INTRODUCTION

Pitvipers are a conspicuous component of most South American snake faunas. They show tremendous morphological and ecological diversity, including extremes in body size (minimum adult total length 45–360 cm), body shape (e.g., slender, Bothrops taeniatus; stout, Bothrocophias hyporora), macrohabitat (deserts to montane forests), microhabitat (subterranean to arboreal), and diet (generalists to highly specialized) (see Martins et al., this volume). Approximately 44–47 species of pitvipers in 5–6 genera (David ans Ineich, 1999; McDermid et al., 1999) are currently recognized from continental South America and nearby islands. The genera differ considerably in terms of species numbers, as well as in morphological and ecological diversity. Several genera are widespread, but others are more restricted in distribution. We present and compare morphological diversity and habitats occupied by the genera in Table 1.

Bothriechis is a genus consisting of small, arboreal or semi-arboreal species that are widespread in Central America, where nine species are currently recognized (Campbell and Lamar, 1989; Solórzano et al., 1998; Campbell and Smith, 2000); in South America Bothriechis is represented by a single species, B. schlegelii, which is restricted to the northwestern part of the continent. Recent studies by Werman (1992), Kraus et al. (1996), and Parkinson et al. (this volume) support the monophyly of Bothriechis.
Lachesis is a genus of large terrestrial pitvipers represented by two species in Central America (*L. melanocephala* and *L. stenophrys*) and one (*L. muta*) in South America (*Zamudio and Greene*, 1977). The affinities of *Lachesis* from the northwest coast of South America, however, remain unresolved. As currently understood, populations of *Lachesis* differ little in morphology and natural history.

*Crotalus* is a speciosum genus (Klauber, 1972) represented in South America by a single widespread species, *C. durissus*; we follow Campbell and Lamb (1989) in treating *pifanoiurn*, *unicolor*, and *vegrandis* as conspecific with *C. durissus*. *Crotalus* is less diverse than *Bothrops* with respect to its morphology and ecology, although North American species of *Crotalus* show considerable differentiation in body size and habitat preferences (Table 1).

Most other South American pitvipers are classified in the genus *Bothrops*. They occur throughout most of the continent except for southwestern South America, the extreme highlands of the Andes, and southernmost Patagonia. *Bothrops* shows great diversity in size, shape, and habitat preferences, which encompasses nearly the entire spectrum of *variation* seen in all other genera of New World pitvipers (Table 1). Three species are endemic to islands (B. caribbicus, B. lucia; B. insularis, Queimada Grande; B. lanceolatus, Martinique), and one species (B.asper) extends northward throughout most of Central America to northeastern Mexico. Some arboreal forms of *Bothrops* have previously been assigned to the genus *Bothriopsis* (Burger, 1971; Campbell and Laman, 1989). However, all phylegetic studies of this group (Caudle, 1992; Wermam, 1992; Kraus et al., 1996; Salomos et al., 1997, 1999; Parkinson, 1999; Vidal et al. 1997; Parkinson et al., this volume) have suggested that *Bothrops* (*sensu Burger, 1971*) is paraphyletic with respect to *Bothriopsis*. Salomos et al. (1997) proposed synonymizing *Bothriopsis* with *Bothrops*. Although some authors have not followed this arrangement (e.g., McDermott et al., 1999; Parkinson et al., this volume), we follow Salomos et al. (1997) in regarding *Bothriopsis* as a synonym of *Bothrops*.

Guterlet and Campbell (2001) assigned the taxon *campaellii, hygrosus, microphilhamus*, and *myerii* to the genus *Bothriophis*. The taxa concerned have been grouped as members of *Bothrops* in mtDNA-based studies (Kraus et al., 1996; Parkinson, 1999; Parkinson et al., this volume; this study), but many of the morphological analyses of Guterlet (1998b) did not support this hypothesis. We have chosen to follow the results of the previous mtDNA studies and treat *Bothriophis* as a synonym of *Bothrops*. The morphological and natural history diversity seen in *Bothrops* is greater than in other clades of New World pitvipers, which provides an interesting opportunity to investigate causes of diversification. Causal hypotheses may include the relative age of these clades (*e.g.*, older clades have had more time to evolve greater differences in phenotype and natural history), at the possibility of rapid adaptive radiation in response to open niches (*e.g.*, the colonization of regions devoid of pitvipers with similar natural history characteristics).

Pitviper diversity in South America is linked to the biotic interchange between North and Central America throughout the Tertiary, particularly after emergence of the Isthmus of Panama. Emergence of the Isthmus 3.5 mya (Coades and Orlando, 1996) does not appear to have been a key factor in the biotic interchange of cetothemine vertebrates, unlike with mammals. Among pitvipers, Vanzolini and Heyer (1985) identified only *C. durissus* as a likely example of a post-Isthmian dispersal event beyond northern South America. Vanzolini and Heyer (1985), however, were unable to provide resolution to the origin and timing of dispersal for *Bothrops* or the "bothriopodid" pitvipers.
due to the lack of phylogenetic understanding of the group at that time. Crother et al. (1992) assumed South American Viperidae to be the result of post-
African colonization.

Resolution of biogeographical problems of this nature requires a robust phylogenetic hypothesis for the taxa concerned. In recent years, the phylogeny of pitvipers has become a subject of intense interest (e.g., Crother et al., 1992; Knight et al., 1992; Werman, 1992, 1997; Kraus et al., 1996; Salomão et al., 1997, 1999; Parkinson et al., 1997, this volume; Vidal et al., 1997, 1999; Guterl et al., 1999a, b; Vidal and Le Coine, 1998; Parkinson, 1999). Studies using mitochondrial DNA sequences have been prominent in pitviper systematics (e.g., Kraus et al., 1996; Salomão et al., 1997, 1999; Vidal et al., 1997, 1999; Wüster et al., 1997, 1999; Zamudio and Greene, 1997; Parkinson, 1999; Puorto et al., 2001; papers in this volume). Although many finer points of pitviper systematics remain to be clarified, these studies have made considerable progress toward our understanding of their relationships.

Molecular markers are particularly promising for this kind of research, since they are unlikely to be affected by the same selection pressures as morphol-
gy, thus providing an independent assessment of relationships unaffected by convergent selection for similar natural history traits (Avise, 1994, 2000). Furthermore, DNA sequences can be used to estimate the age of different clades, and provide a temporal basis for historical biogeography and the evolution of morphological diversification (e.g., Zamudio and Greene, 1997). Existing databases (e.g., GenBank) of mitochondrial DNA sequences are particularly useful for future studies, as new sequences can be added to existing databases.

In this study, we used our own mtDNA sequence data, as well as published databases (Kraus et al., 1996; Parkinson, 1999) to assess the origin and biogeography of the South American pitvipers, as well as the ecological correlates of diversification within this group.

MATERIALS AND METHODS

Sampling and Molecular Techniques

Individuals of a variety of taxa of Bothriechis, Bothrops, C. durissus, and Bothriopsis were obtained through fieldwork or from captive collections. For DNA analysis, we collected tissue (ventral scale clip-
pings or liver tissue) and/or blood (from the caudal vein) from living or recently dead specimens. Tissue samples were stored in 70–100% ethanol, whereas blood samples were collected into 0.5M EDTA and stored in a solution of 0.1M EDTA, 0.1M Tris, 2% sodium dodecyl sulfate (SDS). All samples were kept refrigerated as soon as possible after collection.

Genomic DNA was extracted from tissue and blood samples using standard molecular laboratory protocols (Hillis et al., 1996b). Two regions of the mtDNA molecule were amplified using the polymerase chain reaction (PCR): a 758 base pair (bp) section of the gene for cytochrome b (cyt-b), and/or a 890 bp region of the gene for NADH dehydrogenase subunit 4 (ND4). Details of primers, PCR conditions, and sequencing protocols are described in Poole et al. (2000). Sequencing was carried out on an ABI373 automated sequencer using the manufacturer's recommended protocols. The sequences were checked against the chromatogram output of the automated sequencer and aligned by eye using WordPerfect 5.1 (WordPerfect Corporation). Samples, vouchers, and GenBank accession numbers are listed in Appendix I.

Information on mtDNA Sequence Divergences

We estimated levels of mtDNA sequence divergence for the cyt-b, ND4 and 12s and 16s rDNA gene regions in a number of clades of pitvipers, depending on sequence availability. We generated ND4 and/or cyt-b sequences for various Bothriechis, Bothrops, Crotalus, and South American Bothriopsis samples and combined our ND4 data with that reported by Kraus et al. (1996); 12s and 16s rDNA data were those of Parkinson (1999).

Since the more divergent sequences are subject to saturation of transition sites, we used Kimura 2-para-
meter distances in all calculations involving distance measures. To estimate the levels of sequence divergence within genera or major clades, we selected the two most-basal clades within each group and calculated the average distance between them using the program PHYTEST (Kumar, 1996).

We did not have access to sequence data of all genes for all taxa; therefore, it was important to com-
pare rates of divergence among genes in order to determine if estimates of divergence in different genes are comparable. While it is well known that 12s and 16s rDNA show a slower rate of sequence evolution than cyt-b and ND4 (e.g., Thorpe et al., 1994), the relative rate of evolution between the latter two is less clearly established. In order to compare the rate of sequence divergence of ND4 and cyt-b for our pitviper data, we calculated a pairwise p-distance matrix for a subset of crystalline taxa for ND4 and cyt-b, and plotted...
ND distances against the corresponding \textit{cyt-b} dis-
tances. We then calculated the regression slope to es-
imate the relative rate of divergence of the two genes.
Lachesis sequences were from Zamudio and
Greene (1997). Because the individual fragments of
\textit{cyt-b} and ND4 used in that study were short, and the
genes appear to evolve at a similar rate in pitvipers (see below), we found joined sequences preferable to
a separate analysis. To assess sequence divergence
within \textit{Lachesis}, we calculated the average distance
between the Central and South American clades.
Sequence divergences between species of \textit{Bothrops}
were calculated separately from our \textit{cyt-b} and ND4 data. We included representatives of numerous
species, including several major radiations within a
species complex (e.g. the \textit{B. atrox} complex; Wüster et
al., 1997, 1999). Within-group divergence was calcu-
lated from the average distance between \textit{B. pictus}
and the remainder of the genus.
For \textit{Crotalus}, we calculated Kimura 2-parameter
distances for \textit{cyt-b}, based on our data on \textit{C. barili-
us}, \textit{C. durissus}, \textit{C. molossus}, \textit{C. scutulatus}, \textit{C. viridis}, and a
published sequence of \textit{C. cerastes}, using \textit{C. cerastes}
as the sister group to other species of \textit{Crotalus}, based
on preliminary analyses (not shown). For ND4, we
combined the published sequences of Kraus et al.
(1996) with our own data on \textit{C. durissus}, \textit{C. scutulatus}, and \textit{C. viridis}. As \textit{C. durissus} appears to be the sister
species to other \textit{Crotalus} species included (not shown),
we calculated distances between this taxon and the
remainder of the genus. We also calculated average
levels of divergence in ND4 and 12s and 16s
sequences between \textit{Crotalus} and \textit{Sistrurus}. Since the
sampling of \textit{Crotalus} species was limited for these
data, these numbers are not representative of the
likelihood of including both branches of what is likely one of the most basal
dichotomies of the rattlesnake phylogenetic tree (Parkinson et al., this volume). Divergences of South
American \textit{C. durissus} were calculated from our own
\textit{cyt-b} data.
For \textit{Porthidium}, we calculated ND4 divergences
for three subsets of taxa: one for \textit{Porthidium (senso stricto)}, one for South American samples of
\textit{Porthidium}, and one for \textit{Porthidium (senso lato)}. We
calculated \textit{cyt-b} divergences for the South American
populations of \textit{Porthidium} from our own data.
In order to test if similar levels of sequence diver-
gence in different clades can be interpreted as evidence
of similar age, it is necessary to test if rates of
nucleotide substitution differ between clades. For this
purpose, we used the relative rate test. If the rate of
sequence evolution in all lineages of the ingroup is
equal, then the distances of different ingroup species
or clades to the outgroup should be equal. As an out-
group, we used sequences from the Malayan Pitviper
(\textit{Calloselasma rhodostoma}), which was rooted outside
the New World pitvipers in all phylogenetic studies of
the subfamily (e.g., Kraus et al., 1996; Vidal et al.,
1997, 1999; Vidal and Lecointre, 1998; Parkinson,
1999). The New World pitvipers were grouped by
genus or clade. The groups used were \textit{Bothrops}
(including \textit{bohirondis} and \textit{Bothrocophias}), \textit{Crotalus}
and \textit{Sistrurus}, \textit{Porthidium (senso lato)} for \textit{cyt-b}, these
and \textit{Bothriechis} for ND4, and all the preceding, as
well as \textit{Ophryanurus and Lachesis} for 12s and 16s RNA.
We carried out pairwise relative rate tests, using
Kimura 2-parameter distances, and the two-cluster test
algorithm of Tapper et al. (1995), implemented
through the program PHYTEST (Kumar, 1996).

Phylogenetic Analysis

We used both maximum parsimony (MP) analysis
of unweighted sequence data and maximum likeli-
hood (ML) analysis in order to infer the phylogeny of
several groupings of Neotropical pitvipers. In the case of
ML, we used the program MODELTEST (Posada and
Crandall, 1998) to calculate the best model of sequence
evolution for each dataset. A ML search using these
parameters was used to construct a phylo-
genic tree for the taxa concerned. The sequence
evolution of pitvipers was calculated and a further tree was constructed based on the recal-
culated parameters. This was repeated until no further
changes in parameter values were found.

\textit{Porthidium}—We aligned 693 bp of ND4 sequence,
corresponding to all but the first bp of Kraus et al.
(1996), of the following taxa: \textit{A. nummifer}, \textit{P. nasutum},
and \textit{P. ophiomystes} (Costa Rica; all from Kraus et
al., 1996). \textit{P. l. arcoae} (Ecuador), \textit{P. l. rezei} (NW
Venezuela), and \textit{P. nasutum} (Ecuador). Based on the
results of Kraus et al. (1996) and Parkinson (1999),
which support the monophyly of Hog-nosed Pitvipers,
we assigned \textit{A. nummifer} to the near outgroup and we
used a sequence of \textit{C. rhodostoma} as the far outgroup.
An Old World pitviper was selected as the distant
outgroup in view of the possibility that \textit{Porthidium}
(\textit{senso lato}) is not monophyletic, and includes some of
the basal-most lineages of New World pitvipers (e.g.,
Wermann, 1992). We used MP analysis of the
unweighted data, implemented through the exhaustive
search algorithm of PAUP* 4.0b8 (Swofford, 2001).
Branch support was assessed using bootstrap analysis
Biology of the Vipers

(Felsenstein, 1985), with 10,000 replications using branch-and-bound searching. Bremer (1994) support for the various branches of the tree was investigated by repeating exhaustive searches while retaining successively longer trees until all nodes were collapsed. Maximum likelihood searching used branch- and-bound searching. Maximum likelihood bootstrap support was calculated over 100 replicates using heuristic searching, a neighbor-joining (NJ) starting tree, and SPR branch-swapping.

Crotalus durissus.—We aligned 701 bp of cyt-b sequence for 15 species from 10 localities, representing 8 nominal subspecies of C. durissus from Central and South America. Parsimony analysis of the unweighted data was implemented using PAUP* 4.0b88 (Swofford, 2001), using the branch-and-bound algorithm. Trees were outgroup-rooted using sequences of C. scutulatus, C. v. viridis (both from C. Poole, pers. comm.) and C. molossus. Branch support was assessed using bootstrap analysis, using 1,000 replicates and the branch-and-bound search algorithm. Bremer (1994) support for the various branches of the tree was investigated by repeating branch-and-bound searches while retaining successively longer trees until all nodes were collapsed. Maximum likelihood searching used an NJ starting tree, heuristic searching, and tree-bisection-reconnection (TBR) branch-swapping. Maximum likelihood bootstrap support was calculated over 100 replicates, using heuristic searching, an NJ starting tree and SPR branch-swapping.

Bothrops.—We used parsimony analysis of 1401 bp of the mtDNA genes for cyt-b and ND4. Our analysis included 28 species of Bothrops (sensu stricto) as well as two species of Porthidium and one of Crotalus. The resulting trees were outgroup-rooted using a sequence of C. rhodostoma. The analyses were carried out using a heuristic search algorithm, TBR branch-swapping, and 10,000 random addition sequence repeats. The levels of sequence divergence observed in the database indicated that saturation of some type of bp substitution, in particular, transitions at third bp codon positions, may be a problem. In order to investigate the effect of this potential problem on the data, we repeated the parsimony analysis under exclusion of third codon position transitions. Branch support was assessed using bootstrap analysis with 500 bootstrap replicates with five random addition sequence repeats each, using TBR branch-swapping. The initial ML search involved heuristic searching, using TBR branch-swapping and a NJ starting tree. Maximum likelihood bootstrap support was calculated over 100 replicates, using heuristic searching, an NJ starting tree, and NNI branch-swapping.

Molecular Clock Calibration

Table of the possible advantages of molecular sequence data over morphological data is that the former can, in some circumstances, allow the timing of divergences among lineages to be estimated. Time estimates for major lineage splits, independent of prevailing geological hypotheses, are particularly important for the reconstruction of biogeographical histories (Cadle, 1985). Appropriate use of a molecular clock, however, requires a number of assumptions that are unrealistic, and involves error margins that are often large compared to the times estimated (Hillis et al., 1996a). Nevertheless, the use of molecular clocks can prove illuminating, and some studies have found what appear to be highly consistent rates of sequence evolution in some groups (e.g., Macey et al., 1998). We compared two sequence evolution rate estimates at opposite ends of the spectrum for ecotermic vertebrates, and the effect of each calibration on our interpretation of the evolution of the South American pitviper fauna. Although the absolute timing of events is subject to the errors associated with clock calibration, use of these clocks allows us to estimate the relative timing of events in pitviper evolution.

The timing of clades in the SQUAMATES remains uncertain, as there is no widely accepted, robust estimate of a “squamate molecular clock” for mtDNA in general, or for specific mitochondrial genes (Avice et al., 1998). Zamudio and Greene (1997) surveyed the literature and concluded that the available data were consistent with rates of between 0.47% and 1.32% myr⁻¹. These estimates, however, are made either for the entire mitochondrial DNA genome, based on restriction-fragment-length polymorphism (RFLP) analysis, or for sets of sequences of several different genes. Of the latter, all included slowly evolving 12s and 16s rDNA or cytochrome oxidase subunit I sequences. Consequently, these estimates are difficult to relate to sequence variation in specific mitochondrial genes. Based on these estimates and observed levels of sequence divergence between clades of Lachesis, Zamudio and Greene (1997) hypothesized that cladogenic events in Lachesis may have been influenced by two vicariance events—the uplifts of the northern Andes and the Cordilleras de Talamanca. If one assumes the Zamudio and Greene (1997) hypotheses of causative vicariant events to be correct (which involves a certain level of circularity),
then correlating sequence divergences among *Lachesis* taxa with the timing of these putative vicariant events results in estimates of 0.60–0.76% myr$^{-1}$ and 0.66–1.06% myr$^{-1}$, respectively (Poes et al., 2000), and the two estimates overlap between 0.66–0.76% myr$^{-1}$.

The data presented in this study offer two alternative calibration points for the squamate cyt-b and ND4 molecular clock. Both are based on the time of the final emergence of the Isthmus of Panama, estimated at 3.5 mya (Coates and Obando, 1996). *Crotalus durissus* almost certainly colonized South America via this landbridge (Vanzolini and Heyer, 1985). Another potential candidate for divergence after the isthmian emergence is the South American group of *Porthidium*. Species of *Porthidium* appear to be poor overwater dispersalists, since toeclaws are found on islands of the coast of Central and South America, despite the fact that several species occur in coastal areas (Porras et al., 1981; Campbell and Latnar, 1989). The one exception to the rule is the presence of *P. lanceolatus* on Isle Margarita (Venezuela), but this continental shelf island was linked to the South American mainland at various times in the Pleistocene, during times of lowered eustatic sea-levels.

In order to assess the rate of sequence divergence within these clades we assumed that the colonization of South America, and the first cladogenic event within South American taxa, occurred approximately at the same time as the emergence of the Isthmus, 3.5 mya. We used the largest measure of pairwise sequence divergence among the South American haplotype lineages to estimate rate divergence, by dividing the p-distance by the time since colonization. We calculated 95% confidence limits for our estimates by assuming the spread of divergence values around the mean rate to correspond to a Poisson distribution (Hillis et al., 1996a).

### RESULTS

#### mtDNA Sequence Divergence

The levels of intrageneric Kimura 2-parameter sequence divergence in cyt-b, ND4 and 12s and 16s rDNA are shown in Table 2. The plot of ND4 divergence against cyt-b divergence (not shown) reveals a regression slope of 1.04, which suggests that, in New World pitvipers these two genes evolve at approximately the same rate, so that rates of divergence across the two genes are comparable.

Several trends are apparent from the divergences. First, bearing in mind differences in species-sampling, the major clades [rattlesnakes; Bothriechis; Bothrops; *Porthidium (sensu lato)*] display similar levels of maximum within-group sequence divergence in the cyt-b and ND4 genes (~ 13.9–20.0%). Divergence levels in 12s and 16s rDNA were also similar among genera, lying between 5.4 and 7.4%. *Porthidium (sensu strico)*, Bothrocophias, and Ophryacus display only slightly lower levels of intrageneric divergence, and only *Lachesis* consistently shows much lower levels of variation.

Second, where information is available, and except in *Bothrops*, levels of sequence divergence within the South American representatives of each group are low, varying from 1.1 to 5.6% in ND4 and cyt-b (no data)

<table>
<thead>
<tr>
<th>Taxon</th>
<th>ND4</th>
<th>Cyt-b</th>
<th>12s and 16s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bothrops</td>
<td>12.9</td>
<td>13.9</td>
<td>5.4 ± 0.6</td>
</tr>
<tr>
<td>Bothrocophias (includes <em>B. campbellii</em>)</td>
<td>10.9 ± 1.3</td>
<td>13.4 ± 1.4</td>
<td>5.9 ± 0.9</td>
</tr>
<tr>
<td>Portidium (sensu strico)</td>
<td>13.5 ± 1.4</td>
<td>4.7 ± 0.7</td>
<td>6.4 ± 0.7</td>
</tr>
<tr>
<td>Portidium (South American taxa only)</td>
<td>14.9 ± 1.2</td>
<td>16.6 ± 1.7</td>
<td>7.4 ± 0.9</td>
</tr>
<tr>
<td>B. schlegelii (Costa Rica and Ecuador)</td>
<td>16.1 ± 1.5</td>
<td>9.6 ± 1.3</td>
<td>6.7 ± 0.7</td>
</tr>
<tr>
<td>Crotalus + Crotalinae</td>
<td>20.0 ± 1.7</td>
<td>19.1 ± 1.6</td>
<td>5.8 ± 0.7</td>
</tr>
<tr>
<td>C. durissus (South America only)</td>
<td>14.8 ± 1.3</td>
<td>8.0 ± 0.9</td>
<td>6.0 ± 0.9</td>
</tr>
<tr>
<td>Lachesis (South America only)</td>
<td>9.0 ± 1.3</td>
<td>1.5 ± 0.4</td>
<td>2.6 ± 0.6</td>
</tr>
<tr>
<td>Ophryacus</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.3</td>
<td>6.0 ± 0.9</td>
</tr>
</tbody>
</table>

*W. Wistow, M. Salomido, J. Quijada-Mascarías, R. Thorpe, and BBBSP*
Biology of the Vipers

Fig. 1. Phylogenetic tree for Porthidium spp., based on ML analysis (-lnL = -2234.21490). The consensus of two equally most-parsimonious trees was topologically identical, except that the relationships among the three South American taxa were unresolved. Numbers on nodes indicate Bremer Support / MP bootstrap support / ML bootstrap support.

are available for 12s and 16s rDNA, nor for sequence variation within South American Bothriechis schlegelii; however, the ND4 sequence divergence between Costa Rican and Ecuadorian B. schlegelii is 9.6%.

The relative rate tests show that the different genera or clades do not differ significantly in their levels of sequence divergence from Callioselasma in their cyt-b and ND4 sequence, suggesting that the rate of sequence evolution is equal across these genera. In the case of the 12s and 16s data, all pairwise comparisons were insignificant, except that Bothriechis differed significantly more from Callioselasma than did Porthidium (sensu lato). Consequently, we cannot reject the hypothesis of essentially clockwise sequence evolution in the cyt-b and ND4 genes, nor for the comparisons between the 12s and 16s rDNA sequences of most clades

Phylogeny

Porthidium.—The Atropoides and Porthidium database contained 90 parsimony-informative characters. Our analysis of the phylogeny of the available sequences of Porthidium resulted in two equally most-parsimonious trees of 307 steps (CI = 0.8145, RI = 0.8187, R1 = 0.4754), which differ in arrangement of the South American taxa. Both agree on the monophyly of the three South American samples, to the exclusion of Costa Rican P nasutum and P phrygianus. This monophyly is supported by reasonable bootstrap and Bremer support values. An exhaustive ML search carried out under the assumptions of the HKY85 model with gamma distribution identified as optimal by

Fig. 2. Bootstrap consensus of six equally most-parsimonious phylogenetic hypotheses for populations of the Crotalus durissus species complex, based on parsimony analysis of 701 bp of cyt-b sequence information (length 273 steps, CI = 0.7426, RI = 0.7574, R1 = 0.5891). Numbers above branches indicate Bremer support / MP bootstrap support / ML bootstrap support.

MODELETEST resulted in a single tree consistent with the consensus of the two MP trees (Fig. 1).

Crotalus durissus.—The Crotalus durissus database contained 116 parsimony-informative characters. Our parsimony analysis resulted in six equally most-parsimonious trees of 274 steps (CI = 0.7445, RI = 0.2555, R1 = 0.7500). The bootstrap support for various nodes is shown in Figure 2. The ML tree generated based on the HKY85 model with gamma distribution identified as optimal by MODELETEST resulted in a single tree consistent with the MP consensus tree (Fig. 2). Bootstrap support values in the ML analysis were similar to those obtained using MP. Our analysis strongly supports the monophyly of all South American populations of the C. durissus complex, including unicolor and vегrandis, whereas the Central American and Mexican populations are paraphyletic. Additionally, sequence divergence among the South American C. durissus populations was consistently low, with a maximum pairwise divergence of 1.5%, whereas divergences among the Central American lineages ranged up to 8.0%.

Bothrops.—The Bothrops database contained 491 parsimony-informative characters. The analysis of the unweighted data resulted in 2 equally most-parsimony

n n
Fig. 3. Phylogenetic tree for the included species of Rhetosus and Bothriocroesus, based on ML analysis (– lnL = 122048.64163). Numbers on nodes indicate MP unweighted bootstrap support / MP go 3rd codon position transition bootstrap support / ML bootstrap support. NS = bootstrap support < 50% or contradicted by relevant analysis.
Eliminating third codon position transitions from the analysis results in eight equally parsimonious trees of 875 steps (CI = 0.5234; HI = 0.4766; RI = 0.6099). The eight trees differ only in the arrangement of the branches within the B. atrox complex. Bootstrap support levels are shown in Figure 3. Although levels of bootstrap support differ for some nodes, there are no strong contradictions with respect to the unweighted tree, and none of the main conclusions enumerated below are affected in any way. The ML search, run using the GTR + 1 + G model of sequence evolution identified as optimal by MODELTEST, yielded the tree shown in Figure 3. Although some nodes differ from those supported by the MP analyses, the major points noted below are supported by this tree.

All analyses support several nodes which substantially affect our understanding of phylogenetic relationships within Bothrops and related genera: (1) the taxa hyophora, microphthalma, and campbelli share a more recent ancestor with other Bothrops, not Porthidium, contrary to Schätti and Kramer (1993); the association between hyophora and other Bothrops was previously noted by Gubertel (1998b), Krass et al. (1996) and Parkinson (1999); (2) the monophyly of Bothrops is not supported, as B. campbelli does not group with B. microphthalma and B. hyophora; (3) all included species of Bothriopsis are rooted within Bothrops, making the latter paraphyletic if Bothriopsis is recognized; (4) Bothriopsis is polyphyletic, as Bothrops punctatus shares a more recent common ancestor with species such as B. jararacussu and B. atrox than with the Amazonian species of Bothriopsis; and (5) the Central American and Caribbean taxa (B. asper, B caribbaeus, B. lanceolatus) are rooted deep within the South American taxa. Bootstrap support for these important nodes is consistently high.

Molecular Clock Calibration

The various South American lineages of C. durissus differ by a maximum of 10 bp positions (1.4%) in their cyt-b sequences. Assuming that colonization of South America and splitting of haplotype lineages of South American Crotalus occurred immediately after the upshift of the Isthmus leads to an estimate of cyt-b sequence evolution of 0.4% myr. Taking the highest pairwise measures of divergence (10 bp positions) and assuming a Poisson distribution for the accumulation rate of substitutions, the 95% confidence limits for the rate of sequence evolution lie between 0.2-1.73% myr. There is no reason, however, why the first cladogenic split could not have occurred long after emergence of

the Isthmus. Since this estimate lies at the lower end of substitution rate estimates for squamate and any faster rate is consistent with the evidence, this estimate cannot be regarded as useful, as it does not constrain the possible rates of substitution beyond what has been estimated elsewhere.

The South American populations of Porthidium, when assessed across 1,388 bp of combined cyt-b and ND4 sequences, differ from each other at 66-70 bp positions (p-distance 4.76-5.04%). If the date of emergence of the Isthmus of Panama of 3.5 mya, as proposed by Coates and Obando (1996), is taken as the earliest date of divergence of the three haplotype lineages involved, this leads to an estimated rate of sequence divergence for these two genes of 1.36-1.44% myr. Since the phylogenetic relationships among the three South American Porthidium sequences are unresolved, taking the mean of the three pairwise measures of divergence (68 bp positions) and assuming a Poisson distribution for the accumulation rate of substitutions, the 95% confidence limits for the rate of sequence evolution lie between 1.09-1.77% myr. This estimate for the mean rate is higher than most previous estimates of a squamate mtDNA molecular clock. Given the apparently limited overwater dispersal capability of Portidium, however, we see little or no a priori reason to dismiss this calibration for a squamate cyt-b and ND4 molecular clock. A more detailed mtDNA phylogeny for a greater number of South American and Isthmian Central American populations of Portidium could further test this hypothesis. Acceptance of a "Porthidium-clock" clearly leads to a later estimate than a "Lachesis-clock" for major events in New World pitviper phylogeny and biogeography.

DISCUSSION

Origins of the South American Pitviper Fauna

The results presented here allow for a number of conclusions regarding origins, evolution, and biogeography of the South American herpetofauna. We present alternative timing for the various events, based on joint cyt-b and ND 4 data (where sequences for both genes are available) and both the Lachesis-clock of 0.66-0.76% myr calculated from the data of Zamudio and Greene (1997) and the Porthidium-clock of 1.36-1.44% myr calculated for that group from our data. In view of the many caveats surrounding the use and interpretation of molecular clocks (Hillis et al. 1996a), we suggest that the two clocks could be interpreted as upper and lower boundaries of the timeframe.
<table>
<thead>
<tr>
<th>Timing based on Lachenro-clock (mya)</th>
<th>Timing based on Porterfield-clock (mya)</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-16</td>
<td>15-8.5</td>
<td>First divergence within major New World pitviper clades</td>
</tr>
<tr>
<td>25-20</td>
<td>11-10</td>
<td>Ancestor of Bothrops divergences in South America</td>
</tr>
<tr>
<td>14-12</td>
<td>6.6-6.2</td>
<td>Split between Central and South American <em>Lachesis</em></td>
</tr>
<tr>
<td>14-12</td>
<td>7-6.6</td>
<td><em>Bothriechis schlegelii</em> lineages</td>
</tr>
<tr>
<td>12-10.5</td>
<td>5.9-5.6</td>
<td>First cladogenesis within <em>C. durissus</em></td>
</tr>
<tr>
<td>7.7-6.7</td>
<td>5.5</td>
<td>First cladogenesis in South American <em>Porthidium</em></td>
</tr>
<tr>
<td>2.2-1.9</td>
<td>1</td>
<td>First cladogenesis in South American C. durissus</td>
</tr>
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when these events may have occurred. Nonetheless, it is worth noting that the estimate of the oldest divergences in *Bothrops* at 13 mya, calculated by Hedges (1996) based on the data of Cade (1992), is more consistent with the faster end of the spectrum of pitviper cyt b and ND2 rates suggested here. The possible timetable of the colonization of South America by pitvipers is summarized in Table 3. The absolute timing of the events should be regarded as of lesser interest than their relative timing.

Among New World pitvipers, Parkinson et al. (this volume) suggest a basal dichotomy (albeit weakly supported) between a Neotropical clade, including rattlesnakes and *Atractaspis*, and a Neotropical pitviper clade, including the remaining New World pitviper genera. This basal division may correspond to the Tertiary Vicariance II event (dispersal of northern taxa into Central America, followed by vicariance between central and northern clades; proposed by Savage, 1982). The latter clade gave rise to the ancestral stocks of today's Neotropical pitviper genera at some point during the Tertiary, presumably in Central America. At least four separate colonizations of the South American mainland must have taken place from within the Neotropical clade; as early colonization by the ancestor of all Bothrops and, much later, one by *Porthidium*, one by *Bothriechis schlegelii*, and one by *Crotalus durissus* (see Parkinson, 1999; Vidal et al., 1999; Parkinson et al., this volume). The origins of *Lachesis* remain uncertain. Zamudio and Greene (1997) showed reciprocal monophyly of Central and South American *Lachesis*, and other studies of mtDNA sequences have failed to converge on a robust estimate of the sister group of *Lachesis*. Different hypotheses have variously placed *Lachesis* as unresolved (Vidal et al., 1999), or as the sister group of all bothropid genera except *Cerrophidia* (Wermel, 1992), of all New World pitvipers (Kraus et al., 1998, transversions parsimony), of *Bothrops* (Kraus et al., 1994, third codon position transitions excluded), of *Bothriechis + Bothrops* (Vidal et al., 1997), of *Bothriechis + Porthidium* (Wermel et al., 1999), of *Bothriechis* (Parkinson, 1999), or of *Ophryurus* and/or *Bothriechis* (Parkinson et al., this volume). Most of the analyses of Sather et al. (1998) found *Lachesis* to be the sister taxon of *Bothrops*. But even if *Lachesis* is the sister taxon of all Bothrops, this does not preclude a Central American origin as *Lachesis* may be the sister to the ancestor of the first pitviper to colonize South America. The origin of *Lachesis* is insufficiently resolved, although most published pitviper phylogenies support a Central American origin.

Although the levels of sequence divergence found within the major clades are similar, the genus *Bothrops* contains considerably more sequence divergence than the South American representatives of other clades. The South American *Bothrops* are paraphyletic with respect to species also found in Central America, and diversification of the genus appears to have taken place in South America. This suggests that the common ancestor of all *Bothrops* was the first vivipar to colonize South America, sometime during the Miozene, 10-23 mya. A single species, *B. asper*, invaded Central America much later, and remains the only widespread species of *Bothrops* there.

More extensive sampling of species and populations of *Porthidium* is needed, but the present data suggest that South American populations of *P. lamarii* and *P. nasutum* form a monophyletic group that represents a single invasion from Central America to South America. The *Lachesis* clock places this event in the late Miozene, 7.7-6.6 mya, whereas the *Porthidium* clock takes an invasion immediately after the emer-
gence of the Isthmus of Panama (3.5 mya) as its calibration point.

We do not have sequence data from sufficient localities to shed light on the history of colonization of South America by Bothriechis, but Crotther et al. (1993) hypothesized that this event followed emergence of the Isthmus of Panama. The high levels of sequence divergence between Ecuadorian and Costa Rican B. schlelegelii identified in this study are compatible with an invasion as long as 14–6.6 mya, pre-dating the emergence of the Isthmus by a considerable margin, but a more detailed phylogeographic study of this wide-ranging species is required to elucidate this problem.

Crotalus durissus is clearly a recent occupant of the South American continent, as noted by Vanzolini and Heyer (1985). The low levels of sequence divergence among South American populations of C. durissus are consistent with the hypothesis that this species invaded the South America during the Pleistocene, 1–2 mya, after the uplift of the Panama landbridge. On the other hand, the Central American lineages (C. d. durissus, C. d. culminatus and C. d. zetalanus) are clearly much older.

In summary, our sequence data suggest that only the colonization of South America by C. durissus can be unambiguously attributed to overland colonization after final emergence of the Isthmus of Panama. Colonization by Porthidium, however, may also post-date this colonization. The ancestor of all Bothrops clearly occupied South America long before the emergence of the Isthmus, and the available data for Lachesis and B. schlelegelii are consistent with pre-Isthmian divergence between Central and South American populations.

Faunistic exchange between Central and South America prior to final emergence of the Isthmus of Panama appears to be a common pattern among ectothermic vertebrates, including amphibians (see Hanken and Wake, 1982) and snakes (see Cadle, 1985). Iurralde-Vinent and MacPhee (1999) note the possibility of a land connection between Central and South America in the late Middle Miocene, 12.9–11.8 mya. Our data can be regarded as consistent with this hypothesis. Depending on rates of sequence divergence, the time of this land connection would correspond either to the first cladogenesis of Bothrops in South America (assuming fast rates of sequence evolution), or to the split between Central and South American Lachesis and B. schlelegelii (assuming slow rates of sequence evolution) (Table 3).

Morphological Diversification of Pitvipers in Central and South America

A comparison of the levels of mtDNA sequence divergence and morphological diversity in the different clades of New World pitvipers reveals an interesting pattern. Several clades (rattlesnakes, Bothriochis, Bothrops and Porthidium (sensu lato) and (sensu stricto)) contain broadly similar levels of sequence divergence in the genes examined here. The same applies to Ophrycus, at least as far as 12s and 16sDNA data are concerned.

The relative rate tests generally revealed approximately equal rates of mtDNA sequence divergence from the outgroup taxon Calloselasma, suggesting that rates of sequence evolution do not differ significantly between these clades. Consequently, the similar levels of DNA sequence divergence within each clade suggest that they are of approximately equal age, dating back to the late Oligocene or the Miocene. Our sampling of Bothrops species was more comprehensive than that for other genera. Consequently, compared to Bothrops, our sequence divergence data are likely to underestimate the pairwise divergence present in other clades.

Whereas these various clades have approximately equal levels of mtDNA sequence divergence, and thus may be inferred to be of similar age, they differ profoundly in levels of morphological divergence. The Central American clades Bothriochis and Porthidium (sensu lato) are most conservatively preserved in their size, body shape, and microhabitat use, and this trend occurs to an even greater extent in Porthidium (sensu stricto). The rattlesnakes (Crotalus and Sistrurus) are relatively conserved with respect to body characteristics and microhabitat use, but these clades are partitioned in terms of body size and microhabitat use. Bothrops, however, is even more variable and contains representatives of extremes in all of these categories. Furthermore, Bothrops is conspicuously more species-rich than the other genera/clades, except the rattlesnakes. If Porthidium (sensu lato) is indeed the sister clade of Bothrops, as suggested by Parkinson et al. (this volume), then the contrast is even more marked (see Table 1).

Our sequence divergence data suggest that greater age is an unlikely explanation for greater diversity of species, body plans, and natural history in Bothrops. Instead, the available data suggest that the common ancestor of all Bothrops was the first viperid to colonize South America, at least 4–10 my before any further pitviper clades. Populations of this common ancestor would have been exposed to environments devoid of viperid
faunas and with open niches to occupy. The mid-
tertiary macrostomasm snake fauna of South America
likely consisted primarily of boines and xenodontines
(Cadle, 1985; Cadle and Greene, 1993), which do not
show the trophic specializations of vipersids. It is
therefore likely that the niches occupied by viperid
snakes elsewhere were at least partially unoccupied in
mid-Tertiary South America, as were many niches
presently occupied by a variety of Central American
xenodontines, particularly the "goe-eaters" (Cadle
and Greene, 1993). Consequently, the cause of the
relatively rapid diversification of Bothrops in South
America may have been adaptive radiations into
niches largely unoccupied. Clades of the same age
from Central America would have lacked similar
opportunities for diversification, perhaps due to the
absence of other pitviper lineages already occupying
relevant niches.

The ecological structure of Neotropical colubrid
snake assemblages appears to be heavily influenced
by historical contingency (i.e., which clades with spe-
cific, relatively conserved natural history parameters
occupy a given area, Cadle and Greene, 1993). The
ecological structure of pitviper communities, however,
may follow a more deterministic pathway with rela-
tively similar ecomorphs evolving repeatedly in dif-
ferent clades (= convergence). This would parallel
the situation seen in Greater Antillean Anolis
islands, which have independently evolved a series of comparable
ecomorphs after independent colonization of different,
previously unoccupied islands (Losos et al., 1998).
A quantitative approach to the definition of ecomorphs
and niche partitioning in pitvipers (Martin et al.,
2001, this volume) across their entire distribution and
taxonomic breadth could be a fruitful field of research
in this context.

The hypothesis of an adaptive radiation in Bothrops in South America, in contradistinction to
constraint by competition among other pitviper genera
in Central America, is also supported by the bioge-
ographical distribution of later invaders to South
America. Both Porthidium and Bothriechis are
restricted to the northwestern regions of South
America, where there are no species of Bothrops of
similar size and habitat use. Where such species
approach the range of Porthidium or Bothriechis, they
 tend to be found in different habitats. Bothrocophias
 campbelli occupies higher elevations than P. lansbergeii
or P. nasutum (and attains a considerably larger maxi-
mum size), and B. medusae occupies higher and moister
habitats than P. lansbergii in northern Venezuela
(Roze, 1966; Campbell and Lamar, 1989). Bothrops
punctatus overlaps macrogeographically with B.
schlegelii, but grows much larger (Campbell and
Lamar, 1989) and the extent of its arborality and
ecological overlap with B. schlegelii is unclear. The
range of B. schlegelii does not overlap with that of
more clearly arboreal species of Bothrops, which are
confined to South America east of the Andes.

Of the more recent colonists of South America,
only C. durissus has achieved a wide distribution.
Although C. durissus is broadly sympatric with several
species of Bothrops, it is different in size, morphology,
behavior, habitat choice, and other ecological factors.
These differences may reduce ecological competition
between these two groups. In many parts of its
range (e.g., northeastern Brazil), C. durissus is the
only large pitviper, and in areas where it is broadly
sympatric with species of Bothrops of similar size, it
often occupies drier, more open microhabitats.
Finally, Lachesis is considerably larger than any
Bothrops, and differs in various aspects of its natural
history (Greene, 1997).

Systematic Implications
Porthidium.—Our analysis found P. nasutum to be
paraphyletic with respect to P. lansbergeii. This suggests
that these two taxa form part of an incompletely
understood species complex. More detailed systematic
studies of these snakes are required for resolution.

Bothriechis schlegelii.—Our analysis revealed a
surprisingly high level of ND4 sequence divergence
(9.6%) between Ecuadorian and Costa Rican B.
schlegelii. This level of divergence is almost identical
to that found between the most divergent species of
Lachesis (Zamudio and Greene, 1997), or between
unquestionably distinct species of Bothrops (e.g.,
between B. jararaca and B. neuwiedi, or between B.
asper and B. jararaca). It is also higher than all but
one of the 47 examples of mtDNA sequence diver-
gence among major intraspecific phylogroups in
amphibians and reptiles considered by Avise et al.
(1998). This suggests that the systematics of various
populations of B. schlegelii should be studied, and that
this form is likely to be polytypic, a view supported
by the work of Solórzano et al. (1998).

Crotaulus durissus.—Our analyses revealed high
levels of sequence divergence (up to 8.3%) between
the Central American lineages of this species complex,
suggesting the existence of lineages with long and
independent evolutionary histories. As in Bothriechis,
these high divergence values suggest that some of
these taxa may merit elevation to specific status. On the other hand, mtDNA divergence values of the South American taxa are extremely low. This includes *C. d. unicolor* and *C. d. vragrandis*, which differ from other South American populations with respect to color pattern and body size. These taxa have been regarded as separate species in the past (e.g., Klauber, 1972). They were treated as subspecies of *C. durissus* by Campbell and Lamar (1989) on the basis that *C. durissus* would be paraphyletic if they were excluded, a situation, confirmed by this study. Other South American subspecies of *C. durissus* have been recognized by various workers, but appear to be poorly defined. The low levels of sequence divergence of South American *C. durissus* suggest that these populations share a recent common past, and do not constitute lineages with long and independent evolutionary histories. Further studies of the *C. durissus* complex are in progress by J. Quijada-Mascareñas and others.

The status of *Bothriopsis*.—There is growing evidence that *Bothrops* (sensu Campbell and Lamar, 1989) is paraphyletic with respect to *Bothriopsis* (Cadle, 1992; Kraus et al., 1996; Parkinson, 1999; Parkinson et al., this volume; Salomão et al., 1997, 1999; Werman, 1992; this study). Salomão et al. (1997) acknowledged this paraphyly and synonymized *Bothriopsis* with *Bothrops*. Despite the evidence for rooting *Bothriopsis* within *Bothrops*, a number of workers have been reluctant to accept this change (e.g., McDiarmid et al., 1999; Parkinson, 1999; Parkinson et al., this volume). Their reluctance appears to be largely based on the untested assumption that *Bothriopsis* is a monophyletic group worthy of recognition. This implies that further splits of the genus *Bothrops* are required to avoid paraphyly of this genus relative to *Bothriopsis*. As stated above, we prefer to retain *Bothrops* as a single, large genus that includes *Bothriopsis* (see Salomão et al., 1997; Wüster et al., 1998).

In our opinion, the results presented in this paper provide further reasons to consider *Bothriopsis* as a synonym of *Bothrops*, and to retain the latter as a single, highly diverse genus. Several of these arguments could also be applied to recognition of *Bothrocophias*. However, uncertainty about the relationships between the species assigned to this genus and the rest of *Bothrops* (Gutterlet, 1998b) suggest that recognition of *Bothrocophias* may be a more conservative approach. Such changes in nomenclature in this group of snakes will undoubtedly cause temporary confusion in the medical community.

Regarding the status of *Bothriopsis*, our data suggest that *Bothriopsis* (sensu Burger, 1971) is polyphyletic, as *B. punctata* shares a more recent common ancestor with various species of *Bothrops* (e.g., *B. atrox*, *B. jararaca*us) than with the Amazonian arboreal forms. The case for recognizing only a subset of the arboreal South American pitvipers as a separate genus seems weak at best.

Second, the genus *Bothrops*, including *Bothriopsis*, comprises a single monophyletic group, resulting from a single radiation in South America. This is in contradistinction to other South American pitviper genera that represent later invasions by Central American groups, and splitting *Bothrops* would obscure this biogeographic pattern. Highlighting interesting biogeographic patterns has been used to justify synonymizing established genera in other groups (e.g., Kluge, 1991).

Third, although *Bothrops* contains greater morphological and natural historical diversity than other pitviper genera (e.g., New World crotalines such as *Bothriechis* and *Crotalus*), it appears to be no older. The cyt-b divergence levels in our data for *Bothrops* cyt-b are similar to those observed in many other genera of reptiles and amphibians (Johns and Avise, 1998), and thus do not in themselves present grounds for splitting the genus.

Finally, the greater diversity of body forms and natural history traits found in *Bothrops* reflect the unique historical circumstances of the evolution of this clade (i.e., adaptive radiation on a continent previously devoid of viperid snakes), in contrast to other New World pitviper clades not exposed to these circumstances.

The ecological and morphological diversity of *Bothrops* may be seen as a justification to split this genus into several entities representing more homogeneous groupings (Parkinson, 1999; Gutterlet and Campbell, 2001). However, sister clades often differ in the number and diversity of species they contain, sometimes as a result of taxonomic bias but often for other reasons (Minelli, 1993). *Bothrops* clearly falls into this latter category. Further splitting of the genus, to create smaller or more homogeneous genera would obscure the evolutionary history of the clades concerned and the pattern of unequal diversity resulting from this history.

In conclusion, we feel that synonymizing *Bothriopsis* with *Bothrops* is supported by conceptual, theoretical, and pragmatic considerations, such as treatment of snakebite and use of venom in medical
research. Recognition of Bothrops as a single, diverse genus is thus the most appropriate reflection of the unique evolutionary and biogeographical history of this clade.

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LITERATURE CITED


The specimen here assigned to *Bohdana punctatus* reared from *B. oxhornii* by Freire-Lamasco (1991) in color pattern, and showed scale counts intermediate between Colombian *B. punctatus* and southwestern Ecuadorian *B. oxhornii*. The status of *B. oxhornii* is uncertain, and it has been regarded as conspecific with *B. punctatus* by some authors (David and Meche, 1999; McDiarmid et al., 1999).


*Crototelsma*: C. rhodostoma: Unknown. Cyt-b - no voucher; ND4 - UMMZ 184314. AF292569, U41787.