 1986, 1988]) and used for phylogenetic analyses an 893-bp fragment of mtDNA that encompassed a 697-bp portion of the 3' end of the nicotinamide adenine dinucleotide dehydrogenase subunit 4 (\textit{Ndh}4, or “\textit{ND4}” gene) and a 196-bp section of three transfer ribonucleic acid (tRNA) genes (tRNA\textit{His}, tRNA\textit{Ser}, tRNA\textit{Leu}), using primers labeled ND4 and Leu (Arévalo \textit{et al.}, 1994). ND4 is a reliable tracer of evolutionary history (Russo, 1997; Russo \textit{et al.}, 1996; Zardoya and Meyer, 1996) and a relatively fast-evolving gene useful for resolving relationships among closely related taxa (Cracraft and Helm-Bychowski, 1991). PCR was carried out in a programmable thermal cycler in 100-µl reactions consisting of 2 µl of template DNA (50 ng/µl), 2.5 µl of primers (40 µM), 10 µl of 10× PCR reaction buffer (Stratagene), 2 µl of MgCl$_2$ (25 mM), 2 µl of deoxynucleoside triphosphates (10 mM), 4 µl of \textit{Thermus aquaticus} DNA polymerase (5 U/µl), and 77.5 µl of H$_2$O. DNA was denatured initially at 94°C for 3 min; then 33 cycles of amplification were carried out under the following conditions: 94°C denaturation for 30 s, 55°C annealing for 30 s, and 72°C extension for 1 min, followed by a final 5-min extension at 72°C. Ten microliters of the resulting PCR product were electrophoresed on a 1% agarose gel and stained with ethidium bromide to verify product band size. For each individual, we cloned its PCR product into a phosphatased EcoRV pBluescript II SK $\pm$ phagemid vector (Stratagene) using \textit{Escherichia coli} as the vector and sequenced both DNA strands in an automated sequencer using the dideoxy chain-termination method (Sanger \textit{et al.}, 1977). The sequence of \textit{Elaphe vulpina} (fox snake) included in this study was provided by R. Lawson (California Academy of Sciences, San Francisco).
Phylogenetic Analyses

Sequences from the light and heavy DNA strands were input into the Sequence Navigator (version 1.0.1) program and aligned to each other and to the reference sequence of the colubrid snake *Dinodon semicarinatus* (Kumazawa *et al.*, 1998). This initial alignment was refined with the MacDNASIS Pro software (version 1.0). Pairwise comparisons of observed proportional sequence divergence (p-distance), corrected sequence divergence (with the Tamura–Nei model; Tamura and Nei, 1993), and number of transitions and transversions by codon position were obtained using the computer program PAUP* 4.0b2a (Swofford, 1999).

To estimate the phylogenetic information content of the mtDNA character matrix, we used the g test (Hillis and Huelsenbeck, 1992; Huelsenbeck, 1991; but see Källersjö *et al.*, 1992) to assess the skewness of the tree length distribution of 100,000 trees randomly generated with PAUP*. Probability of phylogenetic structure was assessed using the values provided by Hillis and Huelsenbeck (1992).

We used two methods of phylogenetic reconstruction: maximum parsimony (MP; Camin and Sokal, 1965; Swofford *et al.*, 1996) and maximum likelihood (ML; Felsenstein, 1981; Huelsenbeck and Crandall, 1997), as implemented by PAUP*. For MP analyses, we used two character weighting schemes: equal-weighting, in which all nucleotide substitutions were weighted equally regardless of type or codon position, and differential codon position weighting, in which we down-weighted third position transitions (see below). Sites with insertion or deletion events were removed from the analyses. Each base position was treated as an unordered character with four alternative states. Ancestral character states were determined via outgroup comparison (Farris, 1982; Maddison *et al.*, 1984; Watrous and Wheeler, 1981). We used *Arizona elegans*, *Bogertophis subocularis*, *Elaphe vulpina*, and *Lampropeltis getula* as outgroups because previous molecular systematic studies (Rodríguez-Robles and De Jesús-Escobar, 1999) identified these taxa as close relatives of *Pituophis*.

Because the number of terminal taxa was too large to permit evaluating all trees or employing the branch-and-bound algorithm (Hendy and Penny, 1982), we used heuristic search strategies for each tree-building methodology. We used 100 repeated randomized input orders of taxa for all MP analyses to minimize the effects of entry sequence on the topology of the resulting cladograms. MP analyses were conducted without the steepest descent option and with accelerated character transformation (ACCTRAN) optimization, tree bisection–reconnection (TBR) branch swapping, save all minimal trees (MULPARS), and zero-length branches collapsed to yield polytomies settings in place. We used nonparametric bootstrapping (100 pseudoreplicates, 10 addition-sequence replicates for MP, 50% majority rule) to assess the stability of internal branches in cladograms (Berry and Gascuel, 1996; Felsenstein, 1985; Felsenstein and Kishino, 1993; Sanderson, 1995). Non-parametric bootstrap values generally are a conservative measure of the probability that a recovered group represents a true clade (Hillis and Bull, 1993; Li, 1997; Zharkikh and Li, 1992). Alternate topologies were tested for significance at the 95% level using the Templeton (Larson, 1994; Templeton, 1983) and Kishino–Hasegawa (Kishino and Hasegawa, 1989) tests for MP and ML trees, respectively, as implemented in PAUP*.

For ML analyses we randomly selected as the starting tree one of the trees found during the MP searches. Using empirical nucleotide frequencies and five rate categories, we fixed the probabilities of the six possible nucleotide transformations \((A \rightarrow C, A \rightarrow G, A \rightarrow T, C \rightarrow G, C \rightarrow T, G \rightarrow T)\), the proportion of invariable sites \(\theta\), and the \(\alpha\) “shape” parameter of the gamma distribution of rate heterogeneity across nucleotide positions (Yang, 1996) to the empirical values calculated from the starting tree in a search for a better ML tree (a tree with a higher log-likelihood value), under the general time-reversible model of nucleotide substitution (Gu *et al.*, 1995; Swofford *et al.*, 1996; Yang, 1994). In other words, we used the most parameter-rich model available to search for ML trees. When a tree of higher likelihood was found, we reoptimized and fixed the parameters for a subsequent ML search. We repeated this procedure until the same tree was found in successive iterations (Swofford *et al.*, 1996).

Because ND4 is a protein-coding gene, we plotted p-distance \((y)\) versus corrected (with the Tamura–Nei model) estimates of proportional sequence divergence \((x)\) for first, second, and third codon positions. This was done separately for transitions and transversions to test for the possibility that some types of nucleotide substitutions have become saturated. Points that fall along the \(y = x\) line have the same observed and estimated numbers of changes and thus have not been subjected to multiple hits. Points that fall below the \(y = x\) line indicate that multiple hits have occurred; saturation is reached when observed sequence divergence does not continue to increase, despite the fact that corrected estimates do. Conventional statistical tests of the relationship between estimated and observed sequence divergence are not appropriate because of nonindependence of the data points, due to the inclusion of each point in more than one pairwise comparison. Accordingly, the plots were used as heuristic devices to help identify classes of changes occurring at different rates, which should be weighted differently in phylogenetic analyses.

RESULTS

Sequence Variation

There were 348 variable and 225 potentially phylogenetically informative characters (sites with at least two shared differences among all taxa) in the 893-bp mtDNA
**TABLE 1**

<table>
<thead>
<tr>
<th>Taxon, Sample Number (If Necessary), GenBank Accession No., Voucher Number (If Available), and Locality of the Taxa Used in This Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxon</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Outgroups</td>
</tr>
<tr>
<td>Arizona elegans</td>
</tr>
<tr>
<td>Bogertophis subocularis</td>
</tr>
<tr>
<td>Elaphe vulpina</td>
</tr>
<tr>
<td>Lampropeltis getula</td>
</tr>
<tr>
<td>Pituophis deppei deppei</td>
</tr>
<tr>
<td>Pituophis deppei jani</td>
</tr>
<tr>
<td>lineaticollis gibsoni</td>
</tr>
<tr>
<td>lineaticollis gibsoni</td>
</tr>
<tr>
<td>melanoleucus complex affinis</td>
</tr>
<tr>
<td>melanoleucus affinis</td>
</tr>
<tr>
<td>melanoleucus affinis</td>
</tr>
<tr>
<td>annectens</td>
</tr>
<tr>
<td>annectens</td>
</tr>
<tr>
<td>bimaris</td>
</tr>
<tr>
<td>bimaris</td>
</tr>
<tr>
<td>bimaris</td>
</tr>
<tr>
<td>bimaris</td>
</tr>
</tbody>
</table>

**TABLE 1—Continued**

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Sample number</th>
<th>GenBank Accession no., voucher number, and locality</th>
</tr>
</thead>
<tbody>
<tr>
<td>catenifer</td>
<td>14</td>
<td>AF141106; CAS 201258; U.S.: California, Mendocino Co., Mendocino National Forest, Forest Road M1, 5.1 mi NE (by road) of Mendocino Pass Road at Eel River Work Center</td>
</tr>
<tr>
<td>catenifer</td>
<td>15</td>
<td>AF141107; JAR 75; U.S.: California, Monterey Co., Hastings Natural History Reservation, Carmel Valley, 38601 E. Carmel Valley Road</td>
</tr>
<tr>
<td>catenifer</td>
<td>16</td>
<td>AF141108; JAR 77; U.S.: California, Alameda Co., 9618 Lupin Way</td>
</tr>
<tr>
<td>deserticola</td>
<td>17</td>
<td>AF141109; U.S.: California, Napa Co., Napa, 1.8 mi W 2930 Redwood Road</td>
</tr>
<tr>
<td>deserticola</td>
<td>18</td>
<td>AF138764; MVZ 137577; U.S.: Nevada, Mineral Co., Hwy. 31, 6.6 mi SW Hawthorne</td>
</tr>
<tr>
<td>deserticola</td>
<td>19</td>
<td>AF141110; MVZ 150216; U.S.: Colorado, Garfield Co., on Colorado River, 40 mi E Grand Junction</td>
</tr>
<tr>
<td>deserticola</td>
<td>20</td>
<td>AF141111; RSR 115; U.S.: California, Kern Co., 5 mi N and 2.5 mi E junction Neuralia and California City Boulevard</td>
</tr>
<tr>
<td>deserticola</td>
<td>21</td>
<td>AF141112; U.S.: Utah, Utah Co. 18–27 m W Hwy. 89, about midway between Provo and Springville</td>
</tr>
<tr>
<td>fuliginatus</td>
<td>—</td>
<td>AF141113; Mexico: Baja California, San Martin Island</td>
</tr>
<tr>
<td>lodingi</td>
<td>22</td>
<td>AF141114; HWG 2651; specimen from the pet trade</td>
</tr>
<tr>
<td>lodingi</td>
<td>23</td>
<td>AF141115; HWG 2652; specimen from the pet trade</td>
</tr>
<tr>
<td>melanoleucus</td>
<td>24</td>
<td>AF1387770; MVZ 150219; U.S.: North Carolina, Brunswick Co., 3.5 mi N Southport</td>
</tr>
<tr>
<td>melanoleucus</td>
<td>25</td>
<td>AF141116; MVZ 225520; U.S.: New Jersey, Cumberland Co., The Nature Conservancy, Warner Site</td>
</tr>
<tr>
<td>melanoleucus</td>
<td>26</td>
<td>AF141117; MVZ 225521; U.S.: New Jersey, Burlington Co., Bass River State Forest</td>
</tr>
<tr>
<td>mugitus</td>
<td>27</td>
<td>AF138769; USNM 211452; U.S.: Florida, Wakulla Co., St. Mark’s Wildlife Refuge, about 1.5 mi SW Otter Lake</td>
</tr>
<tr>
<td>mugitus</td>
<td>28</td>
<td>AF141118; MVZFC 13063; U.S.: Florida, Hillsborough Co., Tampa, 1 mi SE University of South Florida campus</td>
</tr>
<tr>
<td>pumilus</td>
<td>29</td>
<td>AF141119; U.S.: California, Santa Barbara Co., Santa Cruz Island, near Stanton Ranch</td>
</tr>
<tr>
<td>pumilus</td>
<td>30</td>
<td>AF141120; U.S.: California, Santa Barbara Co., Santa Cruz Island</td>
</tr>
</tbody>
</table>
of the informative characters, 29 were at first codon positions, 13 at second positions, 144 at third positions, and 39 at noncoding positions. Within *Pituophis* there were 21, 9, 121, and 33 informative characters at first, second, third, and noncoding positions, respectively. Significant phylogenetic signal was present in the data set \((g_1 = -0.6222, P << 0.01; \text{mean} \pm \text{SD tree length} = 1817.3 \pm 31.3, \text{range} 1625–1905)\); thus, inferring cladograms was justified.

Scatter plots of observed versus estimated sequence divergences indicated that these relationships are linear for first and second position transitions and transversions and third position transversions (Fig. 4). Third position transitions deviated greatly from a linear pattern, suggesting that these mutations are saturated. To estimate the transition-to-transversion bias for third position transitions, we fitted a least-squares regression line, forced through the origin, to the part of the curve of third position transitions versus third position transversions that is roughly linear. The slope of the regression line, 0.648, is an estimate of the transition-to-transversion ratio (Lara et al., 1996; Moore and DeFilippis, 1997). Therefore, we down-weighted third codon transitional changes by a factor of 6 using a 1:1:0.17 codon position weighting (first, second, and third codon position, respectively) to correct for the biased substitution rates at this position.

**Phylogenetic Relationships**

The two phylogenetic methods that we used recovered the same major nodes (Figs. 5 and 6), regardless of the weighting scheme used, which suggests that the groupings represent true clades. Most of these nodes were supported by relatively high bootstrap values (≥80%) in the two MP consensus trees but the relationships among the clades were almost completely unresolved. Although the single ML tree obtained (Fig. 6) resolved nearly all the relationships among the clades of *Pituophis*, some of the internal branches of this tree are short, indicating that they are supported by few characters, which may explain why these nodes collapse during the random resampling of characters that takes place during bootstrapping. The short internodes also suggest that the divergence of the major lineages of *Pituophis* occurred rapidly and near simultaneously. MP and ML trees constrained to include only monophyletic subspecies of the *melanoleucus* complex were significantly poorer estimates of phylogenetic relationships than the unconstrained trees (Table 2).

MP and ML methods identified monophyletic clades of the two species of middle American gopher snakes (*P. deppei* and *P. lineaticollis*). Nonetheless, the lack of specimens of *affinis* and *sayi* from Mexico and our limited sampling of the two subspecies of *P. deppei* prevented us from evaluating the disputed monophyly of the latter relative to the *melanoleucus* complex (Conant, 1965; Dixon et al., 1962; Morafka, 1977). Clearly, further studies that include representatives of Mexican *affinis* and *sayi*, of *P. d. deppei* and *P. d. jani* from additional localities, and of *P. l. lineaticollis* from southern Mexico are needed to better understand the evolutionary history of middle American *Pituophis*.

The MP and ML trees also showed that the eastern pinesnakes, *lodigii*, *melanoleucus*, and *mugitus*, form a monophyletic clade, and these trees are significantly shorter or have a higher likelihood ratio, respectively, than those constrained to include nonmonophyletic eastern pinesnakes (Table 3). The MP bootstrap tree inferred with equally weighted characters and the ML tree identified the eastern pinesnake lineage as the sister taxon to all other *Pituophis*, but this topology was not significantly different from that on which the eastern pinesnakes were constrained to not be the sister taxon to all other *Pituophis* (Table 4).

All the topologies recovered by the MP and ML methods identified separate, monophyletic clades of the *melanoleucus* complex (Figs. 5 and 6). Within these, there is clear geographic structuring. As mentioned, the eastern pinesnakes formed a distinct clade, and so

---

**TABLE 1—Continued**

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Sample number</th>
<th>GenBank Accession no., voucher number, and locality</th>
</tr>
</thead>
<tbody>
<tr>
<td>ruthveni</td>
<td>31</td>
<td>AF138771; U.S.: Louisiana, Bienville Parish, 2 km E Kepler Creek Lake Bridge</td>
</tr>
<tr>
<td>ruthveni</td>
<td>32</td>
<td>AF141121; U.S.: Louisiana, Bienville Parish, 7 km E Kepler Creek Lake Bridge</td>
</tr>
<tr>
<td>ruthveni</td>
<td>33</td>
<td>AF138772; U.S.: Louisiana, Bienville Parish, 2 km S junction of LA 154 and 507</td>
</tr>
<tr>
<td>sayi</td>
<td>34</td>
<td>AF141122; MVZ 150218; U.S.: Texas, Jeff Davis Co., on U.S. Route 90, 43.8 mi SE junction of I-10 and U.S. Route 90</td>
</tr>
<tr>
<td>sayi</td>
<td>35</td>
<td>AF141123; U.S.: Colorado, Jef ferson Co., SE junction of W. Coal Mine Road and Wadsworth Boulevard</td>
</tr>
<tr>
<td>sayi</td>
<td>36</td>
<td>AF141124; MVZ 226247; U.S.: Missouri, Saint Louis Co.</td>
</tr>
<tr>
<td>sayi</td>
<td>37</td>
<td>AF141125; U.S.: Oklahoma, Cleveland Co.</td>
</tr>
<tr>
<td>vertebralis</td>
<td>38</td>
<td>AF141126; Mexico: Baja California Sur, Rancho Buena Vista</td>
</tr>
<tr>
<td>vertebralis</td>
<td>39</td>
<td>AF141127; JAR 78; Mexico: Baja California Sur, Cape region</td>
</tr>
<tr>
<td>vertebralis</td>
<td>40</td>
<td>AF141128; specimen from the pet trade</td>
</tr>
</tbody>
</table>

**Note.** Museum and collector abbreviations are: CAS, California Academy of Sciences, San Francisco; MVZ, Museum of Vertebrate Zoology, University of California, Berkeley; MVZFC, MVZ frozen collection; USNM, National Museum of Natural History, Smithsonian Institution, Washington, DC; CII, Carl J. Franklin; CME, Curtis M. Eckerman; HBS, H. Bradley Shaffer; HWG, Harry W. Greene; JAR, Javier A. Rodriguez; RSR, Randall S. Reiserer.
did the gopher snakes from southern Baja California (\textit{vertebralis} and southern \textit{bimaris}); \textit{affinis} from southern Arizona; \textit{catenifer} and \textit{deserticola} from northern California and western Nevada; \textit{annectens}, \textit{bimaris}, \textit{fuliginatus}, and \textit{pumilus} from southern California, northern Baja California, and offshore islands; and \textit{sayi} and \textit{ruthveni} from the central US. To test whether the two recovered clades of endemic Baja Californian gopher snakes (\textit{bimaris} and \textit{vertebralis}) represent a reliable estimate of the relationships of these peninsular snakes, we compared the MP and ML trees (Figs. 5 and 6) with trees obtained using the same search parameters but constrained to form a single monophyletic clade of \textit{bimaris} and \textit{vertebralis}. The unconstrained trees were significantly better estimates of relations than the constrained phylogenies (Table 5).

**DISCUSSION**

Our analyses indicated that some populations of the \textit{melanoleucus} complex are more closely related to geographically closer populations of different subspecies than to more distant, consubspecific populations. Taxa are recognized as subspecies, not full species, precisely because they intergrade with neighbors, and this gene flow will blur the boundaries of subspecies, keeping them from attaining reciprocal monophyly at the mtDNA level (Patton and Smith, 1994). Consequently, the observed pattern of geographic distribution of genealogical lineages within \textit{Pituophis} from the US is not surprising, as there is evidence of intergradation between \textit{affinis} and \textit{sayi}, \textit{affinis} and \textit{deserticola}, \textit{affinis} and \textit{annectens}, \textit{annectens} and \textit{catenifer}, \textit{annectens} and \textit{deserticola}, \textit{bimaris} and \textit{vertebralis}, \textit{catenifer} and \textit{deserticola}, \textit{and among lodingi, melanoleucus}, and \textit{mugitus} (Grismer, 1997; Klauber, 1946, 1947; Sweet and Parker, 1990). Furthermore, the subspecies of the \textit{melanoleucus} complex, especially those from the western US, were originally recognized on the basis of rather minor differences in squamation, number of tail spots, and number, shape, and coloration (e.g., brown versus black) of body blotches (Klauber, 1947), which suggests that some of these taxa may not deserve taxonomic recognition.
The mtDNA sequences resolved several lineages within the *melanoleucus* complex, reflecting the evolutionary history of independent units within this group. We used a conservative approach and based the following discussion of phylogenetic and biogeographic patterns within *Pituophis* on those clades common to the MP and ML trees.

### The Taxonomic Status of the Eastern Pinesnakes

Our genetic data, irrespective of the phylogenetic method used, indicate that the eastern pinesnakes, *lodgingi*, *melanoleucus*, and *mugitus*, form a distinct clade within *Pituophis*. Collectively, these three taxa are allopatric and diagnosable from other subspecies of the *melanoleucus* complex using a combination of morphometric characters (Reichling, 1995). We interpret these findings as evidence that the eastern pinesnakes represent a distinct evolutionary lineage, and therefore we agree with previous suggestions (e.g., Cope, 1900; Fugler, 1955; Klauber, 1947; Wright and Wright, 1957) that they should be considered as a distinct species, for which the name *Pituophis melanoleucus* (sensu stricto) is available. By recognizing *P. melanoleucus* (sensu stricto), we effectively imply the existence of at least one more species within the *melanoleucus* complex, for which the name *Pituophis catenifer* is available, and which includes the subspecies *annectens*, *affinis*, *catenifer*, *coronalis*, *deserticola*, *fuliginatus*, *insulanus*, *pumi-

---

FIG. 5. Maximum parsimony bootstrap consensus trees for 42 mtDNA haplotypes of *Pituophis*. Numbers on the tree indicate percentage of nonparametric bootstrap support for nodes retained by more than 50% of bootstrap replicates; (a) with all characters weighted equally; (b) with third position transitions down-weighted by a factor of 6.1 and with the proposed specific taxonomy indicated for each taxon.

FIG. 6. Maximum likelihood tree (LnL = -5539.18903) for 42 mtDNA haplotypes of *Pituophis*. Branches are drawn proportional to branch lengths (expected amount of character change) estimated by the maximum likelihood algorithm. The proposed specific taxonomy is indicated for each taxon.
Knight (1986) suggested that the shape of the premaxilla–nasal articulation in the skull of Pituophis from the US was a useful character to distinguish between “eastern” (melanoleucus, mugitus, and sayi) and “western” (affinis and deserticola) taxa. Regardless of the fact that our results unambiguously indicate that sayi does not belong within the eastern pinesnake clade, our examination of several skulls from most of the range of the melanoleucus complex indicates that the shape of the premaxilla–nasal articulation is so variable that it cannot be reliably used to differentiate between snakes from this species complex.

The ranges of lodingi and ruthveni, the geographically closest taxa of Pituophis melanoleucus (sensu stricto) and the rest of the melanoleucus complex, respectively, are separated by the broad alluvial plain of the Mississippi River. The alluvium, the swamps, and the periodic flooding to which the big river valley is subjected seemingly combine to form an effective barrier to contact between P. melanoleucus (sensu stricto) and the rest of the melanoleucus complex. The Mississippi River, especially its lower drainage in the southeastern US, also delimits the eastern or western limits of the distribution of subspecies of other amphibian and reptile taxa (e.g., Scaphiopus holbrookii [eastern spade-foot toad; Wasserman, 1968], Deirochelys reticularia [chicken turtle; Zug and Schwartz, 1971], Ophisaurus attenuatus [slender glass lizard; Holman, 1971], Micruroides fulvius [harlequin coalsnake; Roze and Tilger, 1983; see also Blair, 1958]), among which there are no known zones of intergradation. It would be of interest to conduct phylogenetic studies of these taxa to determine whether populations on each side of the Mississippi River also represent different evolutionary lineages.

**The Phylogenetic Position of the Louisiana Pinesnake**

The taxonomic status of ruthveni, the Louisiana pinesnake, has long been controversial. Some authors (e.g., Stull, 1940; Wright and Wright, 1957) have claimed that ruthveni is more closely related to the pinesnakes from the eastern US, whereas others (e.g., Fugler, 1955) stated that its closest relatives are the gopher snakes from the central and western US and Mexico. Espousing Wiley’s (1978:18) version of the evolutionary species concept (“a species is a single lineage of ancestral descendant populations of organisms which maintains its identity from other such lineages and which has its...
own evolutionary tendencies and historical fate”), Reichling (1995) elevated *ruthveni* to the species level.

Both morphologically and geographically, the Louisiana pinesnake lies between the eastern pinesnakes and the gopher snakes, with *lodingi* its nearest neighbor to the east and *sayi* to the west (Reichling, 1995). Apparently, there is no evidence that *ruthveni* intergrades with *sayi*, and there are only three specimens from southeastern Louisiana and western Mississippi to suggest that intergradation may take place between *ruthveni* and *lodingi* (Conant, 1956; Crain and Cliburn, 1971). (The specimen discussed in Conant’s article was until recently believed to be missing but it is at the herpetological collection of Cornell University [CU 12953].) Nonetheless, our analyses (as well as those of allozyme data; J. Himes, pers. comm.) unambiguously support placement of *ruthveni* within the clade of gopher snakes from the central and western US and northern Mexico; they indicate as well that *ruthveni* and three of the four *sayi* included in this study form a well-supported clade (Figs. 5 and 6). Considering that the ranges of *ruthveni* and *sayi* probably were parapatric in the recent past (Conant, 1956; Reichling, 1995), their close relationship could be the result of recent gene flow or of stochastic lineage sorting of shared ancestral polymorphisms.

Is *ruthveni* a distinct species? The answer to this question is the most difficult phylogenetic problem within *Pituophis*, and the arguments presented in support of either position once again underscore that the criteria that we use to identify species and delimit species boundaries in nature are determined by our particular philosophy about species (e.g., de Queiroz and Donoghue, 1988; Frost and Hillis, 1990; Patton and Smith, 1994; Wake and Schneider, 1998; Zink and McKitrick, 1995). Provided that our gene tree represents the evolutionary relationships of *Pituophis*, our data indicate that the recognition of *Pituophis ruthveni* would render *P. catenifer* a paraphyletic species (Figs. 5 and 6). Consequently, systematists who prefer to define species by monophyly would probably disagree with the elevation of *ruthveni* to species status. On the other hand, authors who adhere to the evolutionary species concept (ESC) perhaps would feel that such a taxonomic decision is warranted, as *ruthveni* is an allopatric taxon diagnosable from its nearest neighbors by a combination of morphometric characters (Reichling, 1995). Proponents of the ESC could also claim that the

### TABLE 4

<table>
<thead>
<tr>
<th>Phylogenetic method</th>
<th>Weighting scheme</th>
<th>Topology</th>
<th>Tree length</th>
<th>RCI</th>
<th>n</th>
<th>$T_S$</th>
<th>$-\ln L$</th>
<th>SD</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP</td>
<td>EW</td>
<td>Unconstrained</td>
<td>851</td>
<td>0.367</td>
<td>22</td>
<td>110</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Constrained</td>
<td>854</td>
<td>0.365</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MP</td>
<td>TS/TV 1:6</td>
<td>Unconstrained</td>
<td>1300</td>
<td>0.416</td>
<td>11</td>
<td>24</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Constrained</td>
<td>1303</td>
<td>0.414</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ML</td>
<td>—</td>
<td>Unconstrained</td>
<td>855</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>5539.19</td>
<td>4.52</td>
<td>0.49</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Constrained</td>
<td>858</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>5541.41</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note.* EW, equally weighted characters; RCI, rescaled consistency index; TS/TV, transition-to-transversion ratio.

### TABLE 5

<table>
<thead>
<tr>
<th>Phylogenetic method</th>
<th>Weighting scheme</th>
<th>Topology</th>
<th>Tree length</th>
<th>RCI</th>
<th>n</th>
<th>$T_S$</th>
<th>$-\ln L$</th>
<th>SD</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP</td>
<td>EW</td>
<td>Unconstrained</td>
<td>851</td>
<td>0.367</td>
<td>36</td>
<td>140</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.0007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Constrained</td>
<td>873</td>
<td>0.350</td>
<td>34</td>
<td>112</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MP</td>
<td>TS/TV 1:6</td>
<td>Unconstrained</td>
<td>1300</td>
<td>0.416</td>
<td>34</td>
<td>112</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Constrained</td>
<td>1327</td>
<td>0.400</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ML</td>
<td>—</td>
<td>Unconstrained</td>
<td>855</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>5539.19</td>
<td>17.52</td>
<td>3.71</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Constrained</td>
<td>878</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>5604.13</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note.* EW, equally weighted characters; RCI, rescaled consistency index; TS/TV, transition-to-transversion ratio.
pattern of genetic relationship between *ruthveni* and *sayi* revealed by our analyses simply reflects the recency of their divergence and lack of time for lineage sorting to result in reciprocal monophyly. However, critics might still contend that Reichling’s taxonomic study of *ruthveni* did not include all the subspecies of the *melanoleucus* complex and accordingly cannot be used to argue in favor of or against recognizing the Louisiana pinesnake as a distinct species.

Two arguments are relevant when considering the aforementioned views regarding the taxonomic status of *ruthveni*. First, the theoretical basis for the emphasis on naming monophyletic species is not clear, as any species has evolved from a preexisting species, and this ancestral species necessarily ceased to be monophyletic at the moment of speciation (Baum, 1992; de Queiroz and Donoghue, 1985, 1990; Graybeal, 1995). Hence, it is impossible to avoid recognizing paraphyletic species. (The practice of assigning new species status to all daughters of a cladogenetic event is simply a taxonomic convention and does not necessarily indicate biological reality.)

Second, although Reichling’s (1995) taxonomic study of *ruthveni* did not include the western-most subspecies of the *melanoleucus* complex (i.e., *annectens*, *bimaris*, *vertebralis*, and the four island taxa), it included the geographically and phenotypically closer relatives of *ruthveni* (*lodini*, *melanoleucus*, *mugitus*, and *sayi*), as well as *affinis*, *deserticola*, and *catenifer*. Hence, we maintain that Reichling’s findings should be taken into account when judging the taxonomic status of the Louisiana pinesnake and that not doing so is tantamount to ignoring available evidence. Therefore, because it is likely that at least some of the morphometric characters shown by Reichling to collectively and unambiguously distinguish *ruthveni* from its closer relatives are independent and genetically inherited, we believe that the Louisiana pinesnake has attained the status of independent evolutionary lineage. We then agree with Reichling’s (1995) suggestion (see also Collins, 1991; Conant, 1956) of recognizing *ruthveni* as a distinct species, *Pituophis ruthveni*. One reason why our mtDNA sequence data do not yet reflect the independent evolutionary trajectory of *P. ruthveni* may be that populations of this snake constitute an example of what Graybeal (1995) called “ferespecies,” interbreeding groups of organisms that do not yet possess exclusivity of descent. Nevertheless, it is possible that DNA sequences of other genetic markers (e.g., from the nuclear genome or even other mitochondrial genes) would indicate a higher degree of genetic cohesiveness among populations of *ruthveni*.

Despite the above arguments, the taxonomic status of the Louisiana pinesnake is likely to remain controversial, not so much due to lack of relevant data, but rather because of different interpretations of available evidence, and although we recognize these snakes at full species rank, we understand why other workers may disagree with our position. If Louisiana pinesnakes are undergoing “the speciation process,” their debatable status illustrates the continuing difficulty in making taxonomic assignments in such cases.

**The Taxonomic Status and Biogeography of the Endemic Baja Californian Gopher Snakes**

The results of our phylogenetic analyses regarding the endemic gopher snakes from Baja California, *bimaris* and *vertebralis*, are in disagreement with the current taxonomy of these serpents. Traditionally, the gopher snakes from central and southern Baja California were assigned to *bimaris*, with snakes from the outskirts of the town of La Paz south to the tip of the peninsula (the Cape region) assigned to *vertebralis* (Fig. 1). Recently, Grismer (1994, 1997) synonymized *bimaris* with *vertebralis* and elevated the latter to species level. We discovered that the southernmost *bimaris* included in our study was more closely related to *vertebralis* (sensu stricto) than to more northern populations of *bimaris*, which nested within a separate clade composed of two island subspecies (*fuliginatus* and *pumilus*), and of *annectens* and *catenifer* (sample 15) from southern California (Figs. 5 and 6). Additionally, the two distinct clades that include the endemic Baja Californian gopher snakes are not sister taxa to each other, and when we constrained all *bimaris* and *vertebralis* to form a monophyletic group, the resulting MP and ML trees were significantly poorer estimates of evolutionary relationships within *Pituophis* (Table 2). These findings led us to reject the recognition of *Pituophis vertebralis* as defined by Grismer.

The notable genetic break between northern and southern Baja Californian populations of *Pituophis* mirrors the phylogeographic pattern displayed by *Uta stansburiana* (side-blotched lizard) on the peninsula (Upton and Murphy, 1997). Upton and Murphy suggested that the disruption of mtDNA haplotype distribution between side-blotched lizards from northern and southern Baja California was consistent with the existence of a temporary midpeninsular seaway (Durham and Allison, 1960; Fig. 1). Presumably, this barrier caused the cessation of gene flow between populations on the northern and southern portions of the peninsula, allowing them to evolve independently. Based on various assumptions, Upton and Murphy hypothesized that the seaway existed one million years ago (Mya). The smallest uncorrected percentage sequence divergence between specimens of *bimaris* and *vertebralis* from the northern and southern clades is 6.2%. Estimates of mtDNA sequence divergence for reptile species for which branching events have been confidently dated using fossil records or geological events range from 0.47 to 1.32% per million years (Zamudio and Greene, 1997). Relying on these lower and upper estimates, the two clades of endemic Baja Californian
The view of a single, widely distributed, polytypic *Pituophis melanoleucus* is inconsistent with the inferred evolutionary history of these snakes. Our phylogenetic analyses indicated that two segments of the *melanoleucus* complex, the *lodinki–melanoleucus–mugitus* eastern pinesnake clade and the *affinis–annectens–bimaris–catenifer–desertica–sayi–ruthveni–vertebralis* clade from central and western United States and northern Mexico, represent divergent, allopatric lineages with no known intergradation zone. We interpret the available evidence as suggesting that the Louisiana pinesnake has attained the status of independent evolutionary lineage. We therefore recognize three distinct species in the *melanoleucus* complex, *P. melanoleucus* (sensu stricto), *P. catenifer*, and *P. ruthveni*; whether additional species should be recognized within *P. catenifer* remains questionable. The quotation at the beginning of this paper was fitting in 1900, and the findings and arguments herein presented demonstrate that, despite several additional taxonomic studies, it remains so a century later.

**ACKNOWLEDGMENTS**

We thank H. W. Greene, D. L. Hard, Sr., J. Gee, L. L. Grismer, B. D. Hollingsworth, the late B. E. Dial, J. A. Campbell, M. Sasa, L. M. Hardy, J. G. Himes, H. K. Reinert, G. M. Bolen, T. Benson, S. M. Deban, A. de Queiroz, W. I. Lutterschmidt, R. E. Jones, M. D. Mateoq,
REFERENCES


...


Russo, C. A. M. (1997). Efficiencies of different statistical tests in