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Diversity of *Loxosceles* spiders in Northwestern Africa and molecular support for cryptic species in the *Loxosceles rufescens* lineage

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ABSTRACT

Until recently, *Loxosceles rufescens* was the only species known from a geographic range including North-eastern Africa, Mediterranean Europe and the Middle East. Rich *Loxosceles* diversity in the New World suggests either that *L. rufescens* is a young lineage or that its diversity is underappreciated. We use a molecular phylogenetic and morphological approach to examine diversity in *L. rufescens* and other *Loxosceles* lineages in Northwestern Africa. Molecular analyses of one nuclear and two mitochondrial genes strongly support a monophyletic clade including *L. rufescens*, the Northern Brazilian *L. amazonica* and three other divergent Northwestern African lineages, though relationships among them remain unresolved. A genetically divergent Moroccan individual morphologically consistent with *L. rufescens* was strongly supported as sister to all other putative *L. rufescens*, consistent with the presence of at least 2 species in this lineage. COI *p*-distances and population structuring among remaining putative *L. rufescens* clades further suggest the absence of gene flow between clades and the possibility that they represent multiple species. Morphological characters of preserved *Loxosceles* collected in a range of African countries provide additional indication that *Loxosceles* are more diverse and have a deeper history in Africa than has been previously understood.

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1. Introduction

Phylogenetics provides a framework for elucidating the evolutionary history of taxa, including the timing and location of their evolutionary origin, and patterns of diversification and dispersal. Molecular phylogenetic inference is particularly insightful in complex systems in which human-mediated dispersal and cryptic diversity confounds our understanding of species' geographic distribution and evolutionary history (Garb et al., 2004; Crews and Hedin, 2006; Stockman and Bond, 2007), as is true for some species in the spider genus *Loxosceles*. Most of the ~100 described *Loxosceles* species, including the Brown Recluse, occur in North and South America (Gertsch, 1967; Gertsch and Ennik, 1983). *Loxosceles* are reclusive and though most species are known from small, sometimes isolated geographic ranges, a few species (e.g., *L. reclusa*, *L. laeta*, *L. rufescens*) are widespread and live in close proximity to humans (Gertsch, 1967; Gertsch and Ennik, 1983). *L. rufescens* (Dufour, 1820), in particular, have been transported worldwide by

humans, making their native distribution unclear (Gertsch, 1967; Gertsch and Ennik, 1983; Platnick, 2009).

The presently understood native range of *L. rufescens* stretches from the Canary Islands through Mediterranean Europe and North-eastern Africa to the Middle East. Furthermore, until the recent discovery of a new species in Tunisia, *L. mrazig* (Ribera and Planas, 2009), *L. rufescens* was the only *Loxosceles* species described from the entire Mediterranean basin. The dearth of known diversity in this region is incongruous with the rich species diversity of *Loxosceles* in the North American *reclusa* group (50 spp.; Gertsch and Ennik, 1983) and the South American *laeta* group (31 spp.; Gertsch, 1967) spanning a similarly large geographical range. Disproportionate diversity in Northern Africa suggests that the *Loxosceles* lineage(s) there is either relatively young, or undersampled.

L. rufescens diversity is not well understood because of its deep and complicated taxonomic history. Vague species descriptions, difficulty distinguishing between taxa and loss of holotypes lead Brignoli (1976) to synonymize *L. compactilis* (Simon, 1881) and *L. distincta* (Lucas, 1846) with *L. rufescens*. For the same reasons, he declared *L. decemnotata* (Franganillo, 1925) and *L. rufescens lucifuga* (Simon, 1910) *nomina dubia*. Spider species are typically delineated by genital morphology (Huber, 2004a) because it usually presents clear intraspecific differences with little interspecific variation (Eberhard, 1985; Eberhard et al., 1998). Delineating *L. rufescens*, however, has been historically challenged by lack of a clear pattern

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of variation in genital morphology among populations (Brignoli, 1969, 1976).

The timing and location of the evolutionary origin of the *L. rufescens* lineage is also difficult to discern with current taxon sampling. Southern African *Loxosceles* are paraphyletic with respect to a monophyletic clade including New World species and members of the *rufescens* lineage. The ancient Southern African/South American divergence has been argued as evidence of a Western Gondwanan origin of *Loxosceles* (Binford et al., 2008). *L. rufescens* and *L. lacta* (Wang, 1994) from China share a more recent common ancestor (MRCA) with South American than with Southern African *Loxosceles*. The *L. rufescens*/*L. lacta* sister relationship with the Brazilian species *L. amazonica* could reflect their MRCA predating the split of Gondwana. However, Binford et al. (2008) estimated that the *L. rufescens*/*L. amazonica* ancestor lived between 11 and 72 million years ago, too young to explain their distribution by Gondwanan vicariance.

The possibility of an ancient presence of *Loxosceles* in Northern Africa, however, is supported by the large molecular divergence between *L. rufescens* and *L. mrazig* (COI pairwise *p*-distance ~20%; Ribera and Planas, 2009). *L. mrazig* is also morphologically divergent from *L. rufescens*, bearing closer resemblance to some South American species. The morphological and genetic differences between the two species inspires consideration that they are tips of an old, diverse and poorly known Northern African lineage. Understanding diversity in Northern Africa and how these taxa are related to one another and to *L. amazonica* will provide helpful pieces for resolving the puzzle of how the North African lineage arrived in this region from a MRCA reconstructed on what is now South America.

Here, we reconstruct the phylogenetic relationships and characterize morphological diversity of a broad sampling of *Loxosceles* spiders. Our molecular sampling is concentrated in Northwestern Africa, Iberia and the Canary Islands, while our morphological sampling covers a wider region of North Africa and beyond. Through this work, we endeavor to (1) refine our understanding of Northwestern African *Loxosceles* diversity, and their relationship to New World species; (2) better understand the timing of origin of *Loxosceles* in Northwestern Africa; (3) identify the location of origin of *L. rufescens*; (4) preliminarily explore the structure of diversity within taxa that are morphologically consistent with *L. rufescens*.

2. Methods

2.1. Taxon sampling and vouchers

We collected molecular data from 91 taxa with broad sampling structured for clarifying relationships among and between putative *L. rufescens*, their relatives in Northwestern Africa, and New World *Loxosceles* (Table 1). For the deeper question, we included the Guinean species *L. foutadjalloni* and representation from every defined *Loxosceles* species group except one (Gertsch, 1967; Gertsch and Ennik, 1983; Binford et al., 2008). We did not include the Southern African *spinulosa* group because they are well supported as the basal lineage of *Loxosceles* and are not close relatives of *L. rufescens* (Binford et al., 2008). We rooted the tree with *L. vonwredei*, a Namibian member of the species group strongly supported as sister to a clade of New World *Loxosceles* and *L. rufescens* (Binford et al., 2008). For resolution in the *L. rufescens* lineage, we included 66 individuals from 33 populations in the Iberian Peninsula, Canary Islands, Morocco, Tunisia, China, the United States, and Australia (filled circles in Fig. 1; Table 1). All taxa were field collected by one of us or by colleagues. Specimens were either fixed in 96% ethanol or we preserved the body in 75% ethanol and a leg in 96% ethanol or RNAlater (Ambion). In the lab, we stored preserved specimens at –20 °C.

We identified taxa to species level based on somatic and genital morphology. In describing our results, we refrain from referring to individuals within our experimental group as “*Loxosceles rufescens*” or “*Loxosceles lacta*”. We instead refer to them collectively as “putative *L. rufescens*”, and individually by a 6-digit code made up of 2 letters (indicating the country/region), and 4 numbers (indicating the population number and individual number for each collection locality). For example, “GC0101” refers to individual 1 of collection locality 1 sampled in Grand Canary. Country/region codes are: TN = Tunisia, MA = Morocco, IP = Iberian Peninsula, GC = Grand Canary, US = United States, AU = Australia, CN = China (see Table 1). Vouchers have been deposited in the Lewis & Clark College arachnid collection (R.P.D., M.R.R., G.J.B.) and the Centre de Recursos de Biodiversitat Animal, Universitat de Barcelona, Spain (C.R.). We will eventually deposit representatives of our data set in the California Academy of Sciences, USA (R.P.D., M.R.R., G.J.B.) and national museums of natural history of their countries of origin.

2.2. DNA extraction and molecular data acquisition

Molecular data were collected either at Lewis & Clark College (LC) by R.P.D. or M.R.R., or by C.R. at Universitat de Barcelona (UB). We extracted total genomic DNA from individual legs or partial body parts in the case of very small juveniles using the DNeasy tissue kit (LC) or the QIamp® DNA Mini Kit (QIAGEN) (UB) following the manufacturer's protocol. We verified the purity of gDNA by agarose gel electrophoresis.

We amplified partial fragments of three genes that are effective at inferring phylogenies in spiders at different levels. For resolution at deeper nodes, we used the 5' portion of the nuclear large ribosomal subunit 28S, and for resolution at shallower nodes, we used the mitochondrial markers 16S/t-leucine and cytochrome oxidase subunit I, or COI (Hedin, 2001; Hedin and Maddison, 2001; Arnedo et al., 2004; Garb et al., 2004; Bond and Hedin, 2006; Hendrixson and Bond, 2007; Binford et al., 2008).

PCR methods at LC followed our previously published protocol (Binford et al., 2008) with two exceptions. First, PCR for *L. rufescens* and *L. foutadjalloni* required 40–50° annealing temperatures for all genes and MasterAmp (Epicentre) PCR premixes E (28S, COI), F (16S) or C (16S) or a 4 mM MgCl₂ concentration (16S). Second, amplifying 16S from Chinese taxa yielded two different sized products. We excised the expected band (~800 bps) from the gel, incubated it overnight at room temperature in PCR grade water and performed a second round of PCR under the same conditions.

PCR amplification conditions at UB differed as follows: 28S was amplified in three overlapping fragments using primer pairs ZX1 and 28Sseq, ZR1 and A56, and A58OP1 and A53 (Bond and Hedin, 2006). For 16S and COI (~1 kb), C.R. used primer pairs LR-N-13398 ‘LOX’ (5'-CGC CCT GTT TAA CAA AAA CAT-3'; designed specifically for *Loxosceles* using the same positions as LR-N-13398 from Simon et al. (1994)) with N1-J-12581 (Hedin and Maddison, 2001) and C1-J-1718 (Simon et al., 1994) with C1-N-2776 (Hedin and Maddison, 2001), respectively. The PCR reaction mix contained a final concentration of 0.2 μM of each primer, 0.2 mM of each dNTP, 0.5 U *Taq* polymerase (Promega), with the supplied buffer, and 1.5–2.5 mM MgCl₂ in a final volume of 25 μL. PCR amplification began with an initial step at 94 °C for 3 min, followed by 35 cycles of 30 s at 94 °C, 45 s at 44–48 °C, and 1 min at 72 °C, and ending with another step of 5 min at 72 °C.

We visualized PCR products using agarose gel electrophoresis and purified them using the QiaQuick column purification kit (Qia-gen) (LC) or MultiScreen 96-well filter plates (Millipore) (UB). Sequencing for LC samples was the same as described previously by Binford et al. (2008). At UB, purified products were directly cycle-sequenced from both strands using ABI BigDye (Applied

Table 1
Taxon sampling for molecular data set.

Species	Voucher code	Locality	28S	COI	16S
<i>L. vonwredei</i>	05081610L01 05081610L02 05081610L03	NAMIBIA: Uisib Farm Caves		EU817681	EU817814
<i>L. sp. nov.^a</i>	05081902L01	NAMIBIA: Wundergat	EU817766		
<i>L. deserta</i>	063410L01 00100801L01	USA: Granite Gap, NM	EU817765 EU817778	EU817682	EU817806
<i>L. arizonica</i>	05080401L01 00031701L01	USA: Stanton, AZ USA: Tucson, AZ	EU817769	EU817667 EU817663	EU817799
<i>L. laeta</i>	06082101L35	PERU: Quebrada Mogollon	GQ279183	GQ279222	GQ279180
<i>L. laeta</i>	06080301L06	PERU: Pisco	GQ279182	GQ279223	GQ279181
<i>L. hirsuta</i>	04122401L01 04122401L02 04122401L03	ARGENTINA: Chaco		EU817678	EU817805
<i>L. spadicea</i>	04122801Ls01	ARGENTINA: Catamarca	EU817787	EU817677	EU817804
<i>L. variegata</i>	04122601L01	ARGENTINA: Corrientes	EU817785	EU817675	EU817797
<i>L. gaucho</i>	CRBA-Lx1024	BRAZIL: San Paulo	GQ279184	FJ986178	GQ279174
<i>L. amazonica</i>	06082501L01	PERU: Loreto, Pevas	EU817779	EU817674	EU817813
<i>L. foutadjalloni</i>	07050201L01 07050201L02 07050201L03	GUINEA: Pref. Kindia, Pastoria	GQ279188		GQ279178 GQ279175 GQ279177
<i>L. foutadjalloni</i>	07050101L01 07050101L02 07050101L03 CRBA-Lx1054	GUINEA: Pref. Kindia, Segueya cave	GQ279189 GQ279187 GQ279190 GQ279186 GQ279185	GQ279241 GQ279242 GQ279238 GQ279239 GQ279240	GQ279179 GQ279176
<i>L. mrazig</i>	TN0101 ^{b,c}	TUNISIA: Douz		FJ986179	GQ279173
<i>TN0102^{b,c}</i>		TUNISIA: Ousseltia, Sidi Bou Zouitine cave		GQ279236 GQ279237	GQ279113
<i>TN0201^b</i>	CRBA-Lx1029	TUNISIA: Beja (1267)		GQ279221	GQ279221
<i>TN0301^b</i>	CRBA-Lx1050	TUNISIA: Beja (1270)			GQ279114
<i>TN0401^{b,c}</i>		TUNISIA: Testour		FJ986186	GQ279157
<i>TN0402^{b,c}</i>				GQ279218	
<i>MA0101^{b,c}</i>	CRBA-Lx1020	MOROCCO: Bouizakarne cave	GQ279191	GQ279220	GQ279171
<i>MA0201^b</i>	07021701L01	MOROCCO: Asni	GQ279195	GQ279226	GQ279170
<i>MA0202^b</i>	07021701L02		GQ279198	GQ279224	GQ279168
<i>MA0203^b</i>	07021701L03		GQ279192	GQ279225	GQ279169
<i>GC0101^c</i>		SPAIN: Gran Canaria, Agaete	GQ279112	GQ279111	GQ279172
<i>GC0201^b</i>	CRBA-Lx1068	SPAIN: Gran Canaria, Firgas		GQ279232	GQ279156
<i>IP0101^b</i>	CRBA-Lx1011	SPAIN: Sierra Gorda, Cartagena		FJ986180	GQ279159
<i>IP0201^{b,c}</i>		SPAIN: Monte Arabi, Yecla, Murcia		GQ279213	GQ279155
<i>IP0301^b</i>	CRBA-Lx1061	SPAIN: Torrejon de Adroz		FJ986183	GQ279148
<i>IP0302^{b,c}</i>				GQ279214	
<i>IP0401^b</i>	CRBA-Lx1057	SPAIN: Alicante		GQ279215	GQ279153
<i>IP0402^b</i>	CRBA-Lx1056			FJ986184	
<i>IP0501^b</i>	CRBA-Lx1035	SPAIN: Denia, Punta de Benimaquia Cave		FJ986187	GQ279151
<i>IP0601^b</i>	CRBA-Lx1063	SPAIN: Ciudad Real		FJ986185	GQ279154
<i>IP0602^{b,c}</i>				GQ279216	
<i>IP0701^b</i>	CRBA-Lx1033	SPAIN: Oropesa, Bellver Hole		GQ279234	GQ279152
<i>IP0801^{b,c}</i>		SPAIN: Chumilla		FJ986181	GQ279160
<i>IP0901^{b,c}</i>		SPAIN: Collado Bermejo, Córdoba		GQ279211	GQ279161
<i>IP1001^b</i>	CRBA-Lx1007	SPAIN: Purias		GQ279212	GQ279150
<i>IP1101^b</i>	CRBA-Lx1008	SPAIN: Barcelona		FJ986182	GQ279158
<i>IP1201^b</i>	CRBA-Lx1058	SPAIN: Mostoles		GQ279233	GQ279118
<i>IP1202^b</i>	CRBA-Lx1060		GQ279199	GQ279217	GQ279125
<i>IP1203^b</i>	07050001L02				GQ279121
<i>IP1301^b</i>	07052301L01	SPAIN: Gavà		^d	GQ279137
<i>IP1401^b</i>	CRBA-Lx1331	SPAIN: Villamarxant, Los Murcielagos Cave		GQ279219	GQ279115
<i>IP1501^b</i>	05120001L02	SPAIN: Almería, Cabo de Gata	GQ279196	^d	GQ279144
<i>IP1502^{b,c}</i>	05120001L01		GQ279209	GQ279230	GQ279116
<i>IP1503^{b,c}</i>	05120001L03				GQ279139
<i>IP1504^{b,c}</i>	05120001L04		GQ279193	^d	GQ279135
<i>IP1505^{b,c}</i>	05120001L05		GQ279194	^d	GQ279141
<i>IP1506^{b,c}</i>	05120001L06			GQ279231	GQ279117
<i>IP1601^b</i>	05120002L01	SPAIN: Almería, Desierto de Tabernas		GQ279228	GQ279145
<i>IP1701^b</i>	00000001L01	SPAIN: Cartagena, Bajo Piedra		^d	GQ279142
<i>IP1801^b</i>	00000002L01	SPAIN: Puerto Bensusjo		^d	GQ279138
<i>IP1901^b</i>	05112701L01	SPAIN: Villacarrillo, Jaen, Cueva de la Morciguilla	GQ279208	GQ279211	
<i>IP2001^{b,c}</i>		SPAIN: Siles, Jaen, Cueva del Castillo		FJ986188	GQ279149
<i>AU0101^b</i>	05110001L01	AUSTRALIA: Adelaide, Hawthorn	GQ279201	GQ279229	GQ279147
<i>AU0102^b</i>	05110001L02		GQ279203	^d	GQ279165
<i>US0101^b</i>	04120201L24	USA: Indianapolis, IN	GQ279204	^d	GQ279136
<i>US0102^b</i>	04120201L25		GQ279207	^d	GQ279166
<i>US0103^b</i>	04120201L26			^d	GQ279162
<i>US0104^b</i>	04120201L27			^d	GQ279163
<i>US0105^b</i>	04120201L28			^d	GQ279164

Table 1 (continued)

Species	Voucher code	Locality	28S	COI	16S
US0106 ^b	04120201L29			d	GQ279167
US0107 ^b	04120201L30			d	GQ279143
US0108 ^b	04120201L33		GQ279206	d	GQ279140
US0201 ^b	07073001L01	USA: Manhattan, NY	GQ279210	GQ279227	GQ279146
CN0101 ^b	05082102L01	CHINA: Gizhou Province, Dong	GQ279200	d	GQ279134
CN0102 ^b	05082102L02		GQ279197	d	GQ279131
CN0201 ^b	05082203L01	CHINA: Gizhou Province, Maolan	GQ279202	d	GQ279133
CN0202 ^b	05082202L02		GQ279205	d	GQ279126
CN0203 ^b	05082202L03			d	GQ279124
CN0204 ^b	05082204L01			d	GQ279119
CN0208 ^b	05082203L04			d	GQ279130
CN0209 ^b	05082203L06			d	GQ279122
CN0210 ^b	05082203L07			d	GQ279120
CN0211 ^b	05082203L08			d	GQ279129
CN0212 ^b	05082202L09			d	GQ279132
CN0213 ^b	05082202L10			d	GQ279123
CN0214 ^b	05082202L11			d	GQ279127
CN0215 ^b	05082202L12			d	GQ279128

CRBA, Centre de Recursos de Biodiversitat Animal, Universitat de Barcelona (contact C.R.: ribera@ub.edu).

Remaining vouchers are currently housed at the Lewis & Clark College Arachnid collection (contact G.J.B.: binford@lclark.edu).

Taxa with no voucher codes were small juveniles that were completely homogenized to extract gDNA.

^a Binford et al. (2008).

^b Morphologically consistent with *L. rufescens* (Gertsch, 1967; Gertsch and Ennik, 1983).

^c Individual is a juvenile.

^d Sequence showed evidence of nuclear introgression and was not included in the presented data set.

Biosystems) chemistry with the forward and reverse PCR primers and one additional pair of internal COI primers, CI-J-2183 and C1-N-2191 (Simon et al., 1994). Sequencing reactions were run on ABI Prism 377 (Applied Biosystems) automated sequencers at the Serveis Científic-Tècnics of the Universitat de Barcelona.

After sequencing, we checked all nucleotides by eye against chromatograms and assembled overlapping fragments either in Sequencher v.4.7 (Gene Codes Corporation) or in BioEdit (© Tom Hall, <http://www.mbio.ncsu.edu/BioEdit/BioEdit.html>). COI and 28S fragments sequenced for GC0101 were non-contiguous, leaving

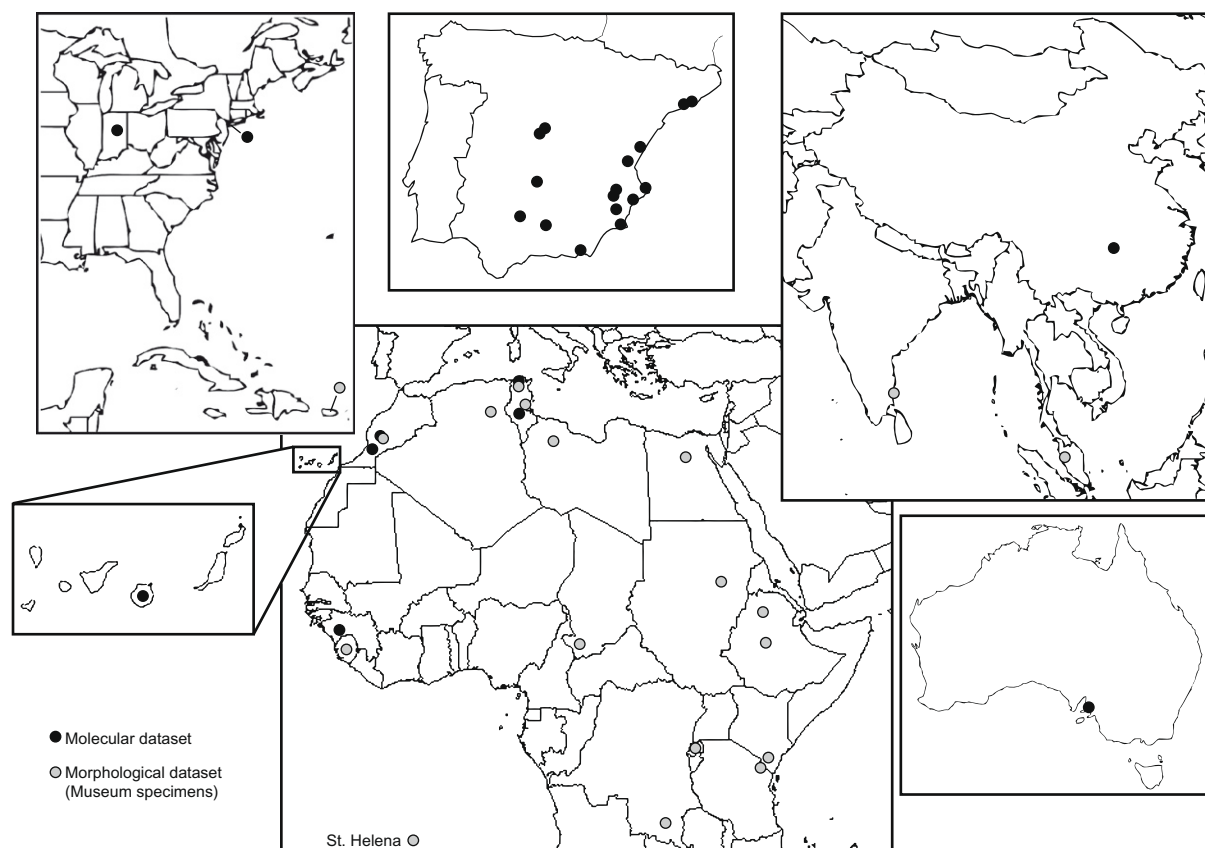


Fig. 1. Geographic distribution of taxa sampled. Filled black circles represent taxa used in our molecular data set and gray circles represent specimens borrowed from museums for morphological examination. Circles represent one or more populations from the country in which they are placed and are meant to approximate the geographic position of the collection locality, except in the case where locality information beyond country is not available (some museum specimens from Egypt and Algeria).

gaps ~50 bp in COI and ~100 bp in 28S. We were unable to amplify the entire marker from this individual since its DNA was partially degraded (it was a small juvenile collected in 2003 and preserved in 70% ethanol). We included the reliable portions of COI and 28S sequences from GC0101 because it is a rare, morphologically divergent taxon. We took the gaps into account when drawing conclusions.

COI sequences from some putative *L. rufescens* were sufficiently ambiguous to preclude assembly. In these sequences we detected evidence of pseudogenes that we are describing in detail elsewhere. Briefly, fragments sequenced in each direction had clear signal except for double peaks in isolated sites at the 5' end and transitioned suddenly to noisy, unreadable signal with at least two overlapping sets of peaks on the chromatogram at the 3' end. The transition occurred such that the clean part of a given fragment overlapped exactly with the noisy part of the opposite fragment, and the position of the transition was the same across taxa showing this pattern. The readable to unreadable transition we observed is consistent with simultaneous amplification of multiple copies of COI from the same conserved priming sites, where at least one copy contains a loss of function insertion in a position that is shared across taxa. We omitted sequences from our data set containing evidence for an insertion, or any other ambiguities that prevented us from assembling sequences (29 omitted, Table 1).

2.3. Sequence alignment and molecular data analysis

There was no internal length variation in COI sequences so we aligned them manually in MacClade 4.06 (Maddison and Maddison, 2003) using the amino acid translation as a guide. Ribosomal genes 16S and 28S varied substantially in length, so we used PRANK (Loytynoja and Goldman, 2005) and Muscle v3.6 (Edgar, 2004) to generate progressive alignments. PRANK alignments began with a Neighbor-Joining tree created in ClustalX v1.83.1 (Higgins and Sharp, 1988).

Taxon representation in our sequence data sets varies across genes because of differences in informativeness for the different temporal scales of our questions. We include less 28S representation for putative *L. rufescens* because sequences are largely invariant among populations. However, we performed phylogenetic analyses on individual 28S alignments to confirm whether they would yield any population structuring of putative *L. rufescens*. COI and 16S sampling is more dense for putative *L. rufescens* to resolve phylogenetic structuring among their populations. We were unable to achieve complete overlap of 16S and COI data sets mostly because of the ambiguity of many COI sequences (mentioned above), which occurred in all individuals from some populations (see Table 1).

Given different representation in data sets, and preliminary evidence of vulnerability of topologies to alignment strategy, we performed separate phylogenetic analyses on individual COI and 16S alignments and on concatenations of the two genes using both 16S alignment strategies. In addition, we performed analyses on concatenated alignments of 28S, COI and 16S for taxa for which all three markers were available to examine relationships between *L. rufescens*, other African species and New World *Loxosceles*. Alignments concatenated across all three markers were extremely reduced because of low taxon inclusion in our 28S data set. To examine the sensitivity of relationships to extreme taxon reduction in concatenated alignments, we generated alignments concatenating 28S, COI and 16S across all taxa. In these full concatenated data sets, gaps replaced sites when a marker was not available for a taxon. Lastly, we were concerned that the missing sections of COI and 28S from GC0101 might misinform relationships, so we also

performed phylogenetic analyses on concatenated ribosomal genes in all possible alignment combinations.

We reconstructed relationships using parsimony and Bayesian analyses. We conducted parsimony analyses in PAUP* v.4.0 (Swofford, 1998) and TNT (Goloboff et al., 2008) using a heuristic search method with tree bisection-reconnection branch swapping and 1000 random addition sequence (RAS) replicates. Gaps were treated as missing data, and we assessed confidence in clades based on 1000 non-parametric bootstrap replicates. Parsimony analyses of the full concatenated data sets were only conducted in TNT, using 1000 bootstrap replicates and 100 RAS. Bayesian analyses were conducted in MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003). We used GTR + I + G as the model of nucleotide substitution for each dataset, as determined by the AIC criterion in Modeltest v.3.7 (Posada and Crandall, 1998) and partitioned concatenated data sets by gene. We conducted 2 runs each with 4 separate Markov Chain Monte Carlo (MCMC) chains and sampled one tree every 100 generations. We initially ran 1,000,000 generations for each data set and subsequently added 1,000,000 generations at a time until the average standard deviation of split frequencies between runs was less than 0.01. We analyzed .p files in Tracer v.1.3–1.4 (developed by Andrew Rambaut and Alexei Drummond, available at: <http://evol.zoo.ox.ac.uk/software.html?id=tracer>) to evaluate the stabilization of parameters (burn-in). We removed trees included in the burn-in determined in Tracer before creating a 50% majority rule consensus tree.

2.4. Haplotypes and network analyses of 16S and COI

We analyzed fine-scale differences and clustering of putative *L. rufescens* COI and 16S sequences by generating haplotype networks in TCS v.1.21 (available from David Posada at: <http://darwin.uvigo.es/software/tcs.html>). TCS collapses DNA sequences into haplotypes and uses parsimony to create a network, showing how many steps (sites where nucleotides differ) separate haplotypes. We used 16S and COI alignments including only putative *L. rufescens* (except MA0101) as input files to generate haplotype networks. We manually aligned 16S sequences for this analysis because they were similar enough that we could do so unambiguously. We treated gaps as missing data and used the default connection limit (95%) for 16S. COI was more genetically diverse than 16S, so we used a 90% connection limit. Of sequences included in phylogenetic analyses, we removed from our network analyses those that were truncated.

2.5. Morphological data collection and analysis from museum specimens

To investigate morphological variation among putative *L. rufescens* and explore their distribution and diversity in Africa, we examined 42 preserved *Loxosceles* specimens from 13 African and 4 non-African countries housed in four museums (Musée Royal de l'Afrique Centrale, Naturalis, Muséum National d'Histoire Naturelle, Paris and Muséum d'Histoire Naturelle, Genève; gray circles in Fig. 1 and outlined in Table 2). The African specimens were from countries within the understood, Northern African native range of *L. rufescens*, or they were from regions adjacent to North Africa (Eastern, Western and Central Africa). Non-African specimens had been previously identified as *L. rufescens* or the synonymous species *L. distincta*. We examined somatic and genitalic morphology of adult specimens to determine if populations or species with morphological affinity to *L. rufescens* occupied these regions. The *L. rufescens* holotype is lost (Gertsch, 1967) and the original description lacks much detail, so we refer to revised descriptions of *L. rufescens* by Gertsch (1967) and Gertsch and Ennik (1983) in assessing morphology of putative *L. rufescens*.

Table 2*Loxosceles* from museum collections used in morphological data set.

Locality	Museum and voucher code	Species identification	Identification reference	Sex	Notes
Algeria ^a	MNHN-Paris AR5567	<i>L. rufescens</i>	Brignoli (1976)	♀	A in Fig. S2
Algeria	MNHN-Paris AR5568	<i>L. rufescens</i>	Brignoli (1976)	♀	
Algeria	MNHN-Paris AR5569	<i>L. rufescens</i>	Brignoli (1976)	♀	
Algeria	MNHN-Paris AR5570	<i>L. rufescens</i>	Brignoli (1976)	♀	
Algeria	MNHN-Paris AR5571	<i>L. rufescens</i>	Brignoli (1976)	♀	
Algeria	MNHN-Paris AR5573	<i>L. rufescens</i>	Brignoli (1976)	♀	
Algeria	MNHN-Paris AR5578	<i>L. rufescens</i>	Brignoli (1976)	♀	
Algeria ^a	MNHN-Paris AR5579	<i>L. rufescens</i>	Brignoli (1976)	♀	B in Fig. S2
Algeria ^a	MNHN-Paris AR5576	<i>L. rufescens</i>	Brignoli (1976)	♂♂	J and K in Fig. S2
Libya ^a	RMNH 7870	<i>L. rufescens</i>	Brignoli (1976)	♀	D in Fig. S2
Sudan: Khartoum ^a	RMNH 7871	<i>L. rufescens</i>	Brignoli (1976)	♀	C in Fig. S2
Tunisia: 10 km outside of Sfax ^a	MHNG	<i>L. gauchoi</i>	Brignoli (1976)	♀	G in Fig. S2
Tunisia: Jendouba, Kef-el-Agab cave ^a	MHNG No. 240	<i>L. rufescens</i> ^b	Brignoli (1976)	♀	F in Fig. S2
Egypt ^a	MHNG No. 207	<i>L. rufescens</i>	Brignoli (1976)	♂	L in Fig. S2
D.R. Congo: Katanga, Kyoralo Lubudi cave	MHNG No.57	Unidentified	Brignoli (1976) (<i>L. sp. A</i>)	j	<i>L. spinulosa</i> group
Malaysia: Gunuung Lanno Perak ^a	MHNG	<i>L. rufescens</i> ^b		♀	H in Fig. S2
Malaysia: Gunuung Lanno Perak ^a	MHNG	<i>L. rufescens</i> ^b		♂♂	M in Fig. S2
India: Madras ^a	MHNG No. 77	<i>L. rufescens</i> ^b	Brignoli (1976)	♂	P in Fig. S2
Puerto Rico ^a	MHNG No. 85	<i>L. rufescens</i> ^b	Brignoli (1976)	♂	O in Fig. S2
Morocco: Imlil	MRAC 154272	<i>L. distincta</i> = <i>L. rufescens</i> ^b		♀	
Egypt: Ein Suchna, Eastern Desert ^a	MRAC 209673	Unidentified ^b		♀	E in Fig. S2
St. Helena	MRAC 129553	<i>L. distincta</i> = <i>L. rufescens</i> ^b		♂♀	
St. Helena	MRAC 1298106	<i>L. distincta</i> = <i>L. rufescens</i> ^b		♂	
St. Helena ^a	MRAC 129115	<i>L. distincta</i> = <i>L. rufescens</i> ^b		♂♀	N and I in Fig. S2
Sierra Leone: Saoulia	MRAC 174665	<i>L. rufipes</i>	This study	♀	
Chad: Bebedjia ^a	MRAC 151444	<i>L. sp. cf. amazonica</i>	This study	♂	Q in Fig. S2
Ethiopia: Addis Ababa	MRAC 207319	Unidentified		♀	<i>L. spinulosa</i> group
	MRAC 207313	Unidentified		♀	<i>L. spinulosa</i> group
Ethiopia: Northern Ethiopia	MRAC 207336	Unidentified		♀	<i>L. spinulosa</i> group
Kenya: Mt. Kasigau, Jora village	MRAC 213028	Unidentified		♂	<i>L. spinulosa</i> group
	MRAC 213031	Unidentified		♂	<i>L. spinulosa</i> group
Kenya: Laikipia, Mpala Ranch	MRAC 214937	Unidentified		♂	<i>L. spinulosa</i> group
	MRAC 212189	Unidentified		♀	<i>L. spinulosa</i> group
Tanzania: Mkomazi Game Reserve, Ibaya camp	MRAC 215676	Unidentified		j j	<i>L. spinulosa</i> group
Rwanda: Astrida	MRAC 66060	Unidentified		♂	<i>L. spinulosa</i> group

RMNH, Naturalis (formerly Rijksmuseum van Natuurlijke Historie, Leiden); MRAC, Musée Royale de l'Afrique Centrale; MHNG, Muséum d'Histoire Naturelle, Genève; MNHN-Paris, Muséum National d'Histoire Naturelle, Paris.

^a Specimens for which photos of genitalia are depicted in Fig. S2.

^b Specimens morphologically consistent with *L. rufescens*.

3. Results

3.1. Morphological species identification and gene sampling for molecular data set

All Iberian, Australian, North American, and Chinese taxa in our ingroup, and most North African taxa, had somatic (juveniles and adults) and genitalic (adults) morphology consistent with *L. rufescens* (Fig. 2) (Gertsch, 1967; Gertsch and Ennik, 1983; noted in Table 1). The individual from Agaete, Grand Canary (GC0101), with a distinctive pattern on its cephalothorax, and *L. mrazig* (Ribera and Planas, 2009) were the only Northern African taxa that were clearly morphologically different from putative *L. rufescens*.

3.2. Molecular data sets and alignments

Taxon sampling for our molecular data set is outlined in Table 1. Our 16S data set was the most inclusive, consisting of 60 putative *L. rufescens* from six countries (Spain, Morocco, Tunisia, Australia, USA, and China). Our COI and 28S data sets included representatives from most of these countries; COI lacked representation from Chinese taxa because all recovered sequences showed evidence of pseudogenes (Table 1; see explanation in Section 2); 28S did not include putative *L. rufescens* from Tunisia, but did include *L. mrazig*. Table 3 summarizes characteristics of alignments used for phylogenetic analyses. The use of different primer pairs at LC and UB re-

sulted in differential overlap of COI, and our final COI data set contained 569 characters. MUSCLE and PRANK alignments differed in length and position of some nucleotides in 16S and 28S rDNA sequences, but variation in proposed positional homology of nucleotides was concentrated in hypervariable regions.

3.3. Phylogenetic analyses

We used individual and combined 16S and COI data sets primarily to examine relationships between putative *L. rufescens*. Our smaller data sets including 28S resolved deeper divergences that were ambiguous in analyses of mitochondrial genes. Bayesian and parsimony analyses supported similar tree topologies, with Bayesian analyses tending toward more resolution and greater support for clades. Exceptions to this rule occurred when parsimony trees resolved relationships that Bayesian analyses either collapsed into polytomies or resolved slightly differently. Later in the results, we mention notable differences between Bayesian and parsimony topologies. PAUP* and TNT produced congruent trees for all data sets. They also generated similar bootstrap support values for well-supported clades, but TNT produced lower values than PAUP* for moderately or poorly supported clades (Figs. 3 and 4). Figures depict the Bayesian tree topology (Figs. 2–4) with Bayesian posterior probabilities represented by branch thickness and color, and parsimony bootstrap support values overlain on the branches (Figs. 3 and 4).

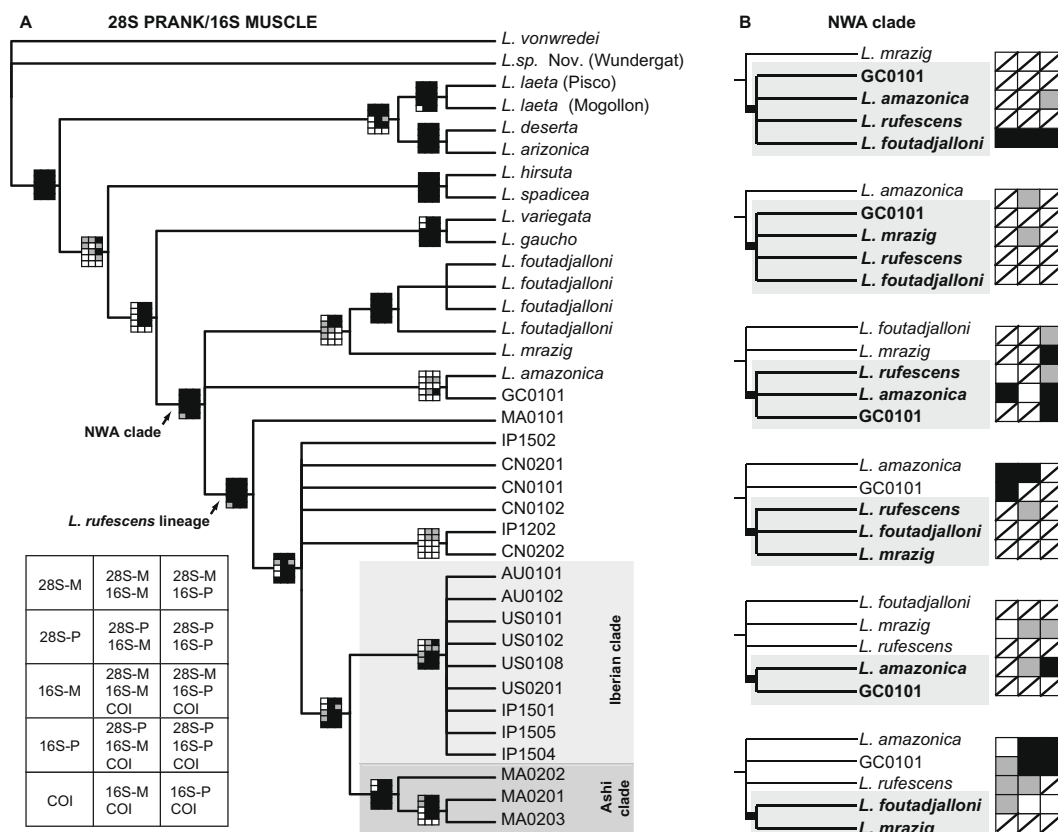


Fig. 2. Skeleton trees showing relationships between the NWA clade and other *Loxosceles*, and among NWA clade taxa. (A) Topology is from Bayesian majority rule consensus tree of combined 28S (PRANK) and 16S (MUSCLE). (B) Six relationships among NWA clade lineages supported by analyses of at least two datasets. Grids show support across all data sets for a given relationship. In (B), diagonal crossed lines in support grids indicate data sets that did not resolve the same relationship as shown in the corresponding tree.

Table 3
Summary of alignment characteristics determined using the Akaike Information Criterion in Modeltest 3.7.

Alignment	Characters Inf./total	A	C	G	T	A<>C	A<>G	A<>T	C<>G	C<>T	G<>T	I	G
COI	211/569	0.28	0.07	0.19	0.47	1	35.05	3.01	3.01	35.05	1	0.34	0.24
16S PRANK	196/421	0.41	0.15	0.14	0.30	3.06	3.31	3.53	0.27	10.57	1	0.28	1.42
16S MUSCLE	193/417	0.41	0.15	0.14	0.30	2.63	3.80	4.16	0.04	11.71	1	0.31	1.38
28S PRANK	126/1538	0.19	0.28	0.35	0.19	0.80	1.67	1.62	0.29	3.98	1	0.78	0.65
28S MUSCLE	119/1547	0.19	0.28	0.34	0.19	0.83	1.31	1.29	0.43	3.37	1	0.76	0.55

Characteristics displayed are the ratio of parsimony informative (Inf.) characters to total characters, base frequencies, nucleotide substitution rates, proportion of invariable sites (I) and the Gamma distribution shape parameter (G).

3.3.1. Deep relationships

Bayesian analyses of all individual and concatenated data sets supported a monophyletic group that we refer to as the Northwest Africa (NWA) clade (Figs. 2–4) (posterior probabilities (PP) COI alone = 0.92, all others >0.95). The NWA clade includes five lineages with variable numbers of terminal taxa included in each: (1) putative *L. rufescens* (the “*L. rufescens* lineage”), (2) GC0101, (3) *L. mrazig*, (4) *L. amazonica* and (5) *L. foutadjalloni* (Figs. 2–4). Parsimony analyses supported monophyly of the NWA clade except analyses of COI. Relationships between the NWA clade and other *Loxosceles* were only resolved in Bayesian analyses of alignments including 28S. Resolutions supported a sister relationship between the NWA clade and either the monophyletic *L. gaucho* species group (including *L. gaucho* and *L. variegata*) or a paraphyletic *L. gaucho* group, with *L. gaucho* being sister to the NWA clade (analyses of 28S alone; Fig. 2a).

Relationships between the five taxa in the NWA clade were weakly supported, unresolved, or inconsistent between genes and alignment strategies (Figs. 2–4). We present relationships sup-

ported by at least two data sets in Fig. 2b with support values above 0.7 PP summarized across data sets in the support grid. The most commonly resolved pair of taxa in the NWA clade was *L. mrazig* and *L. foutadjalloni*, supported by 11 of 15 datasets. *L. amazonica* and GC0101 were supported as sister in five analyses, one of which supported the pairing with less than 0.7 PP (Fig. 2a and b). Most notable in the lack of resolution is that South American *L. amazonica* was only supported as the basal lineage of the NWA clade in analyses of two data sets with support between 0.73 and 0.82 PP. There is no agreement in resolution of a sister taxon to the *L. rufescens* lineage.

3.3.2. Relationships within the *Loxosceles rufescens* lineage

Analyses of all alignment combinations except COI alone (Bayesian and parsimony) support the monophyly of the *L. rufescens* lineage. Within this clade, most analyses support a basal position of MA0101, a Moroccan individual with a relatively long branch. Given the limited data set and informativeness for 28S at this level, we first describe representative patterns based on the

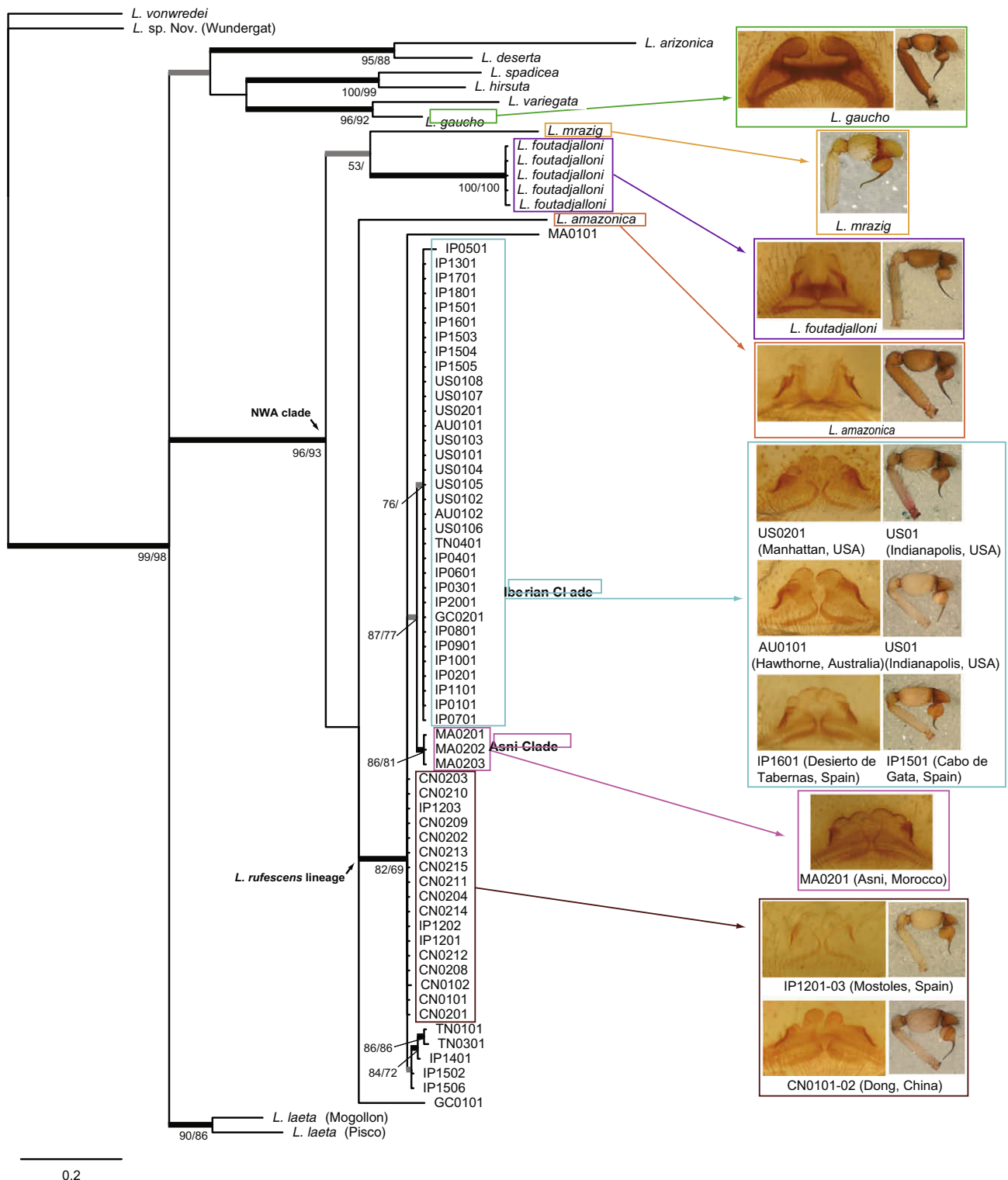


Fig. 3. Bayesian majority rule consensus tree of 16S (MUSCLE) analysis. Support represented by branch weight and color. Black weighted branches lead to nodes that are supported with posterior probabilities of 0.95 or greater. Weighted grey lines lead to nodes that are supported with posterior probabilities between 0.70 and 0.95. Parsimony bootstrap values (PAUP*/TNT) are overlain below nodes. Photographs of genitalic morphology from representative males and females of taxa in the NWA clade appear beside clades. *L. gaucho* genitalia are shown for reference.

COI/16S data sets and then point out deviations from other data-sets. North American and Australian individuals all fall within a large clade of mostly Iberian taxa, one Canarian individual (GC0201) and specimens from one Tunisian population (TN0401-02). We refer to this clade as the Iberian clade. Bayesian analyses of COI/16S strongly support the Iberian clade, and

its sister status with a clade of individuals from Asni, Morocco (MA0201-03; Asni clade). Parsimony analyses also recovered the Iberian clade as sister to the Asni clade, but with lower support. The Iberian/Asni clade is weakly supported as nested within three clusters of two individuals each from three Iberian (IP1201-02; IP1401; IP1502, 06) and one Tunisian (TN0101-02)

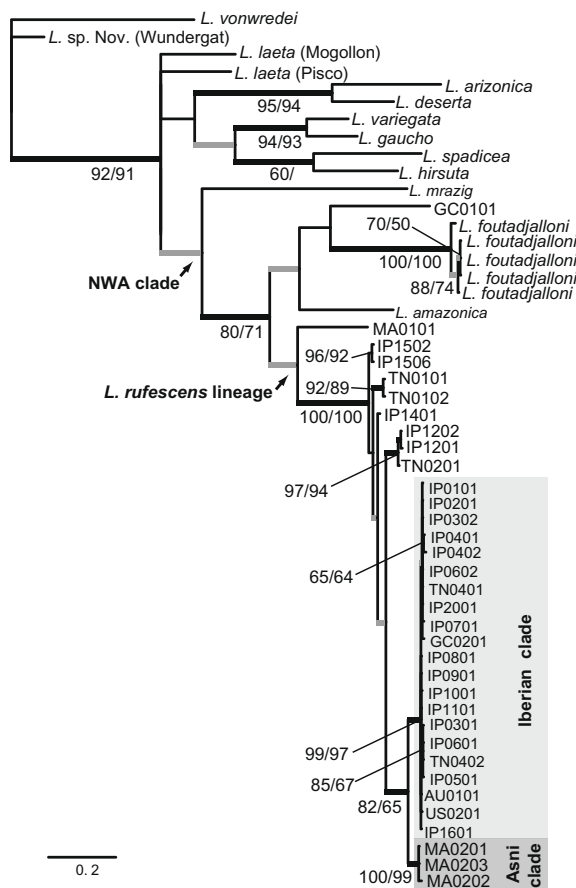


Fig. 4. Bayesian majority rule consensus tree of COI analysis. Support represented by branch weight and color. Black weighted branches lead to nodes that are supported with posterior probabilities of 0.95 or greater. Weighted grey branches lead to nodes that are supported with posterior probabilities between 0.70 and 0.95. Parsimony bootstrap values (PAUP*/TNT) are overlain below nodes.

population. These basal clusters have the same structure as in the 16S MUSCLE tree (Fig. 3).

Individual mitochondrial gene analyses support similar relationships (Figs. 3 and 4). Exceptions in the COI tree are minimal (Fig. 4); the exact placement of the clusters outside the Iberian/Asni clade differed from analyses of concatenated mitochondrial genes, though these relationships were only weakly supported. The basal clusters of individuals also included an additional taxon, TN0201, not included in the 16S data set (Figs. 3 and 4). Exceptions in the 16S tree were more substantial, and it was somewhat sensitive to alignment strategy. First, support for the Iberian/Asni clade is only moderate in individual 16S analyses (0.83–0.85 PP). Second, 16S analyses included Chinese taxa, which it placed individually in a polytomy with other individuals and clusters of putative *L. rufescens* outside of the Iberian/Asni clade. They included another Tunisian individual (TN0301) in the large basal polytomy of the *L. rufescens* lineage (Fig. 3). Third, Bayesian analyses of 16S did not resolve MA0101 as sister to the rest of putative *L. rufescens*, a relationship with weak to strong support in parsimony analyses (82–98% bootstrap support in PAUP*, 69–95% in TNT). Instead, analysis of the 16S MUSCLE alignment resolved MA0101 as a divergent member of the *L. rufescens* polytomy (Fig. 3). Analysis of the 16S PRANK alignment places a polytomy of some Chinese individuals and the three individuals from Mostoles, Spain (IP1201–03) outside of a weakly supported polytomy containing the remaining putative *L. rufescens*, including MA0101 (not shown).

Though taxon sampling for 28S was limited within the *L. rufescens* lineage, it contained representatives of most of the groups that

were supported by mitochondrial genes (Table 1). 28S analyses supported MA0101 as sister to a reasonably supported (0.92–0.99 PP) polytomy of remaining putative *L. rufescens* (Fig. 1). Lack of resolution within the *L. rufescens* lineage (with the exception of MA0101) is probably due to little variation among them (1452 sites contained 20 variable and 3 parsimony informative characters).

Concatenated analyses including 28S also resolved the basal position of MA0101 with strong support (Fig. 2) and generally reflected relationships supported in analyses of mitochondrial genes. They supported the monophyly of the Iberian clade with PP values greater than 0.91 and supported its sister status with the Asni clade with PP values greater than 0.93. Analyses including 28S also placed the rest of putative *L. rufescens* in a basal polytomy with the Iberian/Asni clade. The 28S/COI/16S alignment was extremely reduced, containing only two individuals in the Iberian clade (AU0101 and US0201) and two individuals in the basal polytomy (IP1202 and IP1502). Analyses of alignments concatenating the three genes across all taxa resolved the NWA clade, the *L. rufescens* lineage, and the basal position of MA0101 within *L. rufescens*. Within the *L. rufescens* lineage, however, Bayesian analyses generated low support for the Iberian clade and its sister relationship to the Asni clade, while parsimony analyses collapsed all putative *L. rufescens* (except MA0101) into a polytomy.

3.4. *p*-Distances

3.4.1. NWA clade

Summarized in Tables 4a and 5a are uncorrected COI and 16S *p*-distances between the five taxa of the NWA clade. *p*-Distances between these taxa tended to be comparable between 16S and COI. They ranged from 13.1% to 19.0% for COI and 11.0% to 22.2% for 16S, and more commonly paired taxa in phylogenetic analyses did not necessarily have lower values. *p*-Distances between the *L. rufescens* lineage and *L. mrazig* were the lowest in the COI data set (13.1%), but among the largest in the 16S data set (19%). The lowest 16S *p*-distance was between the *L. rufescens* lineage and GC0101 (11.4%/11.0%; MUSCLE/PRANK). The largest COI *p*-distance was between *L. amazonica* and *L. rufescens* (19.0%), while the largest 16S *p*-distance was observed for *L. amazonica* and *L. mrazig* at 21.1%/22.2%. *p*-Distances between *L. mrazig* and *L. foutadjalloni*, the most commonly paired two NWA clade taxa in phylogenetic analyses, were among the highest in both mitochondrial data sets (18.0% for COI and 17.3%/17.7% for 16S). *p*-Distances were mid-range for *L. amazonica* and GC0101, the second most common pair

Table 4a
Average COI *p*-distances within and between NWA clade lineages.

Group	<i>L. mrazig</i>	GC0101	<i>L. amazonica</i>	<i>L. foutadjalloni</i>	<i>L. rufescens</i>
<i>L. mrazig</i>	–	17.2%	17.7%	18.0%	13.1%
GC0101	–	–	15.1%	16.9%	17.3%
<i>L. amazonica</i>	–	–	–	17.4%	19.0%
<i>L. foutadjalloni</i>	–	–	–	–	14.0%
<i>L. rufescens</i>	–	–	–	–	–

Table 4b
Average COI *p*-distances within and between groups in the *L. rufescens* clade.

Group	IP1401, 1502, 06, TN0101–02	IP1201–02, TN0201	Asni clade	Iberian clade
MA0101	16.1%	15.6%	12.9%	12.8%
IP1401, 1502, 06, TN0201–02	2.4%	3.7%	7.0%	7.3%
IP1201–02, TN0301	–	1.1%	8.4%	6.6%
Asni clade	–	–	–	0.0%
Iberian clade	–	–	–	–

of taxa resolved in phylogenetic analyses: 15.1% for COI and 14.5%/16.5% (PRANK/MUSCLE) for 16S.

3.4.2. *L. rufescens* lineage

We calculated mean, uncorrected pairwise *p*-distances in COI and 16S (summarized in Tables 4b and 5b) between MA0101 and four other groups of putative *L. rufescens* (1) the basal group containing IP1401, IP1502, IP1506, TN0101 (the COI data set also included TN0102 and the 16S data set also included TN0301); (2) the basal group containing IP1201-02 (the COI data set also included TN0201 and the 16S data set also included IP1203 and Chinese taxa); (3) MA0201-03 (Asni clade); (4) the Iberian clade.

p-Distances were lower for 16S than for COI. Distances within the four more derived groups ranged from 0.0% to 2.4% for COI and 0.0% to 0.7% for 16S. Average *p*-distances between the two basal groups were 3.7% for COI and 0.8% for 16S. Average *p*-distances between basal groups and the Asni clade ranged from 7.0% to 8.4% for COI and 0.9% to 2.3% for 16S. Between the two basal groups and the Iberian clade, they ranged from 6.6% to 7.3% for COI and 0.3% to 2.0% for 16S. Average *p*-distances between the Asni and Iberian clades were 4.8% for COI and 0.6%/0.9% for 16S (PRANK/MUSCLE). Mean uncorrected *p*-distances between the four groups and the basal lineage MA0101 ranged from 12.8% to 16.1% for COI and 9.1% to 10.1% for 16S.

3.5. Haplotype networks

COI and 16S haplotype networks (Fig. S1) supported groupings of individuals within the *L. rufescens* lineage that appeared in trees, but the 16S haplotype network provided more structure than the phylogeny to the separation of certain groups. In the 16S network, 32 of 58 taxa shared a haplotype, corresponding to the Iberian clade. Most Chinese individuals shared a haplotype with *Loxosceles* from Mostoles, Spain (IP1201-03), while haplotypes of remaining Chinese taxa were within two steps of the main group. Additionally, two Iberian individuals (IP1502 and IP1506) had a haplotype that was 2–4 steps from the Chinese haplotypes, and 6 steps away from the Iberian clade haplotype (including other individuals from

the same locality). Their divergent haplotype mirrors their placement outside of the Iberian clade in phylogenetic analyses, even when other taxa from the same population were placed in the Iberian clade.

Reflecting the larger *p*-distances between groups of putative *L. rufescens* in the COI dataset, the COI network analysis produced four subnetworks that were at least 25 steps from each other and had more steps between haplotypes than the 16S network. In the COI network, Iberian individuals IP1502 and IP1506 were three steps away from each other and 34–35 steps away from the Iberian individual from Mostoles (IP1202). They were 39–42 steps away from the Iberian clade subnetwork (represented by six haplotypes within four steps of each other).

3.6. Morphological assessments of museum specimens

3.6.1. Previous species identifications

Among the museum specimens we borrowed for morphological assessments, most Northern African taxa were most recently identified as *L. rufescens*, or a synonymous species (*L. distincta*), though a few were unidentified. One Tunisian female was identified as *L. gaucho* (Brignoli, 1976). Western, Eastern, and Central African taxa were unidentified. *Loxosceles* from India, Malaysia, and Puerto Rico were identified as *L. rufescens*, and *Loxosceles* from St. Helena were identified as the synonymous species *L. distincta* (Table 2).

3.6.2. Northern African and non-African females

We examined one female specimen each from Morocco, Libya, Sudan, Egypt, and Malaysia collections, two from Tunisia and St. Helena collections, and eight from Algeria. With the exception of the *L. gaucho* from Tunisia, of which the somatic and genitalic morphology was indistinguishable from Brazilian *L. gaucho* (Figs. 3 and S2), the genitalia of females were morphologically similar to that of *L. rufescens*. Spiders morphologically consistent with *L. rufescens* had epigyna composed of two large, subtriangular receptacles pointing medially, each with two pouch-like anterior lobes and a sclerotized band along the lateral face (see Figs. 3 and S2). Lobe morphology varied greatly among females, some with two pronounced lobes and others with receptacles that were nearly contiguous across the anterior edge and showed only two slight bulges. Some well-pronounced lobes were long and relatively thin while others were short and round. The relative size of lobes also varied, with some individuals' lobes being about equal in size and shape and others possessing on each receptacle one larger or taller lobe, always medial to the smaller/shorter lobe (Fig. S2).

Eye patterns in some individuals corresponded to those found in *L. rufescens*, while in others they were inconsistent in some or all respects. In females from Morocco, Tunisia, Egypt, St. Helena and Malaysia, eye diads were separated by ~2–2.5 median eye (ME) wide diameters. Their eyes were more recurved than in other North African females, with a line drawn across the anterior edge of lateral eyes (LEs) falling posterior to the median diad. These females were more similar than other North African females to *L. rufescens* (Gertsch, 1967; Gertsch and Ennik, 1983), in which diads are separated by 1.25–2 ME long diameters and LEs fall 1.25–1 ME diameter posterior to the median diad. Females from Libya, Sudan, and Algeria had more closely spaced diads, separated by roughly 1 (in Algerian and Libyan females) or 1.5 (in Sudanese female) ME wide diameters. While spacing between the Sudanese female's eye diads was consistent with *L. rufescens*, her eye row, and that of the Libyan female, was much less recurved, with a line drawn across the anterior edge of the LEs intersecting the MEs 1/3 their length from their posterior margin. The eye rows of Algerian females were also less recurved, with a line drawn across the anterior edge of LEs either crossing the posterior margin of MEs or intersecting MEs 1/4 their diameter from their posterior edge.

Table 5a

Average 16S *p*-distances within and between NWA clade lineages (PRANK/MUSCLE).

Group	<i>L. mrazig</i>	<i>L. GC0101</i>	<i>L. amazonica</i>	<i>L. foutadjalloni</i>	<i>L. rufescens</i>
<i>L. mrazig</i>	–	16.9%/16.8%	21.1%/22.2%	17.3%/17.7%	19.0%/19.0%
<i>L. GC0101</i>		–	14.5%/16.5%	16.4%/16.3%	11.4%/11.0%
<i>L. amazonica</i>			–	19.7%/19.9%	15.1%/15.7%
<i>L. foutadjalloni</i>				–	18.5%/17.3%
<i>L. rufescens</i>					–

Table 5b

Average 16S *p*-distances within and between groups in the *L. rufescens* clade (PRANK/MUSCLE).

Group	IP1401, 1502, 06, TN0101, 0301	IP1201-03, China	Asni clade	Iberian clade
MA0101	9.2%/10.1%	9.1%/9.9%	9.1%/9.2%	9.1%/9.9%
IP1401, 1502, 06, TN0201, 0601	0.0%/0.7%	0.8%	1.7%/2.3%	1.1%/2.0%
IP1201-03, China		0.0%	0.9%/1.5%	0.3%/1.2%
Asni clade			0.0%	0.6%/0.9%
Iberian clade				0.1%

3.6.3. Northern, North-Central African and non-African males

We examined one male specimen from each of Egypt, Chad, Malaysia, India, and Puerto Rico (one pedipalp only) collections, three from St. Helena, and five from Algeria. Palps of non-African males were morphologically consistent with *L. rufescens*, possessing long, thin femora, longer tibiae, suboval tarsi and an enlarged bulb with an apically curved embolus (rather than tapered as in *L. amazonica*; see Figs. 3 and S2; Gertsch, 1967; Gertsch and Ennik, 1983). Their eye position also concurred with that of *L. rufescens* males, with diads separated by 1–2 ME wide diameters and a line drawn across the anterior edge of LEs crossing or falling slightly behind the ME posterior margin.

Males from Egypt, Algeria and Chad were morphologically distinct from *L. rufescens* (Fig. S2). The Chadian male had the dark, dentate bands along the sides of the cephalothorax that are present in *L. mrazig*, *L. gaucho* and *L. amazonica* and characteristically absent in *L. rufescens*. North African and Chadian males' pedipalps also differed markedly from those of *L. rufescens*; the femora of their palps were wider and their tibiae shorter, and they had very distinct embolus curvatures and lengths (Fig. S2). The eye diads of Egyptian and Algerian males were separated by slightly more than 1 ME wide diameter and eyes were less recurved than in males resembling *L. rufescens*, with a line drawn across the anterior edge of LEs crossing the posterior margin of MEs or intersecting MEs about 1/4 their long diameter from their posterior edge. The eye position of the Chadian male was the most distinct, with diads separated by less than one ME wide diameter and a line drawn across the anterior edge of LEs intersecting MEs about 1/2 way through.

The relative shape and size of the femora and tibiae of the palps of Chadian, Algerian, and Egyptian males more closely resembles the morphology of *L. amazonica* males than *L. rufescens* males (Figs. 3 and S2), and in fact characteristically distinguish *L. amazonica* from *L. rufescens* (Gertsch, 1967). They also recall the distinctive morphology of *L. mrazig*, with its broad, short segments, though the length of their tibiae is longer relative to their tarsi (longer than the tarsus length) than the tibia of *L. mrazig* (roughly equal to the tarsus length). The Chadian male's palp is nearly identical to that of *L. amazonica*, with a very slight difference in embolus length (Figs. 3 and S2) and curvature from the apical view (not shown). The eye position of the male from Chad is identical to *L. amazonica* males from Brazil (Gertsch, 1967).

3.6.4. Western, Eastern, and Central African *Loxosceles*

We examined one specimen each from Sierra Leone, Rwanda, and Democratic Republic of Congo (DRC), two from Ethiopia and Tanzania, and four from Kenya. These spiders were clearly not related to *L. rufescens*, *L. gaucho*, *L. amazonica*, or any of the morphologically distinct African individuals discussed above. The unidentified Sierra Leonean female was morphologically indistinguishable from *L. rufipes*, a member of the South American *Loxosceles laeta* species group found in Colombia and Panama. Taxa from Ethiopia, Kenya, Tanzania, Rwanda and DRC were also extremely different from North African and Chadian *Loxosceles* from museum collections and all *Loxosceles* in our molecular data set. They closely resembled the divergent southern African species of the *L. spinulosa* group (Newlands, 1975; Binford et al., 2008) in their closely positioned and weakly recurved eye groups, patches of dark brown setae on the cephalothoraces and abdomens, and the elongate, ventrally pointed tarsi of male palps.

4. Discussion

Phylogenetic analyses in this study recovered a monophyletic clade of *Loxosceles* with a range that minimally includes parts of Spain, and Northern and Western Africa (NWA). Our results further

indicate that an assemblage of taxa morphologically consistent with *L. rufescens* evolved from within this NWA clade. The NWA clade contains more taxonomic and genetic diversity than has been previously realized, both in the larger clade and within the lineage of putative *L. rufescens*. Consistent with previous work, given current taxon sampling, this lineage is sister to a clade of *Loxosceles* from South America (Binford et al., 2008), rendering the New World fauna paraphyletic, and African fauna polyphyletic. However, the relative timing and dynamics of the divergence of NWA clade members from South American species remain elusive because phylogenetic analyses of our data set failed to resolve relationships among NWA clade taxa. Despite ambiguity in the relationships between the lineages of the NWA clade, our data consistently support certain patterns of the structure of diversity within the *L. rufescens* lineage.

4.1. The NWA clade includes deep genetic diversity

In previous work, we presented evidence supporting an evolutionary origin of *Loxosceles* that predates the break-up of Western Gondwana. Evidence for a Gondwanan origin of *Loxosceles* included paraphyly of Southern African species with respect to South American species, and inference of divergence dates between South American lineages and their sister Southern African taxon that were older than 95 million years (Binford et al., 2008). The alliance of *L. rufescens* and *L. lacta* with Northern Brazilian *L. amazonica* hinted at the possibility of a Gondwanan origin for their common ancestor as well. However, estimates of the date of their common ancestor were younger than the last connection between Africa and South America, leading us to consider the possibility of a post-Gondwanan colonization of Africa by the *L. rufescens* common ancestor (Binford et al., 2008). Here, we present robust support for a diverse and likely old clade that includes *L. amazonica*, the *L. rufescens* lineage, *L. mrazig* (Tunisia), GC0101 (Canary Islands), and *L. foutadjalloni* (Guinea) (Figs. 2–4).

Comparison of genetic distances between the five NWA clade lineages with well-established clades in *Loxosceles* suggests that this genus has a deep history in Northern and Western Africa. COI and 16S *p*-distances between taxa in the NWA clade (15.1–18% for COI and 14.5–22.2% for 16S) are comparable to *p*-distances among species in the *reclusa* group in N. America and the West Indies (14.7 ± 0.02% for COI and 17.1 ± 0.04% for 16S; unpublished data). The monophyletic *reclusa* lineage is minimally 20 million years old and potentially much older (Binford et al., 2008). COI *p*-distances among NWA lineages are also similar to *p*-distances between *L. gaucho* and *L. laeta* (16.53%; Ribera and Planas, 2009), two South American species from distinct species groups. Thus, 16S and COI *p*-distances suggest that *Loxosceles* taxa in the NWA clade represent comparable genetic diversity to that between species groups in South America, and among species in the diverse (~50 spp.) North American *reclusa* group. Though COI and/or 16S *p*-distances among divergent taxa could be influenced by saturation, the morphological diversity we observed in African museum specimens (Fig. S2) provides further evidence for an old *Loxosceles* lineage in Northern Africa. Given the extent of diversity in Northwestern Africa, it will be interesting to add dense taxon sampling, identify reliable calibration points, and apply molecular dating analyses to test the possibility a Gondwanan MRCA of the NWA clade.

4.2. *Loxosceles* diversity in Northwestern Africa

4.2.1. Divergent Northwestern African and Canarian taxa

One of our more interesting results is the discovery of sufficient diversity among the five taxa in the NWA clade that our analyses failed to resolve them. Poor resolution among NWA clade taxa

could have resulted from divergence levels that are too deep to be captured by our genetic markers, as resolving relationships among divergent South American *Loxosceles* species groups has proved difficult using the same markers (Binford et al., 2008, unpublished data). Relationships among NWA clade lineages that might be predicted by morphology are not resolved. For example, *L. foutadjalloni* has similar genitalic morphology to *L. rufescens* (Millot, 1941), but it never allies with the *L. rufescens* lineage in our analyses. *L. mrzigi* is markedly different morphologically from *L. rufescens* (Ribera and Planas, 2009) and has some similarities with *L. amazonica* (Gertsch, 1967), but never allies with *L. amazonica*. Ribera and Planas (2009) proposed that *L. mrzigi* and *L. foutadjalloni* might form a distinct group based on morphology, a pairing supported inconsistently in our analyses. Clearly, the structure of diversity of species in Northwestern Africa is much more complex than has been understood based on previous sampling including only *L. rufescens* and *L. amazonica* (Binford et al., 2008).

4.2.2. The *L. amazonica* species group in Africa

L. amazonica, a clear NWA clade member, is currently understood to be native to Northern Brazil (Gertsch, 1967), a range in the region of South America last known to share a land connection with Central/Western Africa (Pitman et al., 1993). Thus, a basal position of *L. amazonica* in the NWA clade would suggest that it diverged by Gondwanan vicariance, but our analyses did not resolve *L. amazonica* as the basal lineage of the NWA clade (Fig. 2b). Lack of resolution in relationships of NWA clade taxa and details of *L. amazonica* morphology and collection records (Gertsch, 1967) invite consideration that this species is derived from within Northwest African *Loxosceles* and dispersed recently from Africa to South America. Four pieces of evidence support an African origin of *L. amazonica*. First, the palpal morphologies of male museum specimens from Egypt, Algeria and Chad closely resemble that of *L. amazonica* in the thickened segments, especially the femurs. In fact, the dimensions of palpal segments that conform to *L. amazonica* are characteristics that distinguish *L. amazonica* from both the closely related *L. rufescens* and *L. gaucho* groups (Gertsch, 1967; Binford et al., 2008). The morphological similarities African museum specimens share with *L. amazonica* raise the possibility that *L. amazonica* has relatives widespread across Northern and into Central Africa. Second, the Chadian male museum specimen so closely resembles *L. amazonica* in somatic and genitalic morphology that they are very likely the same species, or at least very closely related (Figs. 3 and S2). Third, *L. amazonica* is prone to human-mediated dispersal and has been introduced in towns in remote, tropical Northern regions of Peru (Gertsch, 1967; Binford et al., 2008). Furthermore, no other similar species is found in South America. Finally, molecular dating analyses of *Loxosceles* reported by Binford et al. (2008) consistently resolved the most recent common ancestor of *L. amazonica* and *L. rufescens* as being too young to be explained by Gondwanan vicariance. They accounted for this discrepancy by citing studies that argued for the existence of ancient cross-continental land bridges. Here, we propose that the break-up of Gondwana did not influence *Loxosceles* distribution at the level of the *L. amazonica*/*L. rufescens* common ancestor but rather at a deeper node, perhaps at the ancestral node of the *L. gaucho* group and the NWA clade. The hypothesis that *L. amazonica* is an African native can be tested, and the source population potentially found, with extensive taxon sampling from across Northern and Central Africa.

The earliest collection record of *L. amazonica* is from 1896 in Amazonas, Brazil, but trade between Brazil and Africa began in as early as the 16th century. Trade is a plausible explanation for the dispersal of *L. amazonica* from Africa to South America, especially in light of the Sierra Leonean *Loxosceles* that is indistinguishable from *L. rufipes*. Known from Colombia and Panama (Gertsch,

1967; Gertsch and Ennik, 1983), *L. rufipes* belongs to the diverse and exclusively New World *L. laeta* group. We recently found that *L. fontainei*, described from Southern Guinea (Millot, 1941), is genetically identical (28S, COI) to *L. rufipes* from Panama (unpublished data). We can therefore confidently identify the museum specimen from Sierra Leone as *L. rufipes*, which has been in West Africa since at least the 1930s, when Millot collected *L. fontainei*. *L. rufipes* is thus another likely candidate for trans-Atlantic introduction via trade.

4.2.3. The presence of *Loxosceles gaucho* in Tunisia

The identification of a female *Loxosceles gaucho* in Tunisia (Brignoli, 1976) is an interesting problem, especially in light of the discovery of *L. mrzigi* in Tunisia, a species showing strong morphological similarity to *L. gaucho* (Ribera and Planas, 2009). Ribera and Planas (2009) argue that the resemblance between the divergent species is convergent, a reasonable explanation because of the simple copulatory structures found in *Loxosceles*. The Tunisian female identified as *L. gaucho* could be another example of morphological convergence in a species divergent from the South American *L. gaucho*, but it also seems likely that she represents yet another population of *Loxosceles* that has dispersed across the Atlantic through human trade. First, though *Loxosceles* genitalia are simple, *L. gaucho* female epigyna have a transverse, sclerotized plate firmly holding the seminal receptacles in place (Fig. 3), which distinguishes *L. gaucho* and related species from all other *Loxosceles* with free-floating receptacles (Gertsch, 1967). The Tunisian *L. gaucho* identified by Brignoli (1976) possesses the transverse plate characteristic of *L. gaucho* epigyna (Fig. S2). Second, the Tunisian *L. gaucho* was found near Sfax (Table 2), a major port and commercial hub in the Mediterranean and a likely place where non-native *Loxosceles* may occur. The question of whether the *L. gaucho* documented in Tunisia is an example of morphological convergence or a recent introduction can be answered with thorough sampling of adult males and females in Tunisia, including individuals sampled from Sfax.

4.3. The *Loxosceles rufescens* lineage

4.3.1. Origin and range

Though they have dispersed all over the world with humans, *L. rufescens* are considered to be native to the Mediterranean (Gertsch and Ennik, 1983). The placement of the divergent MA0101 as the basal lineage of the *L. rufescens* group (Figs. 2–4) and the genetic and morphological diversity found in Northwest Africa offers support for a Northern African origin of the *L. rufescens* lineage.

An interesting and consistent pattern is that relationships between putative *L. rufescens* are not predictable by geographic location. Tunisian and Canarian individuals group with genetically indistinct Iberian, North American, and Australian individuals in the Iberian clade, while other Iberian, Northern African, and Chinese individuals form various, sometimes geography-independent clades outside of the Iberian clade. These patterns suggest multiple independent dispersal events from North Africa to the Iberian Peninsula, and very recent (re-)colonization occurring between Spain and North Africa, the Canary Islands, and the rest of the world. Consequently, the *L. rufescens* introduced range seems to overlap with what we can interpret to be, based on our taxon sampling, part of their North African native range.

Overlap of *L. rufescens* native and introduced ranges could contribute to historical problems systematists of the past two centuries have faced when attempting to delimit species in the Mediterranean basin. A molecular phylogenetic study including thorough sampling from across the geographic range of *L. rufescens* would help determine whether they occupy this range because of ancient, independent dispersal or recent, human-mediated dis-

persal. Such a study would also refine our understanding of the location of origin of the *rufescens* group.

4.3.2. Diversity

Our phylogenies support a monophyletic group of all *Loxosceles* that morphologically resemble *L. rufescens*, within which there are divergent clusters of individuals and populations (Figs. 2–4). Though relationships among some clades in the *L. rufescens* lineage are not well resolved, mitochondrial genes recover structuring of four consistent patterns: (1) The Iberian clade is always sister to (2) a clade of Moroccan individuals MA0201-03 (the Asni clade). Outside of those clades, there is (3) one or more basal groups containing various poorly resolved clades of other Iberian, Tunisian and Chinese individuals. Lastly, (4) MA0101 is sister to all other putative *L. rufescens* (Figs. 2–4). 28S alone and combined with mitochondrial genes also supported MA0101 as sister to the rest of the *rufescens* lineage (Fig. 2).

Genetic distances between some of the groups described above are high enough to inspire consideration that the lineage contains cryptic species (Tables 4b and 5b). DNA barcoding benchmarks have been proposed for genetic distances between sister species in spiders (Barrett and Hebert, 2005; Robinson et al., 2009); however, other studies suggest that the most appropriate reference points may be lineage and gene specific (Bond, 2004; Paquin and Hedin, 2004; Astrin et al., 2006; Huber and Astrin, 2009). The genitalic simplicity of *Loxosceles* has made morphological delineations of many taxa within this lineage challenging and our accumulating genetic data do not always help to clarify standards. Within nominal species for which we have a range of populations sampled, Binford and colleagues (unpublished data) have found maximum uncorrected CO1 *p*-distances ranging from 1.2% (mean $0.3 \pm 0.1\%$) in *L. apachea* (four populations in SW New Mexico, USA), to 9.3% (mean $3.1 \pm 0.6\%$) in *L. arizonica* (six populations near Tucson, Arizona, USA), and as great as 16.0% (mean $13.2 \pm 2.5\%$) in *L. laeta* (ten geographically disparate populations in Peru). While this disparity could be influenced by inclusion of nuclear copies of CO1, the wide-ranging divergences are mirrored by divergences in 16S. Clearly multiple lines of evidence need to be incorporated for species delineation in *Loxosceles*.

Despite these complexities, genetic divergences and monophyletic groupings help point to candidate clades that warrant detailed attention to identify cryptic species. Most notably, distances between the basal MA0101 and other *rufescens* clade taxa range from 12.9% to 16.1%, nearly as high as the mean distance among species in the North American *reclusa* group. Mean uncorrected *p*-distances of COI between other clades in the *L. rufescens* lineage range from 2.4% to 8.4%. The clustering we observed in the *L. rufescens* group (Figs. 2–4 and S1) suggests that it may contain as many as three species.

Interestingly, COI and 16S analyses placed two Iberian individuals (IP1502 and IP1506) outside of the Iberian clade while 16S, our more inclusive molecular dataset, placed additional individuals from the same population (IP1501, IP1503-05) in the Iberian clade. We were unable to confirm inclusion of IP1501 and IP1503-05 in the Iberian clade with COI because sequences from those individuals showed evidence of nuclear integration and we excluded them from analyses (see explanation in Section 2). However, IP1502 and IP1506 were genetically divergent from the Iberian clade in our COI data set (8.1%), suggesting gene flow does not occur between all individuals at the site where they were found and there may be two species there.

4.3.3. Taxonomic difficulties

Despite substantial genetic variation among populations of putative *L. rufescens*, systematists have historically faced difficulties delimiting *Loxosceles* species in the Mediterranean basin based

on morphology (Lucas, 1846; Simon, 1881, 1910; Franganillo, 1925; Brignoli, 1969, 1976). Brignoli (1969, 1976) examined many populations across Northern Africa and Mediterranean Europe, some represented by multiple individuals and some by only one individual, and concluded that they were all *L. rufescens* based on homogeneity of genitalic structures. We agree that many of the individuals he identified as *L. rufescens* that we also examined closely resemble one another, but we also detected differences that may be sufficient for species delineation. For example, a male from Egypt and males from Algeria identified as *L. rufescens* (Brignoli, 1976) had palpal segments that were shorter and/or thicker relative to their length than those of *L. rufescens* (Gertsch and Ennik, 1983), and a distinct curvature of the embolus we have not observed in any putative *L. rufescens* (Figs. 3 and S2). Many of the Northern African populations were only represented by females in collection (see Table 2), which generally have fewer reliable characteristics for species delineation than males. These females were not fully consistent with *L. rufescens* (considering somatic morphology), and we are skeptical of their inclusion in *L. rufescens* without examining corresponding males.

In some individuals we observed striking differences in the curvature of the embolus in male palpi that were not congruent with genetic relationships (Fig. 3; note the opposite curvature of the embolus in males from Indianapolis and China compared to others in the *L. rufescens* lineage). These differences may be due to spontaneous rotation of the bulb during preservation in alcohol. Although bulbal rotation has not been reported in male *Loxosceles*, it is known to occur during courtship in other haplogynes (Huber and Eberhard, 1997) and *Loxosceles rufescens* possess the same muscles responsible for bulbal rotation in other spiders (Huber, 2004b). Studies in which the palpal bulb is removed and oriented to correspond to overall palpal orientation may resolve some of the morphological ambiguity among divergent *L. rufescens* populations.

In combination with genitalic characters, Gertsch (1967) and Gertsch and Ennik (1983) found somatic characters (such as relative eye position) to be useful for delimiting *Loxosceles* species. We detected variation in eye position in museum specimens that are promising for species delimitation in Northern African *Loxosceles*, such as eye groups that were less recurved and closer together than reported for *L. rufescens* (Gertsch, 1967; Gertsch and Ennik, 1983) in *Loxosceles* from Egypt, Libya and Sudan. However, given the historical difficulties in distinguishing between populations of *L. rufescens*, other approaches could aid in delimiting species. Recent works have delimited species and detected morphological/ecological structuring in groups of cryptic or morphologically variable spiders through careful morphometric analysis with replicate individuals (e.g., Crews and Hedin, 2006; Crews, 2009; Huber and Astrin, 2009) and examination of ecological divergence among populations (Stockman and Bond, 2007).

4.3.4. *Loxosceles lacta*

Our 16S and 28S analyses placed *L. lacta* individuals from China (Wang, 1994) in the *L. rufescens* lineage, consistent with the findings of Binford et al. (2008). This is not surprising given the striking morphological similarity of *L. lacta* individuals to the putative *L. rufescens* in our data set (Fig. 3). The presence of this taxon in China is also consistent with human-mediated dispersal of *L. rufescens* around the world (Gertsch and Ennik, 1983). The placement of Chinese individuals in a basal polytomy of the *L. rufescens* lineage with the clusters of Tunisian and Iberian individuals in 16S analyses and their overlap in haplotypes with *Loxosceles* from Mostoles, Spain (IP1201-03; Fig. S1) suggests that *L. lacta* was introduced to China from Mostoles or a closely related population. The question of whether *L. lacta* should remain a bona-fide species can only be answered by a careful taxonomic study of putative *L. rufescens* from

Spain and Northern Africa, including representatives from the *L. rufescens* type locality (Sagunto, Spain), which we have not included here.

5. Conclusions

Loxosceles have been relatively well documented and classified in North and South America, but African diversity is poorly understood. The dearth of species described from Africa reflects the underappreciated diversity of species such as *L. rufescens* and large collection gaps throughout the continent. Divergent genes and morphology of taxa from within the putative native range of *L. rufescens* indicates a need to peruse *Loxosceles* diversity in North-western Africa. The failure of some genetically and morphologically divergent taxa in our data set to ally with *L. amazonica* or *L. rufescens* indicates the presence of multiple divergent lineages in the region. Our genetic and morphological data also provide evidence that *L. amazonica* may have relatives throughout Northern Africa and into Central Africa. We propose that humans introduced *L. amazonica* to Brazil from Africa, perhaps via inter-continental trade, prior to the 20th century. More thorough taxon sampling across Northern, Western and Central Africa would allow us to test these hypotheses. Moreover, the extensiveness of diversity among NWA clade taxa invites reconsideration of a Gondwanan origin of their common ancestor, a hypothesis that can be tested through rigorous dating with denser taxon sampling and reliable calibration points. Support for a Gondwanan origin of the NWA clade MRCA would considerably change our understanding of the age of *Loxosceles*.

Delineating species in the *Loxosceles rufescens* lineage has been historically challenging for systematists. Though clear morphological differences are difficult to detect, especially in females, molecular phylogenetic analyses reveal that the *L. rufescens* lineage contains population structuring and genetic divergences not expected for conspecifics. In addition, the standards Brignoli (1969, 1976) used when classifying *L. rufescens* were too liberal and did not include somatic characters that are generally useful for delineating other *Loxosceles* species (Gertsch, 1967; Gertsch and Ennik, 1983). The patterns we observed are consistent with *L. rufescens* being a species group comprising at least two, but probably more, species. A careful revision of *Loxosceles* from across the geographic range of *L. rufescens* is currently in progress by Ribera and colleagues. Revising the taxonomy of the *L. rufescens* lineage will help determine its size and the extent of its natural and introduced geographic ranges in Africa and Mediterranean Europe.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jympev.2009.11.026.

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