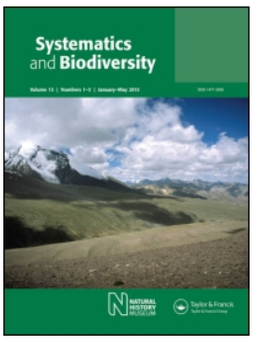

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Research Article

Re-evaluating conservation priorities of New World tarantulas (Araneae: Theraphosidae) in a molecular framework indicates non-monophyly of the genera, *Aphonopelma* and *Brachypelma*

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We present a mtDNA gene tree of tarantula spiders (Araneae: Mygalomorphae: Theraphosidae) based on the mitochondrial *16S-tRNA (leu)-ND1* gene region as a promising initial molecular hypothesis to clarify the taxonomy of the largest subfamily, Theraphosinae. Many species of this New World subfamily are traded widely as exotic pets, yet few scientific studies on them exist, and the robustness of many supposed taxonomic groupings is debatable. Yet the validity of taxon names and knowledge of their distinctiveness is vital for trade regulation, most notably for the Neotropical genus *Brachypelma* Simon 1891, which is listed under CITES (Appendix II, see online supplemental material, which is available from the article's Taylor & Francis Online page at https://doi.org/10.1080/14772000.2017.1346719). The use of molecular markers for tarantula taxonomy has been limited until recently, with most previous studies relying on morphological methods. Our findings, from newly collected molecular data, have several nomenclatural implications, suggesting a need for a rigorous overhaul of Theraphosinae classification at multiple hierarchical levels. Here, we take steps toward a revised classification, favouring division of Theraphosinae into three tribes: the Theraphosini trib. nov., the Hapalopini trib. nov., and the Grammostolini trib. nov. We also make conservation recommendations for two non-monophyletic genera. Firstly, we recover *Aphonopelma* Pocock 1901 as polyphyletic, finding that the large radiation into the USA and Mexico is taxonomically distinct from at least three other lineages distributed throughout Central America, one of which includes the type species of the genus. Secondly, and importantly for conservation, we find diphyly in the CITES listed genus *Brachypelma* Simon 1891, where our data strongly favour a division into two distinct smaller genera. We consider only the lineage with endemics in the Pacific coastal zone of Mexico to be of conservation concern. Finally, we also make suggestions on the future direction of revisionary research for the Theraphosidae as a whole.

http://zoobank.org/urn:lsid:zoobank.org:pub:B37F7795-3F92-4334-A0C7-65C8026EE1FB

Key words: *Aphonopelma*, *Brachypelma*, classification, mtDNA, tarantula, Theraphosidae, tribe

Introduction

Overexploitation of natural resources increasingly threatens biodiversity, particularly organisms where our understanding of evolutionary uniqueness is poor, including many highly sought after for commercial trade (Hafernik, 1992). Consequently, excessive harvesting, habitat loss, and climate change are of increasing concern for the conservation of many organisms. The exotic pet trade has exacerbated threats, as species are exploited with little understanding of the reproductive biology of natural populations. To further compound matters, the evolutionary history and taxonomy of many traded organisms is poorly understood, with organisms often collected before being scientifically described and no reliable knowledge to protect them (Stuart, Rhodin, Grismer, & Hansel, 2006).

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When factored with other anthropogenic driven influences, extinction risk greatly increases (Sarkar, 2011). In recent years, tarantulas (Araneae: Mygalomorphae: Theraphosidae) have become a popular choice of exotic pet, and many wild caught individuals have been collected; including a large number of undescribed species (Molur, Silliwal, & Daniel, 2008). Furthermore, in several countries worldwide, most notably South East Asia, there are additional pressures on natural populations of tarantulas as a source of food and/or traditional medicine (Machkour-M'Rabet, Hénaut, Winterton, & Rojo, 2011; Yen, & Ro, 2013). In many of these areas there has also been a historical exploitation of other taxa such as reptiles and amphibians for food and/or medicine, as well as for the pet trade – either legal or illegal (Menegon, Davenport, & Howell, 2011; Phimmachak, Stuart, & Sivongxay, 2012).

There is currently little scientific understanding about many of the tarantulas actively targeted for commercial exploitation, particularly regarding their evolutionary history (i.e., species limits, population structure, and abundance of natural populations). Only recently has research begun to shed light on the natural population structure for tarantula species in the New World (e.g., Graham, Hendrixson, Hamilton, & Bond, 2015; Hamilton, Formanowicz, & Bond J., 2011; Hamilton, Hendrixson B., Brewer M., & Bond, 2014; Hamilton, Hendrixson, & Bond, 2016a; Hendrixson, DeRussy, Hamilton, & Bond, 2013; Hendrixson, Guice, & Bond, 2015; Mendoza & Francke 2017; Montes de Oca, D'Elía, & Pérez-Miles, 2016; Ortiz & Francke, 2015; 2016; Pérez Miles, Costa, Toscano-Gadea, & Mignone, 2005), yet still almost nothing remains known on the sustainability threshold for any wild populations being exploited. This effectively means that the ability to determine sustainability is unknown for most tarantulas; an issue that has been shown to complicate conservation plans for other wildlife collected for commercial trade (e.g., Courchamp et al., 2006).

Historically, several species of the New World tarantula subfamily Theraphosinae – most notably the Mexican/Central American genus *Brachypelma* Simon 1891 – were heavily exploited for commercial trade during the rise in exotic pet popularity. Wild populations of *Brachypelma* from the Pacific regions of Mexico were thought to have experienced an especially high risk of over-exploitation due to their slow reproductive life cycle, endemic ranges, and their highly sought-after colouring and calm temperament (Locht, Yáñez, & Vázquez, 1999; Longhorn, 2014; Smith, 1994; West, 2005). This led to the first step in theraphosid conservation with *Brachypelma smithi* (F.O. Pickard-Cambridge 1897) given Red List status in the 'Near Threatened' category, and the entire genus *Brachypelma* placed on CITES Appendix II to regulate international trade (Longhorn, Nicholas, Chuter, & Vogler, 2007). These changes may have helped to reduce exploitation of natural populations of *Brachypelma* for international trade, but have failed to impact the now growing pet-trade within Mexico. Moreover, other genera with similar evolutionary history may face a similar extinction risk, most notably the even slower-growing *Aphonopelma* Pocock 1901 from North America and *Grammostola* Simon 1892 from South America. A recent example of poaching was detailed in an open letter published on 1 March 2016 regarding a recently described species of Uruguayan tarantula from the genus *Grammostola* being subject to illegal trafficking through the Caribbean and on to Europe (Aisenberg & Pérez-Miles, 2016). This highlights the need to clarify the distinctiveness of all potentially vulnerable lineages, especially for such high-profile genera, plus define species boundaries and population structure. It is especially important to understand unique isolated populations or narrow endemic species, some of which may now have been exploited beyond the point of recovery (Longhorn et al., 2007; Petersen et al., 2007; West, 2005).

Taxonomic uncertainty has serious implications for the effectiveness of conservation strategies for such vulnerable lineages (Lim, Balke, & Meier, 2012; Siliwal, Molur, & Raven, 2013), leading to ineffectual conservation efforts. The current classification scheme for spiders in the family Theraphosidae is largely reliant on the attributes of a handful of morphological characters, many of which may be plesiomorphic and/or homoplasious, a problem seen in all mygalomorph families (see Bond, Hendrixson, Hamilton, & Hedin, 2012; Goloboff, 1993; Raven, 1985). Despite subsequent efforts that utilized multiple character systems to resolve the evolutionary history of the wider Mygalomorphae, few studies have focused within the Theraphosidae. Some of these have applied molecular data to specific New World genera (Hamilton et al., 2011; 2014; 2016a; Hendrixson et al., 2013; 2015; Longhorn et al., 2007; Montes de Oca et al., 2016; Ortiz & Francke, 2015; 2016; Petersen et al., 2007; Wilson, Gunnell, Wahl, & Pitts, 2013), but with poor understanding of wider generic boundaries, which have been historically inferred solely from morphological data (e.g., Bertani et al., 2001; Bertani, 2012; Gabriel, 2016; Guadanucci, 2014; Pérez-Miles, Lucas, da Silva, & Bertani, 1996; Pérez-Miles, 2000).

Within the family, Theraphosinae is the most diverse of all theraphosid subfamilies, currently with 67 genera distributed across most New World temperate and tropical zones. This subfamily is particularly notable for the synapomorphic presence of urticating hairs on the abdomen that can be kicked free by action of the hind legs as a predator defence. There are several distinct structural forms of urticating hairs, although types III and IV can be confused (see Bertani & Guadanucci, 2013; also Perafán, Cifuentes, & Estrada-Gomez, 2015). Generic relationships were proposed by Pérez-Miles et al. (1996) and Bertani (2001), though each inferred their phylogenetic hypothesis based on Maximum Parsimony of a limited set of morphological characters. Both studies favoured the monophyly of the

Theraphosinae (Fig. 1), but neither was particularly successful in resolving inter-generic relationships, with many nodes only weakly supported by single character changes and/or conflicted by homoplasy. Furthermore, with the exception of some focal genera in Bertani (2001), neither study extensively sampled multiple species for most of the genera, making it impossible to test their monophyly. Pérez-Miles (2000) increased taxon sampling in his revised morphological analysis by the addition of a newly described genus (since renamed as *Bumba*), but results remained largely as in previous data. Overall, taxon sampling in Pérez-Miles et al. (1996) and Pérez-Miles (2000) was considerably less than in Bertani (2001) who focused only on specific South American lineages but demonstrated that a more thorough taxon sampling can provide improved phylogenetic signal between genera. Together, their studies highlight several important areas of congruence regarding generic relationships, with some similar groupings (i.e., the affinity of *Acanthoscurria*, *Phormictopus*, and *Cyrtopholis* (see Fig. 1). Recently, further studies have investigated smaller sub-groups of genera in the context of phylogeny, e.g., Perafán and Pérez-Miles (2014) who re-evaluated *Euathlus* in the context of other supposedly allied South American genera, again based on morphology alone.

Yet elsewhere, molecular data have provided critical information towards resolving the various mygalomorph groups' evolutionary relationships (Arnedo & Ferrández, 2007; Ayoub et al., 2007; Bond et al., 2012; 2014; Garrison et al., 2016; Hamilton et al., 2011; 2014; 2016a; Hamilton, Lemmon, Lemmon, & Bond, 2016b; Hedin & Bond, 2006; Hendrixson & Bond, 2005; Hendrixson et al., 2013; 2015; Kornillios, Thanou, Kapli, Parmakelis, & Chatzaki, 2016; Leavitt, Starrett, Westphal, & Hedin, 2015; Longhorn et al., 2007; Machkour-M'Rabet et al., 2009; Machkour-M'Rabet, Hénaut, Calmé, & Legal, 2012; Mendoza & Francke, 2017; Montes de Oca et al., 2016; Mora, Paspati, Decae, & Arnedo, 2017; Ortiz & Francke, 2015; 2016; Opatova & Arnedo, 2014; Opatova, Bond, & Arnedo, 2016; Petersen et al., 2007; Xu et al., 2015; 2016). Unfortunately, until now we have lacked any such broad molecular phylogenetic studies across the most speciose mygalomorph family Theraphosidae, nor the majority of its subdivisions, leaving significant uncertainty regarding their higher classification. Yet, a growing number of studies clearly demonstrate how molecular markers are useful to assess the evolutionary distinctiveness of theraphosid populations, finding rampant species crypsis and paraphyly, resulting in the designation of novel species and revisions of wider relationships (e.g., Hamilton et al., 2011; 2014; 2016a; Hendrixson et al., 2013; 2015; Montes de Oca et al., 2016; Mendoza & Francke, 2017; Ortiz & Francke, 2015; 2016).

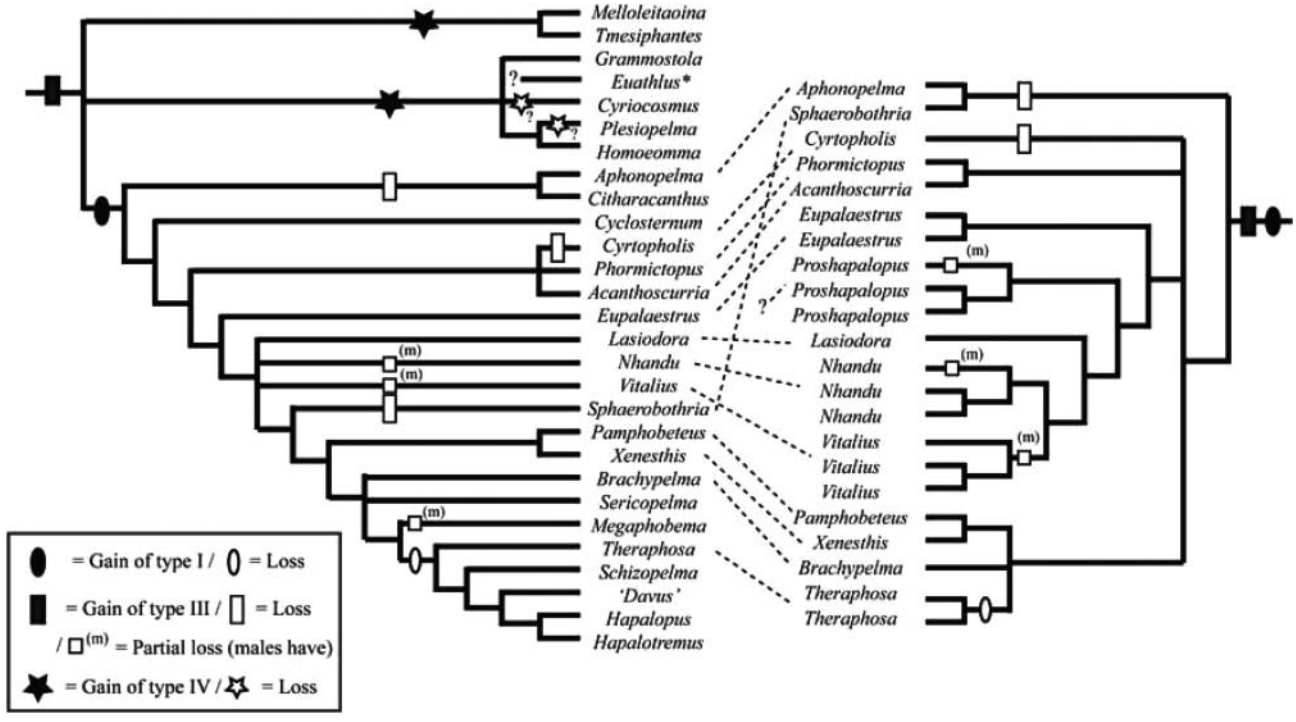

Fig. 1. Previous phylogenetic hypotheses of relationships amongst select Theraphosinae genera based on morphology; (left) redrawn from Pérez-Miles et al. (1996); and (right) from Bertani (2001) [with some genera condensed]. Note: *Euathlus** is labelled in source as tree of Pérez-Miles et al. (1996) as *Paraphysa*, since synonymized, along with other nomenclatural edits such as '*Davus*' from *Metriopelma*. Gains or losses of urticating hair types are mapped as per the key.

Herein we use a 1009 base pair (bp) fragment of the *16S rRNA-tRNA (leu)-ND1* mitochondrial region, frequently used as a taxonomic 'barcode' in spiders, to build a gene tree for the inference of relationships within the tarantula family Theraphosidae, initiate a revision of the Theraphosinae subfamily and redefine the taxonomic limits and evolutionary relationships between some genera and species by using a broad taxon sampling. This initial molecular framework will be vital to focus further in-depth studies on redefined lineages, such as those of conservation importance. Specifically, we re-evaluate the distinctiveness of lineages with great conservation concern, particularly the genus *Brachypelma*, and clarify relationships with other New World taxa. Additionally, we focused on re-evaluating the broader phylogenetic context for those currently placed in the most speciose genus, *Aphonopelma*. And finally, we provide recommendations for conservation priorities based on our findings.

Materials and methods

Sampling of tissue from preserved and live specimens

Tissue for DNA was sampled from both ethanol-preserved museum specimens (EtOH) and live specimens. The sampled museum specimens are deposited in the collections of the Oxford University Museum of Natural History, UK (OUMNH); the Facultad de Ciencias, Uruguay (FCY-MY); Auburn University Museum of Natural History, Alabama USA (AUMNH); and the Natural History Museum, London UK (NHM) [Table S1, see online supplemental material, which is available from the article's Taylor & Francis Online page at https://doi.org/10.1080/14772000.2017.1346719]. Additional alcohol-preserved specimens are in the private collection of S. Longhorn, UK (SJLC) pending museum deposition; predominately comprising specimens that were alive at the time of DNA sampling (see below). Several of the specimens used in this study were obtained through the pet-trade, often by generous donations from interested hobbyists or trader-breeders. Muscle tissue was extracted from alcohol-preserved vouchers after making an incision along the femur of the third leg. Care was taken to prevent damage to potentially informative morphological characters. Muscle tissue was extracted using sterilized forceps and placed into absolute ethanol and frozen at −20°C until extraction, when ∼50 mg of air-dried muscle was placed into extraction buffer. Extra tissue was sampled for future reference wherever possible. To obtain the best quality DNA, several live specimens were also sampled for tissue by the non-lethal method of Longhorn (2002) via induced autospasy of leg III – the breaking at the coxa-trochanter joint, a natural response for predator evasion (after Breene, 1998) – and now common in the sampling of theraphosids for molecular investigations. Each isolated leg was stored in absolute ethanol and frozen at −20° or −80°C until use. Tissue was extracted from the fresh isolated legs as with museum material. A novel method of non-lethal sampling was also trialled on four individuals via extraction of haemolymph from the leg III femur of a restrained spider using a sterile syringe. Between one and three drops of haemolymph were placed directly into EDTA buffer solution. In these cases no extra stock of 'tissue' was available for long-term preservation, but subsequently after death each of these animals was sampled for muscle tissue as per museum specimens. All spiders sampled by use of non-lethal techniques (either leg autotomy or direct haemolymph extraction) appeared to undergo very little stress. All specimens survived the respective procedures (until preserved for vouchers). Extraction of haemolymph was performed by SJL and SPT in the NHM, London.

Extraction, amplification, and sequencing

DNA was extracted from the majority of tissue samples using a Qiagen DNeasy Tissue Kit™ (Qiagen, Valencia, CA) in a vacuum manifold. The *16S rRNA-tRNA (leu)-ND1* region (1009 bp) of the mitochondrial genome was then amplified from the majority of samples in 96 well format. The PCR mix used reagents ($MgCl_2$, buffer and Taq polymerase) from the GOTAQ kit (Promega) and Bioline dNTP kit, plus 'universal' primers 16Sar LR-N-13398 (CGCCTGTTTAACAAAAACAT) and N1-J-12261 (TCRTAAGAAATTATTTGA). Each master mix consisted of 15.2 ul ddH20, 5ul of buffer, 2.5 ul MgCl2, 0.5 ul of each primer, 0.2 ul of dNTPs and 0.1 ul of DNA polymerase, per 25 ul DNA sample. The PCR Profile used an initial denaturing step of 94°C for 1 minute, 39 cycles of denaturing 94°C for 1.5 minutes, annealing 52°C for 45 seconds, and extension at 72°C for 1 minute. Final extension was 10 minutes at 72°C. PCR products were sequenced on ABI3700 Sanger sequencers at various host institutions. For a subset of taxa [indicated in the supplement as 'PARTINT' and deposited in the FCE-MY], a shorter internal region (of about 500 bp) was generated under similar conditions with the primers LR-J-12864 and LR-N-13398, which were sequenced at The University of The Republic of Uruguay.

Alignment, phylogenetic analyses, and hypothesis testing

Sequences were edited using Sequencher® Vs. 5.2. (Gene Codes Corp). Forward and reverse primed sequences were assembled into contigs and manually edited to improve quality or remove ambiguity, and sections matching primers were removed. The corresponding sequence from

Table 1. Likelihood Based Hypothesis Tests Performed in CONSEL.

Tree Constraint	-ln L	Diff –ln L (From best Tree)	AU-test P	SH-test P
Best ML tree	38748.46855	Best Tree		
Brachypelma monophyletic	38865.41869	116.95014	< 0.001	<0.001
Aphonopelma monophyletic	39036.58924	288.12069	< 0.001	< 0.001
Aphonopelma 'group1+group 2' monophyletic	38854.43113	105.96258	< 0.001	0.002
Aphonopelma 'group 1 + 3' monophyletic	38830.49622	82.02766	< 0.001	0.015
Aphonopelma 'group2+3' monophyletic	38964.71655	216.24800	< 0.001	< 0.001

the complete mitochondrial genome of the nemesiid, *Calisoga aff. longitarsus* (Simon, 1891), was used as outgroup to determine the root of the wider Theraphosidae (Masta, Longhorn, & Boore, 2009). Representatives of other theraphosid subfamilies were then used as further outgroups to determine the monophyly of Theraphosinae. New data were combined with published sequences for North American *Aphonopelma* from Hamilton et al. (2011) (Supplementary Figs, see supplemental material online). All sequences were aligned using the automated sequence alignment software package MUSCLE (Edgar, 2004). Mesquite version 2.75 (Maddison & Maddison, 2011) was then used to manually check and adjust sequences, to optimize putative homology of aligned bases. The alignment is available on request from authors. Phylogenetic analyses were performed in both Bayesian and Maximum Likelihood frameworks. Prior to tree searches, the most appropriate model of evolution was evaluated in ModelTest 3.7 (Posada & Crandell, 1998), which indicated the General Time Reversible was the best fitting model with Gamma rate variation and Invariant sites (GTR+Γ+I). Partitioned Bayesian analyses were conducted using MrBayes (Huelsenbeck & Ronquist, 2001) firstly for the total data (all sites), with the alignment partitioned into RNA for *16S* plus *tRNA* (*leu*) and another for the protein-coding *ND1* sub-divided by codon position (1st + 2nd and 3rd). Four MCMC chains were run for 50 million generations, sampling every 2000. A second analysis was run excluding 3rd codon positions of *ND1*, i.e., sites where an elevated substitution rate can masque phylogenetic signal. For each completed Bayesian run, output files were checked to ensure for stationarity (i.e., split frequency of 0.01 or lower). A large burnin value was set to exclude the first 25 million generations to avoid suboptimal tree samples before the chains had reached stationarity. Posterior probabilities were taken as measures of clade support. The program Tracer (Rambaut, Suchard, Xie, & Drummond, 2014) was used to visualize the parameter means from the MCMC analysis. Maximum likelihood was performed using RAxML Vers 7.2 (Stamatakis, 2006) with the same data partitions as Bayesian analysis. Two analyses were performed using the GTR GAMMA model, the first again for the total dataset, the second excluding 3rd positions of *ND1*. We ran 100 ML tree searches for each dataset using random addition sequence. 1000 bootstrap replicates were resampled simultaneously with the best tree search to evaluate nodal support. On the best-scoring ML tree (from all data, i.e., including 3rd sites of *ND1*), constraint analyses were then implemented using the programs CONSEL (Shimodaira & Hasegawa, 2001) and PAUP* (Swofford, 2003) to re-evaluate the monophyly of our two focal genera *Aphonopelma* and *Brachypelma.* Here, we employed Approximately Unbiased (AU) and Shimodaira–Hasegawa (SH) tests to assess six constraint trees with alternate hypotheses against our best ML estimate (Table 1), evaluating differences in statistical significance (using P values) of –ln Log likelihoods. To further evaluate non-monophyly of *Aphonopelma* and *Brachypelma* in a Bayesian context, we took all 11,000-post burn-in trees and filtered them per our monophyly constraints, and the percentage of trees matching the monophyly constraint was used to assess our alternate hypotheses about these two focal genera. All phylogenetic analyses were repeated using the GTRCAT model to compare against the GTR GAMMA analyses.

Results

For ML and Bayesian analyses, the resolution of our mtDNA gene trees obtained using the GTR GAMMA model out-performed those conducted using GTR CAT. The reason for this is probably the low complexity of this single fragment dataset, which consists of relatively few phylogenetically informative molecular characters. Thus, we discuss only the findings of the GTR GAMMA analyses. Bayesian analyses of all codon positions achieved stationarity after 17 million generations, when split frequencies reached a minimum value of 0.0091. Analysis excluding 3rd codon positions of *ND1*, under the same starting parameters, reached stationarity after 12 million generations with a split frequency of 0.0093. A total of 11,000 post burn-in trees were sampled during both analyses. For ML, the optimal likelihood score analysing all codon positions was −31996.735769; after removing the 3rd positions, the best scoring tree was −35811.576315 (see Supplemental figures for detailed results within Theraphosidae). Removal of the 3rd positions generally

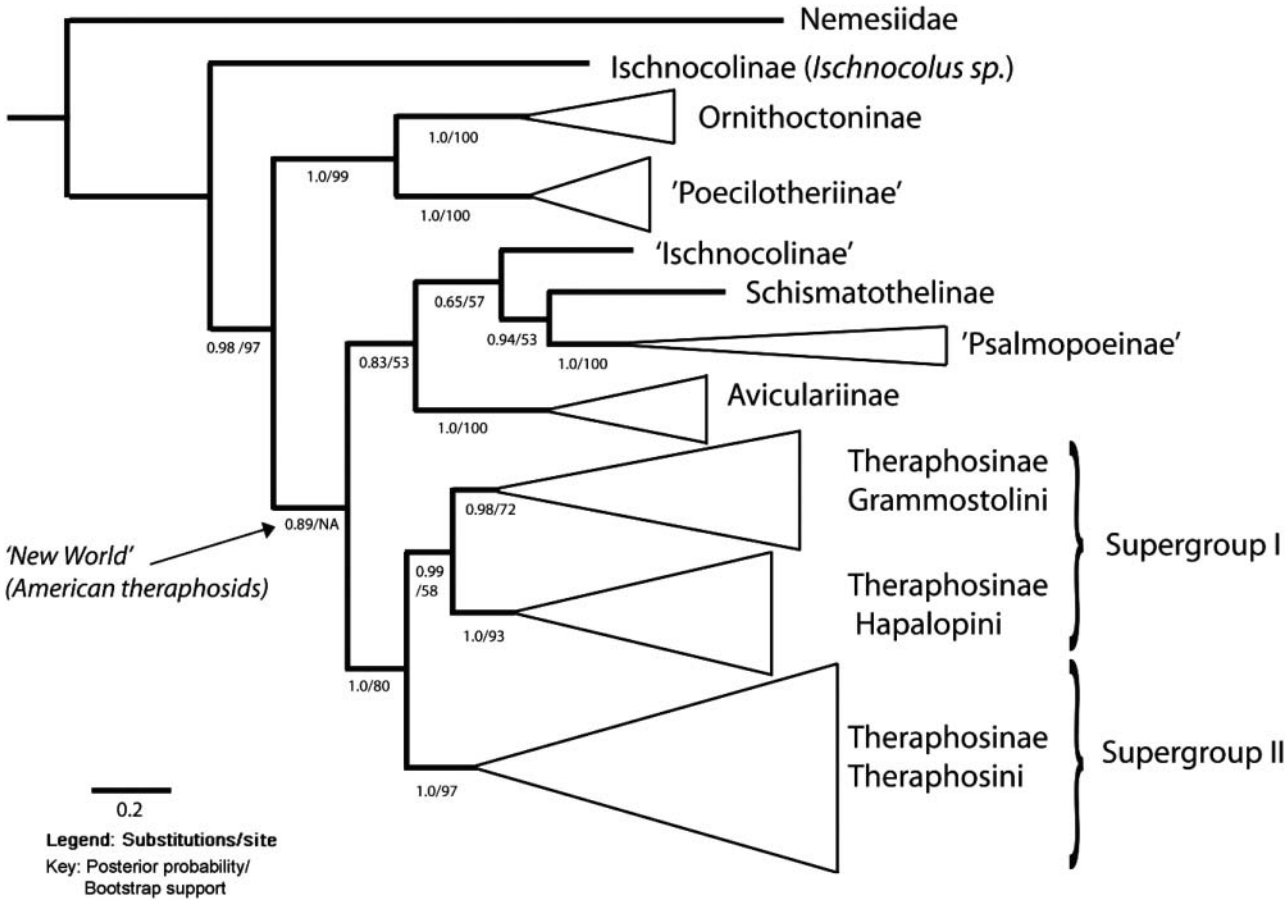

Fig. 2. Broadest phylogenetic relationships across the family Theraphosidae from this study from all included positions, showing separation of sampled subfamilies, and our newly proposed tribal subdivisions of the New-World subfamily Theraphosinae. Nodal support shows posterior probability/maximum likelihood bootstrap. Scale = substitution/nucleotide.

resulted in a less resolved tree structure (particularly in ML), and generally lower nodal support. Due to better tree structure and nodal support values, we hereafter rely primarily on trees inferred from all codon positions to further discuss evolutionary relationships within the Theraphosinae (Figs 2–5).

In all analyses the Old World Ischnocolinae [*sensu stricto* (*s.s.*), represented by *Ischnocolus* sp.], is recovered as sister to all other sampled theraphosid lineages (Fig. 2), when rooted with the outgroup family Nemesiidae. The remaining theraphosid subfamilies form a well-supported monophyletic group (PP = 0.98, BS = 97). Old World genera from the 'Poecilotheriinae' + Ornithoctoninae are recovered as sister to a weakly supported node (PP = 0.89, BS < 50) for all New World representatives, comprised of the Avicullariinae, Psalmopoeinae, 'New World' Ischnocolinae/Schismatothelinae, and our focal subfamily – the Theraphosinae. All other sampled 'New World' lineages form a weakly supported group (PP = 0.83, BS = 53) sister to Theraphosinae. The focal subfamily Theraphosinae is recovered as monophyletic with strong support (PP = 1.0, BS = 80). Bayesian and ML analyses all indicate deep divergence within this subfamily between two newly defined supergroups (Fig. 2), both of which are well-supported (Supergroup I – PP = 0.99, BS = 58; Supergroup II – PP = 1.0, BS = 97). Within Supergroup I there is a further deep subdivision (Fig. 3), with one branch leading to a strongly supported group (PP = 1.0, BS = 93), which we term as Hapalopini trib. nov. for many genera of 'dwarf tarantulas' from Central and Southern America such as *Cyriocosmus*, *Davus*, and *Hapalopus*, plus the 'non-dwarf' genus *Thrixopelma.* Several of these genera have previously been recovered together in cladistic morphology analyses (Pérez-Miles et al., 1996; Pérez-Miles, 2000). A second group, which we term as Grammostolini trib. nov., is comprised of a less well-supported collection of South American genera (PP = 0.98, BS = 72) including *Grammostola*, *Euathlus*, and others, with Hapalopini as their sister lineage. Perafán and Pérez-Miles (2014) similarly found a close relationship between *Phrixotrichus* and *Euathlus,* but instead placed *Grammostola* closer to the Hapalopini (as defined

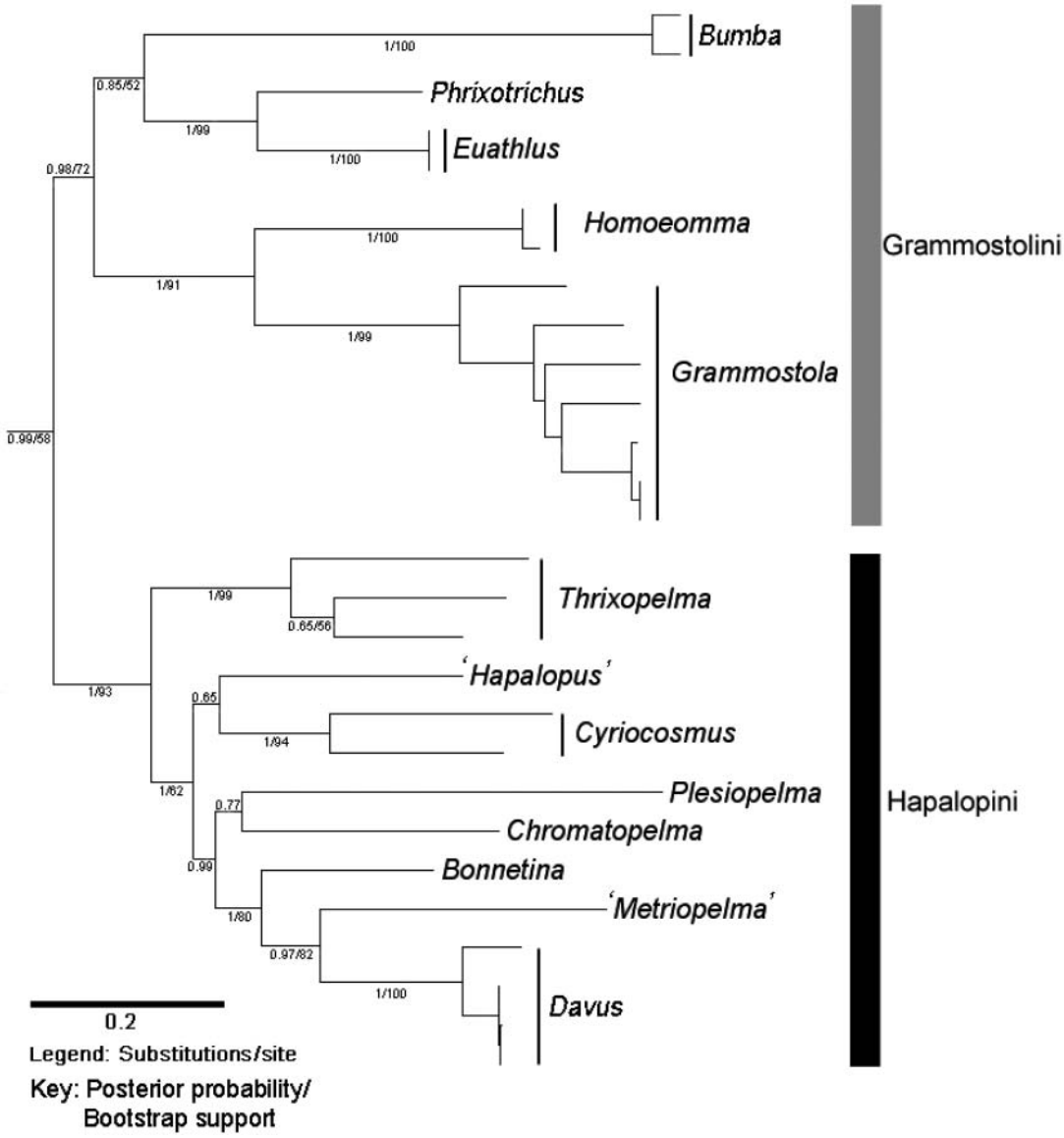

Fig. 3. Phylogenetic relationships of sampled genera within newly proposed tribes Grammostolini and Hapalopini from molecular analysis of all positions of 16S/ND1. Note – the taxon '*Metriopelma*' is from *Davus ruficeps* (formerly called *Metriopelma zebratum*), a revised species that was recently transferred to the genus *Davus* based on morphology (Gabriel, 2016). The identity of the generic specimen here labelled '*Hapalopus*' is also ambiguous, and may perhaps be assigned to *Catanduba.* Nodal support shows maximum likelihood bootstrap/posterior probability.

here) rather than *Homoeomma* – as is strongly supported with these molecular data (PP = 0.91, BS = 100). Notably, although our molecular results were largely congruent in recovered nodes across different analyses, the most unstable node concerned the placement of *Bumba*, which varied in its position, jumping from either sister to *Phrixotrichus* plus *Euathlus* and weakly supported (PP = 0.85, BS = 52), or sister to all other lineages of the Grammostolini (in Bayesian analysis excluding 3rd positions). Overall, most of our recovered relationships within Supergroup I were generally well supported, however, judging from low node support, the placement of *Plesiopelma* and *Chromatopelma* within Hapalopini also appears unstable. Such uncertainties may be resolved by the addition of further molecular data, both from number of nucleotides as well as other unsampled, but morphologically similar, genera (e.g., see Perafán et al., 2015), and marked with an asterisk in the taxonomy section below). The remainder Theraphosinae comprise Supergroup II (Fig. 4), defined here as Theraphosini trib. nov. However, deeper (supra generic) relationships amongst several genera in this newly defined tribe remain uncertain due to

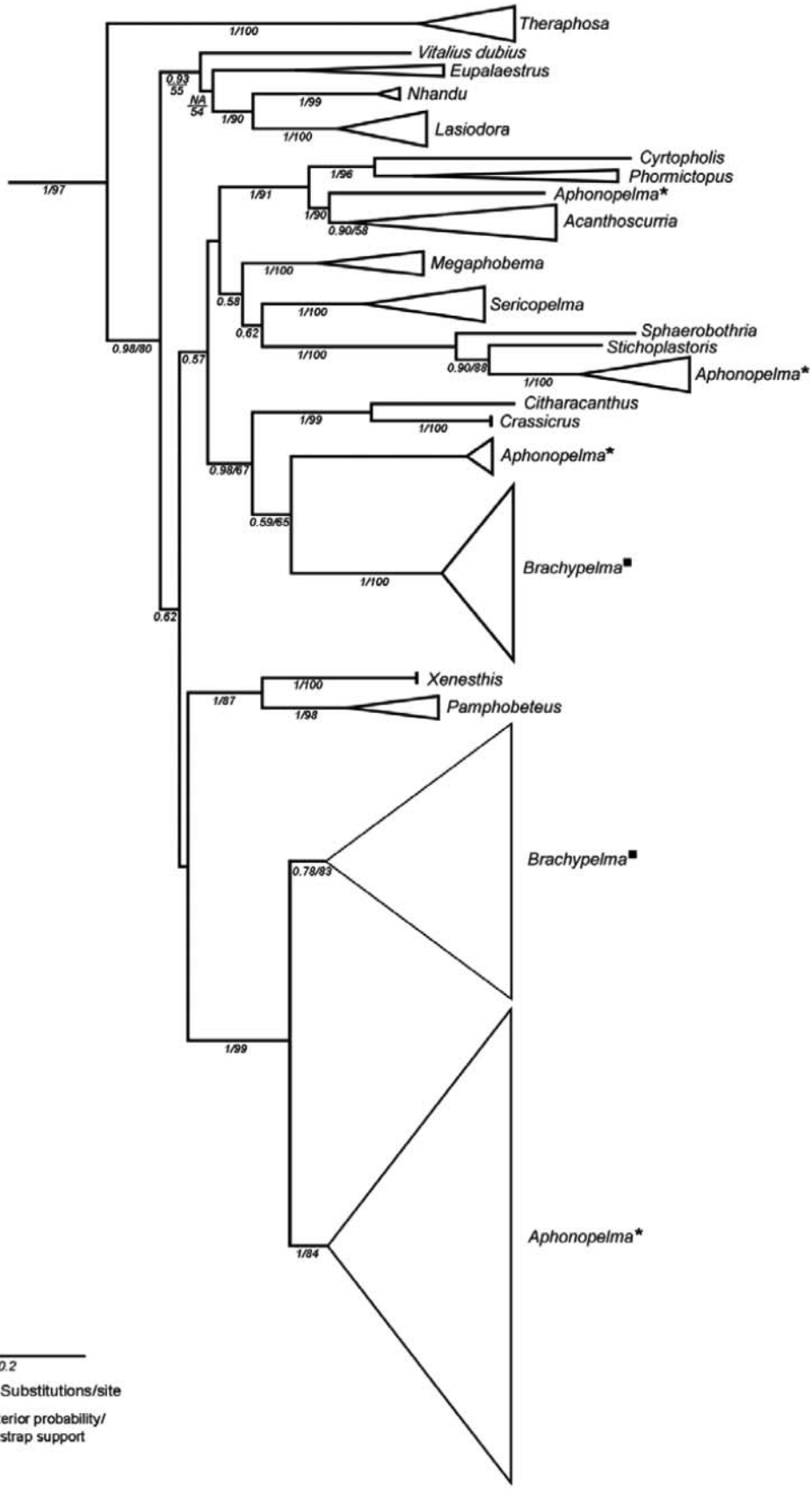

Fig. 4. Phylogenetic relationships amongst genera within proposed tribe Theraphosini from molecular analysis of all positions of 16S/ND1. Nodal support shows maximum likelihood bootstrap/ posterior probability. *denotes *Aphonopelma*, ■denotes *Brachypelma*.

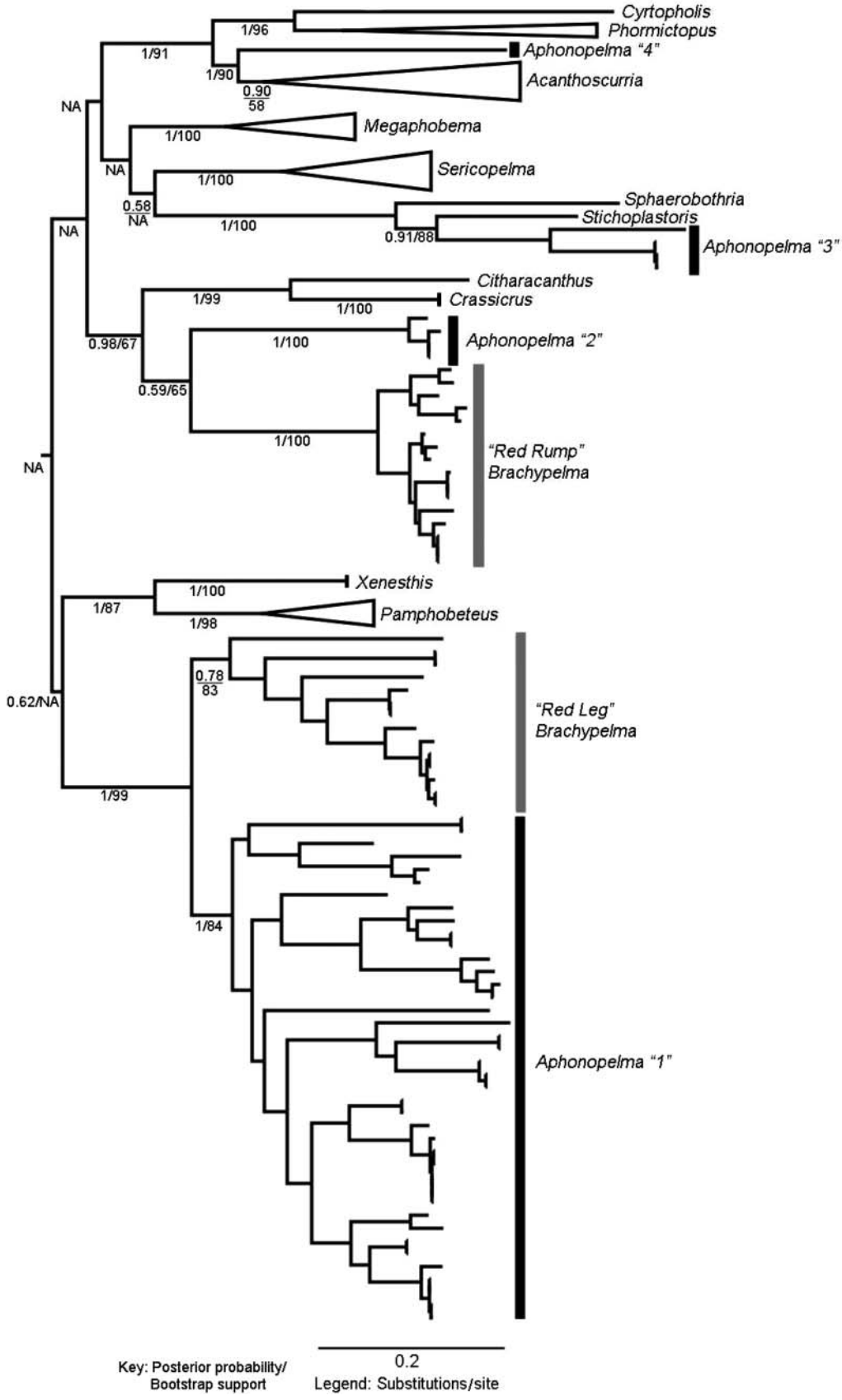

Fig. 5. Reduced sampling of Theraphosini from Bayesian analyses (all positions), highlighting the non-monophyly of *Brachypelma* and *Aphonopelma* and the relative position of type species, plus select taxa of specific interest. Nodal support shows maximum likelihood bootstrap/ posterior probability for each suggested genus and higher groups, but not shown for nodes within genera (for nodal supports and terminal taxa names, see Supplementary Figure 2C).

unresolved polytomies, weakly supported nodes, or conflict between our analyses. Despite this, we recovered several well-supported relationships between several genera, and novel findings for *Aphonopelma* and *Brachypelma* of particular relevance to conservation. Within Supergroup II (= Theraphosini), the genus *Theraphosa* was recovered as sister to all other taxa (PP = 0.98, BS = 80). The South American genera *Eupalestrus*, *Lasiodora*, *Nhandu*, and *Vitalius* were recovered together (PP = 0.93, BS = 55), concordant with morphological analyses (i.e., Pérez-Miles et al., 1996; Pérez-Miles, 2000; Bertani, 2001). A well-supported group of Caribbean genera *Phormictopus* and *Cyrtopholis* was also recovered (PP = 1.0, BS = 96), together with the South American/Caribbean *Acanthoscurria* (PP = 1.0, BS = 91), again in agreement with morphological studies. However, surprisingly, *Acanthoscurria* also groups (PP = 1.0, BS = 90) with nominal *Aphonopelma* from Central Mexico – a unique lineage we hereafter refer to as *Aphonopelma* 'group 4'. Elsewhere, the Central American *Sericopelma* and South American *Megaphobema* together form a weakly supported group affiliated with three other Central American genera (PP = 0.58, BS = <0.50) which comprises of *Sphaerobothria*, *Stichoplastoris*, and *Aphonopelma* (hereafter 'group 3') together strongly supported (PP = 1.0, BS = 100). The latter importantly includes the type species *Aphonopelma seemanni* (F.O. Pickard-Cambridge, 1897), which defines the genus *Aphonopelma* (*sensu stricto*).

Another notable distinct evolutionary lineage was recovered for several Mexican and Central American genera, including several *Brachypelma* species (hereafter 'Red Rump' due to predominance of taxa having long red abdominal setae) with *Crassicrus*, *Citharacanthus* plus *Aphonopelma* (hereafter 'group 2'), together forming a moderately well supported group (PP = 0.98, BS = 67). Both 'Red Rump' *Brachypelma* and *Aphonopelma* 'group 2' are each strongly supported monophyletic lineages (each with PP = 1.0, BS = 100), together forming a less well-supported group (PP = 0.59, BS = 65). Elsewhere, two South American genera (*Xenesthis* and *Pamphobeteus*) group with the remaining *Brachypelma* plus the North American *Aphonopelma* (hereafter 'group 1') (PP = 0.62; BS = <50). This may reflect support for an affinity between these *Brachypelma* with *Xenesthis* + *Pamphobeteus* as seen in some morphological trees (e.g., Bertani, 2001). In our data, *Xenesthis* and *Pamphobeteus* are strongly supported as sister taxa (PP = 1.0, BS = 87), a finding consistent with morphology. Most importantly, there was very strong nodal support (PP = 1.0, BS = 99) for a novel sister group comprising remaining *Brachypelma* and *Aphonopelma* 'group 1'. This distinct group of 'Red Leg' *Brachypelma* (= *Brachypelma sensu stricto*) includes the type species *B. emilia* (White 1856). This group was only moderately supported (PP = 0.78. BS = 83), perhaps due to some uncertainty about which species is sister to all others, though the grouping as a whole appears robust as the next internal node was extremely well supported (not shown, but PP = 1.00, BS = 99). The North American *Aphonopelma* 'group 1' was strongly supported as a distinct lineage (PP = 1.00, BS = 84), as well as strong support for several internal nodes (not shown).

The non-monophyly of *Brachypelma* and *Aphonopelma* is strongly supported in both Bayesian and ML analyses of our data, and remained as the preferred interpretation after re-evaluation of the topologies through hypothesis testing in a likelihood framework. In pairwise comparisons against multiple constraint trees – with monophyly of either or both genera enforced, our best ML topology scored significantly better than all given alternates (Table 1). These results, coupled with the strong support (of both our Bayesian and ML analyses) at these key nodes of interest indicate that neither *Brachypelma* nor *Aphonopelma*, as currently recognized, are monophyletic (Fig. 3, and expanded in Fig. 4). Further evaluation of the Bayesian analyses, by filtering post burn-in topologies, did not yield a single tree that matched any alternate candidate hypotheses of monophyly (Table 1) in any sub-optimal topologies. This again clearly indicates support for non-monophyly of the various lineages of *Aphonopelma* and *Brachypelma*, and that their recovered sub-lineages are deeply divergent.

Discussion

Broadest groupings and sister group of the Theraphosinae

We rooted our mtDNA trees with the nemesiid *Calisoga*, and due to its taxonomic distance, the relative affinities of the other theraphosid subfamilies recovered here should be interpreted with caution. The taxa of focus for this study, subfamily Theraphosinae, are instead polarized and determined to be monophyletic by the inclusion of several other theraphosid subfamilies. Additional mygalomorph samples are needed to ascertain the monophyly of the wider Theraphosidae and its sister-group/s, as other studies suggest that the family Barychelidae is perhaps the closest ally of theraphosids (after Bond et al., 2012, 2014; Garrison et al., 2016; Goloboff, 1993; Raven, 1985), and both families may be mutually polyphyletic. Within the family Theraphosidae, uncertainty remains regarding relationships between subfamilies due to limited sampling of genes and phylogenetically informative characters, limited taxon sampling from Old World theraphosids, and few New World 'Ischnocolinae'. Several unsampled lineages remain as plausible sister-groups to the Theraphosinae. Based on morphology, the included arboreal South American Aviculariinae (if excluding Psalmopoeinae) has been proposed as the preferred sister group to the

Theraphosinae, with both subfamilies sharing the presence of abdominal urticating hairs (Type II in Aviculariinae) and defensive display with abdominal movements as synapomorphies (see Pérez-Miles et al., 1996). Our new molecular data (Fig. 2) instead favour the Aviculariinae (excluding Psalmopoeinae) together with New World 'Ischnocolinae', Schismatothelinae + Psalmopoeinae as a novel group, and this whole assemblage as sister to the Theraphosinae. However, nodes relating to the placement of these subfamilies were weakly supported, and Aviculariinae (excluding Psalmopoeinae) may remain as the preferred sister-group of Theraphosinae. Uncertainty about subfamilial relationships is compounded by the lack of sampling of various African theraphosids, and probably requires more complete sampling of New World 'Ischnocolinae' (i.e., as per Guadanucci, 2014). Overall, as with many taxonomic questions, better resolution of deeper-level evolutionary divergences within Theraphosidae, and inter-relationships between subfamilies, will need additional molecular data and more complete taxon sampling, ideally as part of integrative revision.

Relationships within Theraphosinae and classification issues

Our molecular data show deep splits within the Theraphosinae, dividing the subfamily into three newly proposed tribes within two supergroups (see Figs 2 & 3). Some aspects of morphology that appear useful to diagnose these three tribes are given in the nomenclatural section below. In particular, certain types of urticating hairs seem especially congruent with our inferred groupings – for example, they can be used to aid in defining a tribal lineage Grammostolini (with *Grammostola* as the type genus) from several genera that ancestrally possess both type III and type IV urticating hairs. These characters, and other outlined features, also allow us to predict inclusion of several unsampled genera into our proposed tribes based on morphological considerations (see summary of nomenclatural changes below). The second major lineage we define as tribe Hapalopini is considered to contain multiple genera with only type III urticating hairs ancestrally (with *Hapalopus* as the type genus – see again summary of nomenclatural changes for additional genera). If we consider the genera in the new Hapalopini for re-rooting the morphological trees in Perafán and Pérez-Miles (2014) (specifically as *Cyriocosmus* and *Plesiopelma*), this would alter their recovered topology for Grammostolini to be (*Grammostola*, (*Phrixotrichus, Euathlus*), (*Homoeomma, Bumba*)), which is congruent with our data except for the position of *Homoeomma*, which we find closer to *Grammostola*. The other major lineage, here defined as the tribe Theraphosini (with *Theraphosa* as the type genus), consists of the remaining genera that we propose ancestrally possess type I urticating hairs (and also type III that appear to often be secondarily lost). Our data support *Theraphosa* as the sister genus to all others in this tribe (see Fig. 4). Because notably *Theraphosa* lacks type I urticating hairs (but are present in all other Theraphosini), these could have evolved after its split from other genera within this tribe – if the basal branching indicated by our mtDNA data is correct. Alternatively, a more derived placement of *Theraphosa* would be more concordant with morphology (compare Fig. 1), then requiring a unique secondary loss of type I hairs within this new tribe.

Our three newly proposed tribes of Theraphosinae are also largely congruent with distinct geographic regions. Members of Grammostolini are found only in South America, predominantly in more temperate regions of southern countries such as Chile, Argentina and Uruguay, but also ranging into Southern Brazil, Paraguay and Bolivia (Pérez-Miles et al., 1996; Perafán & Pérez-Miles, 2014). Perhaps at their northern limit is the Amazonian genus *Bumba* (Pérez-Miles, 2000). The Hapalopini occur across much of South America with *Cyriocosmus* and *Hapalopus* both widespread, such as through the Amazonian and into the Andean regions, where *Thrixopelma* occur. At the northern limits, *Davus* extends throughout Central America and into Mexico where *Bonnetina*, *Schizopelma* (and several other small allied genera currently being revised) are found (Gabriel, 2016; Ortiz & Francke, 2015, 2016). Members of the third tribe Theraphosini also have many South American representatives, mostly in more tropical regions, but also throughout Central America and the Caribbean, and then dominate in North America from across Mexico into the southern United States (Hamilton, Hendrixson, & Bond, 2016; Pérez-Miles et al., 1996; West, 2005; Yáñez et al., 2000). The phylogeographic patterns of these three tribes and their sub-lineages reflect the limited dispersal ability of theraphosid spiders, apparently strongly influenced by the complex historical biogeography of the New World, such as the presence of a Central American land-bridge, the uplift of the Andes mountains (e.g., perhaps splitting *Thrixopelma* from remaining Hapalopini), the Trans-Mexican volcanic belt, glacial retreat, and ongoing influence of eustatic changes in sea levels (Cody, Richardson, Rull, Ellis, & Pennington, 2010; Mastretta-Yanes, Moreno-Letelier, Piñero, Jorgensen, & Emerson, 2015; Wegner, Wörner, Harmon, & Jicha, 2011). The large degree of inferred genetic divergence between the two supergroups of Theraphosinae indicates an ancient split, as seen in their relatively long branch lengths (Fig. 2).

Non-monophyly of *Brachypelma* and *Aphonopelma*

Aphonopelma Pocock, 1901 and *Brachypelma* Simon 1891 were each recovered as polyphyletic in this study,

the first molecular systematic study where both genera have been extensively sampled. Previous morphological studies (Bertani, 2001; Pérez-Miles et al., 1996; Pérez-Miles, 2000) only sampled a single exemplar from each genus, making it impossible to assess their evolutionary history and reciprocal monophyly. Our recovered mtDNA gene tree and monophyly tests reveal that *Aphonopelma* forms at least four distinct groups, namely '1', '2', '3', and '4', and *Brachypelma* forms two divergent groups, namely 'Red Leg' and 'Red Rump' (Table 1, Figs 4 & 5).

For *Brachypelma*, the existence of two discrete and divergent lineages was not recognized in the initial published study for the genus using data from the Cytochrome oxidase 1 (*CO1*) gene (Petersen et al., 2007). In their results, two subgroups corresponding to our 'Red Rump' and 'Red Leg' (= *sensu stricto*) groups were recovered, but were simply (mis)-grouped together because no other allied genera were compared. Recently, Mendoza & Francke (2017) have conducted further molecular phylogenetic analyses with additional *CO1* data to better delineate species of *Brachypelma* alongside revised nomenclature, but only include a selection of species from the 'Red Leg' subgroup, leaving the monophyly of the genus unquestioned. However, phylogenetic analysis of other unpublished genetic data from *CO1* (Longhorn, 2001) with additional genera shows a similar division of *Brachypelma* into two distinct lineages as for our presented data, giving us increased confidence in the results presented here. The placement of *B. emilia* (White 1856) as the type species for *Brachypelma* is especially critical, which our molecular data confirm within the 'Red Leg' grouping. We therefore propose retention of the genus name *Brachypelma* for the 'Red Leg' lineage redefined here as *Brachypelma sensu stricto* (which includes *B. emilia*). In contrast, we suggest the other 'Red Rump' group must be recognized as a distinct evolutionary lineage, which we propose warrants a separate genus. However, further redefinition of this latter group requires careful morphological treatment alongside other data sources, which is beyond the scope of this study.

The newly inferred diphyly of *Brachypelma* here has immediate implications for conservation. Some wild populations of *Brachypelma* are threatened by habitat destruction, with losses exacerbated by illegal collection for the pet-trade (Petersen et al., 2007; West, 2005). Consequently, the genus is blanket protected under CITES (Appendix II, see supplemental material online) due to the alleged threats from the exotic pet trade. All spiders included in this genus, until now, have been treated as having similar life history traits that make them prone to extinction, slow growth to sexual maturity and a natural high juvenile mortality rate (Locht et al., 1999; Hénaut, Machkour-M-Rabet, Weissenberger, & Rojo, 2015). We suggest if ecological characteristics such as reproductive success and vulnerability to habitat loss are reconsidered, the 'Red Leg' lineage (i.e., *Brachypelma sensu stricto*) must become even higher conservation priorities than present, while status of the remaining 'Red Rump' taxa reconsidered, perhaps even downgraded. The majority of 'Red Leg' *Brachypelma* are restricted to narrow endemic ranges, mostly along the Pacific coast of Mexico – areas that are often increasingly subject to human development. This leaves several species vulnerable to habitat loss (Petersen et al., 2007; West, 2005), which is recently highlighted in re-assessment by the IUCN Red List, which now moves to propose several 'Red Leg' species as Vulnerable (VU) or Endangered (EN). The 'Red Leg' group also includes the red-knee tarantula *B. smithi*, which has long been cited as a flagship for invertebrate conservation, but has long remained in urgent need of both taxonomic and ecological re-assessment (see Mendoza & Francke, 2017). The close inferred relationship of the North American/Mexican *Aphonopelma* 'group 1' and these Pacific Mexican 'Red Leg' *Brachypelma* (*s.s.*) shown by our data indicates a common evolutionary history. This is also reflected in similar life histories such as relatively slow growth and long time to reach reproductive maturity. In the USA, commercial collection of large numbers of native *Aphonopelma* 'group 1' for the pet trade is becoming increasingly commonplace. As a result, there is urgent need of conservation/impact assessment of targeted species within the USA to quantify resilience to collection, in particular those restricted to the 'sky islands' region of South-eastern Arizona and South-western New Mexico. Here we advocate that several species with the North American *Aphonopelma* 'group 1' should also become an urgent priority for conservation initiatives within the United States and Mexico, based on their close evolutionary history and shared ecological similarities to the vulnerable or endangered Mexican 'Red Leg' *Brachypelma*. Finally, given that a large number of unsampled Mexican species are currently ascribed to '*Aphonopelma*' (i.e., *sensu lato*), it is important to consider that additional distinct evolutionary lineages associated with 'group 1' may be recovered once further taxon sampling is conducted.

The Central American 'Red Rump' *Brachypelma* appear closely allied with the genera *Crassicrus* and *Citharacanthus* (also noting the need for their further taxonomic revision), along with multiple samples of *Aphonopelma* 'group 2'. This latter lineage can be largely ascribed to the Costa Rican/Nicaraguan *Aphonopelma crinirufum* (Valerio, 1980) along with a misplaced El Salvadorian species. These appear most closely associated with the Central American 'Red Rump' *Brachypelma*, here represented by a broad sample of species including the supposedly wide-ranging *B. vagans* (Ausserer, 1875). However, due to the lack of availability of new samples and low number of existing specimens in molecular collections around the world for these taxa, many of our genetic samples were gathered from captive-bred stock

obtained through the commercial pet-trade, without exact provenance to the geographic origins. Most specimens within this group were closely related (i.e., little genetic divergence), and several putative species – most notably those allegedly ascribed to '*Brachypelma vagans*', were not monophyletic (Supplementary figs, see supplemental material online). Despite limited information on several sample origins, our data suggest the possibility of a relatively recent on-going radiation within the 'Red Rump' group, which may explain the lack of clear resolution. Additionally, the close affinity and low sequence divergence across sampled individuals may be due to mitochondrial introgression and incomplete lineage sorting, which have been shown to confound other tarantula molecular studies (Hamilton et al., 2016a). Although beyond the scope of this study, we highlight the 'Red Rump' lineage as a whole is in dire need of thorough taxonomic revision with all available tools, although these preliminary data suggest there will be significant challenges due to hybridization, introgression, etc. Additional molecular data from the nuclear genome may be vital to provide much needed information about their diversification, including estimates of both population and species-level divergence, ideally based on broad sampling of natural populations.

Caution must also be taken about our definitions of '*Aphonopelma*' (*sensu lato*), due to recovery of other evolutionary lineages outside of the North American 'group 1' in our analyses. Importantly, the 'group 3' lineage includes the type species *A. seemanni*, and hence can be redefined as *Aphonopelma sensu stricto*, which was found to be closely allied to *Stichoplastoris* and *Sphaerobothria*. Another noteworthy finding was that *A. seemanni* appears to be broken into two cryptic species. Genetic samples from multiple specimens from the Costa Rican type species formed a sister relationship with a divergent individual from Guatemala. Together, our results indicate that further taxonomic revision is also needed here and nomenclature revised (see below). The sister group of the wider grouping (*Sphaerobothria*, *Stichoplastoris* and *Aphonopelma s.s.* 'group 3'), however, is not yet clear with current data. These three genera occur in overlapping ranges with the candidate sister groups *Sericopelma* and *Megaphobema*, and additional molecular data and/or taxon sampling may provide clearer resolution relative to these or other genera. Finally, the recovery of a fourth isolated lineage of *Aphonopelma* 'group 4' (here represented by the species *A. anitahoffmannae* Locht et al. 1999) also can have important implications for the definition of *Aphonopelma.* We suggest the unique placement of this taxon in isolation from other supposed congeners here perhaps represents the historical genus *Dugesiella* Pocock, 1901, currently synonymized with *Aphonopelma* (in Raven, 1985), which these data may indicate requires revalidation.

Increased taxon sampling and additional molecular characters will probably reveal additional novel lineages and cryptic species throughout the subfamily Theraphosinae. Such data will be especially important to fully understand species limits and population dynamics of taxa with both wide ranges and variable geographic habitats, and could have more implications for conservation (Longhorn et al., 2007). Recent mtDNA work greatly helped understand the species limits of North American ('group 1') *Aphonopelma* (Hamilton et al., 2011; 2014; Hendrixson et al., 2013, 2015) leading to a much needed taxonomic revision (Hamilton et al., 2016a), but such in-depth studies remain rare for tarantula genera. Given the apparent need to redefine limits of several genera from the current classification, we also highlight the need for refocused protection with greater awareness of distinct evolutionary lineages. This is especially critical given that our understanding of the current status of many wild populations is often extremely limited. For Theraphosinae, we here highlight the need for taxonomic revision alongside future conservation initiatives, as suggested for other protected arthropods (e.g., Arnedo & Ferrández, 2007; Lin, Huang, Lee, & Chen, 2011), here highlighting the 'Red Leg' *Brachypelma* and group 1 '*Aphonopelma*' (*s.l.*) as amongst the highest priorities.

Conclusion and future directions

As with several other invertebrates harvested for the exotic pet trade, the evolutionary history and taxonomic uniqueness of most tarantulas is not well known. The advent of modern molecular techniques now allows a wealth of novel information to be quickly gathered to help resolve longstanding taxonomic and systematic questions, most urgently those of conservation importance. Such genetic information has already laid the foundations to improve protective legislation for an array of diverse taxa including several arthropods (e.g., Arnedo & Ferrández, 2007; Lin et al., 2011). This study is the first attempt to use molecular data to re-evaluate the evolutionary relationships of tarantulas at the subfamily-level and for generic boundaries, focused on the CITES protected *Brachypelma* and the highly diverse *Aphonopelma*. Whilst a promising starting point, further in-depth study is required to resolve phylogenetic relationships within these and many other genera, particularly the speciose and wide ranging *Acanthoscurria* and *Pamphobeteus* – neither densely sampled in this study. Several predominantly monotypic genera are also not yet included, although would be vital for broadest resolution, notably several Mexican and Brazilian endemics, requiring collaborations with local researchers. However, the highly conserved body plans of these spiders often makes it difficult to assign diagnostic phenotypic characteristics, particularly

at generic and species levels (Hamilton et al., 2011; 2014; 2016a; Hendrixson et al., 2013). We also acknowledge that, despite the many useful insights into the Theraphosinae provided by this mtDNA fragment, that any phylogenetic hypothesis based on any single mtDNA region should be treated with caution. Additional sampling of nuclear and other mtDNA loci is needed to reach improved tree resolution and establish a strong phylogenetic framework. This, coupled with informative morphology and phylogeography could provide a stable classification system (Dayrat, 2005; Hamilton et al., 2016a).

We recommend that future studies explore next generation sequencing methods such as transcriptome sequencing, targeted-sequencing approaches (e.g., Anchored Hybrid Enrichment, UCE), or reduced representation library sequencing (e.g., RAD-tag, ddRAD (Burns et al., 2016)) to resolve phylogenetic problems and other evolutionary questions (e.g., Bond et al., 2014; Garrison et al., 2016, Hamilton et al., 2016a; 2016b; Lemmon, Emme, & Lemmon, 2012; Starrett et al., 2016). Of note, tarantulas were a focal group in the development of the new AHE spider probe kit (Hamilton et al., 2016b), including '*Aphonopelma*' (*s.l.*), providing a valuable resource for future genetic investigations of the focal lineages presented here. Finally, we also recommend that the conservation status of all species of '*Aphonopelma*' be reassessed, particularly in the USA. Future work should focus on expanding genetic and taxon sampling to further evaluate the limits of genera, species, and population structure of as yet under-sampled taxa. This information can then be used in a solid comparative framework that considers shared evolutionary histories, biogeography, life histories, population ecology, reproductive strategies, and human impact to together re-inform modern conservation decisions within the revised subfamily Theraphosinae and beyond.

Summary of nomenclatural changes for Subfamily Theraphosinae Thorell 1870.

Grammostolini nov. trib. Turner *et al*. The constituent genera are: *Agnostopelma**, *Aguapanela**, *Bistriopelma**, *Bumba*, *Euathlus*, *Grammostola* [as type genus], *Homoeomma*, *Magulla**, *Melloleitaoina**, *Phrixotrichus*, and *Tmesiphantes**.

Diagnosis:

(1) Typically without abdominal patterning into adulthood (but faint at rear in some *Euathlus*).
(2) Tibial apophysis of adult male dual (but lost in *Aguapanela*).
(3) Metatarsus I of adult male never with retrolateral nodule with megaspines, typically without basal process (except basal process present in some *Homoeomma*).
(4) Femur IV without retrolateral scopula (all).
(5) Lack type I abdominal urticating setae (all).
(6) Presence of type III abdominal urticating setae, either intermixed with large area of type IV (e.g., *Bumba*), in broad dorso-posterior patch of type III surrounded at margins by type IV (e.g., *Grammostola*), or divided (or nearly so) into two more lateral patches, again typically with type IV at margins (e.g., *Homoeomma*, *Euathlus*).
(7) Lack trochantal stridulating setae on leg bases (all, except *Aguapanela*).
(8) Typically lack coxal stridulating setae on leg bases (all, except *Grammostola*).
(9) Reduced labial cuspules i.e., <15 (all, except *Grammostola*, *Homoeomma*, *Tmesiphanthes*).
(10) Male palpal bulb typically with filiform non-spatulate embolus, with few keels except distinct posterior superior [PS] and posterior inferior [PI] which may not be well developed.
(11) Spermathecae unfused, often with twin narrow seminal receptacles and widely separate bases (although broader receptacles in *Bumba*).

Hapalopini nov. trib. Turner *et al.* The constituent genera are: *Aenigmarachne**, *Bonnetina*, *Cardiopelma**, *Catanduba**, *Chromatopelma*, *Cyriocosmus*, *Davus*, *Hapalopus* [as type genus], *Haplotremus**, *Kochiana**, *Magnacarina**, *Munduruku**, *Plesiopelma*, *Schizopelma**, and *Thrixopelma.*

Diagnosis:

(1) Often with strong abdominal patterning such as lateral banding into adulthood (but can be reduced or absent in several *Thrixopelma*, *Plesiopelma*, *Schizopelma*, *Hapalopus* and others).
(2) Tibial apophysis of adult male often dual, occasionally modified (singular with megaspine, e.g., *Schizopelma;* or accessory apophysis e.g., *Bonnetina*, etc.).
(3) Metatarsus I of adult male can have retrolateral nodule with megaspines (e.g., *Bonnetina*, *Magnacarina*), occasionally with basal process (e.g., *Plesiopelma, Catanduba*).
(4) Femur IV without retrolateral scopula (all, note: misreported for *Schizopelma* in some matricies).
(5) Lack type I abdominal urticating setae (all).
(6) Most with only type III abdominal urticating setae, predominantly in dorso-medial oval patch, although several cases of debate about co-presence of type IV (e.g., *Aenigmarachne*, *Plesiopelma*,

Chromatopelma) and some where both types III and IV have been characterized (e.g., *Munderuku*, *Thrixopelma*).
(7) Lack trochantal stridulating setae on leg bases (all).
(8) Lack coxal stridulating setae on leg bases (all).
(9) Numerous labial cuspules i.e., >15 (all, except *Hapalotremus*).
(10) Male palpal bulb typically rather compact, can have highly modified keels and other developed attributed such as notable paraembolic apophysis (although *Catanduba* can be more filiform on embolus)
(11) Spermathecae often either simple single fused dome medially sclerotized, or separate twin receptacles with many complex intricate extensions.

Theraphosini nov. trib. Turner *et al.* The constituent genera are: *Acanthoscurria*, *Acentropelma**, *Ami**, *Aphonopelma*, *Barropelma**, *Brachypelma*, *Citharacanthus*, *Clavopelma**, *Coztetlana**, *Crassicrus*, *Cubanana**, *Cyclosternum**, *Cyrtopholis*, *Eupalaestrus*, *Eurypelmella**, *Lasiodora*, *Lasiodorides**, *Longilyra**, *Megaphobema*, *Mygalarachne**, *Metriopelma**, *Miaschistopus**, *Neischnocolus**, *Neostenotarsus**, *Nesipelma**, *Nhandu*, *Pamphobeteus*, *Phormictopus*, *Proshapalopus**, *Pseudhapalopus**, *Pterinopelma**, *Reversopelma**, *Sericopelma*, *Sphaerobothria*, *Stichoplastoris*, *Theraphosa* [as type genus], *Vitalius*, and *Xenesthis.*

Diagnosis:

(1) Typically without abdominal patterning into adulthood (can be notably present in some e.g., *Ami*, *Neostenotarsus*, etc,).
(2) Tibial apophysis of adult male variable, ranging both in placement and structure from various presentations of dual structures, to singular (e.g., *Acanthoscurria*, *Pseudhapalopus*, etc,) through to complete absence (e.g., *Acentropelma*, *Theraphosa*, *Sericopelma*, etc.).
(3) Metatarsus I of adult male without retrolateral nodule with megaspines nor basal process.
(4) Femur IV often with retrolateral scopula as distinctive pad, but many taxa without.
(5) Presence of type I abdominal urticating setae (all except *Theraphosa*).
(6) Typically with type III urticating setae, predominantly in broad oval patch (but can be absent in some genera e.g. '*Aphonopelma*', *Cyrtopholis*, or other cases just females not males), but notably never with type IV setae.
(7) Often with trochantal stridulating setae on leg bases, but many taxa without.
(8) Often with coxal stridulating setae on leg bases, but many taxa without.
(9) Numerous labial cuspules i.e., >15 (all).
(10) Male palpal bulb varies massively, often apically broad or spatulate embolus with numerous keels some of which can be serrated or have loose denticulations, but several genera notably with much of the palpal bulb filiform and extended with inconspicuous keels).
(11) Spermathecae often fused broad dome or twin wide receptacles with fused bases, sometimes with many sclerotized ridges, but several genera notably with narrow twin receptacles.

Theraphosinae *insetae sedis.* *Hemirrhagus**, *Kankuamo**, *Ozopactus**

Nomenclatural notes: Those genera marked by * are not explicitly included here in our presented molecular data, hence their suggested placement to the above tribes is based on morphological considerations alone. *Metriopelma** refers to type species *M. breyeri*, rather than other species subject to further taxonomic revision elsewhere. The genus *Hemirrhagus** does not appear to fit well into any of these three tribes, many aspects of their morphology are complicated by adaptation to troglophilic environments, but may be sister to one of the wider groupings within this subfamily. *Ozopactus* is known only from a single poorly preserved specimen, so its placement is highly uncertain. For *Kankuamo** – see description for discussion of possible taxonomic affinities where placement in Theraphosini may be most suitable in a relatively basal position, or even elsewhere basally within the wider subfamily. Consequently, these three genera are more cautiously treated as Theraphosinae *incertae sedis*.

***Brachypelma sensu stricto* (i.e., our 'Red Leg' group).**

Composition: *Brachypelma albiceps* Pocock, 1901; [*Brachypelma annitha* Tesmoingt, Cleton & Verdez, 1997]; *Brachypelma auratum* Schmidt, 1994; *Brachypelma baumgarteni* Smith, 1994; *Brachypelma boehmei* Schmidt & Klaas, 1994; *Brachypelma emilia* (White, 1856); *Brachypelma hamorii* Tesmoingt, Cleton & Verdez, 1997; *Brachypelma klaasi* (Schmidt & Kraus, 1994; *Brachypelma smithi* (F. O. Pickard-Cambridge, 1897)

Nomenclatural notes: All other currently recognized species of *Brachypelma* as defined elsewhere are herein proposed as misplaced in this genus, pending further taxonomic revision. *Within this group, *B. annitha* is treated as a junior synonym of *B. smithi* in accordance with Mendoza and Francke (2017), which is also supported by our molecular data.

***Aphonopelma sensu stricto* (i.e., our 'Group 3').**

Composition: *Aphonopelma seemanni* (F. O. Pickard-Cambridge, 1897); *Aphonopelma belindae* Gabriel, 2011; *Aphonopelma burica* Valerio, 1980; *Aphonopelma latens* (Chamberlin, 1917)

Nomenclatural notes: All other currently recognized species of *Aphonopelma* as defined elsewhere are herein proposed as misplaced in this genus, pending further taxonomic revision.

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