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Phylogenetic relationships of *Loxosceles* and *Sicarius* spiders are consistent with Western Gondwanan vicariance

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ABSTRACT

The modern geographic distribution of the spider family Sicariidae is consistent with an evolutionary origin on Western Gondwana. Both sicariid genera, *Loxosceles* and *Sicarius* are diverse in Africa and South/Central America. *Loxosceles* are also diverse in North America and the West Indies, and have species described from Mediterranean Europe and China. We tested vicariance hypotheses using molecular phylogenetics and molecular dating analyses of 28S, COI, 16S, and NADHI sequences. We recover reciprocal monophyly of African and South American *Sicarius*, paraphyletic Southern African *Loxosceles* and monophyletic New World *Loxosceles* within which an Old World species group that includes *L. rufescens* is derived. These patterns are consistent with a sicariid common ancestor on Western Gondwana. North American *Loxosceles* are monophyletic, sister to Caribbean taxa, and resolved in a larger clade with South American *Loxosceles*. With fossil data this pattern is consistent with colonization of North America *via* a land bridge predating the modern Isthmus of Panama.

1. Introduction

Phylogenetic studies provide the framework necessary to determine historical species distributions and to reconstruct patterns of change in their phenotypic characters. Here, we present a global-scale phylogenetic analysis of spiders in the family Sicariidae (Keyserling, 1880), providing new insights into large-scale biogeographical patterns and a framework for analyses of venom diversity in this medically important lineage.

The family Sicariidae includes the genus *Loxosceles* (brown or violin spiders) (Heineken and Lowe, 1832) and its sister genus, *Sicarius* (Walckenaer, 1847) (Platnick et al., 1991). There are ~100 described species of *Loxosceles* the majority of which are in the Americas, West Indies, and Africa (Gertsch, 1967; Gertsch and Ennik, 1983; Newlands and Atkinson, 1988). The 23 described species of *Sicarius* are currently found in Southern Africa, and Central and South America (Gerschman and Schiapelli, 1979) (Fig. 1). The ranges of two species *L. laeta* and *L. rufescens* have been extended by recent human-transport and *L. rufescens* is nearly cosmopolitan, however, *L.rufescens* is considered native to the Mediterranean (Gertsch, 1967; Brignoli, 1969; Brignoli, 1976) and *L. laeta* native to South America (Gertsch, 1967). Two species have been described from China (Wang, 1994), one resembles *L. rufescens*, and the other *L. laeta*.

Loxosceles are notorious for the ability of their venoms to cause dermonecrotic lesions in mammalian tissues, an effect termed Loxoscelism that has been documented across the geographic distribution of the genus (Newlands and Atkinson, 1990; Binford and Wells, 2003; da Silva et al., 2004; Vetter, 2008). *Sicarius* are commonly called six-eyed sand spiders in reference to their habit of burying themselves under sand and the ability of their bodies to adhere fine sand particles (Duncan et al., 2007). Although less is known about the effects of *Sicarius* bites, venoms of some species cause serious dermonecrotic lesions (Newlands and Atkinson, 1990; Van Aswegen et al., 1997; but see Alegre et al., 1977).

Sicariid taxonomy, particularly that of New World *Loxosceles*, has historically benefited from relatively careful attention due to the toxic potential of their venom. Systematic revisions of *Loxosceles* have been conducted for North American (Gertsch and Ennik, 1983) and South American taxa (Gertsch, 1958; Bücherl, 1961, 1964; Gertsch, 1967; commentary in Brignoli, 1976). These works recognized a single species group in North America and the West Indies (Gertsch and Ennik, 1983) and four species groups in South America (Gertsch, 1967). *Sicarius* and African *Loxosceles* have not

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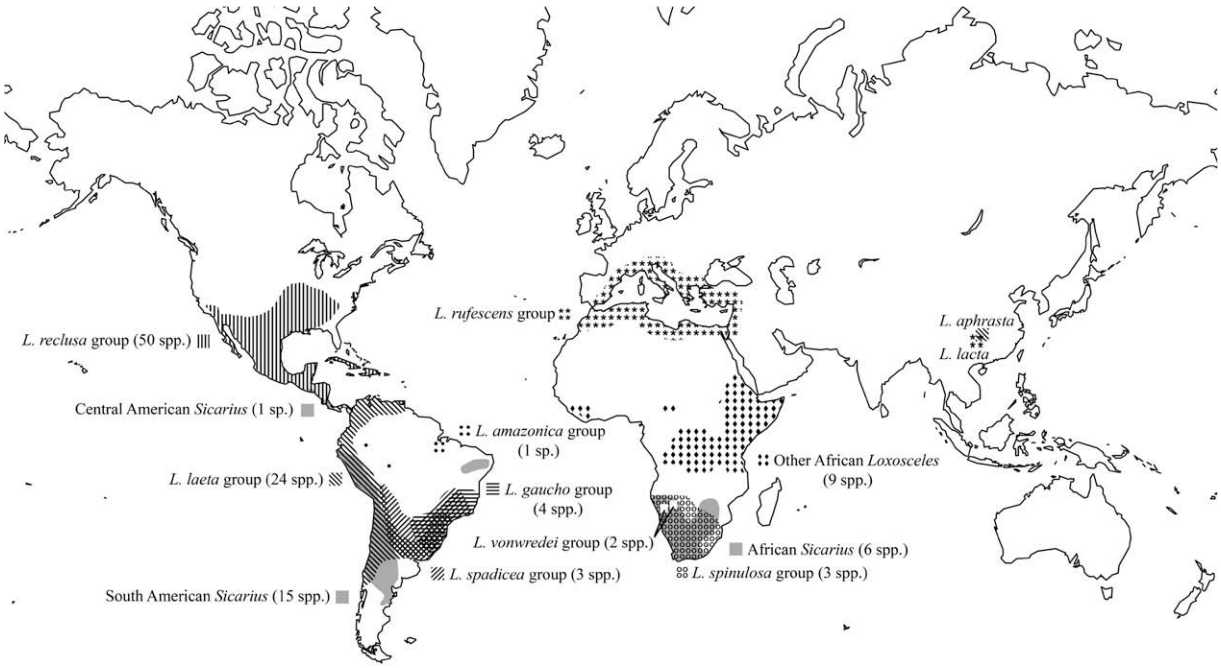

Fig. 1. Native geographic distribution of *Loxosceles* and *Sicarius*. Defined species groups of *Loxosceles* are indicated separately with species numbers included in parentheses.

undergone unified systematic revision (see Newlands, 1975; Brignoli, 1976; Gerschman and Schiapelli, 1979 for more recent regional attention to species groups). Current species counts and species groups are summarized in Fig. 1. On all continents to which these species are native, researchers continue to discover undescribed species (particularly for *Loxosceles*, e.g. Martins et al., 2002).

Despite systematic attention within *Loxosceles*, proposed generic and family-level relationships within this lineage have been in considerable flux (Brignoli, 1976; Alayón-García, 1981; Lehtinen, 1983; Platnick et al., 1991; Goloboff and Ramirez, 1991). The current status of *Loxsceles* and *Sicarius* as unified within the family Sicariidae was proposed by Platnick et al., 1991, based on synapomorphies in spinneret morphology. These two genera also uniquely share the dermonecrotic venom toxin sphingomyelinase D (Binford and Wells, 2003). The proposed sister-taxon to Sicariidae includes Drymusidae, Scytodidae (Platnick et al., 1991), and Periegopidae (Forster, 1995; Coddington et al., 2004). Members of the monogeneric family Drymusidae are poorly known, but currently described from Africa, South and Central America, and the West Indies (Valerio 1971; Alayón-García, 1981; Goloboff and Ramirez, 1991; Platnick, 2008). Scytodidae are found worldwide and Periegopidae are Australasian (Forster, 1995; Platnick, 2008). Molecular phylogenetic studies that would provide independent evidence of the relationships proposed by morphology have not been published to date.

Knowledge of dispersal biology is central to understanding biogeographic patterns. Sicariids are not naturally long-range dispersers (Binford, personal observation). *Loxosceles* are found in caves or on or near the ground in crevices made by natural debris such as cracks in dry hillsides, between rocks or plant debris and the ground. Human debris also creates crevices that are ideal habitat (Hite et al. 1966; Gertsch, 1967; Gertsch and Ennik, 1983; Fischer and Vasconcellos-Neto, 2005 and personal observation). *Sicarius*, like *Loxosceles*, live in shallow caves and in crevices made between natural debris and the ground, but they are often in highest abundance in patches of fine sand, such as at the base of cliffs or under rocky overhangs (Binford, personal observation). Unlike some spiders, sicariids do not undergo long-distance dispersal by ballooning, but rather, disperse by walking on the ground. We frequently find series of molted exoskeletons of increasing size next to single mature *Loxosceles* or *Sicarius* in natural retreats, suggesting that individuals remain in the same locations for most, if not all, of their lives. We have also seen individuals outside of retreats and wandering at night. With this type of dispersal biology, natural range expansions likely occur slowly and over short distances.

The extant distribution of sicariid species suggests that their diversification was influenced by vicariance events resulting from continental drift. The concentration of species diversity of *Loxosceles* and *Sicarius* on Africa and the Americas is consistent with a common sicariid ancestor on Western Gondwana with early divergence initiated by separation of these continents which was complete 95 MYA (Pitman et al., 1993). Recovery of reciprocal monophyly of taxa on each continent, and divergence times that predate this split would be consistent with such a biogeographic influence. Furthermore, the hypothesis of a common ancestor for *Loxosceles* on Western Gondwana would require colonization of North America from South America. This scenario would be supported by a monophyletic North American *Loxosceles* clade with a sister-taxon relationship to South American *Loxosceles*. In addition, the presence of native *Loxosceles* species on the islands of the West Indies (Gertsch and Ennik, 1983) and the discovery of a fossil *Loxosceles* in Dominican amber (ca. 20 MYA) (Wunderlich, 2004) are consistent with a presence in the region that predates the modern Isthmus of Panama (Morley, 2003; Pennington and Dick, 2004). There are a series of proposed temporary land connections that could serve as dispersal corridors between South America and North America. Dates of these connections range between 70 and 15 MYA (reviewed in Sanmartin and Ronquist, 2004).

The goal of this work is to use molecular phylogenetics and molecular dating analyses to test the hypothesis that large-scale divergence patterns within sicariid genera have been influenced by vicariance events caused by continental drift. In addition to biogeographic insight, these data provide a framework for further systematic attention to this group. While the focus of this manuscript is biogeographic, an upcoming paper will apply the patterns we infer here to analyses of venom evolution.

2. Methods

2.1. Taxon sampling

Our dataset includes *Sicarius* and *Loxosceles* representatives from all known geographic regions to which they are native except *Loxosceles* from Northern and Central Africa. Our *Loxosceles* taxa include representatives of all distinct species groups described to date (Table 1 and Fig. 1). We include only a few *Drymusa*, and *Scytodes* taxa, however, they span a large geographic range. For more remote outgroups, we include a few representative haplogyne taxa that are proposed to be the closest relatives of sicariids and scytodoids (Platnick et al., 1991; Coddington et al., 2004) (Table 1).

For some populations, we have mature specimens that do not morphologically resemble any described species and are genetically divergent from all other taxa in our analysis. We do not describe those here, but list them in Table 1 as "sp. nov." and differentiate them in discussion by collecting locality. We place all of our *Loxosceles* in species groups that were defined by Gertsch 1958, 1967 or Gertsch and Ennik, 1983, or in species groups that we propose. Some *Loxosceles* and *Sicarius* individuals had genitalic and somatic characteristics that were not morphologically consistent with, but were similar to, currently described species. We refer to an individual as "sp. cf. X" when we consider it likely to be a divergent member of another species (X), or as "sp. aff. X" when we consider them likely to be a different species that shares an affinity with another species. We include three *Loxosceles* individuals that represent distinct geographic regions, but for which we only had juvenile specimens (Cayman Islands; Dominican Republic; Oorlogskloof, South Africa). We were able to identify the Dominican Republic specimen based upon somatic characters, but we refer to the other two as "sp". Upon completion of our ongoing studies, we will deposit voucher specimens in the California Academy of Sciences and duplicates in the National Museums of Natural History in the respective countries of origin.

All specimens were collected in the field by ourselves and colleagues (see acknowledgments). When we collected mature spiders we either preserved them whole in 95% EtOH, or we removed a leg (usually the 3rd left leg) which we stored in RNAlater (Ambion) or 95% EtOH and then preserved the body in 75% EtOH. When we only found immatures in the field for a particular population, we brought them to Lewis & Clark College where we reared them to maturity in a 25 °C temperature controlled room (with constant 40% humidity) and a light cycle with 13 h on and 11 h off.

2.2. Molecular analyses

From at least two individuals per population included in the analysis, we extracted genomic DNA from a single leg using a DNAeasy kit (Qiagen Inc.). Genomic DNAs were eluted with buffer EB and stored at 4 °C. We visualized DNA quality by running 5 μl on a 1% agarose gel. From these DNA samples, we amplified up to three gene fragments that have been informative about species-level and/or generic–level relationships in spiders: a ~1800 bp fragment of the large nuclear ribosomal subunit 28S; a ~600 bp fragment of the mitochondrial gene cytochrome oxidase 1 (COI); an ~900 bp fragment of the nuclear region that includes the ribosomal subunit 16S, *t*-leucine, and the 5′ portion of nitrate dehydrogenase 1 (16S/ND1). 28S has been informative for resolving relationships among genera and species groups (e.g. Hedin, 2001; Hedin and Maddison, 2001; Arnedo et al., 2004; Bruvo-Mađarić et al., 2005; Hedin and Bond, 2006; Hendrixson and Bond, 2007); while COI, 16S, and ND1 evolve faster than 28S and have been useful for analyses of more recent divergences (see above and Garb et al., 2004). Primers and PCR conditions are summarized in Table 2. All reactions used buffers from the MasterAmp PCR optimization kit (Epicentre Technologies) and Taq Polymerase (New England BioLabs). Because of the limitations of the sequencing reaction, smaller fragments of 28S were sequenced using primers listed in Table 2. All PCR products were cleaned-up, quantified, normalized and sequenced in 96-well format at the University of Arizona Genomic Analysis and Technology Core (Applied Biosystems 3730xl DNA Analyzer). While our goal was thorough overlap of molecular markers for all taxa in our analysis, after many attempts to optimize we were unable to amplify all genes for all taxa. Therefore, our taxon inclusion varies across genes with 28S and COI having the most thorough coverage and 16S/ND1 limited to *Loxosceles* with *Sicarius* as an outgroup (Table 1).

2.3. Sequence analyses

We assembled sequences using Staden Package (Staden et al., 2000), or Sequencher v4.7 (Gene Codes Corporation) and checked nucleotide base calls by eye against chromatograms. For protein-coding genes (COI and ND1), we eliminated sequences from the data matrix that had premature stop codons or frameshifting indels.

2.3.1. Alignment

We used different approaches for aligning the rDNA and coding sequence datasets. We manually aligned the nucleotide sequences for protein-coding regions (COI and ND1) in MacClade v.4.06 (Maddison and Maddison, 2003) using the translated amino acid sequence as a guide. For the rDNA sequences (16S and 28S), we estimated optimal alignments using a variety of static optimization approaches. First, we did an elision analysis by constructing several alignments using the program ClustalX (Higgins and Sharp, 1988) with varying gap opening/extension costs (8/2, 20/2, 24/4, and 24/6) along with a manual alignment in MacClade 4.0 following the approaches of Hedin and Maddison (2001). We then made a concatenated matrix (elision matrix) (Wheeler et al., 1995) of all five alignments. We also generated progressive alignments using kPrank (Loytynoja and Goldman, 2005) and Muscle v3.6 (Edgar, 2004). The kPrank analysis started with a neighbor-joining tree generated with Clustal X and used a Jukes Cantor model. We subjected each of these alignments to a full heuristic parsimony analysis with 1000 bootstrap replicates (PAUP* 4.0b10, Swofford, 1998) and Bayesian analyses under the conditions detailed below. We quantitatively compared the similarity of parsimony trees resulting from each individual alignment by using quartet distance analyses in Component 2.0 (Page, 1993), and symmetric-differences distances computed in PAUP*.

2.3.2. Dataset characteristics

For each individual gene, we estimated the best-fit model of evolution using Akaike Information Criterion (AIC) as computed by ModelTest v3.7 (Posada and Crandall, 1998). We tested for significant heterogeneity in nucleotide base composition using the χ^2 test in PAUP*. We tested for potential saturation in each of the data partitions using the Xia et al. (2003) test of substitution saturation and by visual analysis of patterns of transitions and transversions both using DAMBE (Xia and Xie, 2001).

2.4. Phylogenetic analyses

We performed analyses on individual genes and on the partition combinations summarized in Tables 1 and 4. For all datasets, we analyzed relationships using parsimony in PAUP* and Bayesian analyses in MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003). Gaps were treated as missing. Parsimony searches were heuristic, with random addition sequence (1000 replicates) and tree bisection–reconnection (TBR) branch swapping. Confidence in the trees

Table 1
Taxon inclusion of individual genes. Combined analyses included all possible combinations for which genes were available within a single population

Species	Locality	28S	COI	16S/ND1
Loxosceles				
reclusa species group				
L. deserta	USA: Stanton, AZ	EU817778	EU817667	EU817799
L. deserta	USA: Granite Mtn, CA			EU817796
L. kaiba	USA: Grand Canyon NP, AZ	EU817774	EU817662	EU817808
L. reclusa	USA: Oxford, MS	EU817776	EU817669	EU817801
L. arizonica	USA: Tucson, AZ	EU817769	EU817663	EU817798
L. sabina	USA: Bill's Cave, Vail, AZ	EU817771	EU817666	
L. apachea	USA: Stein's Ghost Town, NM	EU817768	EU817665	EU817793
L. blanda	USA: Carlsbad, NM	EU817770	EU817664	EU817818
L. baja	Mexico: El Triunfo, Baja Sur	EU817775	EU817661	EU817792
L. boneti	Mexico: Iguala, Guerrero	EU817772		EU817807
L. colima	Mexico: Coquimatlan, Colima	EU817777	EU817668	EU817800
L. chinateca	Mexico: Apazapan, Veracruz		EU817670	EU817802
L. sp.	Cayman ISL: Queen Eliz. II Pk.	EU817773	EU817660	
L. caribbaea	Dominican Republic: Oviedo		EU817659	EU817819
laeta species group				
L. laeta	Argentina: Buenos Aires	EU817784	EU817680	
L. laeta	Peru: Pisco	EU817783		EU817794
L. laeta	Peru: Lima			EU817812
L. sp. nov.	Argentina: Catamarca		EU817679	EU817811
L. sp. nov.	Bonaire: Uruzjan Blanco Cave	EU817782	EU817658 EU817671	EU817809
spadecia species group				
L. intermedia	Argentina: El Palmar	EU817786	EU817676	
L. hirsuta	Argentina: Chaco	EU817788	EU817678	EU817805
L. spadecia	Argentina: Catamarca	EU817787	EU817677	EU817804
gaucho species group				
L. variegata	Argentina: Corrientes	EU817785	EU817675	EU817797
amazonica species group				
L. amazonica	Peru: Loreto, Pevas	EU817779	EU817674	EU817813
rufescens species group				
L. rufescens	USA: Indianapolis, IN[a]	EU817780	EU817673	EU817803
L. lacta	China: Gizhou Province, Dong	EU817781	EU817672	
spinulosa species group				
L. sp.	South Africa: Oorlogskloof NR, N. Cape	EU817760	EU817694	EU817815
L. speluncarum	South Africa: Greensleeves cave	EU817751	EU817692	EU817816
L. speluncarum	South Africa: Groenkloof Fountain Valley, Gauteng	EU817752 EU817753	EU817691	EU817822
L. sp. aff. *speluncarum*	South Africa: Strydpoort Mtns, Northern Province	EU817754	EU817693	EU817821
L. spinulosa	South Africa: Kwazulu-Natal	EU817756	EU817688	
L. spinulosa	South Africa: Borakalalo	EU817759	EU817683	
L. spinulosa	South Africa: Kruger	EU817757	EU817689	
L. spinulosa	Namibia: Grootfontein	EU817758	EU817690	EU817795
L. sp. cf. *spinulosa*	Namibia: Waterburg 1	EU817761	EU817686	
L. sp. cf. *spinulosa*	Namibia: Waterburg 2	EU817763		
L. sp. aff. *spinulosa*	Namibia: Ruacana Falls	EU817755	EU817687	EU817820
L. sp. aff. *spinulosa*	Namibia: Munsterland	EU817764	EU817685	EU817823
L. sp. aff. *spinulosa*	Namibia: Windhoek	EU817762	EU817684	
vonwredei species group				
L. vonwredei	Namibia: Uisib Farm Caves	EU817766 EU817767	EU817681	EU817814
L. sp. nov.	Namibia: Wundergat	EU817765	EU817682	EU817806
Sicarius				
Africa				
S. damarensis	Namibia: Daan Viljoen	EU817749	EU817702	
S. damarensis	Namibia: Waterburg 1	EU817748	EU817701	
S. damarensis	Namibia: Waterburg 2	EU817747		
S. sp. aff. *damarensis*	Namibia: Munsterland Farm	EU817746	EU817699	
S. sp. cf. *damarensis*	Namibia: Uisib Farm Caves	EU817745	EU817700	
S. sp. cf. *damarensis*[b]	South Africa: Oorlogskloof	EU817744	EU817697	
S. dolichocephalus	Namibia: Ruacana Falls	EU817741	EU817698	
S. sp.	Namibia: Wundergat	EU817743	EU817703	EU817824
S. albospinosus	Namibia: Gobabeb	EU817750		
S. sp. cf. *hahni*[c]	South Africa: Strydpoort Mtns, Northern Province	EU817742	EU817696	
South/Central America				
S. rugosus	Costa Rica: Palo Verde	EU817736	EU817706	
S. peruensis	Peru: Lima	EU817730 EU817731		
S. rupestris	Argentina: Corralito	EU817733	EU817695	
S. patagonicus	Argentina: Picun Leufu	EU817734	EU817707	
S. patagonicus	Argentina: Arroyito	EU817732	EU817708	
S. sp. aff. *patagonicus*	Argentina: Merlo	EU817735	EU817709	EU817817
S. terrosus	Argentina: Sierra de las Quijades	EU817740	EU817704	EU817810
S. sp.	Argentina: Paso Cordoba	EU817738		
S. terrosus	Argentina: Catamarca	EU817739		

(*continued on next page*)

Table 1 (*continued*)

Species	Locality	28S	COI	16S/ND1
S. terrosus	Argentina: Salta	EU817737	EU817705	
Drymusa				
D. capensis	South Africa: Capetown		EU817711	
D. serrana	Argentina: Sierra de la Ventana	EU817728 EU817729	EU817712	
D. serrana	Argentina: Merlo	EU817726 EU817727	EU817713	
D. dinora	Costa Rica: Osa Peninsula	EU817718 EU817719	EU817714	
Scytodes				
Scytodes sp.	Argentina: Sierra de la Ventana	EU817722		
Scytodes sp.	COSTA RICA: Osa Peninsula	EU817720		
Scytodes sp.	MEXICO: El Rosario, Baja	EU817725	EU817710	
Scytodes sp.	NAMIBIA: Wundergat	EU817723		
Scytodes sp.	NAMIBIA: Waterburg	EU817724		
Outgroups				
Usofila pacifica	USA: Eagle Creek, OR	EU817721		
Diguetia canites	USA: Baboquivari Mtns. AZ	EU817717	EU817715	
Dysdera crocata	USA: Portland, OR	EU817789		
Plectreurys	USA: Anza-Borrego, Park, CA	EU817790	EU817716	
Kibramoa	USA: Anza-Borrego, Park, CA	EU817791		

Taxa are organized by genus and species group (*Loxosceles* only). Complete collecting locality information is available upon request from G.J.B. GenBank Accession Nos. are included for individual gene fragments.

[a] Confirmed morphologically and genetically indistinct from *L. rufescens* native to Spain.
[b] Same population referred to as *Sicarius testaceus* in Binford and Wells (2003).
[c] Same population referred to as *Sicarius hahni* in Binford and Wells (2003).

Table 2
PCR conditions used for amplifying genes

Gene	Primers		Master amp buffer	Annealing temperature (°C)	Fragment length (bp)
ND1/	N1-J-12261[a]	5′-TCA TAA GAA ATT ATT TGA GC-3′	B	43–51	~900
16S	LR-N-13398[b]	5′-CGC CTG TTT AAC AAA AAC AT-3′			
COI	C1-N-2568[c]	5′-GCT ACA ACA TAA TAA GTA TCA TG-3′	E	47	~580
	C1-J-1751 'SPID'[c]	5′-GAG CTC CTG ATA TAG CTT TTC C-3′			
28S	ZX1[d]	5′-ACC CGC TGA ATT TAA GCA TAT-3′	H	56	~1900
	A58OP1[d]	5′-AGA GCC AAT CCT TGT CCC GA-3′			
	Internal primers for sequencing				
	A53[d]	5′-CCG AAG TTT CCC TCA GGA TAG C-3′			
	A56[d]	5′-TCT TAG GAC CGA CTG ACC-3′			
	ZR3[d]	5'-GAA AAG AAC TTT GAA GAG AGA GTT CA-3'			
	ZR1[d]	5′-GTC TTG AAA CAC GGA CCA AGG AGT CT-3′			
	A50[e]	5′-TAG TTC ACC ATC TTT CGG GTC-3′			

[a] Hedin (1997).
[b] Simon et al. (1994).
[c] Hedin and Maddison (2001).
[d] Bond and Hedin (2006).
[e] This study.

Table 3
Summary of data set characteristics and model parameters as estimated from Modeltest (whole fragments), and nucleotide frequency analyses on PAUP (separate codon positions)

Gene	Model	A	C	G	T	A<>C	A<>G	A<>T	C<>G	C<>T	G<>T	G	I	Homo p	# Char inf/all
28S muscle	GTR + I + G	0.19	0.29	0.33	0.20	0.94	1.98	1.60	0.61	4.84	1	0.43	0.48	0.99	566/2098
16S 24/6	GTR + I + G	0.43	0.15	0.12	0.31	4.11	5.13	5.16	2.25	17.62	1	0.94	0.19	0.02	232/563
ND1	TVM + I + G*	0.41	0.22	0.04	0.32	0.35	2.18	0.50	1.36	2.18	1	0.69	0.1	0.00	258/362
1st		0.45	0.21	0.11	0.23									0.78	121
2nd		0.21	0.22	0.09	0.48									1.00	121
3rd		0.45	0.21	0.02	0.32									0.00	120
COI	TVM + I + G	0.25	0.05	0.21	0.49	1.86	22.66	1.64	7.42	22.66	1	0.30	0.27	0.00	275/569
1st		0.26	0.11	0.33	0.29									1.00	189
2nd		0.15	0.21	0.19	0.46									1.00	190
3rd		0.25	0.03	0.19	0.53									0.00	190

The number of characters is reported for both the parsimony informative "inf" and total characters in the aligned dataset "all". Homo *p* refers to the *p*-value from χ^2 test of nucleotide homogeneity as implemented in PAUP* 4.0b.
*Delta value of difference between 1st AIC and 2nd AIC selected model (GTR + I + G) is 0.87.

was assessed using 1000 bootstrap replicates. For all Bayesian analyses, we used the model of substitution indicated by AIC, and searches were done using MrBayes. For analyses of concatenated data, we used separate model parameters for each data partition. All Bayesian analyses consisted of two simultaneous runs each with four simultaneous Markov Chain Monte Carlo (MCMC) chains. We initially ran these for a minimum of 1 million generations and we continued each run until we considered the sampling

Table 4
Bayesian posterior probability support of particular clades

dataset / clade	28S							COI		16S ND1	28S COI	28S 16S ND1	COI 16S N16	all
	M	kP	82	202	242	246	Ma	nuc	aa					
Loxosceles								p	pa		s			
spinulosa group									s					
(NW *Lox*, *vonwredei* grp)								p		§				
NW *Lox* + *ruf* group														
(*L. amaz., L. rufescens)*														
(*L.var.,* (*L amaz., L ruf*))								p	X	X			X	
(*L.rufescens, L. lacta*)								X	X					
spadicea group														
(*L.* WI, N. Am *reclusa*)						¶		p	X					
N. Am *reclusa* group								*	*					
(*L.*sp nov Bonaire, *L laeta*)								X	X					
(*L.laeta*, *reclusa* group)								p	X	p			p	
Sicarius		p						pa	pa					
NW *Sicarius*														
OW *Sicarius*														
Drymusa		us	X	u	X		u	X						
Scytodes														
Drymusa + *Scytodes*		u	u	u	u	u	u	pa	X		X			
Sicariidae	pa	pa		pa		p	p		s		X			

p - group is one possible resolution of a basal polytomy
pa - taxa are paraphyletic
§ - *L. vonwredei* is derived from within the S. American clade.
* - *L.* sp nov Bonaire is a polytomy with members of the reclusa group.
u - *Usofila* is derived from within the clade
s - *Scytodes* is derived from within the clade
¶ - *L.* sp. Cayman Isles is derived from within the continental *reclusa* group

An empty cell indicates monophyly of that group was not testable with taxon composition in the analysis. An X indicates the clade is not supported as monophyletic, a box shaded grey indicates clade is resolved, but posterior probabilities are <0.95, and a black box indicates support between 0.95 and 1.0. For 28S, support of focal clades by Bayesian analyses of different alignment strategies is indicated in small boxes: M, Muscle; kP, kPrank; the four columns with numbers the upper number is gap insertion penalty and the lower number is gap extension penalty; Ma, manual. For COI, nuc, nucleotide; aa, amino acid. NW, New World; *Lox*, *Loxosceles*; *ruf*, *rufescens*; *var*, *variegata*; *amaz*, *amazonica*; WI, West Indies.

to be adequate (average standard deviation of split frequencies <0.01) (Ronquist et al., 2005). We stopped runs after 10 million generations if the average standard deviation of split frequencies did not reach the 0.01 threshold (necessary only for CO1 analyses). The current tree at every increment of 100 generations was saved to a file. We used default cold and heated chain parameters and compared the separate runs every 1000 generations to facilitate convergence. We determined the burnin period as prior to log likelihood stabilization and convergence which we visually inspected and verified using the program Tracer 1.3 developed by Andrew Rambaut and Alexci Drummond (http://evol.zoo.ox.ac.uk/software.html?id=tracer).

2.5. Divergence time estimation

To test temporal hypotheses corresponding to vicariance events, we estimated divergence times for 28S and CO1/16S/ND1 datasets in r8s v1.71 (Sanderson, 2003, 2006) and the multidivtime (multidistribute package; Thorne, 2003; software available at http://statgen.ncsu.edu/thorne/multidivtime.html). We selected these datasets because they include the most thorough taxonomic coverage for testing our hypotheses. Likelihood ratio (LR) tests performed in PAUP* rejected the molecular clock hypothesis (28S: LR = 165.68, df = 27 for reduced dataset, $p < 0.005$; COI/16S/ND1: LR = 147.96, df = 28, $p << 0.005$), so we used methods that account for rate heterogeneity in r8s and the multidistribute package.

2.5.1. Dataset preparation and clock calibration

We reduced the 28S dataset to 28 taxa (rooted with Dysderidae; Fig. 7), including South American and African *Sicarius* and at least one representative in each *Loxosceles* species group. The COI/16S/ND1 dataset included 29 taxa in the ingroup (rooted with *Sicarius* from Wundergat, Namibia; Fig. 6b). For each dataset using all molecular dating approaches, we constrained the ancestral node age of Caribbean and North American *Loxosceles* to be at least 20 MYA based on a fossil *Loxosceles* in Dominican amber (Wunderlich, 2004). The root age of the 28S tree was calibrated to be 240 MYA (based on mean date obtained by Ayoub et al. 2007 for the ancestor of Plectreuridae and Diguetidae) and the root age of the COI/16S/ND1 tree was calibrated to be 130 MYA (based on a conservative estimate of the ancestral node of *Sicarius* and *Loxosceles* using the 28S dataset; see Table 5). Roots were fixed in r8s analyses but since multidivtime does not allow node ages to be fixed, we set the above numbers to be the upper bounds for the age of the roots. We also tested the influence of an imposed Gondwanan time frame on the divergence time estimate of the *reclusa* species group by omitting the fossil constraint and constraining the age of either (1) the ancestral node of *L. vonwredei* and New World *Loxosceles* or (2) the node in (1) and the ancestral node of *L. rufescens* and *L. amazonica* to be at least 95 MYA, corresponding to the last physical connection between South America and Africa.

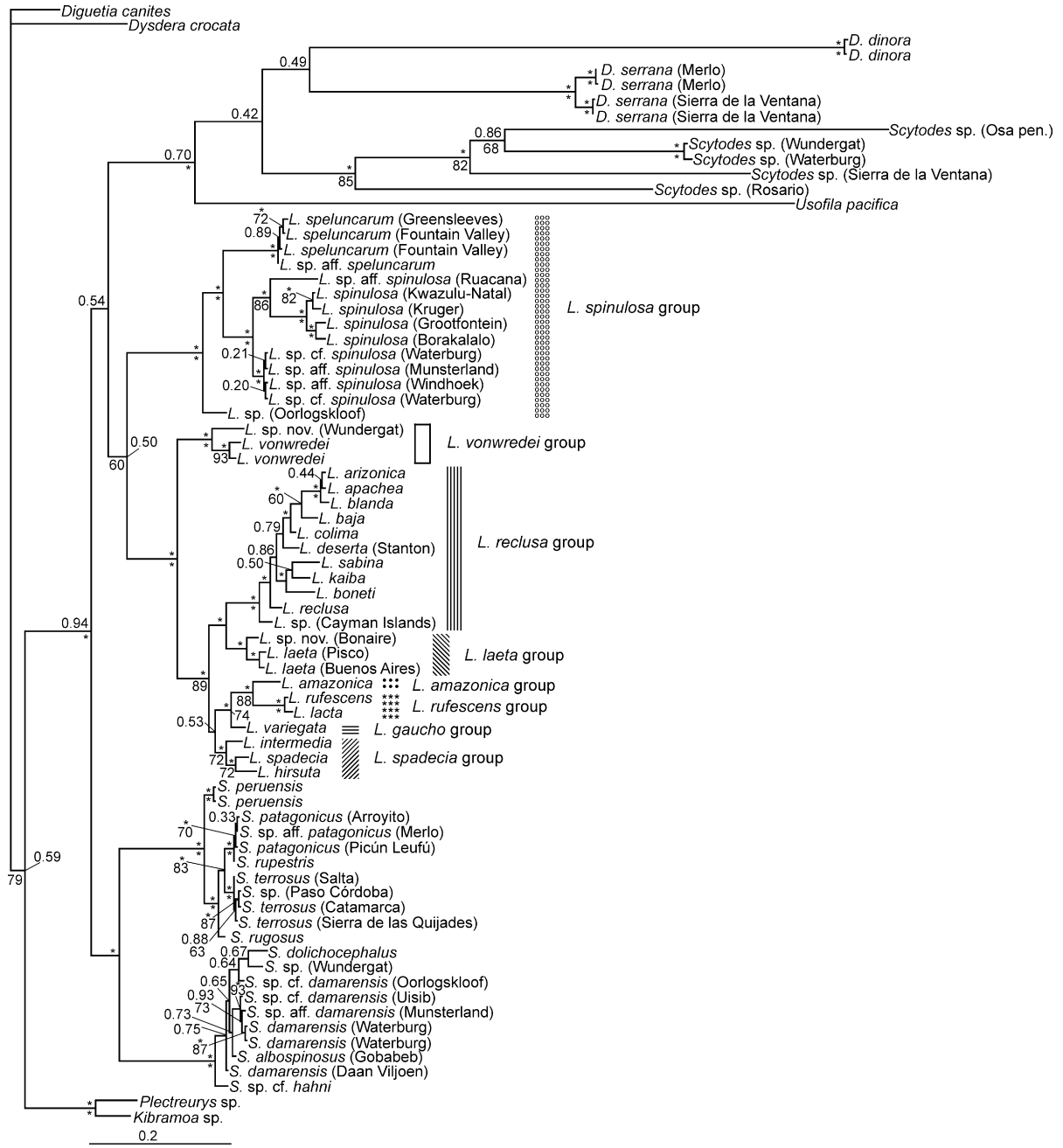

Fig. 2. Phylogeny of Sicariidae based on Bayesian analyses of 28S sequence data aligned using progressive alignment (Muscle). Numbers above branches represent posterior probabilities of the nodes to their right. Numbers below branches represent bootstrap support (1000 reps) from parsimony analyses. In all cases, stars indicate values > 95%. Posterior probabilities < 0.5 and parsimony bootstrap values < 50 are not shown. Bars to the right of clades indicate *Loxosceles* species groups using the same shading patterns as in Fig. 1.

2.5.2. Estimating divergence times using r8s

For analyses in r8s, we pruned the outgroup before estimating divergence times (Sanderson, 2003, 2006). We selected Langley–Fitch (LF) (Langley and Fitch, 1974) and Penalized Likelihood (PL) (Sanderson, 2002) methods to estimate divergence times based on results from cross-validation analyses (described in Sanderson, 2006). Cross-validation analyses allow the user to objectively select an appropriate smoothing value for a particular dataset (Sanderson, 2003, 2006). For both datasets, cross-validation scores were lowest for the PL method using an additive penalty function (Sanderson, 2003; Sanderson, 2006) and a smoothing value ranging from 100–1000 (28S) to 1000–10,000 (CO1/16S/ND1). Because high smoothing values performed best for the CO1/16S/ND1 dataset, we estimated divergence times using both PL and LF.

To produce confidence intervals (CIs) of our divergence time estimations, we followed the suggestion in the r8s user's manual (Sanderson, 2006) and made 100 bootstrapped character matrices of each dataset in seqboot v3.5e (from the PHYLIP package, Felsenstein, 2002). We performed a maximum likelihood analysis on each matrix in PAUP*, using likelihood settings calculated from the original character matrix and constraining the tree topology, to produced 100 trees with identical topologies and different branch lengths. We analyzed these trees in r8s from 25 different starting points to optimize the results. In the 28S dataset, relatively few variable sites caused the trees we made from bootstrapped character matrices to contain zero-length internal and terminal branches. r8s is limited in how it can work with zero-length branches so before estimating divergence times we assigned all

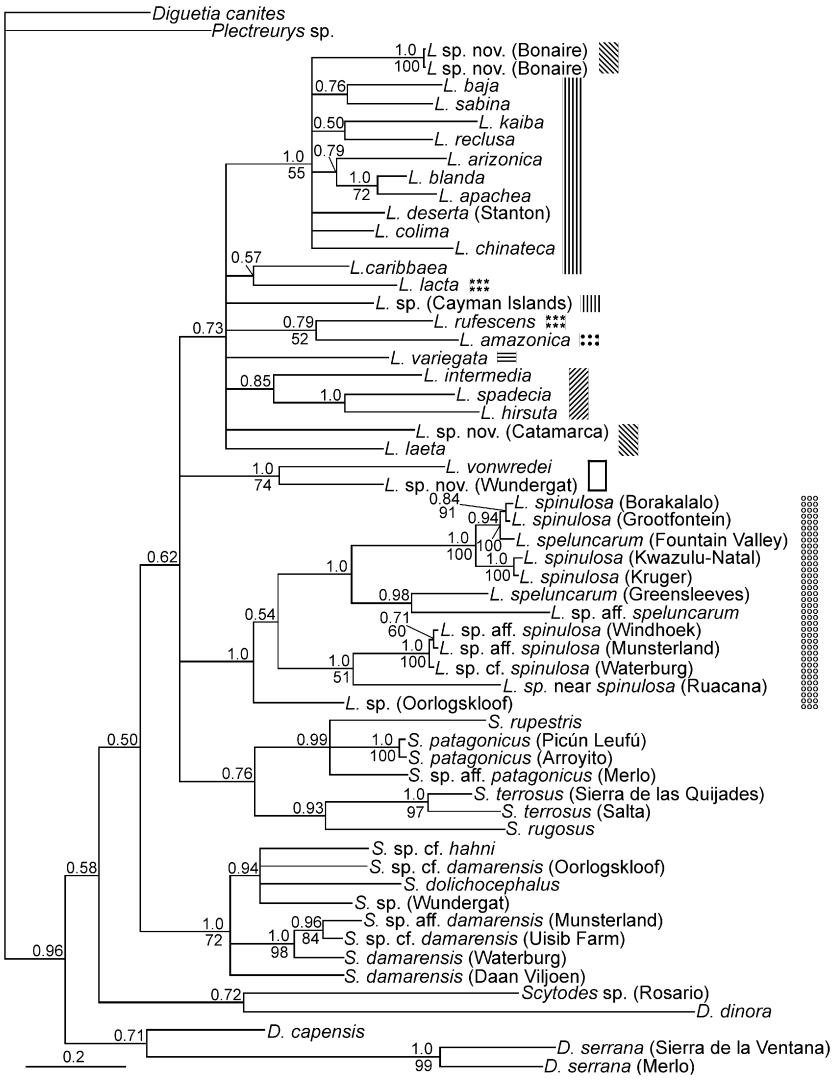

Fig. 3. Phylogeny of Sicariidae based upon Bayesian analyses of COI nucleotide sequence data. Numbers above branches represent posterior probabilities of the nodes to their right. Numbers below branches represent bootstrap support (1000 reps) from parsimony analyses. Posterior probabilities < 0.5 and parsimony bootstrap values < 50 are not shown. Bars to the right of clades indicate *Loxosceles* species groups using the same shading patterns as in Fig. 1.

zero-length branches a value of 0.1% of the shortest branch length in the corresponding tree. Only one taxon in the COI/16S/ND1 dataset had a zero-length terminal branch (*L. speluncarum* from Fountain Valley, South Africa) and because there were many *spinulosa* group representatives present in the dataset we pruned it from trees before estimating divergence times.

2.5.3. Estimating divergence times using a Bayesian approach

For an independent test of our hypotheses we estimated divergence times using a Bayesian MCMC approach. We used multiple programs in the multidistribute package to prepare the dataset and followed the guidelines outlined by Thorne and Kishino (2002), Rutschmann (2005) and the multdivtime manual. First, we estimated transition/transversion ratios, nucleotide frequencies and shape parameters for the discrete γ model of rates among sites using the F84 model in baseml (PAML package, Yang, 1997). We then used estbranches (multidistribute package) to estimate the maximum likelihood of branch lengths for the rooted tree along with a variance–covariance matrix. estbranches removes the outgroup from the dataset for divergence time estimation in multidivtime. Multidivtime estimates posterior distributions of substitution rates and divergence times and requires that prior probabilities of certain parameters be specified before running. For our 28S dataset, we used the following settings for priors: rttm and rttmsd = 240 MYA (based on Ayoub et al. 2007); rtrate and rtratesd = 3.95 (substitutions per million years; median length of branches from root to tip divided by 240 MY); brownmean and brownsd = 0.625 (so that rttm * brownmean = 1.5); bigtime = 390 MYA (based on oldest recorded spider fossil in Selden et al., 1991). For the COI/16S/ND1 dataset we set the above priors to be 130 MYA, 47.1 substitutions per million years, 1.19, and 390 MYA, respectively. We ran the chain for a total of 210,000 generations, using 200,000 as the burnin value and sampling every 100 generations for the last 10,000 generations to obtain our divergence time estimates. We performed each analysis twice from different starting points to check for convergence of the MCMC.

3. Results

3.1. Data characteristics and model choice

The taxonomic composition of our datasets is summarized in Table 1. The dataset for 28S and COI are the most inclusive while 16S and ND1 are limited to only *Loxosceles*, with *Sicarius* as an

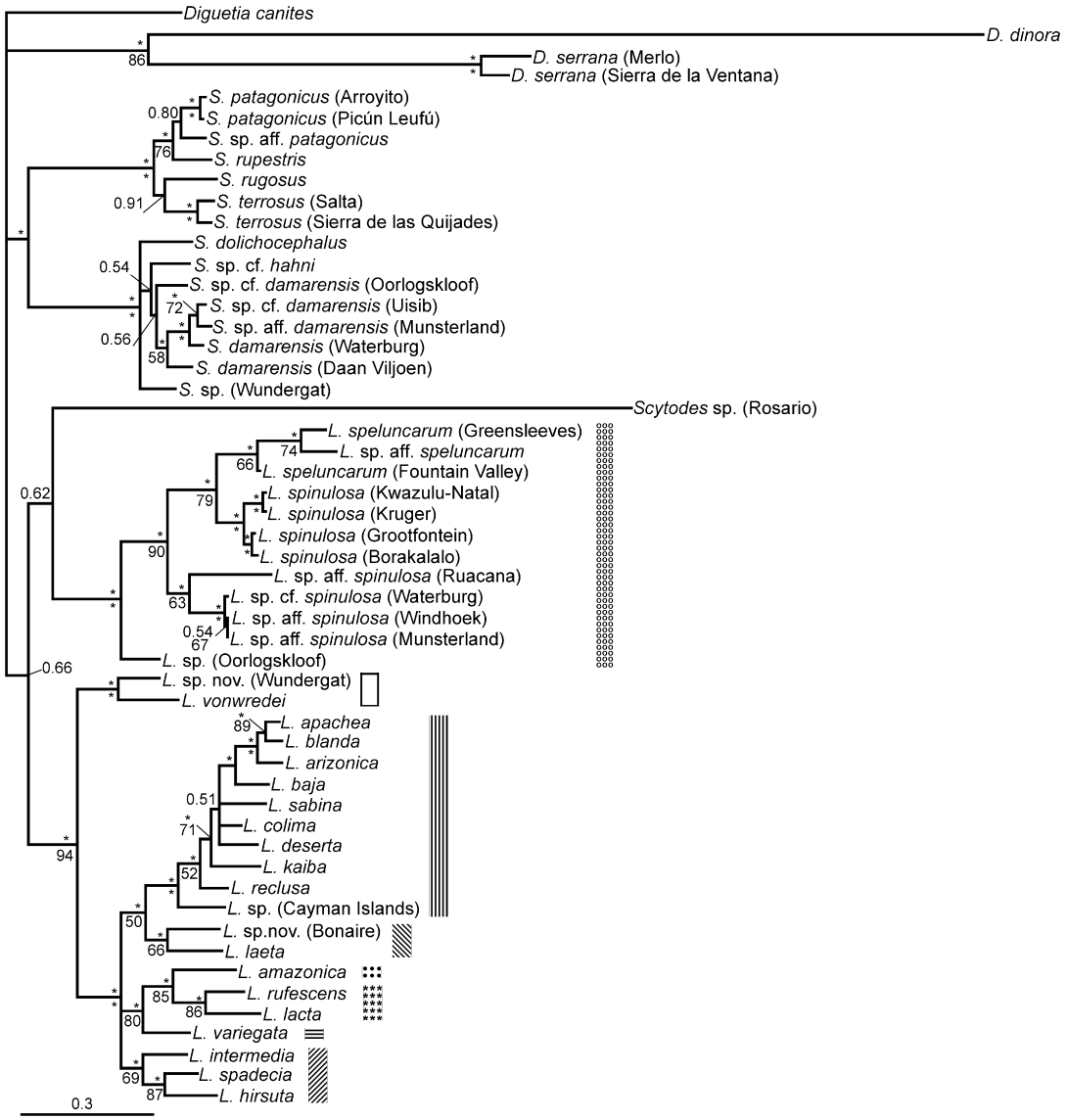

Fig. 4. Phylogeny of Sicariidae based upon Bayesian analyses of combined 28S and COI nucleotide data. Numbers above branches represent posterior probabilities of the nodes to their right. Numbers below branches represent bootstrap support (1000 reps) from parsimony analyses. In all cases, stars indicate values > 95%. Posterior probabilities < 0.5 and parsimony bootstrap values < 50 are not shown. Bars to the right of clades indicate *Loxosceles* species groups using the same shading patterns as in Fig. 1.

outgroup. The numbers of bases in the final alignments, uncorrected base frequencies and model characteristics are summarized in Table 3. The mitochondrial sequences, COI, 16S, and ND1 have significant heterogeneity of base compositions with an A/T bias (~0.73 for each) that is particularly acute in nucleotides in the third codon position of the protein-coding genes (Table 3). In contrast to the mitochondrial genes, the 28S dataset is GC rich (0.64). In all three mitochondrial genes, transitions outnumber transversions, particularly in third codon positions of the protein-coding genes, a pattern that is consistent with saturation (data not shown).

Tests of substitution saturation (Xia et al., 2003) indicated that saturation could be an issue for resolving relationships for some of our data partitions. In particular, we detect indices of substitution saturation (Iss) that were either statistically indistinguishable or greater than Iss values at which sequences are predicted to fail to recover the true tree (Iss.c). The following situations are listed from less to more severe repercussions for utility of the data: 28S (32 OTUs, asymmetrical trees only); 16S (16 and 32 OTUs, asymmetrical trees only); COI, all nucleotides (32 OTUs, asymmetrical trees only), 3rd positions (all circumstances except 4 OTUs); ND1 (all circumstances).

3.2. Alignment analyses of ribosomal genes

The parsimony tree from the 24/6 alignment of the 16S dataset had the smallest symmetric distances from the trees resulting from the concatenated data (8 branch differences, others had between 10 and 13) and had the shortest average symmetric distance from the topologies resulting from all other alignment strategies. Thus, we selected the 24/6 alignment for all subsequent analyses.

For the 28S dataset there was no single static alignment within the range of gap/extension penalties that had the shortest tree distance from the tree resulting from the concatenated alignment. Analyses of symmetric tree distances between parsimony topologies resulting from the static alignments used in the elision analyses, kPrank, and Muscle resulted in the tree from the Muscle alignment having the shortest average distance to all other topologies (16.3). The manual alignment was a close second (18.6).

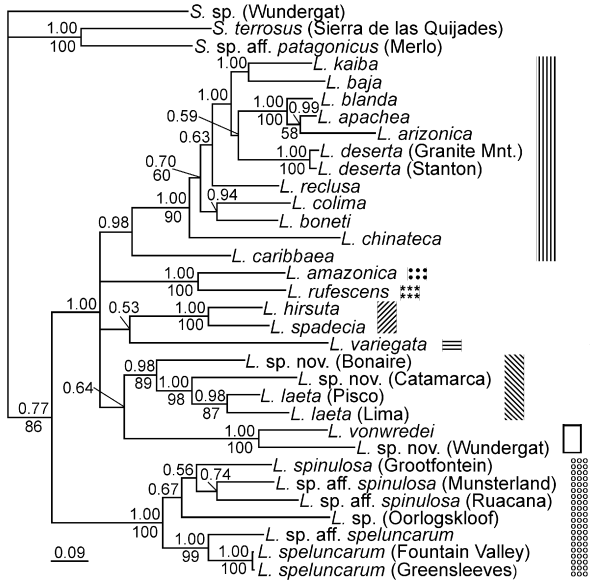

Fig. 5. Phylogeny of *Loxosceles* based upon Bayesian analyses of 16S/ND1 nucleotide data. Numbers above branches represent posterior probabilities of the nodes to their right. Numbers below branches represent bootstrap support (1000 reps) from parsimony analyses. Posterior probabilities < 0.5 and parsimony bootstrap values < 50 are not shown. Bars to the right of clades indicate *Loxosceles* species groups using the same shading patterns as in Fig. 1.

The 24/2 alignment had the greatest distance from all others (38.6). Thus, we present the Bayesian analysis of the Muscle alignment of 28S (Fig. 2) and we used the Muscle alignment in datasets that combine 28S with the other data partitions.

Both parsimony and Bayesian analyses of each of these different alignments consistently supported the same species group level topologies of relationships within *Loxosceles* (Fig. 2 and Table 4). The higher-level relationships (generic and above) were the most vulnerable to different alignment strategies. None of the alignments produce trees that consistently support the monophyly of Sicariidae (Table 4).

3.3. Phylogenetic analyses

The degree to which relationships were resolved varied across the genes used in the analysis. Within genes, analysis types consistently resolved the same clades, however, the degree of resolution varied with Bayesian analyses tending toward more resolution and parsimony toward less. Generally, 28S (Fig. 2), 16S/ND1, and the concatenated analyses (Figs. 4–6) resulted in trees with fewer polytomies and more concordance among resolved nodes than did COI nucleotide or amino acid analyses (Fig. 3 and Table 4). Concatenated analyses that included COI had more resolved nodes than independent analyses of COI (Figs. 3, 4, and 6). Bayesian posterior support values for particular clades from different datasets are summarized in Table 4.

3.3.1. Relationships of species groups within Loxosceles and Sicarius

Loxosceles are resolved as monophyletic in most analyses, but with weak support (Figs. 2–6 and Table 4). COI includes New World *Sicarius* in an unresolved polytomy with major *Loxosceles* clades described below (Fig. 3). 28S/COI also places the sole *Scytodes* taxon as derived from within an otherwise monophyletic group of *Loxosceles* (Fig. 4). There are a number of clades within *Loxosceles* that are consistently resolved across genes and analysis types (Figs. 2–6 and Table 4). For example, all analyses strongly support the monophyly of a distinct Southern African clade that we refer to as the '*spinulosa*' clade that includes a wide geographic sampling of *L. spinulosa*, *L. speluncarum*, and other undescribed species. This clade is weakly supported as sister to all other *Loxosceles* in resolved analyses. A second distinct clade from Africa includes two Namibian species, *Loxosceles vonwredei*, and an undescribed species from western Namibia (sp. nov. Wundergat). This clade is supported as sister to New World *Loxosceles*, making Southern African *Loxosceles* paraphyletic, except with COI, which places the *vonwredei* clade within an unresolved basal polytomy with the

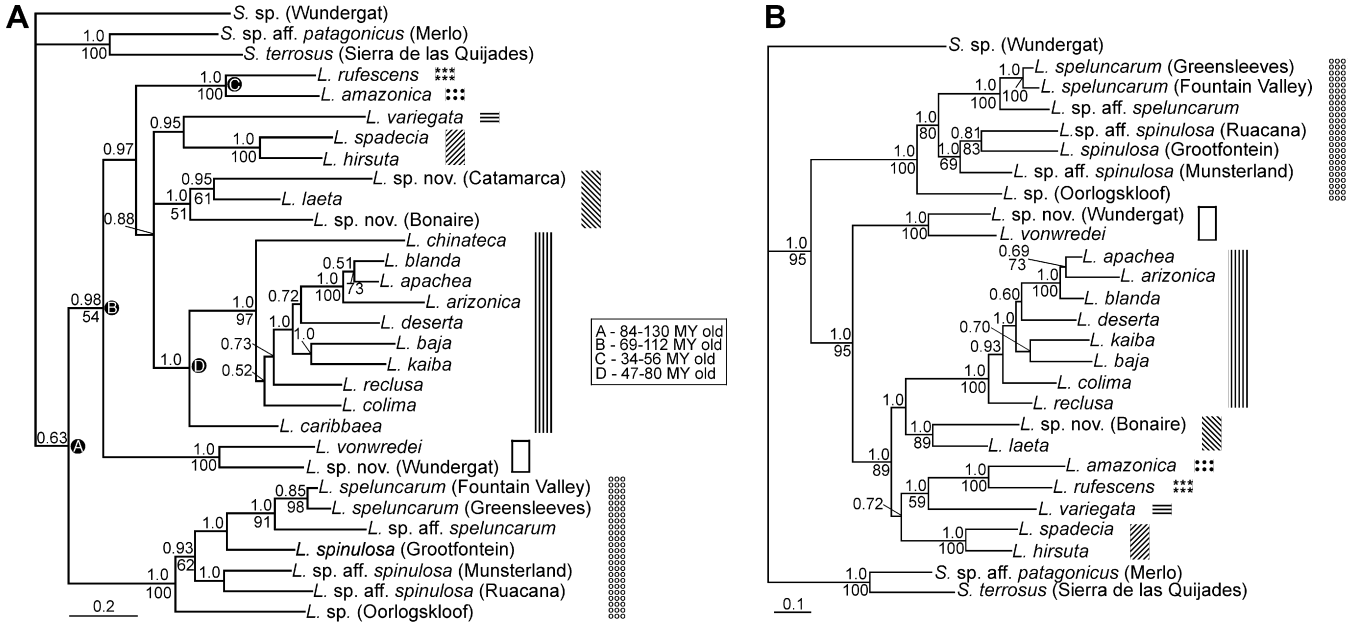

Fig. 6. Phylogenies of *Loxosceles* based upon Bayesian analyses of (a) combined COI and 16S/ND1 nucleotide data and (b) combined 28S, COI, and 16S/ND1 data. Numbers above branches represent the posterior probability support of the nodes to their right. Numbers below branches represent bootstrap support (1000 reps) of parsimony analyses. Posterior probabilities < 0.5 and parsimony bootstrap values < 50 are not shown. We have overlain on the phylogeny in (a), estimates of divergence dates of the nodes labeled A–D from analyses of the CO1/16S/ND1 combined data set. The ranges in dates are based on estimated mean dates from Langley–Fitch (r8s) and Bayesian (multidivtime) analyses. Bars to the right of clades indicate *Loxosceles* species groups using the same shading patterns as in Fig. 1.

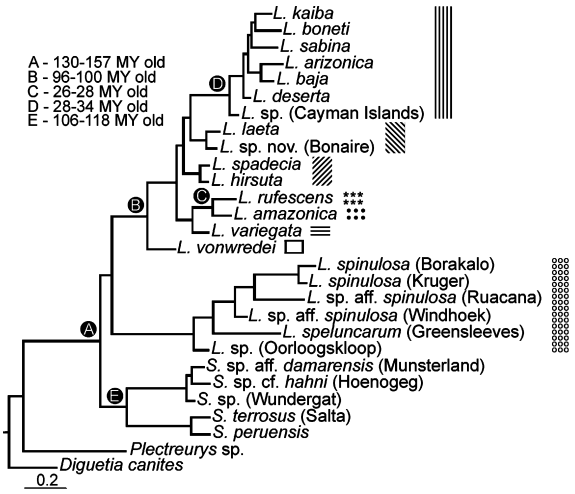

Fig. 7. Phylogeny of Sicariidae based on a reduced dataset of 28S. Overlain on the tree are ranges of divergence dates of the nodes labeled A–E. The ranges of dates are based on the mean dates estimated from penalized likelihood (r8s) and Bayesian (multidivtime) analyses. Bars to the right of clades indicate *Loxosceles* species groups using the same shading patterns as in Fig. 1.

spinulosa group, New World *Loxosceles* and *Sicarius* (Fig. 3); and 16S/ND1 which places the *vonwredei* clade as derived from within the American clade (Figs. 2–6 and Table 4).

All analyses except COI support the monophyly of South American species groups as defined by Gertsch, 1967; however, patterns of relationships among the representatives of these species groups are inconsistent. All analyses that include 28S (individual and combined) support a sister-taxon relationship of members of the *laeta* species group to North American *Loxosceles* (Figs. 2, 4, and 6b and Table 4). Undescribed species from Bonaire in the Netherland Antilles and from San Fernando de Catamarca in Argentina have genitalic features that are characteristic of the *laeta* species group and are resolved in a clade with *L. laeta* except in COI analyses (Figs. 2–6 and Table 4). The widespread species *L. rufescens* is resolved as sister to *L. lacta* from China (except with COI), and is unambiguously supported as sharing a more recent common ancestor (MRCA) with South American *Loxosceles*, specifically *L. amazonica*, than with species from Southern Africa (Figs. 2–6 and Table 4).

Members of the *reclusa* species group (Gertsch and Ennik, 1983) are generally supported as monophyletic with Caribbean members (*L. caribbaea* or *L.* sp. Cayman Islands) being sister to all North American species in analyses that included them (Figs. 2–6 and Table 4). Independent analyses of COI are the exception, placing *L.* sp. nov. from Bonaire in a polytomy with *reclusa* group species and failing to resolve relationships between *L. caribbaea*, *L.* sp. from the Cayman Islands and other New World taxa. In addition, the Cayman Islands individual is weakly supported as derived from within the North American *reclusa* group in one 28S alignment (Table 4). All analyses that included broad geographic sampling of *Sicarius* (28S and COI only) strongly support distinct and monophyletic American and African clades (Figs. 2–4 and Table 4). Support of monophyletic *Sicarius* is inconsistent, with concatenated 28S/COI (Fig. 4) and 28S under some alignment parameters supporting monophyly, while other analyses result in either a basal polytomy (some alignments of 28S, Table 4) or continental clades in separate parts of the tree (COI alone) (Fig. 3).

3.3.2. Higher-level relationships

Only the 28S and COI datasets include sufficient outgroup sampling to test hypotheses of the monophyly of the genera *Sicarius* and *Loxosceles* and their current family-level taxonomic status. Neither of these genes, nor the concatenated analyses consistently supported the monophyly of sicariids (Figs. 2–4 and Table 4). Monophyly was either violated by inclusion of *Scytodes*, *Drymusa*, and *Usofila* in an unresolved basal polytomy with *Sicarius* and *Loxosceles* (many 28S alignments), or a clade of *Scytodes*, *Drymusa*, and *Usofila* was resolved as sister either to *Loxosceles*, *Sicarius*, or the *spinulosa* clade of *Loxosceles*. *Scytodes* are consistently supported as monophyletic (taxon inclusion is scant in this group), however, our only representative of *Usofila* is nearly always resolved as derived from within *Drymusa*, or a combined *Scytodes/Drymusa* clade. *Scytodes*, *Drymusa*, and *Usofila* taxa have extremely long branches in our 28S analyses (Fig. 2) and some are also long in COI (Fig. 3), in particular *Drymusa dinora* (Costa Rica).

3.4. Divergence time estimations

Molecular dating analyses produced four sets of results. LF and PL methods in r8s resulted in age estimates within less than one million years of each other in the COI/16S/ND1 analyses, so we report only results from analyses using the LF method. Table 5 summarizes the mean ages and CIs obtained for key ancestral nodes for analyses in which we calibrated the clock using the *Loxosceles* fossil in Dominican amber. Ages for these nodes are mapped onto the trees for each dataset in Figs. 6a (COI/16S/ND1) and 7 (28S). Of the nodes that would be > 95 MYA under a hypothesis of Gondwanan Vicariance (OW/NW Sicarius, *Sicarius*/*Loxosceles* and *L. vonwredei*/NW *Loxosceles*), all had mean ages > 95 MYA except the ancestral nodes of *Sicarius*/*Loxosceles* and *L. vonwredei*/NW *Loxosceles* under the COI/16S/ND1 multdivtime analysis (Figs. 6a, 7 and Table 5). However, most of these ages lack support in the confidence intervals. All estimates of the date of the node of the MRCA of *L. amazonica* and *L rufescens* (means and CIs) are considerably younger than 95 MYA.

When we constrained the *L. vonwredei*/New World *Loxosceles* common ancestor to be at least 95 MYA without using the fossil calibration, the *reclusa* group maintained a very similar range of mean ages (80–29 MYA) as in analyses including the fossil calibration (Table 5), with the minimum CI value being 9 MYA. The *rufes-*

Table 5
Summary of results for ages of ancestral nodes for key clades in molecular dating analyses using the fossil calibration

Node	28S r8s	28S multidivtime	CO1/16S/ND1 r8s	CO1/16S/ND1 multidivtime
Plectreurys/Diguetia	**240**	**188 (111–238)**	—	—
OW *Sicarius*/NW *Sicarius*	118 (28–166)*	106 (58–157)*	—	—
Sicarius/*Loxosceles*	157 (110–187)	130 (76–182)*	**130**	**84 (41–127)****
L. vonwredei/NW *Loxosceles*	100 (29–155)*	96 (54–142)*	112 (100–123)	69 (33–108)**
L. reclusa group	**28 (20–45)**	**34 ± (21–61)**	**80 (68–93)**	**47 (22–76)**
L. amazonica/*L. rufescens*	26 (11–41)**	28 (10–58)**	56 (42–72)**	34 (15–58)**

Ages are displayed as mean (95% CI range). Ages in bold belong to nodes that were constrained or fixed in dating analyses. A single asterisk (*) marks those ages of putative nodes that occurred before the split of Western Gondwana for which the minimum age in the 95% confidence interval was younger than 95 MYA and a second asterisk (**) indicates which of those nodes has a mean age which is less than 95 MYA. For r8s analyses, ages for 28S were obtained using PL with an additive penalty function and those for CO1/16S/ND1 were obtained using the LF method.

cens group common ancestor also maintained a similar mean age range of 56–27 MYA, with the maximum CI value being 67 MYA. Constraining the *L. amazonica*/*L rufescens* ancestral node to be at least 95 MYA caused the *reclusa* group ancestral node to be older (mean range 89–57 MYA, maximum CI = 101 MYA) than analyses in which this node was left unconstrained.

4. Discussion

Our data support patterns of relationships that are consistent with a hypothesis that the global distribution and diversity of extant species of *Loxosceles* and *Sicarius* was influenced by the separation of South America and Africa and subsequent contact between South America and North America via the Caribbean plate. Relevant to the language we use throughout the discussion is the unresolved sister-taxon status of *Loxosceles* and *Sicarius* in our analyses. Because of this lack of resolution, we refer to each of these genera independently and to the family Sicariidae as putative. Because the mitochondrial genes suffered from issues of saturation, we place more confidence in the results of analyses of 28 S, concatenated analyses, and analyses that utilized appropriate models for these datasets (Bayesian).

Tree topologies resulting from the COI dataset conflict with topologies from analyses of other genes, a pattern that has been reported in other molecular systematic studies of spiders (e.g. Hedin and Maddison, 2001; Bruvo-Mađarić et al., 2005). We cannot empirically address reasons for this conflict, but candidate problems are saturation and/or nuclear pseudogenes (Bensasson et al., 2001). If saturation was the only source of conflict, we would also expect ND1 and 16S topologies to conflict with 28S, which is not the case. Patterns that raise concern about potential nuclear introgression include several sequences with frame-shifting insertions, and one sequence with an 11 bp microsatellite insertion. While the conflict between COI and the other markers makes us view patterns resulting from COI as suspect, we are optimistic that signal from COI will provide some useful information for resolving relationships within species groups. Benchmarks have been proposed for COI divergences that correspond to species limits in spiders (Barrett and Hebert, 2005). Therefore, while acknowledging the potential of estimates being confounded by nuclear pseudogenes, we consider percent divergence of COI instructive for delimiting species in this group. We are most confident in COI divergence estimates that parallel relative degrees of divergence in the other genes in our dataset.

4.1. Gondwanan vicariance

The combined results of phylogenetic analyses support the most recent common ancestor(s) of the genera *Loxosceles* and *Sicarius* (putative MRCA of Sicariidae) having been present on Western Gondwana. This pattern is most clearly seen in the robust reciprocal monophyly and deep divergences between New World and Old World *Sicarius* (Figs. 2 and 4 and Table 4). The strong and consistent recovery of near monophyly of New World *Loxosceles* (with the exception of the derived Old World taxa *L. rufescens*, and *L. lacta* (China)) is further support of this scenario. Mean estimates of divergence dates are largely consistent with this scenario, but large confidence intervals, and differences between estimation methods make these data inconclusive (Table 5).

Interestingly, patterns of relationships among the African *Loxosceles* in our analyses suggest the potential for substantial diversity within this genus that predated the separation of Africa and South America. African *Loxosceles* have at least two clades that are paraphyletic with respect to New World *Loxosceles*. All analyses strongly support the monophyly and deep divergence of the African *spinulosa* group from all other *Loxosceles* in the dataset. In fact, the divergences of this group are sufficient that many analyses of 28S and COI that include broad outgroup sampling do not resolve them as sister to other *Loxosceles*, but rather as an unresolved polytomy with *Sicarius*, *Drymusa*, and *Scytodes*.

All analyses also strongly support *Loxosceles vonwredei* (Newlands, 1980) and *Loxosceles* sp. nov. Wundergat as a distinct clade, referred to here as the *vonwredei* clade, members of which share a MRCA with New World species than with the other African species in the analyses. This scenario could have resulted from either *Loxosceles* diversifying before the break-up of Gondwana or ancestors of the *vonwredei* clade dispersing from South America back to Africa after the break-up. Only three of four estimates of mean divergence dates for the split between the *vonwredei* clade and New World *Loxosceles* are older than 95 MYA and all but one have confidence intervals that include dates younger than the Gondwanan split (Figs. 6a and 7 and Table 5). Therefore, we consider these estimates inconclusive in their support of the hypothesis that this divergence predates the separation of Western Gondwana. However, human mediated dispersal is unrealistic given the divergence date, and the absence of ballooning behavior in this group render natural dispersal across oceans unlikely. Thus, based upon current evidence we argue that the most plausible explanation is that the *L. vonwredei* clade diverged from the New World lineage before the split of the continents.

It is highly possible that *L. vonwredei* and *L.* sp. nov. Wundergat are members of a larger lineage with representatives in regions where *Loxosceles* collections are limited, in particular to the North and East of Namibia (e.g. Angola, Zambia, The Democratic Republic of the Congo). Despite being collected roughly 400 km from *L. vonwredei*, sp. nov. Wundergat is both morphologically and genetically distinct. Uncorrected COI percent divergence between these taxa is 16.9% (60% GTRIR corrected) compared to a conservative estimate of average intraspecific divergence in this gene for spiders of 4% (Barrett and Hebert 2005). Uncorrected 28S divergence is 1.98%, which is much higher than estimates of 28S divergence that have been calculated from sibling species in other spider genera. For example, Hedin (2001) estimates percent divergence of 28S at 0.13–0.54% between two closely related species of *Hypochilus* with divergence dates estimated at 12–10 MYA. Thus, unless these two species are relicts of a largely extinct group, there is likely more diversity in the *vonwredei* group even within Namibia.

4.2. Loxocseles rufescens shares more recent common ancestry with American Loxosceles than with Southern African species

One of the more interesting and robustly supported patterns is a sister-taxon relationship between *L. amazonica* (representative of the monotypic South American *amazonica* species group (Gertsch, 1967)) and two species in our analyses that have distinct geographic ranges, *L. rufescens* and *L. lacta* (Fig. 1). The modern range of *L rufescens* has been influenced by human dispersal (Gertsch, 1967) and this complicates our understanding of the native range, however, we detail below why it is evident that their Old World presence predates human dispersal. Patterns of relationships between populations of *L. rufescens* and the Chinese species *L. lacta* are under fine-scale analysis but preliminary data suggest that *L. lacta* have recently diverged and are morphologically similar to *L. rufescens*. Thus, their relationship is consistent with dispersal of the *rufescens* lineage across Eurasia. Morphological similarity between *L. rufescens* and *L. amazonica* was recognized by Gertsch (1967). Despite this similarity and the strong evidence of a sister-taxon relationship between these species, we propose that *L. amazonica* remain the sole member of its own monotypic species group and *L. rufescens*, *L. lacta*, and their relatives be united as the *rufescens* species group. This makes sense given that the *rufescens*

group likely includes much undescribed diversity in the Old World (see below). Moreover, this will minimize confusion about the relatively high profile species *L. rufescens* in medical literature.

Loxosceles rufescens is widespread and genetically diverse in the Mediterranean region (data not shown; Carles Ribera, personal communication). Moreover, this species group probably extends into Western Africa and possibly beyond. Systematists have noted that West African species of *Loxosceles* bear morphological resemblance to *L. rufescens* (Millot, 1941). Furthermore, RPD recently collected specimens of *L. fouta-djalloni* from Guinea and preliminary analyses of 28S sequence data place *L. fouta-djalloni* as sister to *L. rufescens* and *L. lacta*.

The depth of diversity in the Old World adds to our surprise that the *rufescens* lineage shares a closer relationship with South American species than it does to Southern African species in our data set. We consider two possible explanations for this pattern that differ in their predictions about the amount of divergence between the taxa in this lineage. Either (1) the MRCA of this clade predates the split of the continents and the *rufescens* lineage is old (>95 MYA) and diverse in the Old World; or (2) there was a natural dispersal event from South America to the Old World after the Gondwanan split. The possibility of dispersal to the Mediterranean region by human-transport is highly unlikely given the length of time it would take to accumulate the observed genetic divergence between *L. amazonica* and *L. rufescens* (28S: 2.1% uncorrected, 2.3% GTRIR; COI: 18.0% uncorrected, 60.8% GTRIR), and the diversity of the *rufescens* species group in the Old World.

While genetic divergences are greater than we would expect for *L. amazonica* and *L. rufescens* to have diverged after dispersal by humans, all of our divergence date estimates (72–11 MYA (Table 5)) are too young for a MRCA of this clade to have existed on Gondwana. The Northeastern South American range of *L. amazonica*, and the North African/Mediterranean range of *L. rufescens* (Fig. 1) are provocative with respect to the potential of a MRCA on Gondwana or a natural dispersal after continental separation because the last physical connection is estimated to have been in the Northeastern corner of South America and coastal Central Africa (modern Nigeria, Cameroon, Equatorial Guinea, Gabon, etc.) (Pitman et al., 1993). There are a number of other terrestrial flora and fauna that do not disperse by air that also have sister-taxon relationships that span Northeastern South America and Western Africa, yet are not sufficiently divergent to be explained by Gondwanan vicariance (Parrish, 1987; Morley, 2003). These patterns have led to proposals of temporary land dispersal corridors connecting South America and Africa through the late Cretaceous and Paleocene (~65 MYA) (e.g. Morley, 2003). Distinguishing between ancient vicariance and more recent dispersal to explain the relationship of *L. rufescens* and *L. amazonica* will be facilitated with broader inclusion of *Loxosceles* from Central and Northern Africa, and Northeastern South America.

4.3. Loxosceles colonization of North America from South America via Proto-Antillean land bridge

The inclusion of a range of North American *Loxosceles* species and a few representatives from the Caribbean allows us to preliminarily test hypotheses about the timing and mechanism of colonization of North America. Patterns in our analyses, fossil data, and extant distributions of *Loxosceles* in this region are consistent with the genus colonizing North America via a land bridge that predates the modern Isthmus of Panama. First, most analyses (COI alone as the exception) support North American and West Indies species as monophyletic with a derived placement within the New World *Loxosceles* clade. This pattern can be most parsimoniously attributed to *Loxosceles* dispersing to North America from South America, since it requires only one dispersal event between continents and any other explanation would require multiple intercontinental dispersal events. Second, analyses that include *L. caribbaea*, and/or *L.* sp. Cayman Islands, generally support these taxa as sister to a monophyletic North American clade. The exception is the analysis of COI alone (Fig. 3), which supports the Cayman Islands individual as part of a large basal polytomy with monophyletic North America and the Caribbean taxa unresolved with the major South American species groups. Despite this discrepancy, our confidence in this relationship is increased by large decay indices supporting the Cayman Islands individual as sister to North America in analyses that do not include COI (data not shown). Third, there are fossil *Loxosceles* from Dominican amber dating to ~20 MYA (Wunderlich, 2004). Finally, preliminary analyses of North American species relationships consistently support patterns that cannot be explained by a radiation that occurred in the last 3 MY (unpublished data) as would have to be the case if colonization occurred via the modern Isthmus of Panama.

Many lines of geological and biogeographical evidence support the presence of possible land connections between North and South America that predate the Isthmus of Panama (Hay et al., 1999; Iturralde-Vincent and McPhee, 1999; reviewed in Sanmartin and Ronquist, 2004). These differ in their proposed continuity and length of connection. To briefly summarize (see above references for details), the earliest proposed discontinuous bridge was an island arc that connected the Yucatan to Northwestern South America from the mid to late Cretaceous (~70 MYA) until the early to mid-Eocene (49–39 MYA). GAARlandia (Greater Antilles and Aves Ridge) has been proposed as a discontinuous bridge lasting 3 MY around the Eocene–Oligocene boundary (35–33 MYA). A more recent brief connection may have existed during the late Tertiary via the Panama Island Arc (15 MYA), and the modern Isthmus of Panama provides the most recent connection (3.5 MYA to present).

Given these hypothesized connections, colonization of North America by *Loxosceles* via migration that began across GAARlandia, or potentially the more ancient island arc, is the most reasonable explanation that reconciles both support of the basal position of West Indian *Loxosceles* relative to North American species, and the 20 MYA *Loxosceles* fossils in Dominican amber. This dispersal scenario would date the MRCA of the North American radiation of the *reclusa* group be minimally 33 million years old. Molecular dating analyses that leave the MRCA node for this clade unfixed yield a range of estimates that include this date. With current information we favor the explanation of dispersal across GAARlandia because it minimizes the number of dispersal events across open water, and it is consistent with a relatively recent common ancestor of the *reclusa* clade, which corresponds with the relative morphological homogeneity within the group (Gertsch and Ennik, 1983). The support of a sister-taxon relationship between the *reclusa* group and the *laeta* group (Figs. 2–6 and Table 4) is consistent with this scenario because the *laeta* species group is the only one with known extant representatives in Northwestern South America (Fig. 1).

In contrast to *Loxosceles*, *Sicarius* do not occur in the West Indies, and *S. rugosa* is the only described species in Central America with a Northern range extending as far as El Salvador, a pattern consistent with more recent colonization of Central America, perhaps across the modern isthmus. While our taxon sampling does not enable detailed biogeographic analyses of *Drymusa*, the potential of this genus in the Caribbean to be influenced by vicariance biogeography has been long noted (Lutz, 1915; Alayón-García, 1981; Penny, 1999). Analyses with thorough taxon sampling of *Loxosceles* and *Drymusa* from the West Indies, Central America and Northwestern South America may yield patterns of divergence that reflect complex and nuanced vicariance events of Caribbean and Central American biogeography (Iturralde-Vincent and McPhee, 1999).

4.4. Systematic implications

The primary goal of this study was to test the influence of Gondwanan vicariance on the present-day distribution of *Loxosceles* and *Sicarius*, but our results also provide a basic scaffold that is useful for a preliminary understanding of the systematics of these genera. While details of systematics within species groups will be explored in subsequent fine-scale studies, our results reveal general patterns that are helpful for focusing these efforts.

4.4.1. Support for recognized species groups

Although we include only a subset of described *Loxosceles*, our taxon sampling (Fig. 1 and Table 1) is sufficient to provide support of monophyly of the *spadecia* and *reclusa* species groups proposed by Gertsch (1967) and Gertsch and Ennik (1983). While we have only included one described species in the *laeta* species group, preliminary analyses of 11 species in this lineage support monophyly of this group (data not included). Furthermore, two undescribed species, *L.* sp. nov. Bonaire, and *L.* sp. nov. San Fernando de Valle Catamarca (Table 1) morphologically conform to the *laeta* species group (Gertsch, 1967) and consistently (except for Bonaire with COI) pair with *L. laeta* in our analyses. Together these patterns suggest that the morphological characters used by Gertsch and colleagues are effective for defining species groups.

4.4.2. Issues of species delimitation

While at least some of the species groups defined by Gertsch (1967) and Gertsch and Ennik (1983) appear to be robust, species delimitations in *Loxosceles* have been problematic (Gertsch, 1958, 1967; Bücherl, 1964; Newlands, 1975; Brignoli, 1976; Lehtinen 1983; reviewed in Gertsch and Ennik, 1983). *Loxosceles* species vary in their inclusiveness with some having wide geographic ranges and variable genitalic morphology (for example *laeta*, *rufescens*, *arizonica*, *deserta*, and *spinulosa*) and others described from only a few specimens from single localities (many examples in Gertsch 1967; Gertsch and Ennik, 1983). Some authors have argued that Gertsch's definitions are too fine and have synonymized species previously described by Gertsch and others (Bücherl, 1964; Newlands, 1975; Brignoli, 1976).

The issues of defining meaningful species in this lineage need to be addressed with fine-scale regionally focused analyses. However, our dataset lends preliminary insight into this issue with respect to populations in Southern Africa. Our results strongly support monophyly of all cave-dwelling taxa that are morphologically consistent with *L. speluncarum* (Newlands, 1975). However, high genetic divergences (average distances between Strydpoort Mountains, Fountain Valley and Greensleeves: COI = uncorrected 16.3% ± 2.0; GTRIR 19% ± 2.8) and distinct differences in genitalic and somatic characters appear to be sufficient to warrant splitting this group into distinct species. *L. spinulosa* likewise contains many populations that are consistent with this species based on somatic characters (Newlands, 1975) (Table 1) but clear differences in genitalic morphology and patterns of genetic similarity and differences suggest multiple divergent lineages in this group. The only clusters of populations with pairwise COI differences < 4% (raw and GTRIR estimates) (Barrett and Hebert, 2005) are Kwazulu-Natal and Kruger (1.7%), and Windhoek, Munsterland, and Waterburg (average = 1.5% ± 0.001). In contrast, the population from Ruacana has an average genetic difference for COI from all other species in this clade of 16% ± 1.0 uncorrected and 20% ± 2.0 GTRIR. Moreover, the tibial apophysis and curvature of the embolus of male pedipalps from Ruacana is distinct. We do not intend to formally propose new species here, but rather to argue that species delineations in this clade warrant revisiting, and attention to genetic and genitalic differences will be helpful in the process.

Preliminary analyses of populations of *L. laeta* (previously 4 distinct species), and *L. rufescens* (previously 5 distinct species) indicate that within their native ranges these species, as currently defined, also contain substantial genetic and genitalic variation and focused studies of these taxa will also lend insight into appropriate species definitions in these groups. At the other extreme, attention is also needed to determine whether finer delineations are appropriate in many closely related taxa (e.g. *arizonica*, *blanda*, and *apachea*) in the desert Southwest of North America.

4.4.3. Systematic implications—Sicarius

In general, *Sicarius* has received far less systematic attention than *Loxosceles* and to date there has been no overarching systematic revision of the genus, which it sorely needs. Our results reveal preliminary patterns that may inform future detailed systematic work. For example, one of the few published species-level keys for *Sicarius*, including only the three species found in Argentina (Gerschman and Schiapelli, 1979), includes cephalothorax dimensions, eye distance, and raised stridulatory tubercles on the medial palpal femur. In our experience these characteristics are helpful for identifying related groups of Argentine *Sicarius*. However, using these criteria, individuals consistent with the species *patagonicus* have variation in the number of epigynal lobes, and high levels of genetic divergence in COI (Merlo–Arroyito = 13.5% uncorrected and 15.4% GTRIR). A similar situation exists in the South African taxa for which morphological characteristics (largely somatic) are consistent with identification of *S. damarensis*, however, pairwise COI divergences range from 8% to 17.8% GTR (7.4–15.0% uncorrected). A genus-wide analysis of morphological characters, in combination with molecular analyses, will likely help identify key species-delineating characters.

4.4.4. Systematic implications—generic level issues

As mentioned previously, our data generally support the monophyly of *Loxosceles* and *Sicarius*, but do not consistently resolve them as sister taxa. This is likely due to some combination of a lack of density of outgroups, inappropriate outgroups, accelerated rates of evolution particularly in 28S of putative outgroups, and depths of divergence that are beyond the ability of 28S to retain informative signal. Only in the 28S individual gene dataset do we have sequences for a telemid (*Usofila*) that represents the putative sister-taxon of the Scytodoidae superfamily (Sicariidae, Scytodidea, Drymusidae, and Periegopidae). We cannot determine whether the long branches of *Usofila* and *Drymusa* in particular are due to rate acceleration, incomplete homogenization of members of the 28S gene family by concerted evolution, or inaccurate systematic placement. We are optimistic that these issues can be resolved with more appropriate phylogenetic characters and more thorough taxon sampling of putative outgroups. Preliminary attempts to resolve these relationships with a fragment of 18S were unsuccessful due to lack of variation in this gene.

5. Summary

With the data presented here it is evident that the genera *Loxosceles* and *Sicarius* are old, having originated and diversified on Western Gondwana before the separation of the African and South American continents. Extant distributions reflect diversification that has occurred subsequently within each of these continents, and colonization of and radiation on North America. While vicariance can explain many of the large-scale patterns, the sister-taxon relationship between the *rufescens* species group and the South American *amazonica* group is difficult to explain without a long-distance dispersal event. There are many regions for which our collections are limited in this analysis, in particular, depth within

species groups of *Loxosceles* and *Sicarius* in South America and Central America; and *Loxosceles* diversity in the West Indies, and Northern and Central Africa. More thorough collecting in these areas will help refine our understanding of diversification patterns in this lineage. We hope the data presented here will provide a solid scaffold and inspiration for more focused regional biogeographic analyses, systematic revisions, and analyses of venom diversification.

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