Zootaxa 3814 (2): 177–201
www.mapress.com/zootaxa/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

http://dx.doi.org/10.11646/zootaxa.3814.2.2
http://zoobank.org/urn:lsid:zoobank.org:pub:185ED0AE-5571-4656-96E1-291E86FEB52D

Descriptions of two new, cryptic species of *Metasiro* (Arachnida: Opiliones: Cyphophthalmi: Neogoveidae) from South Carolina, USA, including a discussion of mitochondrial mutation rates

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Abstract

Specimens of *Metasiro* from its three known disjunct population centers in the southeastern US were examined and had a 769 bp fragement of the mitochondrial gene cytochrome *c* oxidase subunit I (COI) sequenced. These populations are located in the western panhandle of Florida and nearby areas of Georgia, in the Savannah River delta of South Carolina, and on Sassafras Mt. in South Carolina. This range extends over as much as 500 km, which is very large for a species of cyphophthalmid harvestmen and presents a degree of physical separation among populations such that we would expect them to actually be distinguishable species. We examined the morphology, including the spermatopositors of males, and sequences from 221 specimens. We found no discernible differences in the morphologies of specimens from the different populations, but corrected pairwise distances of COI were about 15% among the three population centers. We also analyzed COI data using a General Mixed Yule Coalescent (GMYC) model implemented in the R package SPLITS; with a single threshold, the most likely model had four species within *Metasiro*. Given this level of molecular divergence, the monophyly of the population haplotypes, and the number of exclusive COI nucleotide and amino acid differences distinguishing the populations, we here raise the Savannah River and Sassafras Mt. populations to species status: *M. savannahensis* **sp. nov.**, and *M. sassafrasensis* **sp. nov.**, respectively. This restricts *M. americanus* (Davis, 1933) to just the Lower Chattahoochee Watershed, which in this study includes populations along the Apalachicola River and around Florida Caverns State Park. GMYC models reconstructed the two main haplotype clades within *M. americanus* as different species, but they are not exclusive to different areas. We estimate COI percent divergence rates in certain cyphophthalmid groups and discuss problems with historical measures of this rate. We hypothesize that *Metasiro* began diversifying over 20 million years ago.

Key words: Sassafras Mountain, Savannah River, harvestmen, DNA taxonomy, barcoding

Introduction

Cyphophthalmid species typically have ranges of only a few kilometers and are distinguishable by subtle body proportions and genitalic differences, even when closely related (Giribet 2000; Giribet *et al.* 2012a). This feature appears to be a result of exceptionally poor dispersal abilities, and, when combined with their old age, allows them a unique role in testing hypotheses of historical landmass movements (see, for example, Boyer & Giribet 2007; Boyer *et al.* 2007b; Clouse & Giribet 2010; Giribet *et al.* 2012a; and Murienne *et al.* 2009). With an increase in cyphophthalmid sequence data, it has been possible to sort species with more confidence, as small differences in morphology and geographical location are usually accompanied by large numbers of molecular synapomorphies. In cases where specimens have been included in molecular phylogenies before a thorough morphological examination could be completed, sequence divergences have been used as a preliminary guide to species diversity (Clouse 2012).

In this context we investigated the strangely widespread US cyphophthalmid species *Metasiso americanus*, which lives in the western panhandle of Florida and nearby areas of Georgia, Sassafras Mountain in the Southern

Accepted by P. Schwendinger: 19 Apr. 2014; published: 9 Jun. 2014

Appalachians of South Carolina, and in the Savannah River Delta area of South Carolina. This range is enormous for a cyphophthalmid, and it has seemed likely that detailed morphological data and DNA sequences would reveal three or more species corresponding to the different localities. The first sequences obtained from across this range (one specimen each from Sassafras Mt., Savannah, and Florida) showed large differences in COI sequences but no differences in over 3,500 bp of nuclear ribosomal sequence (2,041 bp of 28S rRNA and 1,763 bp of 18S rRNA; single bp differences in each reported earlier now recognized as miscalls) nor any obvious morphological differences. Thus, we here reassess the taxonomic status of what has previously been included in *M. americanus*, using a larger number of specimens from each locality and a more detailed morphological examination than in previous published examinations of the genus (Boyer *et al.* 2007b; Davis 1933; de Bivort and Giribet 2004; Giribet *et al.* 2012a).

Material and methods

Specimen collection. *Metasiro* collections were assembled from the major known localities, beginning with the type locality in Torreya State Park, as well as other sites along the Apalachicola River (Table 1). In addition, three other sites from where no specimens were collected in many years were sampled to obtain material for molecular study. These are Florida Caverns State Park in Jackson County, Florida (last sampled by S. B. Peck in 1981); a few miles north of the Georgia border in Jasper Co., South Carolina, now part of the Savannah River National Wildlife Refuge (last sampled by R. Norton in 1974); and Sassafras Mountain, 750 m high in Pickens Co., South Carolina (sampled once by S. B. Peck and A. Fiske in 1969). Collections have been made in Georgia just north of the Florida border, in Grady Co. by W. Sober in 1965 and in Decatur Co. by P. Kovarik, but we did not have sufficiently specific enough information to identify recollection sites.

Specimens were collected by sifting leaf litter into white pans, which were then examined for live animals. These were then preserved in 95% EtOH and kept cool. Specimens are deposited in the Museum of Comparative Zoology (MCZ), Harvard University, and data are accessible in the MCZbase (http://mczbase.mcz.harvard.edu). Each collection has two accession code numbers, an original one used through the course of this study (prefix "MCZ DNA) and a more recent code from the new MCZ cataloging system (prefix "Invertebrate Zoology, here shortened to "IZ-"). Each specimen within a collection was also entered into the Giribet Lab Biota database (Colwell 2012) for Cyphophthalmi and received a unique specimen number (prefix "SPM"). Individual specimens are reported by a shortened combination of the collection and specimen numbers (e.g., "IZ-134535(105644)-7171" corresponds to MCZ Invertebrate Zoology 134535, MCZ DNA105644, and Biota specimen SPM007171), or, for greater readability in some sections, just the shortened specimen numbers, the collection numbers for which can be seen in Table 1.

DNA sequencing and analysis. DNA was extracted using the Qiagen DNeasy tissue kit (Qiagen, Valencia, CA, USA) and amplified using illustra Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, UK). Mitochondrial fragments of cytochrome *c* oxidase subunit I (COI) were amplified and sequenced at 45–47˚C (usually 46˚C) using the primers listed in Table 2. So that there were no missing data, and because some specimens were amplified using longer primers, the first 42 bases after the primer LCO and the last 4 bases before the reverse primer HCOoutout were removed.

Phylogenetic analyses were conducted under the parsimony optimality criterion in POY4 (Varón *et al.* 2009), with the data read as prealigned (as it had no length variation), transversion costs set at 2, and transition costs set at 1. The resulting tree was read into TNT (Goloboff *et al.* 2008), and both the nucleotide and amino-acid sequences were optimized on it. TNT's report of synapomorphies was used as a guide to finding unique characters for each species. Some reconstructed states had back mutations, which were rejected, and those which were unique in each species, the autapomorphies, were found manually. The data were also read by the program SPIDER (Brown *et al.* 2012), which is implemented in the R statistical package (R Core Team 2012); this was used to assist in quantifying and visualizing the distribution of pairwise COI distances. We exported two kinds of distances from SPIDER, ones corrected using the Kimura 2-parameter model (the default output) and uncorrected *p*-distances (using the argument [model="raw"] when creating the distance matrix).

TABLE 1. MCZ accession numbers, original MCZ accession numbers, specimen identification numbers, GenBank accession codes for COI sequences, and localities for all specimens examined in this study. MCZ and original collection accession numbers are for whole collections, which consist of various numbers of specimens (seen in the range of Biota specimen numbers). Not all specimens from each collection were sequenced, thus the smaller range of GenBank numbers.

Species	MCZbase accession no.	Original MCZ accession no.	Biota specimen numbers	GenBank	Location	Latitide, Longitude
M. americanus	IZ-133791	DNA103805	SPM006432–6	KJ405965–8	Torreya State Park, Liberty Co., Florida, USA	30.568, -84.95167
"	IZ-133792	DNA103806	SPM006437–53	KJ405969–76	"	"
"	IZ-133796	DNA103810	SPM006491	–	"	"
"	IZ-133797	DNA103811	SPM006492–505	KJ405986–992	"	"
"	IZ-133798	DNA103812	SPM006506		"	"
"	IZ-134557	DNA101532	SPM006379–427	KJ405955–964	"	30.56472, -84.95139
"	IZ-134558	DNA101533	SPM006428–31	–	"	"
"	IZ-133793	DNA103807	SPM006454–6	KJ405977	Liberty Co., Florida, USA	30.52558, -84.93667
"	IZ-133794	DNA103808	SPM006457–9	KJ405978–80	"	"
"	IZ-133795	DNA103809	SPM006460–90	KJ405981–85	"	"
"	IZ-134492	DNA103814	SPM006522–55	KJ406000–009	Chattahoochee, Gadsden Co., Florida, USA	30.70232, -84.82556
"	IZ-134493	DNA103813	SPM006507–21	KJ405993–999	Flat Creek, Gadsden Co., Florida, USA	30.62793, -84.83528
"	IZ-133808	DNA105654	SPM007369–88	KJ406107–26	Florida Caverns State Park, Jackson Co., Florida, USA	30.82119, -85.24639
"	IZ-133809	DNA105655	SPM007389–98	KJ406127–36	"	"
"	IZ-133810	DNA105656	SPM007399–405	KJ406137–43	"	"
"	IZ-133811	DNA105657	SPM007406–415	KJ406144–53	"	"
"	IZ-133812	DNA105662	SPM007416–35	KJ406154–163	"	30.81433, -85.24667
"	IZ-133813	DNA105663	SPM007436–68	KJ406164–175	"	30.81408, -85.24722
M. savannahensis **sp. nov.**	IZ-133799	DNA 105645	SPM007184–213	KJ406022–30	Kingfisher Pond, Savannah National Wildlife Refuge, Jasper Co., South Carolina, USA	32.19, -81.08*
"	IZ-133800	DNA 105646	SPM007214–229	KJ406031–40	"	"
"	IZ-133801	DNA 105647	SPM007230–239	KJ406041–9	"	"
"	IZ-133802	DNA 105648	SPM007240–265	KJ406050–8	"	"
"	IZ-133803	DNA 105649	SPM007266–294	KJ406059–67	"	"
"	IZ-133804	DNA 105650	SPM007295–314	KJ406068–77	"	"
"	IZ-133805	DNA 105651	SPM007315–334	KJ406078–87	"	"
"	IZ-133806	DNA 105652	SPM007335–354	KJ406088–96	"	"
"	IZ-133807	DNA 105653	SPM007355–368	KJ406097–106	"	"
M. sassafrasensis **sp. nov.**	IZ-134534	DNA105643	SPM007168–70	KJ406010–2	Jocosse Gorge, Sassafras Mt., Pickens Co., South Carolina, USA	35.06228, -82.795
"	IZ-134535	DNA105644	SPM007171–83	KJ406013–21	"	"

*Coordinates differ slightly among accession numbers IZ-133799–807, which were collected within 100 m of each other. These differences are mostly due to the error in GPS readings and are here treated as the same locality.

TABLE 2. Primers used in this study to amplify COI, written 5'to 3'.

Primer name	Reference	Sequence (5' to 3')
forward		
LCO1490	(Folmer *et al.* 1994)	GGT CAA CAA ATC ATA AAG ATA TTG G
LCOdeg1		TCH ACD AAY CAY AAA GAT ATT GGW ACW AT
LCOMeta2		KKT CAA CGA ATC ATA AAG ATA TTG G
LCOMeta8		TKK TCA ACG AAT CAT AAA GAT ATT
reverse		
HCOoutout	(Prendini, 2005)	GTA AAT ATA TGR TGD GCT C
HCOooOpi2		GTA AAT ATA TGR TGD GCT CAT AC
HCOoutMeta		ATA GTG ACA ATA AAA TTA ATA GCT CC

The phylogeny recovered from POY was also read into BEAST (Drummond & Rambaut 2007), where the COI branch lengths were optimized and the tree made ultrametric using an uncorrelated relaxed clock and GTR substitution model. The swapping operators were manually removed from the xml input file to maintain the tree topology, and five runs of 10,000,000 generations were carried out on the CIPRES computing cluster (Miller *et al.* 2010). The run which best achieved stability (highest ESSs) was used to generate the maximum clade credibility tree with mean node heights after a burnin of 1,000,000. This tree was then read into the SPLITS package (v. 3.0.2) (Ezard *et al.* 2009), which is implemented in the R statistical package (R Core Team 2012). This program traces the number of lineages that appear over time within a single locus and compares this to a gene tree built from the coalescent. It then tests whether the accumulation of lineages through time can be better explained by a model that incorporates both species and populations (a General Mixed Yule Coalescent, GMYC, model) (Fujisawa & Barraclough 2013). Options include using a GMYC model that has only one speciation threshold or multiple, and a scaling parameter that defines a per lineage branching rate (constant, accelerating, or decelerating).

Imaging and measurements. Color photographs were made using a complete Leica imaging system (www.leica-microsystems.com), including an M125 optic carrier, MC170 HD camera and SW kit, and LAS Image Builder software. Scanning electron micrographs (SEMs) were done on a JEOL 6480 microscope after coating with gold in a Denton desktop sputtering system. Spermatopositors were embedded in glycerine gel and the dorsal and lateral views photographed on a Laser Scanning Confocal Microscope (LCSM) (Zeiss LSM 710 Axiovert Z.1) as described in Clouse (2012). Ventral views of the spermatopositors were photographed at a single focal plane through a compound microscope. The depth of the chelicer was measured just proximal to the dorsal crest and following the outer ridge, and tarsal depths were measured at their deepest points. Cuticular sculpturing follows the terminology of Murphree (1988). Appendage and article measurements were not taken from holotypes, as the legs are prone to breaking between the trochanter and femur, and we wanted to keep the holotypes intact; appendage measurements were taken from paratypes.

Results

Molecular analysis

We recovered a 769 bp fragment of COI from 221 individuals of *Metasiro*, and this translated to a sequence of 256 amino acids (using the second reading frame and trimming all sequences to the same length). This amino acid sequence began 43 nucleotide base pairs after the forward primer LCO with threonine, alanine (proline in one specimen of *M. americanus*), methionine, and serine. We found four haplotypes from the Sassafras Mt. specimens (hereafter *M. sassafrasensis* **sp. nov.**), 23 haplotypes from the Savannah River delta specimens (hereafter *M. savannahensis* **sp. nov.**), and 30 haplotypes from the Florida populations (*M. americanus*). Each species was found to have a large number of exclusive nucleotide identities and 1–6 exclusive amino acid identities (Table 3). Sequences have been deposited in GenBank under accession numbers KJ405955–KJ406175 (Table 1).

TABLE 3. Locations and identities of unique nucleotides (nuc.) and amino acids (a.a.) in the 221 *Metasiro* COI sequences obtained here. Nucleotide positions marked with an asterisk are distinct in each species and can be used to diagnose all three. Amino acid position 115 in *M. americanus* has two possible states, methionine or threonine, the latter of which was found in only one individual.

M. americanus (n=124)				*M. savannahensis* **sp. nov.** (n=85)				*M. sassafrasensis* **sp. nov.** (n=12)			
nuc.		a.a.		nuc.		a.a.		nuc.		a.a.	
79	T			13	C			14	A		
127	T			68	T			22	T		
158	A	53	I	100	T			28	C		
163	C			112	C			43	T		
178	A			121	C			85	C		
196	C			130	C	43	I	91	C		
247	C			145	C			97	T		
253	T			184	C			115	T		
286*	A			190	T			142	G		
307	C			202	G			259	C	87	V
340*	A/G			239	A	80	T	260	G		
344	A	115	M(T)	242	T			262	T		
364	C			256	T			286*	T		
370*	A			271	T			289	G		
443	C			274	G			310	T		
458	T			283	A			313	C		
484*	T			286*	G			325	C		
500	C			340*	T			328	C		
502	T			370*	T			340*	C		
514	C			373	G			349	T		
517	G			394	A			358	C		
520	A			406	G	135	M	361	C		
562	A			433	A			370*	C		
631	C			435	A	145	N	451	G		
667	C			460	C			454	C		
700*	T			484*	A			457	T		
712	C			488	G	163	V	484*	C		
715	C			508	T			490	C		
739	C			544	C			532	A		
743	C/G	248	V	577	T/A			553	C		
				598	C			599	C		
				611	C			601	C		
				625	C			628	C		
				634	T			637	G		
				640	T			676	A		
				655	C			700*	C		

.....continued on the next page

TABLE 3. (Continued)

M. americanus (n=124)		*M. savannahensis* **sp. nov.** (n=85)				*M. sassafrasensis* **sp. nov.** (n=12)			
nuc.	a.a.	nuc.		a.a.		nuc.		a.a.	
		664	C			751	C		
		688	T			766	T		
		691	C						
		694	A	231	Q				
		700*	G						
		721	T/C						
		736	C						
		742	G						
		745	T						
		755	T						
		758	T						

Corrected distances for COI were, on average, 0.1% and 0.4% within *M. savannahensis* **sp. nov.** and *M. sassafrasensis* **sp. nov.**, respectively, and 4.0% within *M. americanus* (Fig. 1; Table 4); these were not notably different from uncorrected distances. The larger percentage divergence in *M. americanus* corresponds with the larger geographic range from which its specimens were collected (50 km vs. less that 100 m for the other two). Among the different species, corrected pairwise distances were around 15%; the closest species were *M. sassafrasensis* **sp. nov.** and *M. americanus*, with an average divergence of 13.1%. Uncorrected distances among the species ranged from 12–14%.

TABLE 4. Corrected COI pairwise divergences within and between species of *Metasiro*.

Species pairwise comparisons	Geographic max. distance	Corrected COI divergence (%) average	range
M. americanus	50 km	4.0	0–8.2
M. savannahensis **sp. nov.**	80 m	0.1	0–0.8
M. sassafrasensis **sp. nov.**	1 m	0.4	0–0.8
M. americanus / *M. savannahensis* **sp. nov.**	400 km	15.6	14.0–16.8
M. savannahensis **sp. nov.** / *M. sassafrasensis* **sp. nov.**	350 km	15.2	14.8–16.0
M. sassafrasensis **sp. nov.** / *M. americanus*	500 km	13.1	12.1–14.0

Analysis of the lineage-through-time plot derived from the ultrametric tree produced by BEAST suggested highly significant improvement to the evolutionary model when there were four clusters (species) that arose after a single threshold, and when the branching rate was allowed to accelerate toward the tips (scaling factor = 5–10) (Fig. 2). When the scaling factor was lower (the default, 0–5), four species were still inferred, but it was only marginally significant (p = 0.095). Using either the low (0–5) or high (5–10) scaling factor with multiple thresholds, the GMYC model showed significant improvement after species splits, but reported an implausible 32 species with very young origins; this is consistent with the finding that single-threshold analyses in this package outperform those using multiple thresholds (Fujisawa & Barraclough 2013). Two of the four putative species originally reported from the single-threshold analysis are not reciprocally monophyletic with respect to locality and are still considered a single species, *M. americanus*.

We could not discern a gap in morphological character states or measurements among the three species, and thus we offer here no guidance on distinguishing these species morphologically. We present some of the SEMs we examined and images of genitalia to demonstrate this interspecific similarity and illustrate some interesting morphological details common to all three (Figs. 3–13). Scanning electron mircographs of *M. americanus* can also

be found in de Bivort and Giribet (2004) and Giribet and Kury (2007). Despite the lack of distinguishing morphological characters, we name two new species in *Metasiro* based on the extent of their distinct molecular characters.

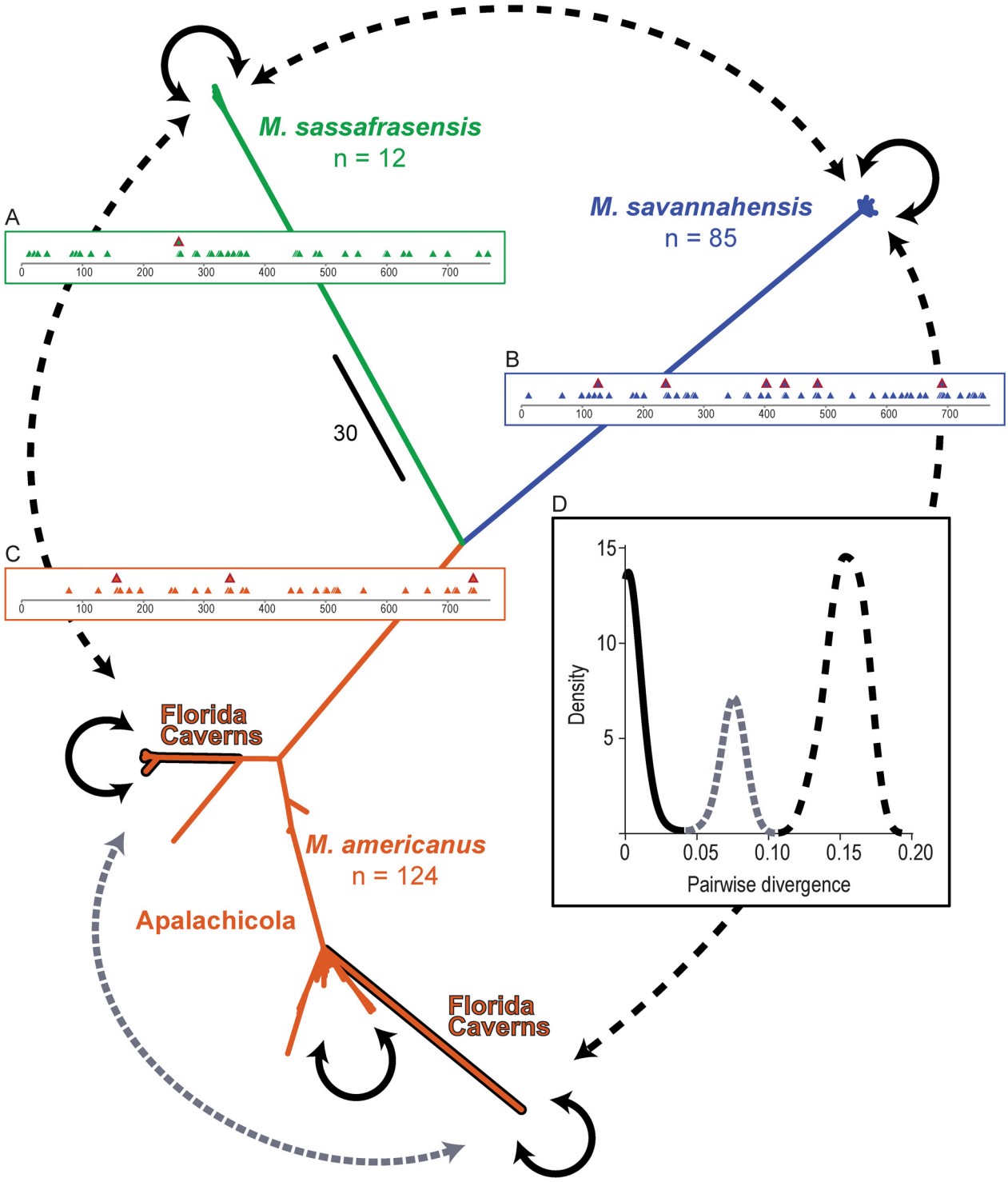

FIGURE 1. Unrooted phylogeny for all *Metasiro* COI sequences, found under parsimony in POY and showing parsimony branch lengths. Insets A, B, and C show the locations of unique nucleotides (smaller, lower triangles) and amino acids (larger, upper triangles) along the 769 bp COI fragment. The frequencies of corrected pairwise divergences are shown in inset D and are divided by the type of comparison: solid black for intraspecific distances, dashed black for interspecific distances, and dashed gray for distances among widespread populations of *M. americanus* in the Florida panhandle.

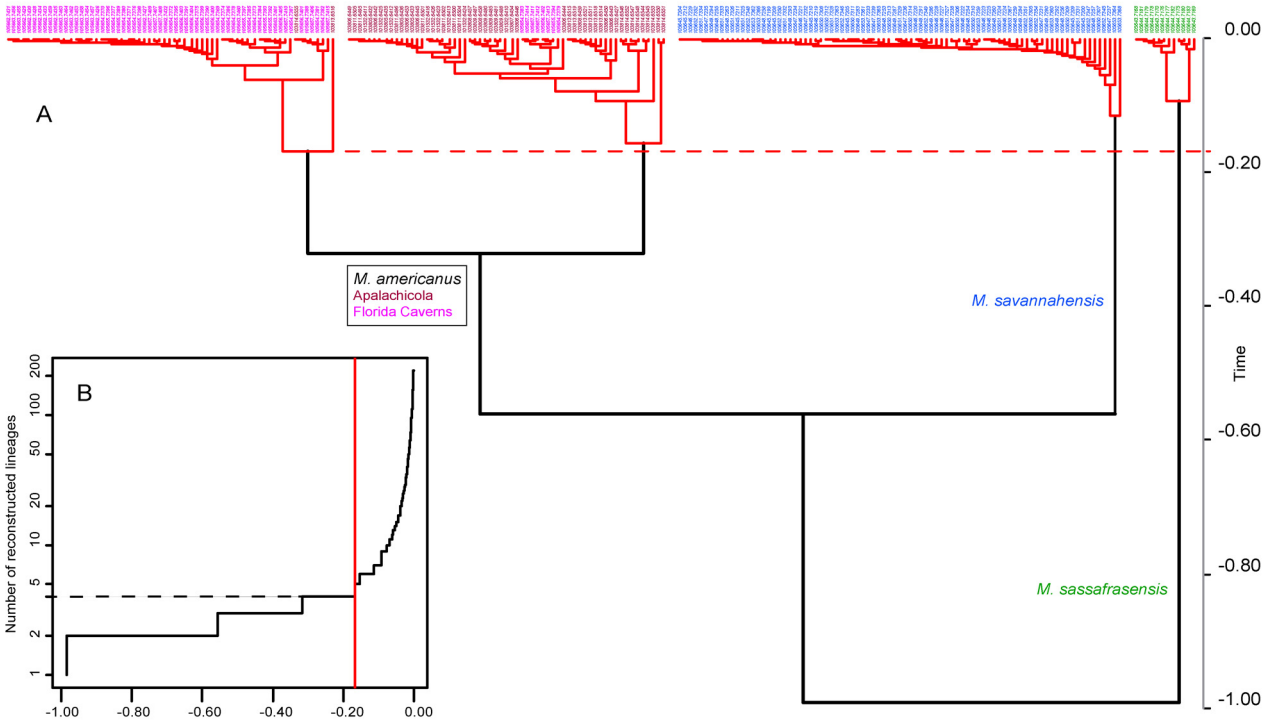

FIGURE 2. A, molecular phylogeny of *Metasiro* COI sequences, made ultrametric using a relaxed clock, showing the four putative species from the GMYC model (red clades above the dashed red line); B, lineage-through-time plot for *Metasiro* diversification, the red line indicating the inferred time of rapid within-species COI diversification resulting from a single-threshold analysis. Time for all plots is expressed as the proportion of time since the present to the root. Specimens of *M. americanus* collected from the Apalachicola River region and Florida Caverns are colored by location.

Taxonomy

Metasiro americanus (Davis, 1933)

(Figs. 3A–C; 4A, D, G, J; 6A; 10E, F, I; 11E, F; 12B)

Siro americanus Davis, 1933
Parasiro americanus Hinton, 1938
Metasiro americanus Juberthie, 1960
Floridogovea americana Hoffman, 1963

Taxonomic notes: Giribet (2000) considered the synonymization of *Metasiro* and *Floridogovea* to have been done by Shear (1980), who noted the latter genus name but did not adopt it. Giribet and Kury (2007) formalized this synonymization and recombined *Metasiro* out of Sironidae into Neogoveidae. Shear (1980) examined the Sassafras Mt. collections by S. Peck and A. Fiske, and determined those specimens to be *M. americanus*, but the same has not been done with the Savannah River specimens, other than informally. Discussions and illustrations of *M. americanus* morphology can be found in these references, as well as in the original description, and as far as we can determine, they apply equally to all three species. Body and appendage article measurements are in Tables 5–6, respectively, and appendage article relative sizes are compared to similar data from Davis (1933) in Table 7.

Material examined: Males (n=112), females (n=131), and juveniles (n=74) collected at the type locality of Torreya State Park in the Florida panhandle (IZ-133791–2, IZ-133796–8, IZ-134557–8), nearby localities east of the Apalachicola River (IZ-133793–5, IZ-134492–3), and at Florida Caverns State Park (IZ-133808–13). Two specimens from collection IZ-133812 were dissected for genitalia (SPM007418 and 7420), the latter of which was disarticulated for appendage measurements and SEMs. A total of 124 specimens covering all localities were sequenced for molecular analysis.

Diagnostic molecular characters: In our COI amino acid fragment (described above) no. 53 is isoleucine (not valine), no. 115 is methionine (rarely threonine; not leucine), and no. 248 is valine or leucine (not isoleucine). See Table 3.

TABLE 5. Body lengths, widths (in mm) and proportions for *M. sassafrasensis* **sp. nov.** (n=3), *M. savannahensis* **sp. nov.** (n=4), and *M. americanus* (n=3). The rightmost column shows the range size in values for males across all three species.

	M. americanus			*M. savannahensis* **sp. nov.**				*M. sassafrasensis* **sp. nov.**			
	male	male	male holotype	male holotype	male paratype	male paratype	female paratype	male holotype	male paratype	female paratype	
	105662-7420	105663-7436	(from Davis, 1933)	105645-7184	105645-7185	105646-7221	105646-7222	105644-7171	105644-7176	105644-7180	range size (males)
Length	2.0	1.8	2.0	1.9	1.9	2.1	2.1	1.9	2.2	2.3	0.4
Width across widest part	1.2	1.1		1.0	1.2	1.1	1.1	1.0	1.2	1.3	0.2
Width across ozophores	1.0	1.0		1.0	1.0	1.0	1.0	0.9	1.1	1.1	0.2
Length / ozophore width	2.0	1.9		1.9	1.9	2.0	2.1	2.0	2.0	2.1	0.1

FIGURE 3. Male specimens of *Metasiro*, showing dorsal (left column), lateral (middle), and ventral (right) views: A–C, *M. americanus* specimen IZ-133813(105663)-7436; D–F, *M. savannahensis* **sp. nov.** holotype, specimen IZ-133799(105645)-7184; and G–I, *M. sassafrasenesis* **sp. nov.** holotype, specimen IZ-134535(105644)-7171.

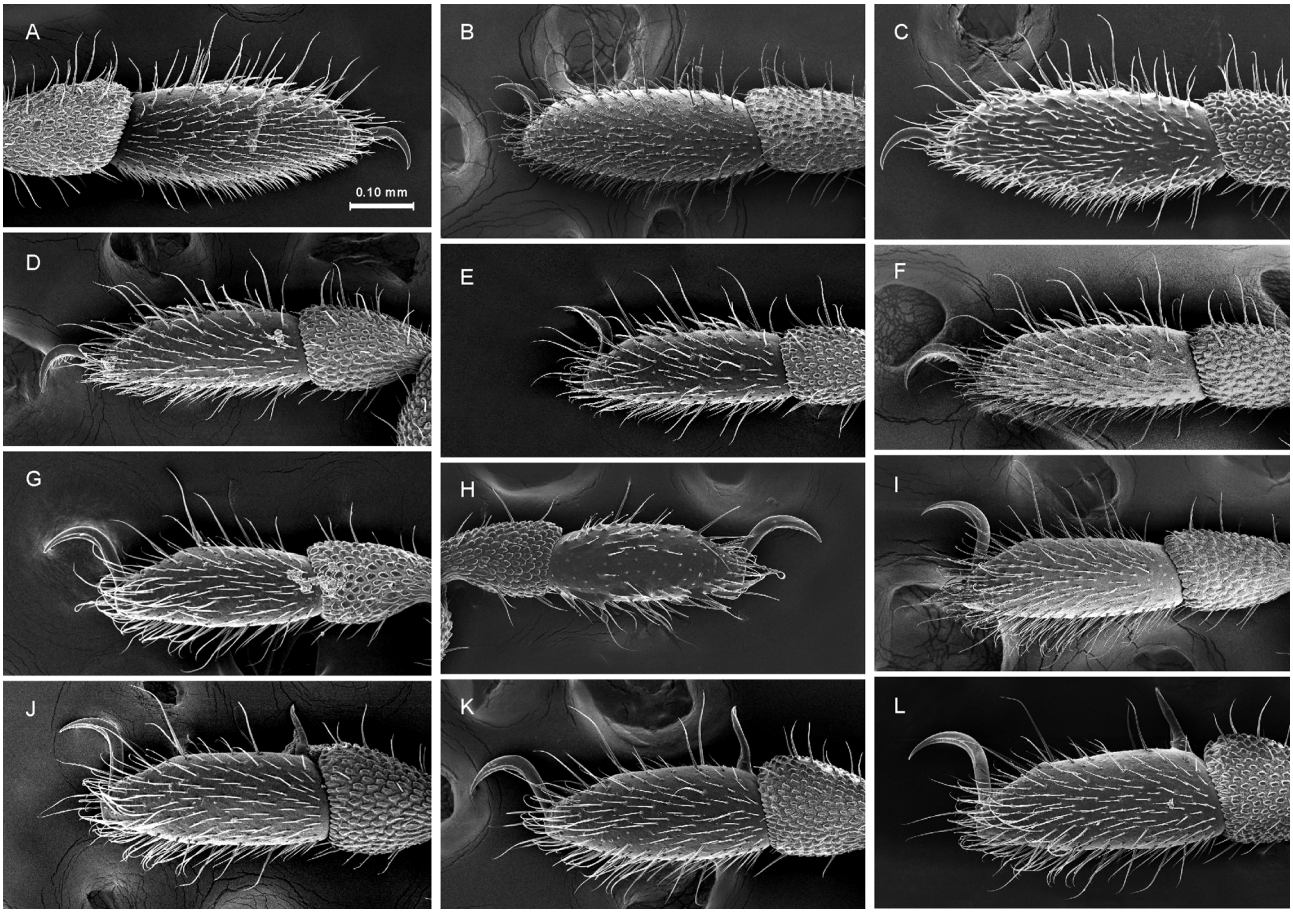

FIGURE 4. Tarsi I (A-C), II (D-F), III (G-I), IV (J-L) for *M. americanus* specimen IZ-133812(105662)-7420 (left column), *M. savannahensis* **sp. nov.** specimen IZ-133799(105645)-7185 (median column), and *M. sassafrasensis* **sp. nov.** specimen IZ-134535(105644)-7176 (right column).

TABLE 6. Appendage and article lengths (in mm) and length / length-to-depth ratios, both values separated by forward dashes, for one specimen each of *M. sassafrasensis* **sp. nov.**, *M. savannahensis* **sp. nov.**, and *M. americanus* (plus values for the holotype taken from literature). The rightmost column shows the range size in length values across all three species.

		M. americanus		*M. savannahensis* **sp. nov.**	*M. sassafrasensis* **sp. nov.**	
		male	male	male	male	
		-	holotype	paratype	paratype	
		7420	(from Davis, 1933)	7185	7176	range size (lengths)
Chelicer	I (whole)	0.6 / 2.9		0.7 / 3.3	0.7 / 3.3	0.1
	I (from crest)	0.4 / 1.9		0.4 / 1.9	0.4 / 2.0	0
	II	0.8 / 5.0		0.8 / 5.0	0.9 / 5.6	0.1
	III	0.2 / 5.7		0.2 / 4.9	0.2 / 4.1	0
	Total length	1.6	1.4	1.7	1.8	0.4
Palp	Trochanter	0.3 / 3.6		0.2 / 2.9	0.3 / 3.4	0.1
	II	0.4 / 5.0		0.4 / 5.0	0.4 / 5.7	0
	III	0.2 / 3.4		0.2 / 3.0	0.3 / 3.2	0.1
	IV	0.3 / 4.0		0.3 / 4.0	0.3 / 3.9	0

.....continued on the next page

TABLE 6. (Continued)

		M. americanus		*M. savannahensis* **sp. nov.**	*M. sassafrasensis* **sp. nov.**	
	Tarsus	0.3 / 3.8		0.3 / 3.3	0.3 / 3.7	0
	Total length	1.5	1.6	1.4	1.6	0.2
Leg I	Trochanter	0.2 / 1.1		/	0.2 / 1.0	/
	Femur	0.5 / 2.9		0.5 / 3.3	0.6 / 3.5	0.1
	Patella	0.3 / 1.7		0.3 / 1.5	0.3 / 1.8	0
	Tibia	0.3 / 2.1		0.3 / 2.2	0.4 / 2.3	0.1
	Metatarsus	0.3 / 1.8		0.3 / 1.9	0.3 / 1.9	0
	Tarsus	0.4 / 2.2		0.4 / 2.3	0.4 / 2.3	0
	Total length	2.0	2.0	~2.0	2.2	0.2
Leg II	Trochanter	0.2 / 1.3		0.2 / 1.1	0.2 / 1.3	0
	Femur	0.4 / 2.7		0.4 / 2.9	0.5 / 2.8	0.1
	Patella	0.2 / 1.5		0.2 / 1.4	0.3 / 1.4	0.1
	Tibia	0.3 / 1.6		0.2 / 1.5	0.4 / 2.1	0.2
	Metatarsus	0.2 / 1.5		0.2 / 1.7	0.2 / 1.7	0
	Tarsus	0.3 / 2.3		0.3 / 2.3	0.4 / 2.5	0.1
	Total length	1.6	1.8	1.5	2.0	0.5
Leg III	Trochanter	/		0.2 / 1.0	/	/
	Femur	0.3 / 2.2		0.3 / 2.1	0.4 / 2.8	0.1
	Patella	0.2 / 1.3		0.2 / 1.5	0.2 / 1.4	0
	Tibia	0.2 / 1.4		0.3 / 1.6	0.3 / 1.8	0.1
	Metatarsus	0.2 / 1.7		0.2 / 1.7	0.3 / 1.8	0.1
	Tarsus	0.3 / 2.6		0.3 / 2.2	0.4 / 2.8	0.1
	Total length	1.4	1.7	1.5	~ 1.8	0.4
Leg IV	Trochanter	/		/	0.2 / 1.4	/
	Femur	0.4 / 2.6		0.4 / 2.7	0.6 / 3.0	0.2
	Patella	0.3 / 1.4		0.3 / 1.6	0.3 / 1.6	0
	Tibia	0.3 / 1.5		0.3 / 1.8	0.4 / 1.9	0.1
	Metatarsus	0.3 / 1.4		0.3 / 1.6	0.3 / 1.5	0
	Tarsus	0.3 / 2.1		0.4 / 2.3	0.4 / 2.5	0.1
	Total length	~1.8	1.9	~1.9	2.2	0.4

***Metasiro savannahensis* sp. nov.**
(Figs. 3D–F; 4B, E, H, K; 6B, C; 7; 8; 10C, D, H; 11C, D; 12A; 13B)

Metasiro americanus (Davis, 1933), partim

Material examined. Males (n=62), females (n=72), and juveniles (n=51), with the following collection details: Jasper County, South Carolina, USA, at Kingfisher Pond, Savannah National Wildlife Refuge, 300 m south of parking lot (lat. 32.18923, long. -81.08008, elev. 3 m), leg. P. Sharma and R. Clouse, 16 March 2010. *Holotype*, male, specimen IZ-133799(105645)-7184. *Paratypes*, from collections IZ-133799–807, 6 males (SPM007215, 7230, 7240, 7266, 7295, 7315) and 7 females (SPM007191, 7222, 7234, 7252, 7275, 7301, 7322); two males dissected for genitalia (SPM007185 and 7335), SPM007185 disarticulated for appendage measurements and SEMs, and SPM7214 and 7221–3 mounted for dorsal and ventral SEMs. A total of 85 specimens were sequenced for molecular analysis. All specimens are deposited in MCZ.

Description. Morphology as in the original description of *M. americanus* (Davis, 1933), with the following recap of characters and additional details. Body and appendage article measurements and proportions available in Tables 5–7. Tuberculate granulations, irregular in shape and spacing, covering entire body (Figs. 3D–F; 7B) and legs with the following exceptions: on dorsal prosoma just anterior to ozophores and above anterior tip of coxa I; on irregular medial strip of anal plate of males (being glossy) (Figs. 6B, C; 7B); on inner distal femur and patella of leg II (being glossy); on inner trochanter of leg IV (being glossy), and on all tarsi (being generally smooth). Anal plate in males with smooth, raised, medial strip, tapering posteriorly to width of anal gland pore on tergite IX, not reaching anterior edge of anal plate (Figs. 6B, C; 7B). Tergite IX distinctly bilobed in males and females (Figs. 6B, C; 8B). Gonostome in males oval, with straight posterior edge and a toothlike projection on either side of anterior edge, edges otherwise smooth (Fig. 7A); gonostome more rounded in females, without large toothlike lateral projections, anterior edge with fringe of small, sharp projections (Fig. 8A). Setation pattern even over most of body and appendages; especially concentrated on tarsi (Fig. 4B, E, H, K), these bearing different kinds of setae, including weakly defined solae on tarsus I (Fig. 4B), and solenidia (thick, curved setae; Willemart & Giribet 2010) on dorsal surface of tarsi I and II (Fig. 4B, E); hairs nearly absent from chelicerae, especially from second article. Microtrichial formula of spermatopositor: 3–4, 6–8, 4+4+4 (Figs. 10C, D, H; 11C, D; 12A; 13B); ventral microtrichia at nearly same level (middle slightly more distal) across middle of ventral surface (Fig. 10C, D, H); apical microtrichia in some cases separated by deep medial cleft (Fig. 11C).

Molecular diagnostic characters. In our COI amino acid fragment (described above) no. 43 is isoleucine (not methionine), no. 80 is threonine (not serine), no. 135 is methionine (not isoleucine), no. 145 is asparagine (not serine or glycine), no. 163 is valine (not isoleucine), and no. 231 is glutamine (not histadine). See Table 3.

Distribution and etymology. Known only from the type locality, the Savannah River delta in South Carolina, for which the species is named.

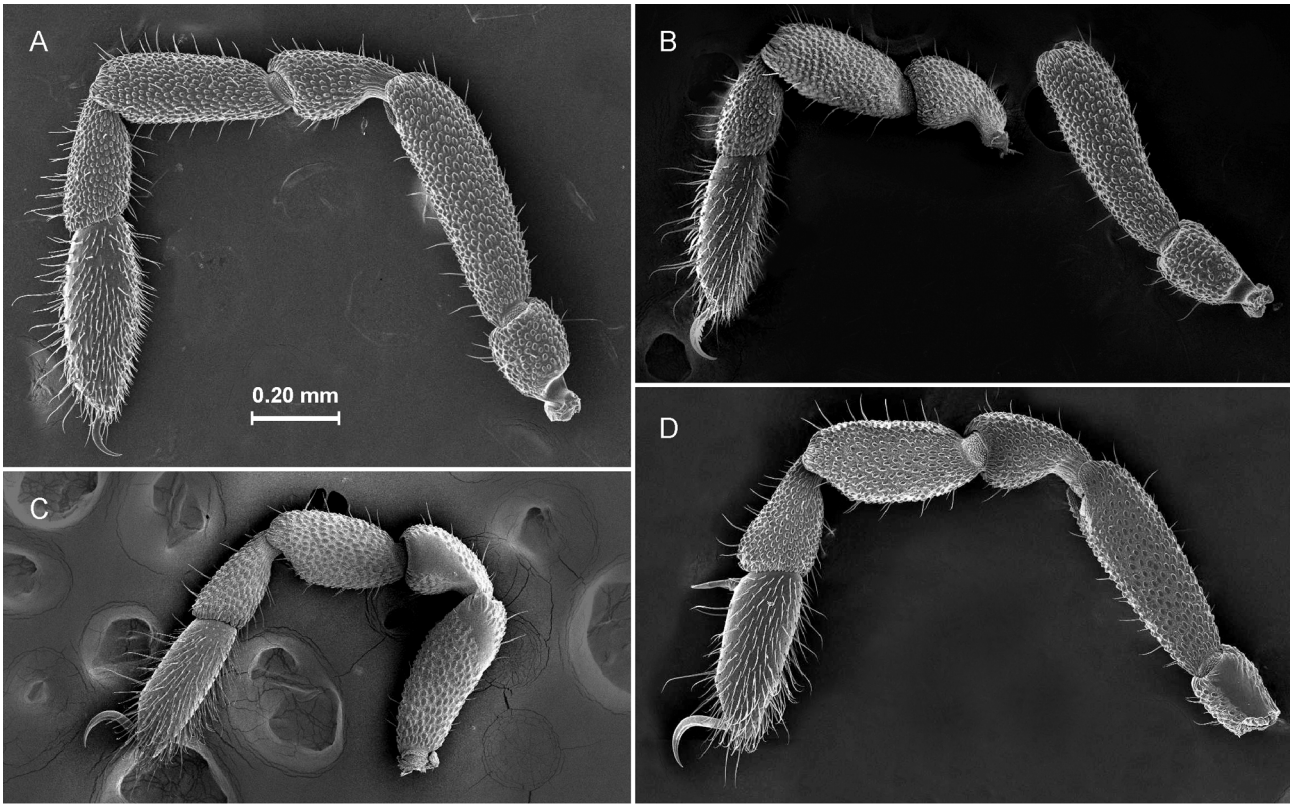

FIGURE 5. Legs of *M. sassafrasensis* **sp. nov.** specimen IZ-134535(105644)-7176: A, leg I; B, leg II; C, leg III; D, leg IV.

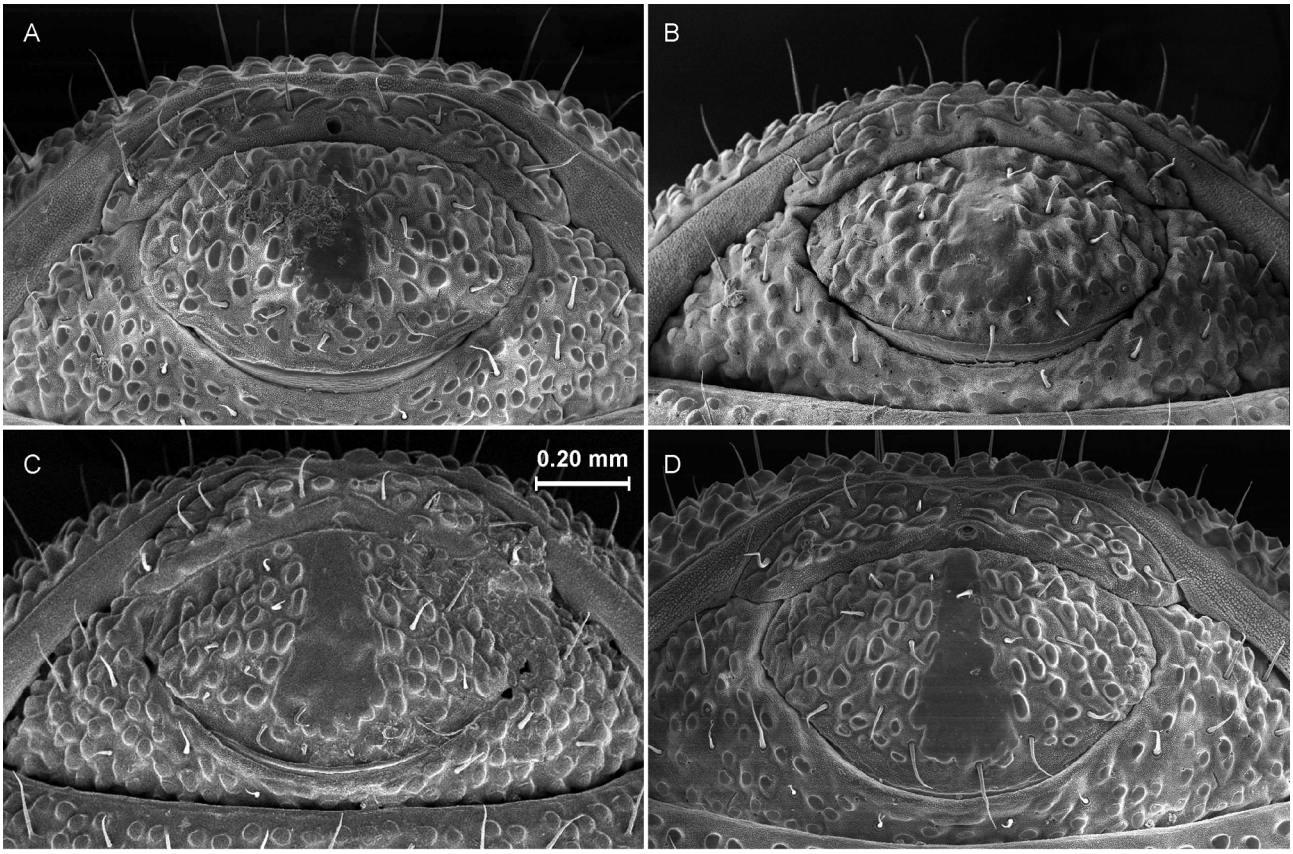

FIGURE 6. Details of anal plate for males of, A, *M. americanus* specimen IZ-133812(105662)-7420, B, *M. savannahensis* **sp. nov.** specimen 105645-7185, C, *M. savannahensis* **sp. nov.** specimen IZ-133800(105646)-7221, and, D, *M. sassafrasensis* **sp. nov.** specimen IZ-134535(105644)-7176.

***Metasiro sassafrasensis* sp. nov.**
(Figs. 3G–I; 4C, F, I, L; 5; 6D; 9; 10A, B, G; 11A, B; 13A)

Metasiro americanus (Davis, 1933), partim

Taxonomic notes. Original 1969 collections by Peck and Fiske examined by Shear (1980) and reported as *M. americanus*.

Material examined. Males (n=8), females (n=7), and juveniles (n=1), with the following collection details: Pickens County, South Carolina, USA, at Jocosse Gorge, Sassafras Mt., off F. van Clayton Highway (lat. 35.06228, long. -82.79500, elev. 760 m), over ridge in old forest, around rotting log, leg. P. Sharma and R. Clouse, 15 March 2010. *Holotype*, male, specimen IZ-134535(105644-7171). *Paratypes*, from collections IZ-134534–5, 7 males (SPM007168 and 7172–7), 7 females (SPM007169–70 and 7178–82); male specimens SPM007176 and 7177 dissected for genitalia, and SPM007176 disarticulated for appendage measurements. Twelve specimens were sequenced for molecular analysis. All specimens are deposited in MCZ.

Description. Morphology as in the original description of *M. americanus* and of *M. savannahensis* **sp. nov.** (see above; leg characters of *M. sassafrasensis* **sp. nov.** illustrated in Fig. 5; chelicer, palp, claw, and solenidia of *M. sassafrasensis* **sp. nov.** illustrated in Fig. 9). Microtrichial formula of spermatopositor: 3, 6, 4+4+4 (Figs. 10A, B, G; 11A, B; 13A). Body and appendage article measurements and proportions available in Tables 5–7.

Molecular diagnostic characters. In our COI amino acid fragment (described above) no. 87 is valine (not leucine). See Table 3.

Distribution and etymology. Known only from the type locality, Sassafras Mountain, South Carolina, for which the species is named.

TABLE 7. Ratios of article lengths relative to the longest article of the same appendage (i.e. article II for chelicer, femur for legs and palp) for males of *M. sassafrasensis* **sp. nov.**, *M. savannahensis* **sp. nov.**, and *M. americanus* (original measurements of the holotype of *M. americanus* by Davis (1933) given in separate column). The rightmost column shows the range size in values across all four males.

		M. americanus		*M. savannahensis* **sp. nov.**	*M. sassafrasensis* **sp. nov.**	
		male	male holotype	male paratype	male paratype	
		7420	(from Davis, 1933)	7185	7176	range size (all)
Chelicer	Article I (whole)	0.7	0.8	0.8	0.8	0.1
	Article III	0.3	0.3	0.3	0.2	0.1
Palp	Patella	0.7	0.7	0.6	0.7	0.1
	Tibia	0.8	0.9	0.8	0.8	0.1
	Tarsus	0.8	0.7	0.8	0.8	0.1
Leg I	Patella	0.6	0.6	0.5	0.5	0.1
	Tibia	0.7	0.6	0.6	0.7	0.1
	Metatarsus	0.6	0.6	0.5	0.5	0.1
	Tarsus	0.7	0.7	0.7	0.7	0.0
Leg II	Patella	0.6	0.5	0.5	0.5	0.1
	Tibia	0.6	0.6	0.6	0.7	0.1
	Metatarsus	0.5	0.5	0.5	0.5	0
	Tarsus	0.8	0.6	0.7	0.7	0.2
Leg III	Patella	0.6	0.6	0.7	0.5	0.2
	Tibia	0.7	0.8	0.9	0.7	0.2
	Metatarsus	0.7	0.8	0.7	0.6	0.2
	Tarsus	0.9	0.8	1.0	0.8	0.2
Leg IV	Patella	0.6	0.6	0.6	0.5	0.1
	Tibia	0.7	0.7	0.7	0.7	0
	Metatarsus	0.6	0.7	0.6	0.5	0.2
	Tarsus	0.7	0.7	0.8	0.7	0.1

Discussion

Variation and speciation in *Metasiro*. *Metasiro* contradicts expected trends in cyphophthalmid character variation within and among species. Within species we typically find little or no variation in genitalic morphology and in body and appendage proportions, as well as no variation in nuclear ribosomal sequences; among species we find clear breaks in these characters, with the occasional exception of the nuclear ribosomal marker 18S rRNA. Here, however, we have found variation in spermatopositor morphology within species but not among them, nearly identical body and appendage measurements within and among species, a complete lack of nuclear ribosomal sequence variation within and among species, and yet COI sequences so divergent among species they are best explained by prolonged isolation.

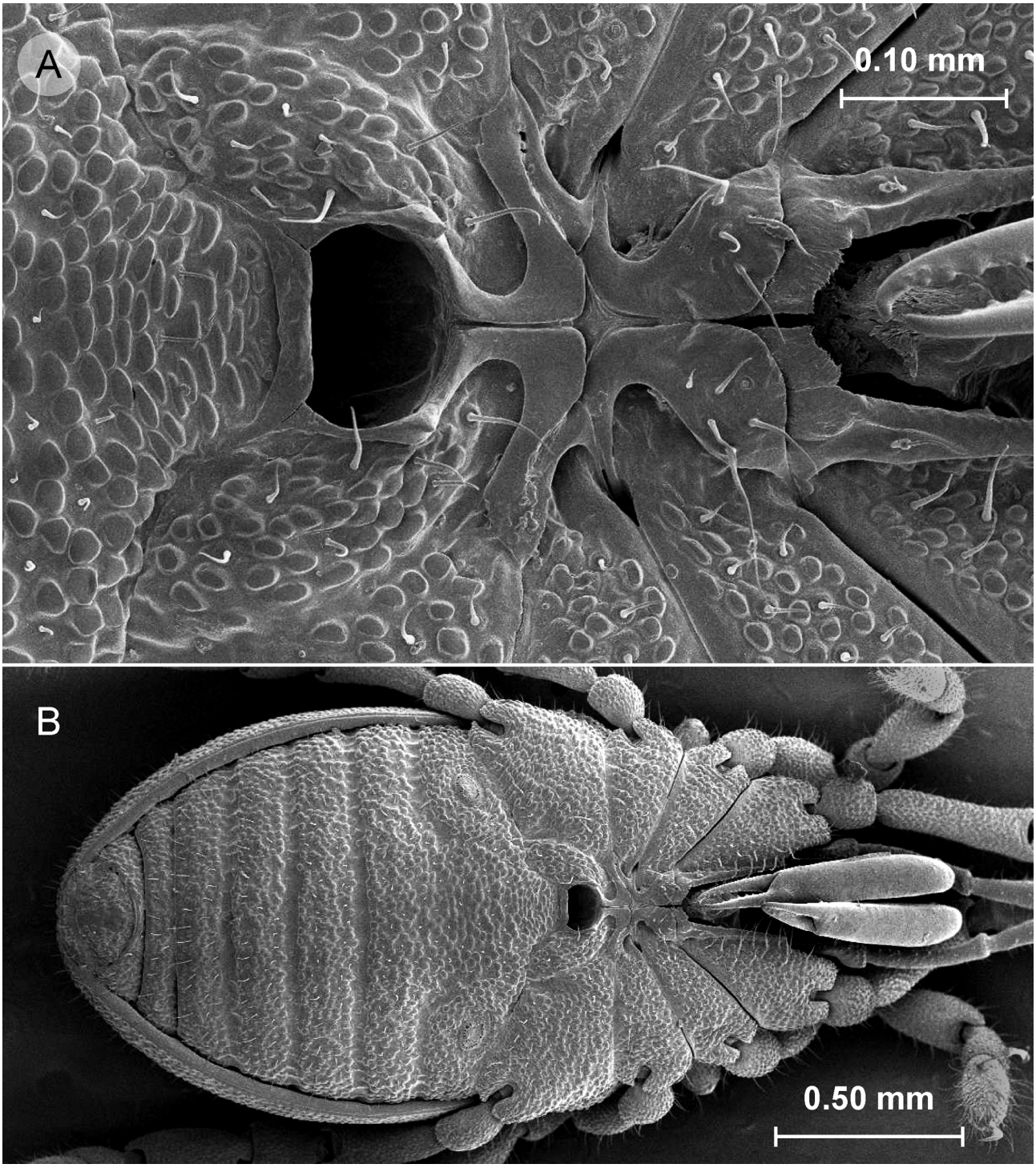

FIGURE 7. A, gonostome and ventral complex detail and, B, full ventral view of *M. savannahensis* **sp. nov.** male specimen IZ-133800(105646)-7214.

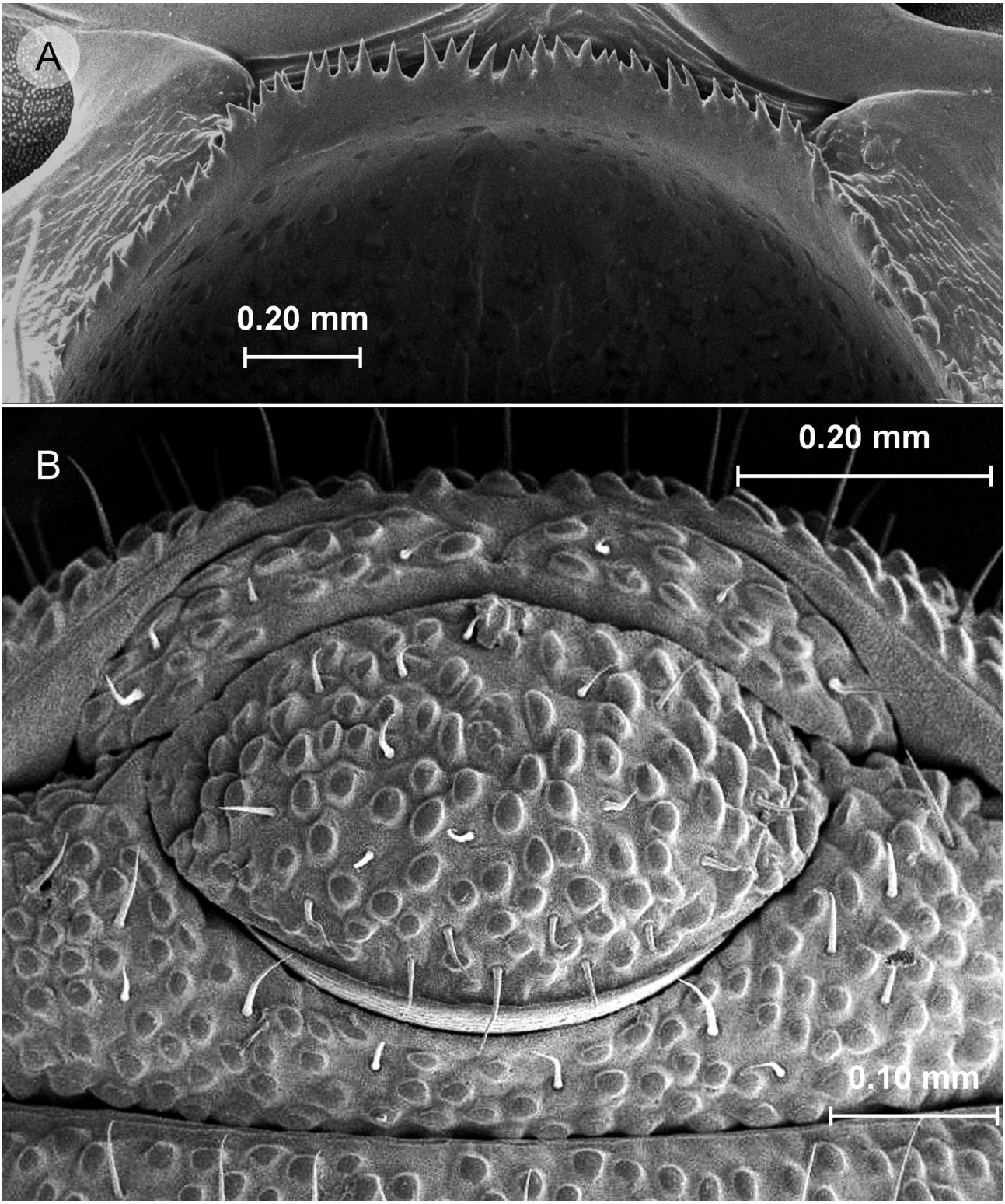

FIGURE 8. A, detail of anterior margin of gonostome and, B, of anal plate of *M. savannahensis* **sp. nov.** female specimen IZ-133800(105646)-7223.

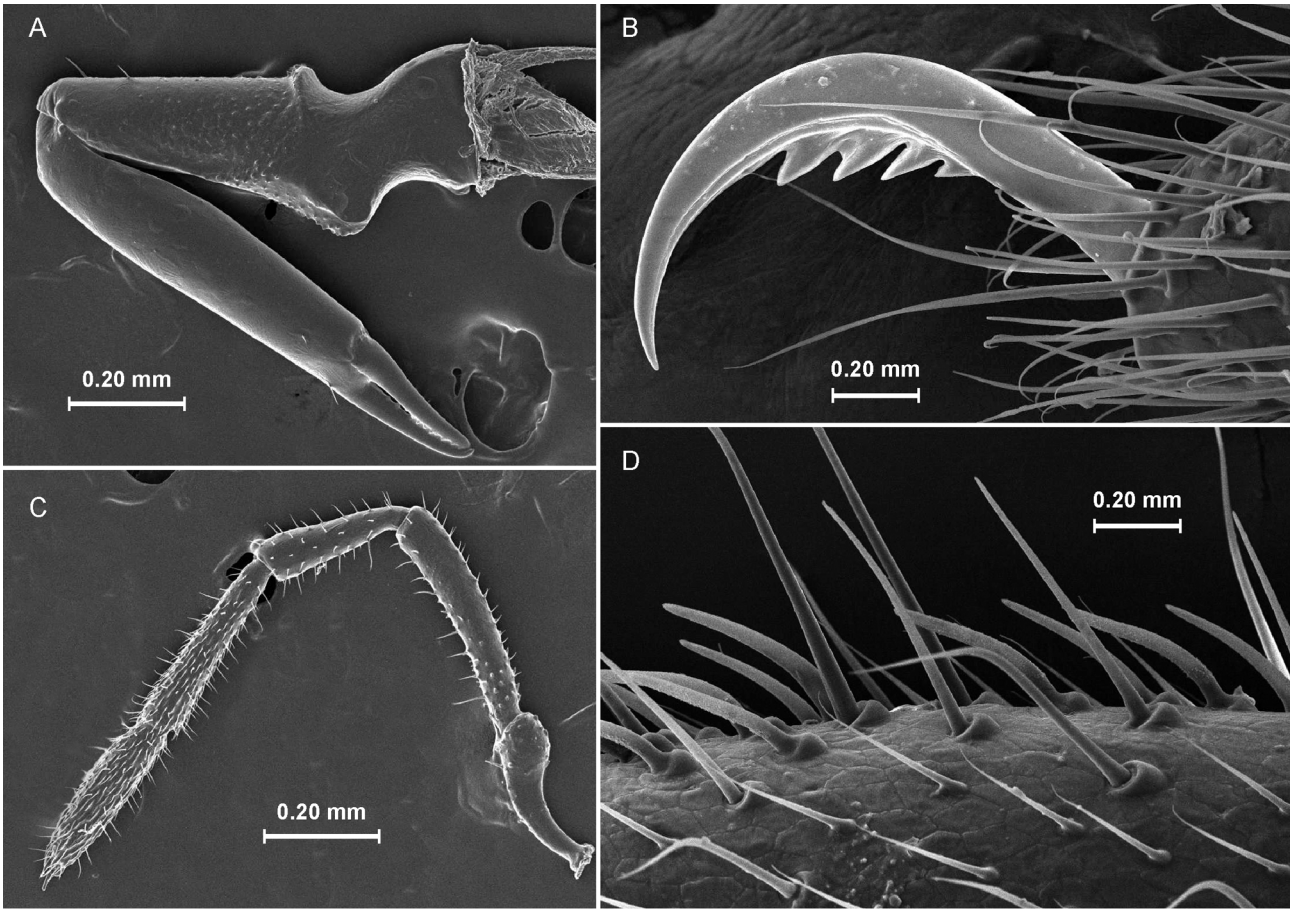

FIGURE 9. A, chelicer, B, claw of leg II, C, palp, and, D, dorsal tarsal setae of leg II of *M. sassafrasensis* **sp. nov.** specimen IZ-134535(105644)-7176.

With the proliferation of DNA sequencing, cryptic species have been suspected in a variety of organisms (Colborn *et al.* 2001), and there is no evidence that they are more prone to be in certain taxa or the result of particular ecological conditions (Pfenninger & Posada 2002). The use of combined phylogenetic and coalescent models has assisted in deciding whether the deeper branching patterns in a clade resemble speciation events (Esselstyn *et al.* 2012; Parnmen *et al.* 2012). Results require interpretation, however; and not all suggested species have been accepted (Hamilton *et al.* 2011). Likewise, we here reject the suggested split in *M. americanus*. What to do with cryptic species taxonomically has been debated, with no clear resolution, since species descriptions have traditionally been based on morphology (Bickford *et al.* 2006; Cook *et al.* 2010; Hebert *et al.* 2004). Nonetheless, there are examples of species described from DNA characters alone (Edgecombe & Giribet 2008; Halt *et al.* 2009).

The analysis of our COI data using the GMYC model, which attempts to optimize the assignment of branching in a single locus to within-species diversification or speciation, found that the COI branching pattern of *Metasiro* is best explained by it having more than one species. However, it also suggested that *M. americanus* is two different species, one centered in the Apalachicola River area, and the other around Florida Caverns, 42 km away. Indeed, the plot of sequence divergences (Fig. 1 D) shows a distinct peak around 5–10%, which corresponds to comparisons between these two clusters. In addition, some of the mutations separating these clades are nonsynonymous. However, the geographic correspondence of these clades is not perfect, the most notable exception being certain specimens from Florida Caverns that carry haplotypes more closely related to those predominantly found in the Apalachicola River area (pink terminals in Fig. 2 A). It seems reasonable to consider this a case of deeply divergent haplotypes being brought together in populations of a single species than a case of sympatry, but the resolution of this will require more study. Although the GMYC approach to single-locus DNA taxonomy was developed as an attempt to move beyond the simplicity of cut-off rules (where a certain percentage divergence is used to delimit species), as well as arguments for gene pool isolation based on *a priori* assumptions of taxonomy (Fujisawa & Barraclough 2013; Pons *et al.* 2006), we are also limited by the abilities of evolutionary

models employed by GMYC analyses to capture the ecology of *Metasiro*. We reject the suggestion of further splitting *M. americanus*, as well as the suggestion from multiple-threshold analyses of 32 recent species, but we note the inclination of the GMYC analysis to model *Metasiro* as consisting of more than one species.

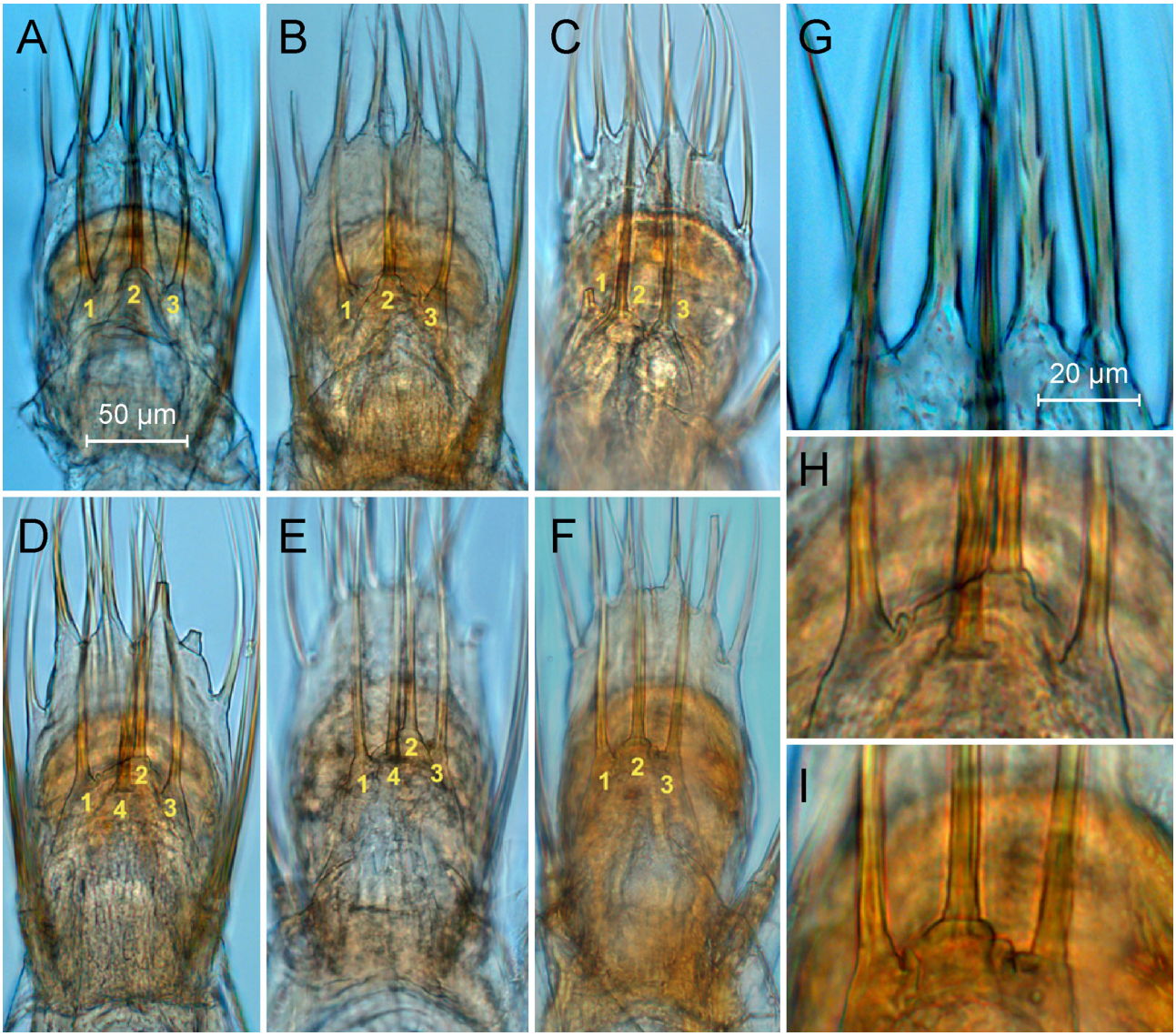

FIGURE 10. Ventral views of spermatopositors from specimens of *M. sassafrasensis* **sp. nov.** (A and G, IZ-134535(105644)-7176; B, IZ-134535(105644)-7177), *M. savannahensis* **sp. nov.** (C, IZ-133799(105645)-7185; D and H, IZ-133806(105652)-7335), and *M. americanus* (E, IZ-133812(105662)-7418; F and I, IZ-133812(105662)-7420). Ventral microtrichia indicated by numbers 1–3 or 1–4 on each specimen. Close-up views show denticles on shafts of microtrichia (G) and the irregular placement of ventral microtrichia (H–I).

Metasiro americanus resembles the New Zealand cyphophthalmid *Aoraki denticulata* (Forster, 1948), which is divided into two subspecies, *A. denticulata denticulata* and the slightly larger, geographically isolated *A. denticulata major* (Forster, 1948). Boyer *et al.* (2007a) found this species to have highly divergent COI haplotypes, around 19% average uncorrected *p*-distances among populations. With no locality or morphological data suggesting more than one species, *A. denticulata* was left as one species, and it was argued that co-occurring, deeply divergent haplotypes generally undermined the rationale for using COI divergences for species identification ("barcoding") and taxonomy (using divergence percentages to delimit species). We agree, not only because of odd species such as *A. denticulata*, but also because such approaches are essentially phenetic and do not consider a wide range of character sources. The proper use of barcoding data in biodiversity studies is the subject of an ongoing debate (Collins & Cruickshank 2013). COI divergences among the three *Metasiro* species recognized here are far more pronounced than among the beetle species in an early call for DNA taxonomy (Pons

et al. 2006) (15% *vs.* 2.2–6.3%), and, unlike *A. denticulata*, the new species named here have haplotypes which are monophyletic and geographically isolated. After separating *M. savannahensis* **sp. nov.** and *M. sassafrasensis* **sp. nov.**, we are still left with a widespread species that, like *A. denticulata*, has strange mixtures of deeply divergent haplotypes, and the interesting question now is what ecological and genetic processes may be at work in both regions to produce these abnormal species. Also, better knowledge of the remaining neogoveids, which are otherwise found in the Neotropics and West Africa, may shed light on whether morphological stasis is common in this family, the species of which remain mostly undescribed (Benavides & Giribet 2013; Kury 2012).

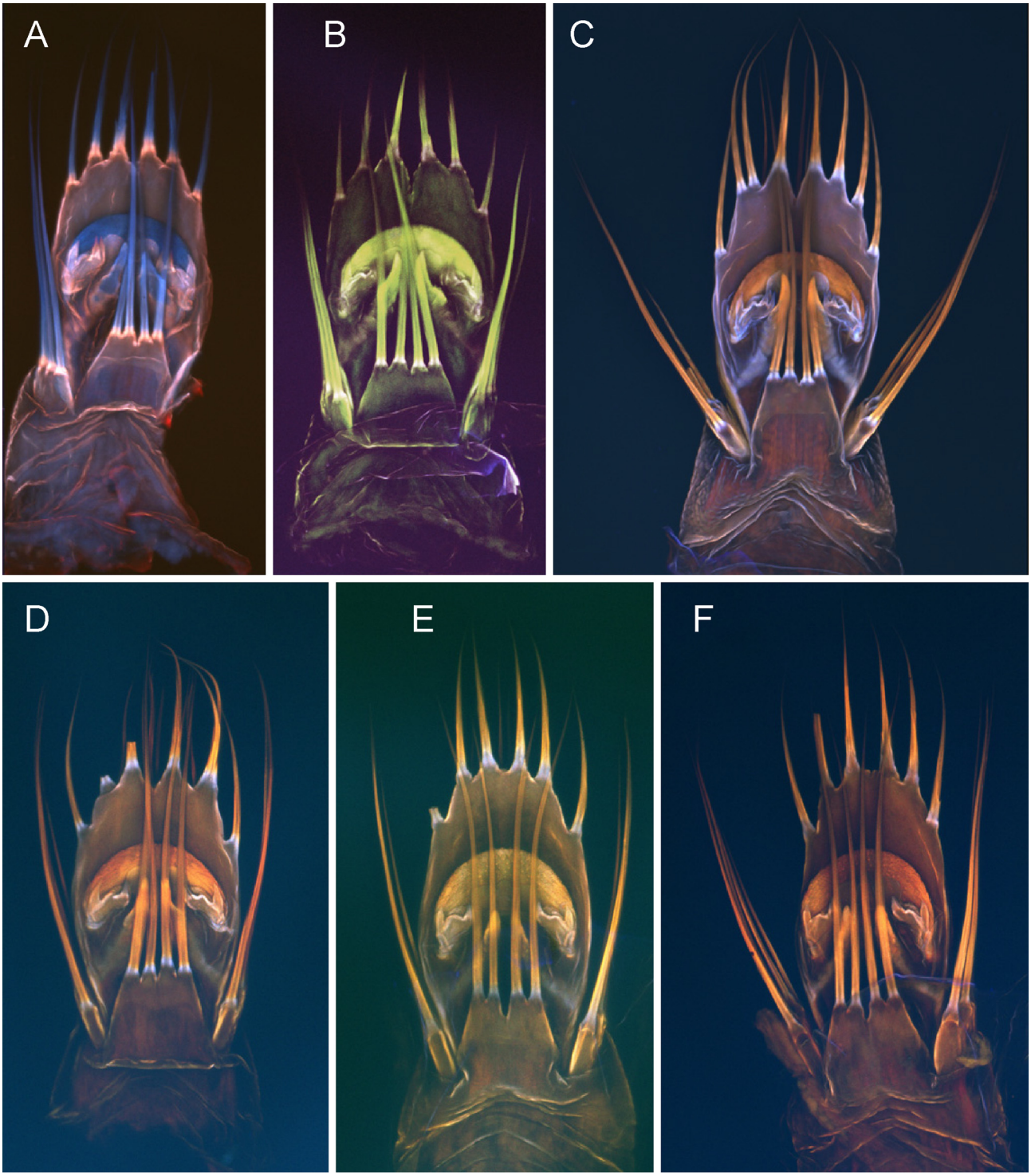

FIGURE 11. Dorsal views of spermatopositors from males of *M. sassafrasensis* **sp. nov.** (A, IZ-134535(105644)-7176; B, IZ-134535(105644)-7177), *M. savannahensis* **sp. nov.** (C, IZ-133799(105645)-7185; D, IZ-133806(105652)-7335), and *M. americanus* (E, IZ-133812(105662)-7418; F, IZ-133812(105662)-7420).

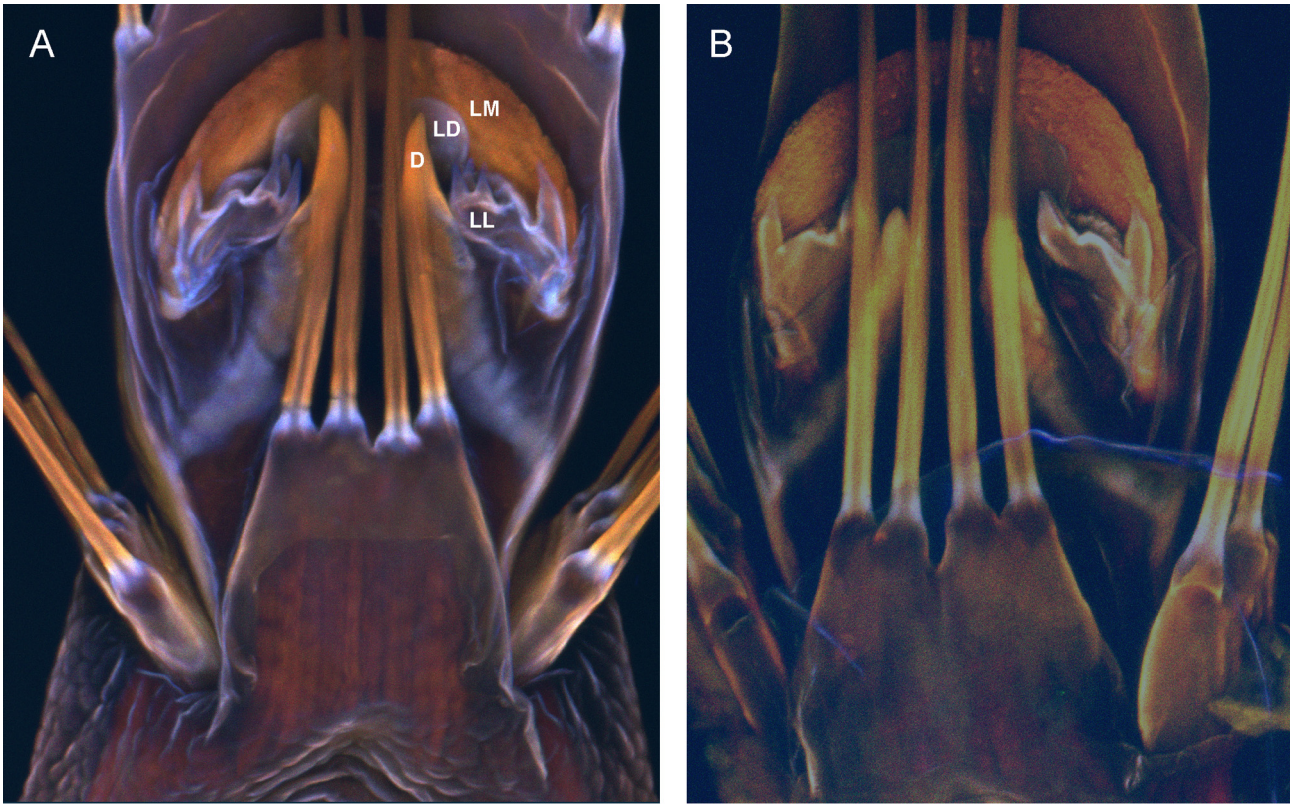

FIGURE 12. Close-up dorsal views of spermatopositors from, A, *M. savannahensis* **sp. nov.** (IZ-133799(105645)-7185) and, B, *M. americanus* (IZ-133812(105662)-7420), showing the lobus medialis (LM), lacinia dorsalis (LD), lobulus lateralis (LL), and digitus (D).

Mitochondrial mutation rates. In Giribet *et al.* (2012a) *Metasiro* was estimated to have begun diversifying around 35 million years (Ma) ago in a dated phylogeny calibrated with well-preserved fossils having highly constrained date estimates (Dunlop *et al.* 2004; Giribet *et al.* 2012b; Poinar 2008). We can use this estimate, pairwise COI divergences, and phylogeny branch lengths to explore mutation rates and ages of cyphophthalmid lineages. It was suggested for *A. denticulata* in New Zealand that their large number of mitochondrial haplotypes was due to an especially fast mutation rate (Boyer *et al.* 2007a), and indeed average uncorrected *p*-distances for all COI sequences among the cyphophthalmid family Stylocellidae (20%, Clouse & Giribet 2010) and among the genus *Aoraki* (14.4%) are similar despite the latter being estimated to have diversified 130 Ma more recently (Giribet *et al.* 2012a). Due to back-mutations, we would expect larger divergences to be more of an underestimate, but the Kimura 2-parameter correction increases their differential only slightly (to 7% from 5.6%). It is also possible that because these are average distances, one or the other is lowered by the inclusion of multiple conspecifics. However, using maximum corrected distances (34.9% and 28.3%), Stylocellidae still has a crudely estimated divergence rate (twice the per-lineage mutation rate) three times slower than *Aoraki*—0.21% per million years (%/Ma) vs. 0.79 %/Ma. Perhaps *Aoraki* has a faster divergence rate compared to all other Cyphophthalmi, which appear to have slow mutation rates relative to the oft-cited arthropod mtDNA divergence rate of 1.5–2.3% per Ma.

With an estimated age of *Metasiro* from Giribet *et al.* (2012a), we can calculate its COI mutation rate in two different ways. One is to take the maximum corrected divergence among all COIs in *Metasiro* (18.21%) and divide it by the fossil-calibrated, estimated age (35.94 Ma), which gives us a divergence rate estimate of 0.55 %/Ma. A second method consists of taking the deepest per-site mutation probability on the ultrametric tree from BEAST, dividing it by the age, and multiplying it by two to get the pairwise divergence, (0.0986/35.94) x 2. This gives a percent divergence rate that matches well with the previous calculation, 0.51%/Ma.

Conversely, we can use divergence rates from other Cyphophthalmi to estimate *Metasiro*'s age. If we use the divergence rates from *Aoraki* and Stylocellidae implied by the dates in Giribet *et al.* (2012a), respectively 0.79 %/Ma and 0.21 %/Ma, and the maximum corrected divergence for *Metasiro*, we estimate that *Metasiro* began

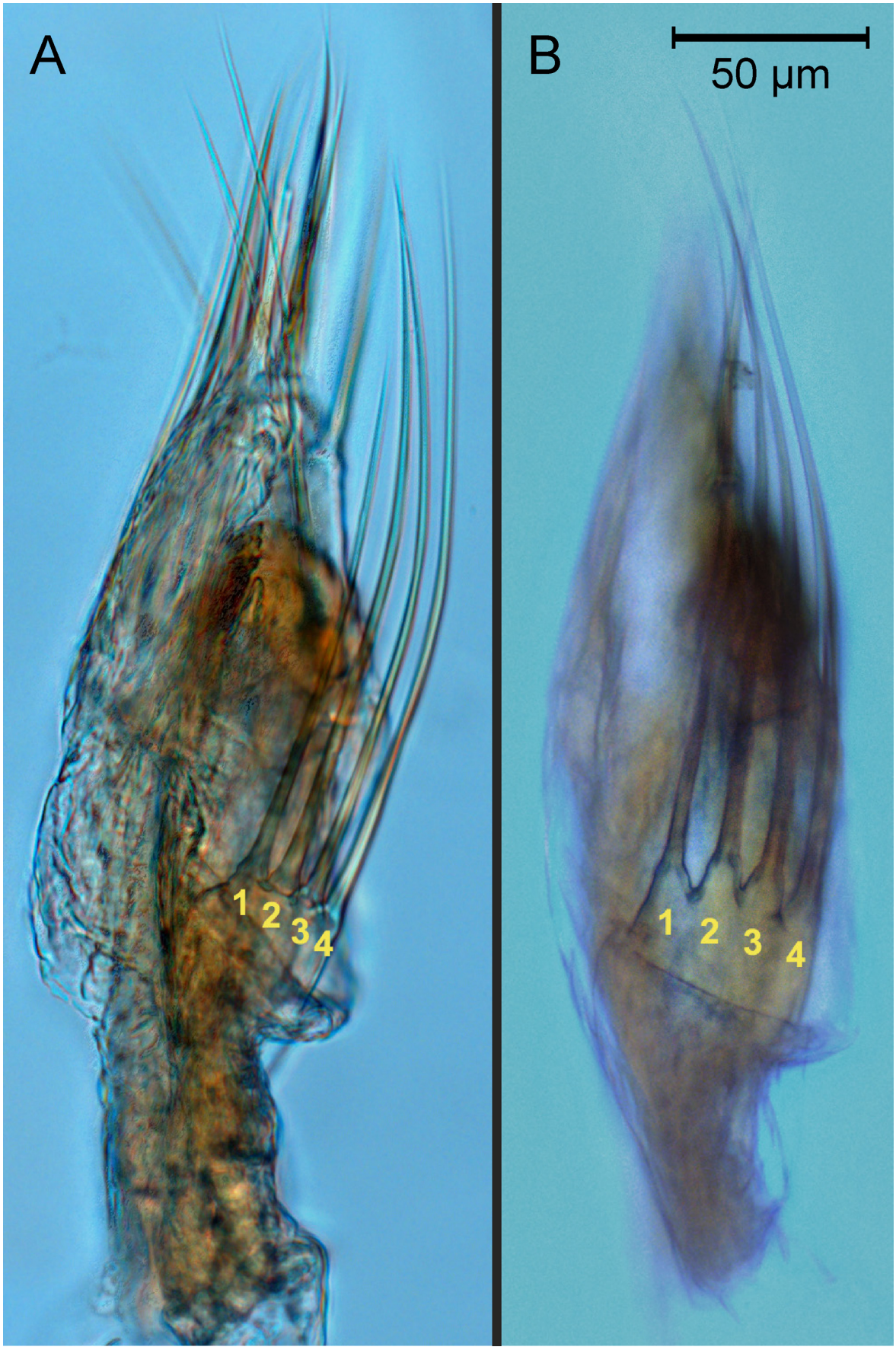

FIGURE 13. Lateral views of spermatopositors from *M. sassafrasensis* **sp. nov.** (A, IZ-134535(105644)-7176) and *M. savannahensis* **sp. nov.** (B, IZ-133806(105652)-7335), showing one set of dorsolateral microtrichia (numbered 1–4).

diversifying between 23 and 87 Ma. This is a large range, but the minimum date allows ample time for speciation in a small arthropod.

One issue this raises is just how low *Metasiro* (and all Cyphophthalmi) divergence rates really are relative to other arthropods, and whether they are anomalously so. The body of evidence against the idea of a consistent molecular clock and easy explanations for its variation has grown. In 2006, for example, it was reported that population size and mitochondrial diversity are not correlated, suggesting a low mutation rate in invertebrates (Bazin *et al.* 2006); that different regions of mtDNA have mutational hotspots, thus making mutation rates contingent upon the sequenced fragments (Galtier *et al.* 2006); that a coral (already known for slow and erratic rates of mtDNA evolution) has no detectable intraspecific rate of mutation and a fossil-calibrated, interspecific, synonymous COI divergence rate of only 0.05 %/Ma (Hellberg 2006); and that invertebrate mutation rates do not correlate with body size (Thomas *et al.* 2006). Since then, considerable variation (two orders of magnitude) has been detected in mtDNA mutation rates in vertebrates (Nabholz *et al.* 2008 and 2009).

Not only have recent data contradicted a general 1.5–2.3 %/Ma divergence rate in invertebrates, the original studies that did so much to establish this rate (Brower 1994; Farrell 2001; Knowlton *et al.* 1993; Knowlton & Weigt 1998) have also been difficult to put into agreement. One of the earliest invertebrate divergence rates reported, 2.2–2.6 %/Ma, was for COI in pairs of snapping shrimp species (genus *Alpheus*) separated by the Isthmus of Panama (Knowlton *et al.* 1993). The closing of the Isthmus of Panama (3.25 Ma ago) was used as a calibration point, and after the three most divergent pairs were excluded due to staggered isolation, the four remaining ones were used to calculate the rate. This rate was later modified to 1.4 %/Ma using the two most recently diverged, mangrove-dwelling pairs in an expanded study (Knowlton & Weigt 1998). Brower (1994) used the first reported *Alpheus* rate as one point in a scatter plot between time and percent divergence, from which was inferred a mutation rate of 2.3 %/Ma. The other points came from two flies, from a homopteran, and from an orthopteran, and they used restriction site data and sequences of 12S and 16S; all the insects diverged between three hundred and 700,000 years ago. Then Farrell (2001) reported a divergence rate for one genus of beetles (*Tetraopes*) as 1.5 %/Ma, which, although being less than Brower (1994) was in close agreement with the latest estimate from *Alpheus* (Knowlton & Weigt 1998). Indeed, a COI divergence rate for scorpions was more recently calculated as approximately 1.4 %/Ma (Gantenbein *et al.* 2005).

However, the axes in Farrell's (2001) Figure 8 are switched, meaning that the measured rate should actually be more like the reciprocal of the slope, 0.66 %/Ma. When the data are re-plotted, the slope of the regression relating percent divergence to age is 0.57 %/Ma (with a correlation coefficient of only 0.56), not very different from what we calculate for *Metasiro* here. The axes do match those of Hillis and Moritz (1990), which is cited as a methodological reference, but those are used for illustrating variation and 95% confidence intervals. Also, it is true that when one simply divides the beetle divergences accumulated in each species pair by their age of separation (Farrell's Table 4), and then averages those across the pairs, one obtains an average divergence rate of 1.57 %/Ma; in a perfectly correlated system the percent divergence of a single species pair divided by its age, the averages among all species pairs, and the slope of the regression relating them should equal each other and be the rate of divergence. However, the three youngest beetle divergences have average divergences per Ma far higher than those inferred from a trendline through the origin connecting them to the oldest pair, so the average is inflated. So possible cyphophthalmid divergence rates of around 0.2 %/Ma in Stylocellidae, 0.5 %/Ma in *Metasiro*, and 0.8 %/Ma in *Aoraki* may be on the low end of estimated mitochondrial divergence rates in arthropods, but what is actually known about these rates is incomplete.

Acknowledgements

We thank Prashant Sharma, Gonzalo Giribet, Sarah Boyer, and Lloyd Davis for collecting specimens and discussing early drafts of this paper. We also thank Peter Schwendinger and G. Giribet for comments and edits that substantially improved the final manuscript. We are also indebted to Jessie Stuart of Stanford University, who sequenced DNA at AMNH. Collecting trips were funded by a grant from the French National Research Council to P. Grandcolas and G. Giribet (for the project BIONEOCAL—Endemism in New Caledonia: a phylogenetic and population-level study of its origins). DNA sequencing was mostly conducted at the American Museum of Natural History (AMNH), where it was funded by a grant from the Defense Advanced Research Projects Agency (DARPA,

W911NF-05-1-0271). We also appreciate the Department of Bioinformatics and Genomics, the Department of Mechanical Engineering and Engineering Science, and the Center for Optoelectronics and Optical Communications at UNC Charlotte for the support of multiuser facilities used to take the images presented here; special thanks for use of these facilities go to Professors Daniel Janies and Haitao Zhang. Confocal imaging was kindly done at the AMNH by Amanda White, and we also thank Estefanía Rodriguez for use of her compound microscope for other spermatopositor photographs. Collecting permits for Florida were facilitated by Tova Spector and Arthur Stiles, and for Savannah River National Wildlife Refuge by Chuck Hayes, to whom we are grateful. Dennis Chastain helped us find the *M. sassafrasensis* **sp. nov.**, population on Sassafras Mountain, where we also received the assistance of Greg Lucas of the South Carolina Department of Natural Resources. Chris Hawthorne and Steve Cutshaw provided important information leading to productive collecting sites at Florida Caverns State Park. Additional assistance was provided by HaWook Song, Diane Sheridan, Mohammed Faiz, and Isabella Kappner.

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