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# Testing species boundaries in the *Antrodiaetus unicolor* complex (Araneae: Mygalomorphae: Antrodiaetidae): "Paraphyly" and cryptic diversity

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**Abstract**

The inability to correctly identify species has far reaching implications in nearly all areas of biology, yet few studies investigate methods for delineating species boundaries. Moreover, once these boundaries have been hypothesized, little thought has been given to how these constructs can be further evaluated. We employ a molecular phylogenetic approach using nuclear 28S rRNA and mitochondrial cytochrome *c* oxidase subunit I genes to test the general efficacy of species boundaries in the *Antrodiaetus unicolor* spider species complex. Our analyses provide evidence that *An. unicolor* is "paraphyletic" with respect to *An. microunicolor*, indicating that morphological criteria used to delineate species boundaries undersplits actual species-level diversity in this group of spiders. These analyses also demonstrate that individuals from geographically proximate populations sometimes exhibit considerable molecular divergence, strongly suggesting that *An. unicolor* is a cryptic species complex. Finally, this molecular approach has provided the phylogenetic framework that is necessary to begin interpreting the vast amount of morphological variation observed in these spiders based upon findings from previous studies. Our approach using multiple genes appears to be a rigorous method to critically examine species boundaries originally based on traditional morphological approaches to spider taxonomy.


*Keywords:* *Antrodiaetus*; Congeneric phylogeography; Cryptic species; Paraphyletic species; Species boundaries; Spider taxonomy

## 1. Introduction

Accurate assessment of species-level diversity is essential to systematic research; conservation decisions and policy, studies of biodiversity, ecology, and natural history necessitate correct species identification. Despite its relative importance, how we define species remains one of the more contentious topics in evolutionary biology. Although considerable attention is devoted to species concepts in the primary evolution literature, far less consideration is dedicated to developing methods for empirically delimiting and assessing species boundaries (Morando et al., 2003; Sites and Marshall, 2003, 2004; Wiens and Penkrot, 2002). Moreover, once species boundaries have been hypothesized, little thought has been given to how these constructs can be assessed in a rigorous manner (Sinclair et al., 2004; Sites and Crandall, 1997).

The study we present in this paper attempts to assess the general efficacy of species boundaries in a group of mygalomorph spiders (tarantulas, trapdoor spiders, and their relatives) using a molecular phylogenetic approach. These spiders are ideal models for studying allopatric speciation due to their limited ability to disperse and extreme habitat fidelity (i.e., ecological niche conservation; Wiens, 2004a,b). Species boundaries in this group

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of spiders are most often defined on the basis of male mating clasper morphology (e.g., Bond, 2004; Bond and Opell, 2002; Coyle, 1971, 1995; Goloboff, 1995; Hendrixson and Bond, 2004, 2005). Mating claspers are modifications to the tibiae and metatarsi of leg I (sometimes leg II) that are used during courtship. These modifications typically consist of various apophyses and cuticular projections, spines and setae, or changes in leg shape. Despite these (often striking) differences in males, females of closely related species tend to be morphologically similar (e.g., Bond, 2004; Coyle, 1971; Goloboff, 1995).

The mygalomorph spider *Antrodiaetus unicolor* provides a challenging model for studying the detection and delimitation of species boundaries; morphological variation within this single "species" is complex and thus has been characterized as difficult to interpret (Coyle, 1971). This species is widely distributed throughout the eastern United States, with populations reaching high densities in the relatively cool mesic forests of the Central and Southern Appalachian Mountains. After examining numerous specimens from many populations across the distribution of *An. unicolor*, Coyle (1971) indicated that the species-level diversity was likely more extensive than previously thought. However, Coyle remained conservative in his diversity assessment because: (1) some populations were morphologically different, however, intermediate forms were common; (2) intrapopulation variation was often as great as interpopulation variation; and (3) some allopatric, and perhaps isolated, populations were indistinguishable from each other. Consequently, *An. unicolor* had been used as a "catch-all" name for *Antrodiaetus* specimens collected in the Southern Appalachian Mountains. Based on recent studies of specimens taken from the Coweeta long-term ecological research (LTER) site in the southwestern mountains of North Carolina, Hendrixson and Bond (2005) identified two discrete morphological forms, both of which fell within the putative range of variation described by Coyle (1971) for *An. unicolor* (e.g., large or small size, mating clasper with presence or absence of metatarsal macrosetae). Based on these compelling morphological differences and differences in ecology (e.g., temporal isolation during breeding seasons), Hendrixson and Bond (2005) recognized the smaller form as a new species ("*An. microunicolor*"). Given that *An. microunicolor* was described and diagnosed based on a "subset" of the original characters used to identify *An. unicolor*, the authors re-diagnosed the latter species (and thus hypothesized new species boundaries) using the following combination of characters: presence of macroseta A on male metatarsus I (rarely absent, or rarely with macroseta B); at least one-fifth of macrosetae on male prolateral tibia I ensiform; and presence of thickened convergent medial setae just posterior to the pedicel on the abdomen (on immature and female specimens).

The primary objective of this paper is to *test* these newly constructed species hypotheses (*An. microunicolor* + *An. unicolor*), based on an accepted morphological approach to mygalomorph taxonomy, by using an alternative molecular-based approach that employs genes surveyed from both the nuclear and mitochondrial genomes. While the use of molecules has revolutionized the field of systematics by providing a large data source, most molecular studies of species boundaries depend on data taken from a single mitochondrial locus. Mitochondrial DNA data should reflect species boundaries more rapidly than nuclear loci (Moore, 1995; Wiens and Penkrot, 2002) because they are haploid and maternally inherited. Despite this recognized advantage, mitochondrial genes by themselves provide only an incomplete picture of evolution and may not always accurately reflect population or species history (Ballard and Whitlock, 2004; Zhang and Hewitt, 2003), especially in cases where females are dispersal limited and males account for the majority of gene flow (as is likely the case in *Antrodiaetus*).

## 2. Materials and methods

### 2.1. Taxon sampling

We have employed a "congeneric" approach to phylogeography (Funk and Omland, 2003) in order to assess intraspecific and interspecific genetic variation, and to test species boundaries in *An. unicolor* and *An. microunicolor*. This method involves sampling multiple populations from two or more closely related species. We sampled several specimens of *An. unicolor* (as designated by Coyle, 1971) throughout its distribution, paying particular attention to highly variable, morphologically divergent, or peripheral populations. Populations of *An. microunicolor* were collected from the type locality (Coweeta LTER) together with nearby localities in southwestern North Carolina, southeastern Tennessee, and northeastern Georgia (Fig. 1). Methods used to collect these spiders have been discussed elsewhere (Coyle, 1971; Hendrixson and Bond, 2005). In total, we sampled 71 specimens (ingroup, *An. unicolor* and *An. microunicolor*) from 50 localities. Table 1 includes a list of specimens, the state and county from where they were collected (complete locality/collection data can be obtained from http://www.mygalomorphae.org), haplotype designations, and GenBank accession numbers. Multiple outgroups were used to estimate the root of the phylogeny (Maddison et al., 1984): two specimens of *An. robustus* (haplotypes ROB1, ROB2) from Indiana and Pennsylvania, respectively; *An. apachecus* (APA) from Arizona; *An. stygius* (STY) from Missouri; *Aliatypus isolatus* (ALI1) from Arizona; and *Al. janus* (ALI2) from California. A label has been included in each vial indicating it as a voucher for this study.

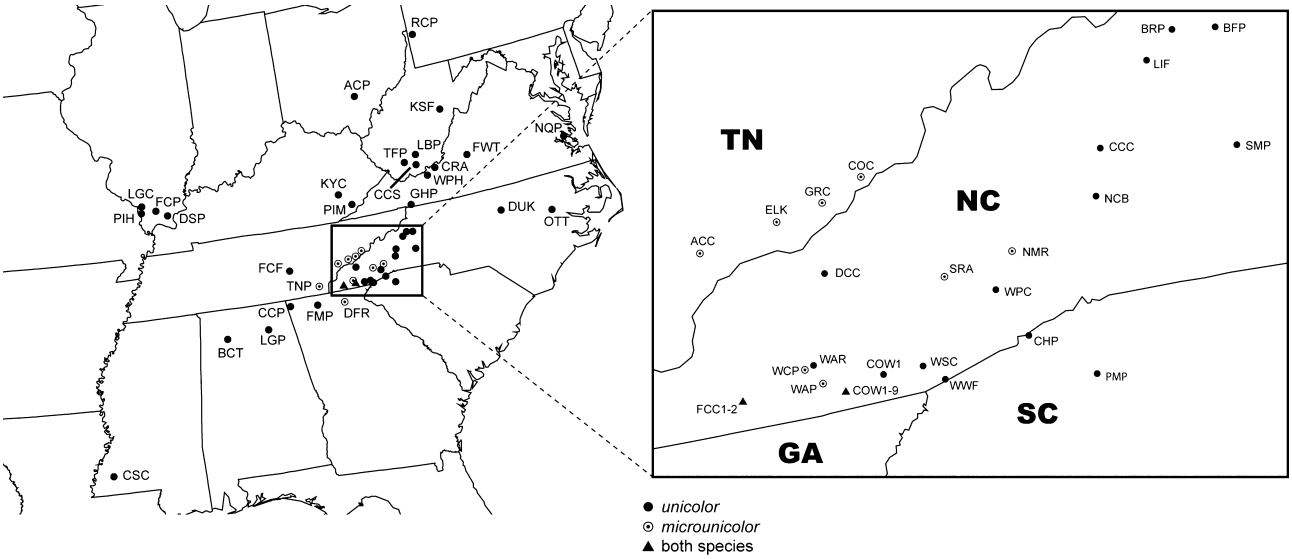


Fig. 1. Map indicating sample localities of the haplotypes used in this study (see Table 1): solid circles, *An. unicolor*; open circles with dot, *An. microunicolor*; and triangles, *An. unicolor* and *An. microunicolor* (collected from the same locality).

### 2.2. Molecular protocols

Specimens were brought back from the field alive or in 100% ethanol. Between two and four legs were autotomized from the right side of each spider to obtain tissue samples. For very small spiders (i.e., carapace length less than 2 mm), the entire cephalothorax (including chelicerae, legs, and pedipalps) was removed from the body and used as a tissue sample. Tissue samples were immediately stored in RNA*later* RNA Stabilization Reagent (Qiagen, Valencia, CA), placed into a 10 °C refrigerator for approximately 24 h, and subsequently transferred to a −80 °C freezer in the laboratory. Total genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen, Valencia, CA) and stored at −20 °C prior to amplification. Voucher specimens were preserved in 80% ethanol and stored at −20 °C for at least 4 weeks. These vouchers, currently housed in the spider collection at East Carolina University, will eventually be deposited in the National Museum of Natural History, Smithsonian Institution, Washington, DC, USA and the California Academy of Sciences, San Francisco, USA.

Two gene fragments [nuclear 28S rRNA, mitochondrial cytochrome *c* oxidase subunit I (COI)] were amplified using the polymerase chain reaction (PCR) for subsequent sequence analysis (Mullis and Faloona, 1987; Saiki et al., 1988). 28S rRNA was amplified using the following PCR cocktail (50 μL final volume): 25 μL FailSafe PCR 2× Premix I (Epicentre, Madison, WI); 14.5 μL ultra pure water (Water Optima, Fisher Scientific, Hampton, NH); 5 μL of each 2.5 pM/μL primer; 0.5 μL *Taq* DNA polymerase (Invitrogen, Carlsbad, CA); and 1 μL genomic DNA. Primers ZR2 and ZR3 (Mallatt and Sullivan, 1998; Winchell et al., 2002) were used for amplification. Thermal cycle parameters were as follows: initial denaturation at 96 °C for 2 min; 30 cycles of denaturation at 96 °C for 45 s, annealing at 54 °C for 45 s, and extension at 72 °C for 45 s; and final extension at 72 °C for 2 min. For COI mtDNA, amplification was performed using a PCR cocktail of: 21.5 μL ultra pure water; 5 μL of dNTPs; 5 μL 10× PCR buffer (200 mM Tris–HCl, 500 mM KCl); 5 μL $MgCl_2$; 5 μL of each 2.5 pM/μL primer; 1 μL dimethyl sulfoxide (DMSO); 1 μL bovine serum albumin (BSA); 0.5 μL Invitrogen *Taq* DNA polymerase; and 1 μL genomic DNA. Primers used to amplify this region of COI included C1J-1751SPID and C1N-2776 (Hedin and Maddison, 2001). Thermal cycle parameters were as follows: initial denaturation at 95 °C for 2 min; 30 cycles of denaturation at 96 °C for 30 s, annealing at 48 °C for 30 s, and extension at 72 °C for 1 min; and final extension at 72 °C for 2 min. Unincorporated dNTPs, primers, and other impurities were removed from the amplified PCR products by using the High Pure PCR Product Purification Kit (Roche Diagnostics, Indianapolis, IN).

Multiple copies of 28S rRNA made it impossible to obtain readable sequences directly from the final PCR products of three specimens (MY 2008, MY 2028, and MY 2038). These PCR templates were cloned using the TOPO TA Cloning Kit for Sequencing with One Shot TOP10 Chemically Competent *Escherichia coli* (Invitrogen, Carlsbad, CA). Transformed colonies were transferred to a kanamycin plate that contained 40 μL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and subsequently incubated overnight at 37 °C. We screened eight colonies per specimen using the same PCR protocols listed above.

Final PCR products were sequenced using an ABI Prism 377 automated DNA sequencer (Applied Biosystems, Foster City, CA) using the ABI Big Dye

Table 1
Specimen and sequence information for taxa included in this study

| Taxon name | Database No. | Collection locality[a] | Haplotype | Accession Nos. | |
|---|---|---|---|---|---|
| | | | | COI | 28S |
| *Antrodiaetus microunicolor* | MY 0760 | NC: Macon Co. | WAP | AY896883 | AY896948 |
| *Antrodiaetus microunicolor* | MY 0772 | NC: Clay Co. | FCC2 | AY896884 | AY896949 |
| *Antrodiaetus microunicolor* | MY 0820 | GA: Lumpkin Co. | DFR | AY896885 | AY896950 |
| *Antrodiaetus microunicolor* | MY 2327 | NC: Henderson Co. | NMR | AY896886 | AY896951 |
| *Antrodiaetus microunicolor* | MY 2349 | NC: Haywood Co. | SRA | AY896887 | AY896952 |
| *Antrodiaetus microunicolor* | MY 2363 | TN: Polk Co. | TNP | AY896888 | AY896953 |
| *Antrodiaetus microunicolor* | MY 2369 | TN: Blount Co. | ACC | AY896889 | AY896954 |
| *Antrodiaetus microunicolor* | MY 2374 | TN: Sevier Co. | ELK | AY896890 | AY896955 |
| *Antrodiaetus microunicolor* | MY 2379 | TN: Sevier Co. | GRC | AY896891 | AY896956 |
| *Antrodiaetus microunicolor* | MY 2384 | TN: Cocke Co. | COC | AY896892 | AY896957 |
| *Antrodiaetus microunicolor* | MY 2401 | NC: Macon Co. | COW3 | AY896893 | AY896958 |
| *Antrodiaetus microunicolor* | MY 2402 | NC: Macon Co. | COW4 | AY896894 | AY896959 |
| *Antrodiaetus microunicolor* | MY 2420 | NC: Macon Co. | COW5 | AY896895 | AY896960 |
| *Antrodiaetus microunicolor* | MY 2422 | NC: Macon Co. | COW3 | AY896893 | AY896958 |
| *Antrodiaetus microunicolor* | MY 2425 | NC: Macon Co. | COW7 | AY896896 | AY896961 |
| *Antrodiaetus microunicolor* | MY 2441 | NC: Macon Co. | COW8 | AY896897 | AY896962 |
| *Antrodiaetus microunicolor* | MY 2448 | NC: Macon Co. | COW9 | AY896898 | AY896963 |
| *Antrodiaetus microunicolor* | MY 2449 | NC: Macon Co. | WCP | AY896899 | AY896964 |
| *Antrodiaetus unicolor* | MY 0649 | NC: Durham Co. | DUK | AY896900 | AY896965 |
| *Antrodiaetus unicolor* | MY 0654 | NC: Pitt Co. | OTT | AY896901 | AY896966 |
| *Antrodiaetus unicolor* | MY 0681 | NC: Swain Co. | DCC | AY896902 | AY896967 |
| *Antrodiaetus unicolor* | MY 0686 | NC: Jackson Co. | WWF | AY896903 | AY896968 |
| *Antrodiaetus unicolor* | MY 0693 | NC: Burke Co. | LIF | AY896904 | AY896969 |
| *Antrodiaetus unicolor* | MY 0750 | VA: York Co. | NQP | AY896905 | AY896970 |
| *Antrodiaetus unicolor* | MY 0755 | NC: Macon Co. | COW1 | AY896931 | AY896996 |
| *Antrodiaetus unicolor* | MY 0771 | NC: Clay Co. | FCC1 | AY896906 | AY896971 |
| *Antrodiaetus unicolor* | MY 0780 | IL: Union Co. | PIH | AY896907 | AY896972 |
| *Antrodiaetus unicolor* | MY 0788 | IL: Jackson Co. | LGC | AY896908 | AY896973 |
| *Antrodiaetus unicolor* | MY 0793 | IL: Johnson Co. | FCP | AY896909 | AY896974 |
| *Antrodiaetus unicolor* | MY 0796 | IL: Pope Co. | DSP | AY896910 | AY896975 |
| *Antrodiaetus unicolor* | MY 0802 | SC: Greenville Co. | PMP | AY896911 | AY896976 |
| *Antrodiaetus unicolor* | MY 0810 | SC: Greenville Co. | CHP | AY896912 | AY896977 |
| *Antrodiaetus unicolor* | MY 2005 | GA: Murray Co. | FMP | AY896913 | AY896978 |
| *Antrodiaetus unicolor* | MY 2008 | GA: Dade Co. | CCP | AY896914 | AY896979 |
| *Antrodiaetus unicolor* | MY 2020 | AL: Marshall Co. | LGP | AY896915 | AY896980 |
| *Antrodiaetus unicolor* | MY 2028 | AL: Lawrence Co. | BCT | AY896916 | AY896981 |
| *Antrodiaetus unicolor* | MY 2038 | MS: Franklin Co. | CSC | AY896917 | AY896982 |
| *Antrodiaetus unicolor* | MY 2156 | KY: Harlan Co. | PIM | AY896918 | AY896983 |
| *Antrodiaetus unicolor* | MY 2170 | TN: Van Buren Co. | FCF | AY896919 | AY896984 |
| *Antrodiaetus unicolor* | MY 2183 | KY: Clay Co. | KYC | AY896920 | AY896985 |
| *Antrodiaetus unicolor* | MY 2193 | OH: Hocking Co. | ACP | AY896921 | AY896986 |
| *Antrodiaetus unicolor* | MY 2198 | PA: Beaver Co. | RCP | AY896922 | AY896987 |
| *Antrodiaetus unicolor* | MY 2215 | WV: Randolph Co. | KSF | AY896923 | AY896988 |
| *Antrodiaetus unicolor* | MY 2227 | VA: Botetourt Co. | FWT | AY896924 | AY896989 |
| *Antrodiaetus unicolor* | MY 2229 | VA: Giles Co. | CRA | AY896925 | AY896990 |
| *Antrodiaetus unicolor* | MY 2236 | VA: Giles Co. | WPH | AY896926 | AY896991 |
| *Antrodiaetus unicolor* | MY 2246 | VA: Grayson Co. | GHP | AY896927 | AY896992 |
| *Antrodiaetus unicolor* | MY 2256 | WV: Mercer Co. | CCS | AY896928 | AY896993 |
| *Antrodiaetus unicolor* | MY 2262 | WV: Wyoming Co. | TFP | AY896929 | AY896994 |
| *Antrodiaetus unicolor* | MY 2266 | WV: Raleigh Co. | LBP | AY896930 | AY896995 |
| *Antrodiaetus unicolor* | MY 2300 | NC: Macon Co. | COW1 | AY896931 | AY896996 |
| *Antrodiaetus unicolor* | MY 2301 | NC: Macon Co. | COW1 | AY896931 | AY896996 |
| *Antrodiaetus unicolor* | MY 2302 | NC: Macon Co. | COW1 | AY896931 | AY896996 |
| *Antrodiaetus unicolor* | MY 2303 | NC: Macon Co. | COW1 | AY896931 | AY896996 |
| *Antrodiaetus unicolor* | MY 2314 | NC: Macon Co. | COW1 | AY896931 | AY896996 |
| *Antrodiaetus unicolor* | MY 2315 | NC: Macon Co. | COW2 | AY896932 | AY896997 |
| *Antrodiaetus unicolor* | MY 2316 | NC: Macon Co. | COW1 | AY896931 | AY896996 |
| *Antrodiaetus unicolor* | MY 2317 | NC: Macon Co. | COW1 | AY896931 | AY896996 |
| *Antrodiaetus unicolor* | MY 2323 | NC: Macon Co. | COW1 | AY896931 | AY896996 |
| *Antrodiaetus unicolor* | MY 2332 | NC: Transylvania Co. | WPC | AY896933 | AY896998 |
| *Antrodiaetus unicolor* | MY 2338 | NC: Jackson Co. | WSC | AY896934 | AY896999 |

Table 1 (*continued*)

| Taxon name | Database No. | Collection locality[a] | Haplotype | Accession Nos. | |
|---|---|---|---|---|---|
| | | | | COI | 28S |
| *Antrodiaetus unicolor* | MY 2358 | NC: Buncombe Co. | NCB | AY896935 | AY897000 |
| *Antrodiaetus unicolor* | MY 2390 | NC: Macon Co. | COW2 | AY896932 | AY896997 |
| *Antrodiaetus unicolor* | MY 2391 | NC: Macon Co. | COW2 | AY896932 | AY896997 |
| *Antrodiaetus unicolor* | MY 2393 | NC: Avery Co. | BRP | AY896936 | AY897001 |
| *Antrodiaetus unicolor* | MY 2407 | NC: Burke Co. | SMP | AY896937 | AY897002 |
| *Antrodiaetus unicolor* | MY 2410 | NC: Caldwell Co. | BFP | AY896938 | AY897003 |
| *Antrodiaetus unicolor* | MY 2415 | NC: McDowell Co. | CCC | AY896939 | AY897004 |
| *Antrodiaetus unicolor* | MY 2452 | NC: Macon Co. | WAR | AY896940 | AY897005 |
| *Antrodiaetus* 'hybrid' | MY 2421 | NC: Macon Co. | COW6 | AY896941 | AY897006 |
| Outgroups | | | | | |
| *Antrodiaetus apachecus* | MY 0275 | AZ: Cochise Co. | APA | AY896942 | AY897007 |
| *Antrodiaetus stygius* | MY 2823 | MO: Dallas Co. | STY | AY896943 | AY897008 |
| *Antrodiaetus robustus* | MY 0798 | IN: Monroe Co. | ROB1 | AY896944 | AY897009 |
| *Antrodiaetus robustus* | MY 0800 | IN: Jackson Co. | ROB1 | AY896944 | AY897009 |
| *Antrodiaetus robustus* | MY 2203 | PA: Westmoreland Co. | ROB2 | AY896945 | AY897010 |
| *Aliatypus isolatus* | MY 0260 | AZ: Yavapai Co. | ALI1 | AY896946 | AY897011 |
| *Aliatypus janus* | MY 0726 | CA: Kern Co. | ALI2 | AY896947 | AY897012 |

[a] Specific locality information can be downloaded at http://www.mygalomorphae.org.

Terminator version 3.1 Cycle Sequencing Ready Reaction Kit. PCR primers served as sequencing primers for both genes; in addition, primers ZR1 and ZR4 (Mallatt and Sullivan, 1998) were used for 28S rRNA. All sequences were edited in the program Sequencher (Genecodes, Madison, WI).

28S rRNA sequences were initially aligned with ClustalX version 1.81 (Thompson et al., 1997) using default parameters (gap opening cost = 15; gap extension cost = 6.66; delay divergent sequences = 30%; and DNA transition weight = 0.50), followed by slight manual adjustments in MacClade version 4.0 (Maddison and Maddison, 2001) to improve assessment of homologous nucleotide positions (Remerie et al., 2004). Alignment for the COI sequences was straightforward and trivial due to a lack of indel events. Datasets in NEXUS format are available for download at http://www.mygalomorphae.org.

### 2.3. Phylogenetic inference

We performed parsimony analyses in PAUP* version 4.0b10 (Swofford, 2002) on three separate datasets (28S, COI, and combined). A heuristic search was performed using 100 random addition sequence replicates with tree bisection–reconnection (TBR) branch swapping, 100 trees held at each step during stepwise addition, all characters of equal weight, gaps treated as missing, and branches collapsed if maximum branch length is zero. Nonparametric bootstrapping (Felsenstein, 1985) was used to evaluate the support of nodes using a heuristic search with the above parameters based on 500 pseudoreplicates.

The program Modeltest 3.06 (Posada and Crandall, 1998) was used to determine the appropriate model of DNA evolution by likelihood ratio test (lrt) for Bayesian analyses. Each dataset was considered as a separate partition. The COI dataset was further partitioned by codon position. MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001) was used to infer the phylogeny using the model of sequence evolution model indicated by lrt. Four concurrent Markov Chain Monte Carlo (MCMC) chains (one heated) were run for 1,000,000 generations, saving the current tree to file every 100 generations. Topologies prior to −ln likelihood stabilization ("burn-in") were discarded and posterior clade probabilities were computed from the remaining trees. These analyses were replicated three times to ensure topological convergence and homogeneity of posterior clade probabilities (Huelsenbeck et al., 2002). The reported likelihood score was calculated by averaging the likelihood scores for all topologies post burn-in using the "sump" command in MrBayes.

### 2.4. Testing species boundaries

Some authors (e.g., de Queiroz, 1998) have argued that most species concepts, though hotly debated, essentially share the general ontological view that species are lineages (i.e., a single line of direct ancestry and descent); they differ primarily through the means by which species are recognized, this largely being an epistemological matter. One underlying theme of phylogenetic systematics requires that taxa be monophyletic; however, a *general* lineage-based approach to viewing species does not necessitate that species be "exclusive" with respect to their gene genealogies (e.g., see Funk and Omland, 2003; Hedin, 1997b; Wiens and Penkrot, 2002) because of incomplete lineage sorting and other phenomena. If these alternative explanations of "polyphyly" or "paraphyly"

can be ruled out, then species should be recognized as monophyletic groups of haplotypes. Using *An. unicolor* as the "focal species," our molecular approach for testing morphological species boundaries follows the "tree-based species delineation with DNA data" methodology outlined by Wiens and Penkrot (2002). In conjunction with geographical data, this technique recognizes well-supported *basal* haplotype lineages as species. Discordance between these two approaches (morphological and molecular) requires further discussion.

## 3. Results

### 3.1. Sequences

Approximately 837 bp of COI and 813 bp of 28S rRNA were sequenced from each specimen. Fifty-nine unique haplotypes (for each dataset) were recovered and analyzed from the 71 ingroup specimens (GenBank Accession Nos. for COI: AY896883–AY896947; for 28S: AY896948–AY897012). Base composition was as follows: A = 0.22934; C = 0.17386; G = 0.26457; T = 0.33223. A $\chi^2$ test of homogeneity of base frequencies across haplotypes, as implemented in PAUP*, indicated that the sequences were not significantly heterogeneous ($\chi^2 = 51.339531$, df = 192, $P = 1.00$). Across the combined dataset, sequence divergence values (uncorrected *P*) ranged from a minimum of 0.001 to a maximum of 0.088 (for COI: 0.001–0.121; for 28S: 0.001–0.059).

### 3.2. Parsimony analysis

A heuristic search on the combined 28S and COI dataset resulted in 30 equally most parsimonious (MP) trees (tree scores reported are after uninformative sites have been excluded): (L = 2564, CI = 0.309, RI = 0.613); of 1650 nucleotides, 509 sites (30.8%) were parsimony-informative. Parsimony analyses also resulted in 19 MP trees (L = 1794, CI = 0.217, RI = 0.575) for the COI dataset and 29,400 MP trees (L = 687, CI = 0.585, RI = 0.776) for the 28S dataset, respectively. In all three analyses (separate analyses not shown), the strict consensus trees recovered a "monophyletic" (i.e., genealogically exclusive) group that included all individuals belonging to *An. microunicolor* (minus haplotype COW6, see discussion below). The *An. microunicolor* clade is strongly supported in the combined dataset (92% bootstrap value), but only moderately supported for COI (64%), and lacks solid support for 28S. Furthermore, the *An. microunicolor* clade is nested within *An. unicolor*, rendering the latter species "paraphyletic." In each case, "paraphyly" resulted from *An. unicolor* haplotypes RCP, CRA, DUK, and NQP falling outside all remaining ingroup haplotypes (see Fig. 2).

### 3.3. Bayesian analysis

The following models of DNA evolution were determined for each partition used in the Bayesian analysis: 28S (K80 + I + G); COI codon 1 (K81uf + G); COI codon 2 (TrN + I + G); and COI codon 3 (HKY + G). Likelihood stabilization occurred at approximately 60,000 generations, and as a consequence, the first 600 trees were discarded. Results from the Bayesian analysis (Fig. 2) are largely congruent with parsimony: (1) *An. microunicolor* is unequivocally supported as a "monophyletic" group (minus haplotype COW6) with 100% posterior clade probability; (2) *An. microunicolor* is nested within *An. unicolor*, rendering the latter species "paraphyletic"; (3) species-level "paraphyly" in *An. unicolor* results from haplotypes RCP, CRA, DUK, and NQP falling outside all remaining ingroup haplotypes. Haplotype COW6 (originally designated as *An. microunicolor* by Hendrixson and Bond, 2005) is sister to clade "*unicolor* 'A"' (Fig. 2).

### 3.4. Testing alternative phylogenetic hypotheses

Evolutionary and taxonomic inferences based on tree topologies can be sensitive to root placement (Graham et al., 2002). When *An. unicolor* "monophyly" was forced as a topological constraint for parsimony (combined dataset only) using PAUP*, the results did not differ statistically from our optimal tree (Wilcoxon matched-pairs signed rank test; Templeton, 1983; 10 extra steps required for constrained topology; $P = 0.3681$). In addition, we performed a Shimodaira–Hasegawa (SH) test for likelihood (Shimodaira and Hasegawa, 1999) based on the results for our Bayesian analysis following the methods outlined by Bonett and Chippindale (2004) using a GTR + I + G model of DNA substitution (as computed by Modeltest for the 28S and COI combined dataset). These results were marginally significant using an alpha value of 0.1 ($P = 0.100$). The "Filter Trees" command in PAUP* was used to assess the proportion of trees (post "burn-in") that retained *An. unicolor* as a "monophyletic" group; of 9400 total trees evaluated, there was not a single instance of genealogical exclusivity for all *An. unicolor* haplotypes.

## 4. Discussion

### 4.1. Species "paraphyly"

The phylogenetic analysis of combined COI and 28S sequence data indicates that *An. unicolor* is "paraphyletic" with respect to *An. microunicolor*. This pattern is inconsistent with currently hypothesized species boundaries based exclusively on morphology (Coyle, 1971; Hendrixson and Bond, 2005). Species-level "paraphyly"

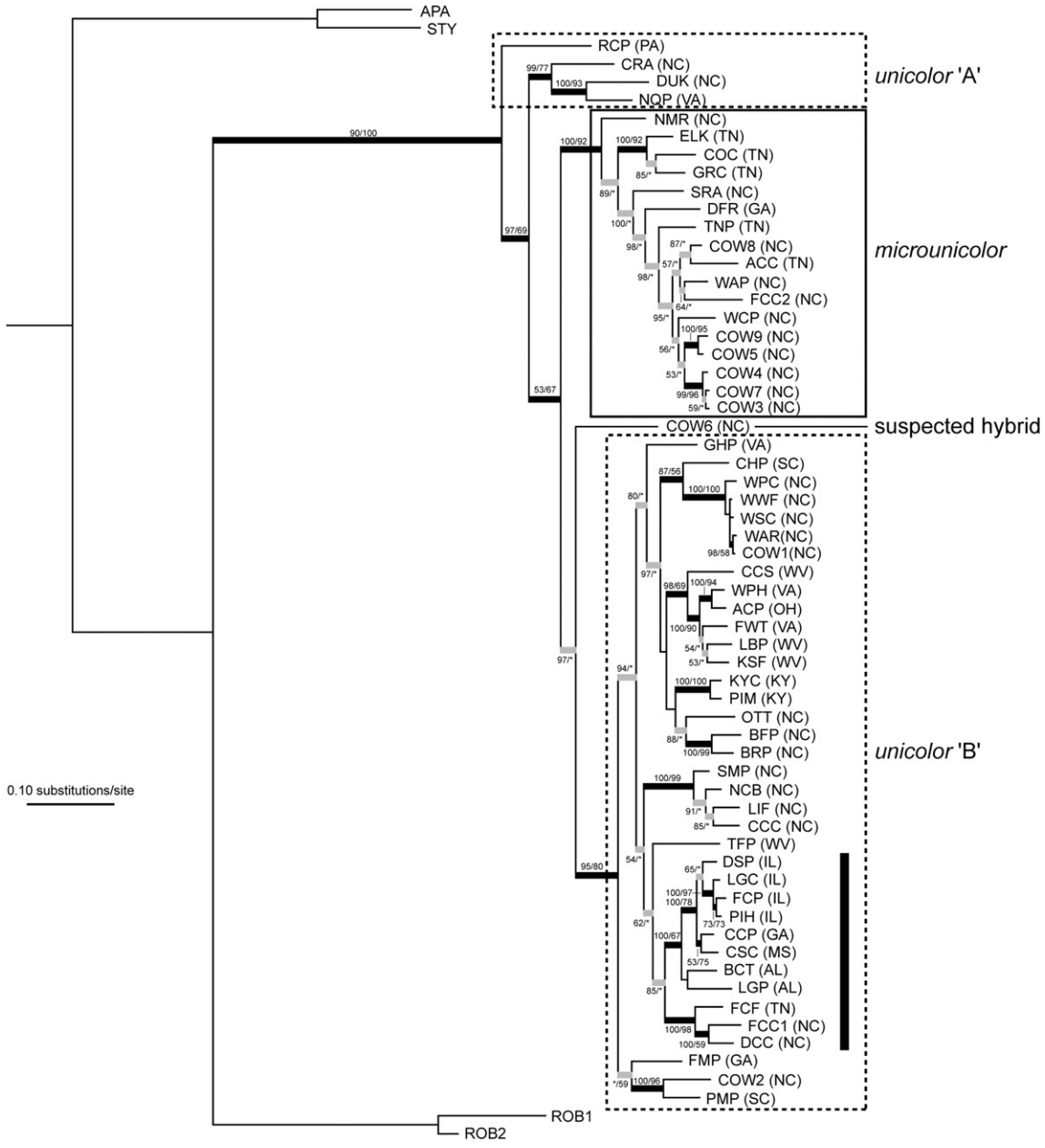


Fig. 2. Phylogenetic tree of haplotypes obtained via Bayesian analysis. Abbreviations inside parentheses designate the state from which specimens were collected. Numbers near branches correspond to posterior clade probabilities/bootstrap values (latter from parsimony analysis). Thick black lines indicate nodes supported by greater than 50% posterior clade probability *and* bootstrap value. Thick gray lines indicate nodes supported by 50% posterior clade probability *or* bootstrap value (∗, no support from particular analysis). Dotted-outlined boxes surround haplotypes of *An. unicolor*; the solid-outlined box surrounds haplotypes of *An. microunicolor*. The thick black vertical bar denotes the clade containing red-colored spiders (see text for discussion). The two remaining outgroups (*Aliatypus* spp.) were removed for presentation.

and "polyphyly" are not unusual phenomena and appear to be fairly common empirical observations. Funk and Omland (2003) found in a review of the recent literature (i.e., 1990–2002) that nearly one in every four animal species studied exhibited species-level "paraphyly" or "polyphyly." In addition, some authors estimate that 50% or more of plant species may show signs of discordant patterns between gene trees and species trees (Crisp and Chandler, 1996).

This study is not the first to document a "paraphyletic" arthropod species. Funk and Omland (2003) identified species-level "paraphyly" or "polyphyly" in 26.5% of arthropod species. When this is combined with the number of invertebrate taxa exhibiting discordant gene tree/species tree topologies, approximately 30% (nearly 1 in 3) of invertebrates show some degree of "paraphyly" or "polyphyly." For select insects, "paraphyly" has been observed in Coleoptera

(Funk, 1999; Funk et al., 1995), Diptera (Brown et al., 1996; DeSalle et al., 1987), and Lepidoptera (Beltrán et al., 2002; Brown et al., 1994). Such a pattern has also been found in similar spider species with putative limited dispersal capabilities (e.g., *Nesticus*, Hedin, 1997b; *Apomastus*, Bond, 2004). This phenomenon likely owes in part to our relative ignorance of arthropod and invertebrate diversity as compared to "higher vertebrates" (e.g., only 17% of birds and mammals show such discordance; determined from Funk and Omland, 2003).

Species-level "paraphyly" and "polyphyly" is most often attributed to improper taxonomy (Funk and Omland, 2003). However, gene tree/species tree incongruence can also be due to inadequate phylogenetic information, interspecific hybridization, incomplete lineage sorting, or unrecognized paralogy. Inadequate phylogenetic information is not a likely candidate to explain our observed case of "paraphyly" in *An. unicolor*. At least one of the major nodes of interest indicating *An. unicolor* "paraphyly" is well supported by posterior clade probabilities and bootstrap values, and no topologies examined post burn-in from the Bayesian analyses retained all haplotypes of *An. unicolor* exclusive of *An. microunicolor*. It appears that interspecific hybridization may have played a minimal role in gene tree/species incongruence, but this does not affect the overall interpretation of our data (discussed in more detail below). Species-level "paraphyly" and "polyphyly" are expected under some models of speciation (see Avise, 2000), due largely in part to incomplete lineage sorting. Mitochondrial markers are expected to sort to reciprocal monophyly four times faster than nuclear markers for reasons mentioned earlier. If we assume that COI has sorted to reciprocal monophyly, we may also assume that 28S has sorted because these two gene trees are largely congruent. Therefore, incomplete lineage sorting does not explain species-level "paraphyly" in *An. unicolor*. Finally, unrecognized paralogy can cause significant discordance between gene trees and species trees. However, the absence of stop codons in the COI data provides some indication that nuclear pseudogenes were not sequenced. Paralogy in nuclear ribosomal DNA has been demonstrated in some taxa (e.g., Hartmann et al., 2001). In this study, haplotypes obtained from specimens exhibiting multiple copies of 28S (which could indicate paralogy) generally resulted from a single indel event and were consistently recovered together in all phylogenetic analyses (not shown). This observation, in addition to congruence between the COI and 28S gene trees, suggests that paralogous genes are not responsible for observed patterns of "paraphyly". Given that the above criteria fail to adequately address species-level "paraphyly" in *An. unicolor*, imperfect taxonomy is a reasonable alternative explanation.

### 4.2. Imperfect taxonomy: An. unicolor is undersplit

Our analyses suggest that species-level "paraphyly" in the *An. unicolor* "complex" is the result of improper taxonomy. In this case, if species should be recognized as monophyletic entities, *An. unicolor* as presently defined (Hendrixson and Bond, 2005) cannot be considered a "good species." Two alternative strategies can be employed to promote phylogenetic and nomenclatural congruence: (1) synonymize *An. microunicolor* and consider all populations of *Antrodiaetus* in the Southern Appalachians a single species; or (2) elevate the lineages that render *An. unicolor* "paraphyletic" (i.e., "*unicolor* 'A'" in Fig. 2) to species status (see Omland et al., 1999; Voelker, 1999). The first option is not a desirable solution because it fails to recognize the unequivocal nature and biological distinctness of *An. microunicolor*. The latter species is clearly divergent from other populations of *An. unicolor*, particularly where they co-occur at the Coweeta LTER facility in southwestern North Carolina (Hendrixson and Bond, 2005) and adjacent areas. Accordingly, the second option for promoting phylogenetic and nomenclatural congruence appears reasonable and justified. A minimum of two nomenclatural changes would be necessary in order to impose *An. unicolor* exclusivity, one comprising haplotype RCP from the southwestern corner of Pennsylvania (Fig. 1) and the other containing haplotypes CRA, DUK, and NQP (coincidentally, these are *basal* lineages that would be recognized as individual species using the Wiens–Penkrot method). It is beyond the intent of this paper to formally address nomenclatural changes (a taxonomic revision of this species complex is in progress), but the biological uniqueness of these populations should be briefly discussed. Coyle (1971) indicated that spiders from Duke Forest, North Carolina (from which haplotype DUK was obtained) were divergent from most populations of *An. unicolor* by a number of male morphological characters. Perhaps this population should in the future be elevated and granted species status. The morphology of adult male spiders from another coastal plain population (haplotype NQP) compare favorably to those from Duke Forest (Hendrixson, unpub. data). Because adult spiders were not collected from the Cascades Recreation Area, Virginia (haplotype CRA), it is premature to speculate on their status based on morphology. However, their molecular divergence and position in our analysis indicate that further investigation is necessary. Finally, haplotype RCP was obtained from a population along the periphery of the distribution of *An. unicolor*. Based on preliminary field observations, this population appears to be separated (and perhaps isolated) from other populations by unsuitable habitat, consequently impeding or limiting migration. Habitat specialization promotes speciation by restricting gene flow between allopatric populations

(Bond et al., 2001; Hedin, 1997a,b; Hedin and Wood, 2002; Wiens, 2004a,b), thus this population may have diverged sufficiently to be warranted species status. Further studies of morphology and sampling of haplotypes from this region are required to completely address this question. For now, recognition of a "paraphyletic" *An. unicolor* must be permissible until additional studies are conducted to fully unravel species-level diversity in this complex using nested clade analysis, data from adult morphology, and notes on their natural history.

To this point, we have discussed species-level "paraphyly" in the absence of aberrant haplotype COW6. This specimen was originally assigned to *An. microunicolor* on the basis of its unusually small size and breeding season (Hendrixson and Bond, 2005). However, this same specimen possessed a macroseta on the distal aspect of metatarsus I (i.e., part of the mating clasper), a character diagnostic of *An. unicolor*. Morphology together with the position of haplotype COW6 in this phylogeny (i.e., "basal," with no geographic association to parental populations) strongly indicates that interspecific hybridization has occurred between the two species at Coweeta (Funk and Omland, 2003). However, the presence of a single hybrid does not affect the interpretation of our results or preclude us from continuing to recognize two distinct species from this area (based on morphology, ecology, and molecules). At least 200 specimens of *Antrodiaetus* have been examined from Coweeta and only one hybrid individual has been observed (Hendrixson, pers. obs.). Based on this information, hybridization appears to occur rarely and does not significantly influence our interpretation that *An. unicolor* and *An. microunicolor* remain "cohesive" species in sympatry.

### 4.3. Cryptic diversity

The molecular data we present are suggestive of a more complex set of problems in species delineation. The most compelling finding is that this phylogeny does not completely reflect or correspond to geography (i.e., haplotypes from the same or nearby localities are not always genealogically exclusive) as might be expected for a *single* species with limited dispersal capabilities (e.g., Bond et al., 2001; Hedin and Wood, 2002). The placement of haplotypes from southwestern North Carolina is noteworthy because they appear in several positions on the gene tree ("*unicolor* 'B"' in Fig. 2). This strongly suggests that multiple, molecularly divergent species are sympatric in the Southern Appalachian Mountains and that *An. unicolor* is a complex of cryptic sibling species (Bond and Sierwald, 2002; Funk and Omland, 2003; Wiens and Penkrot, 2002).

In addition to identifying conflicts regarding species boundaries in *An. unicolor* and recognizing the potential for multiple cryptic species, this molecular phylogenetic approach has provided the framework that is necessary to begin interpreting the morphological variation observed in these spiders. For example, Coyle (1971) identified several populations with lightly colored individuals from the western and southern portions of the distribution of *An. unicolor*, whereas darker forms were found elsewhere. However, in parts of the Southern Appalachian Mountains, the ranges of these two color morphs overlap considerably and some populations contain both morphs living along the same stream banks in burrows separated by a minimal distance (Coyle, 1971; Hendrixson, pers. obs.). Coyle (1971) suggested that these lightly colored spiders potentially belonged to a species of their own, but refrained from describing them as such because of the difficulty of interpreting variation across the entire range of *An. unicolor*. The current study included 11 haplotypes taken from lightly colored individuals and these were recovered as a monophyletic group (Fig. 2). These haplotypes are exclusive of haplotypes obtained from dark individuals that were collected from the same or nearby localities. This indicates that there may be a genetic basis to coloration, and also provides corroborative evidence that these lightly colored specimens are a different species (for a similar example in Lepidoptera, see Hebert et al., 2004).

Despite prior views suggesting that only one species occupied the Southern Appalachian Mountains (Coyle, 1971), it is not surprising that these spiders are appreciably more diverse (Fig. 3). This ecoregion has a rich and complicated geologic history (summarized by Fisher, 1970; Rodgers, 1971) that has provided ample opportunities for speciation, is a biodiversity "hotspot," and is among Earth's most biologically valuable because of its extraordinary biota (Irwin and Andrew, 2000; Olson and Dinerstein, 1998).

## 5. Conclusions

This study provides a very compelling example of species-level "paraphyly" and adds to a growing body of literature suggesting that traditional methods for delineating species boundaries in arthropods may underestimate *actual* species-level diversity (Bond and Sierwald, 2002, 2003; Bond et al., 2001; Hedin, 1997a, 2001; Hedin and Wood, 2002; Wilcox et al., 1997). The amount of molecular variation recovered in this study indicates a significant level of species diversity not before demonstrated by studies of morphology alone. However, despite the advantage of a molecular approach for generating hypotheses about species boundaries in *Antrodiaetus* (e.g., presence of morphologically cryptic species), arguments advocating a strict molecular approach to taxonomy (e.g., Hebert et al., 2003a,b; Tautz et al., 2003) should be viewed with caution (e.g., see Bond, 2004; Paquin and Hedin, 2004) because the relationship between morphological and molecular

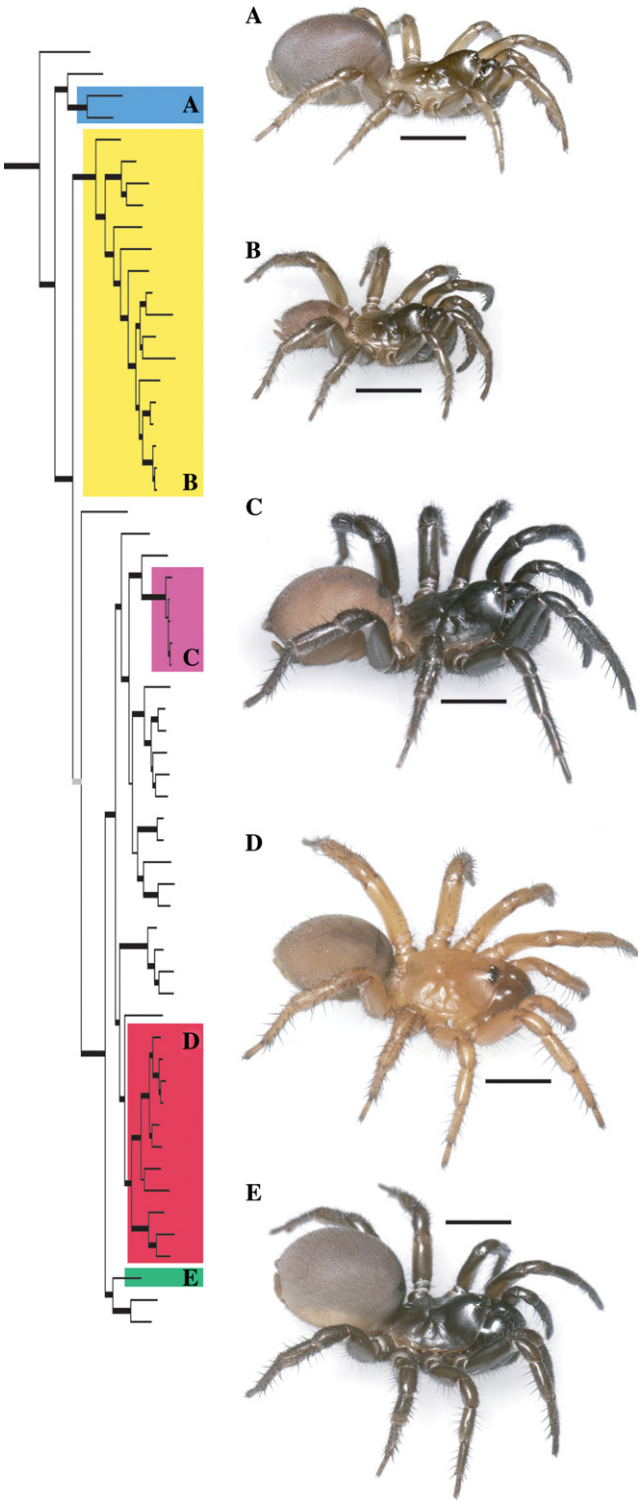


Fig. 3. Tree topology (same as Fig. 2) showing representative color habitus photographs of adult female *Antrodiaetus* spp.: (A) *An. 'unicolor'* from the coastal plain (VA: York Co.); (B) *An. microunicolor* from the Coweeta LTER site (NC: Macon Co.); (C) *An. unicolor* from the Coweeta LTER site (NC: Macon Co.); (D) reddish-colored *An. unicolor* (TN: Sequatchie Co.; individuals from this population were not included in this study); and (E) *An. unicolor* (GA: Murray Co.). Scale bars = 5 mm.

taxonomy is not always straightforward. Conflicting results must be addressed and explored in greater detail. As agreeably stated, "molecular phylogenetic analyses do not free systematists from a thorough inclusion of morphological and ecological data" (Kiefer et al., 2002). This certainly holds true for the *An. unicolor* species complex; further data are required to place confidence in where to "draw species boundaries" in this group. Several lines of evidence support the validity of *An. microunicolor* (e.g., morphology, genealogical exclusivity, breeding behavior at Coweeta), but we cannot place the same confidence in *An. unicolor* (sensu lato) at this time. A total-evidence approach including data from morphology, molecules (multiple genes), natural history, and geography should be advocated as the most robust approach to delimiting species boundaries (Bond, 2004; Bond and Sierwald, 2002, 2003; Bond et al., 2001; Doan and Castoe, 2003; Hebert et al., 2004; Kiefer et al., 2002; Lee, 2004; Lipscomb et al., 2003; Paquin and Hedin, 2004; Puorto et al., 2001).

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