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# Monophyly of the subfamily Neobisiinae (Pseudoscorpiones: Neobisiidae)

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**Abstract.** Members of the Neobisiidae are currently classified in two subfamilies, Neobisiinae and Microcreagrinae. Taxonomic assignment to subfamily is based upon two morphological characters, neither of which is consistently found within either subfamily. The form of the galeae is elongate and hyaline in the Microcreagrinae, but reduced and sclerotic in the Neobisiinae. However, some members of the Microcreagrinae also have reduced galeae. The position of trichobothrium *ist* located on the fixed finger of the pedipalp chelae is generally positioned subdistally and closer to trichobothrium *est* in Neobisiinae but sub-basally and closer to trichobothrium *ib* in Microcreagrinae. However, members of the genus *Parobisium*, currently assigned to the subfamily Neobisiinae, have a microcreagrine-like subbasal trichobothrium *ist*. Since neither subfamily is defined by an undisputed apomorphy, the monophyly of both groups has long been questioned. In this study, we tested whether or not the two subfamilies are monophyletic by inferring the phylogeny of the family using DNA sequence data from the mitochondrial protein-coding gene, COI, and the nuclear ribosomal gene 28S. Results of the molecular phylogenetic analyses indicate that neither of the subfamilies is monophyletic as presently defined. We transfer the genus *Parobisium* to the Microcreagrinae in order to simultaneously obtain a monophyletic Neobisiinae and resolve character inconsistency for the position of trichobothrium *ist*, which is sub-distal in all Neobisiinae taxa included in our study. We also find that reduction of the galea is not a reliable character state at the subfamily level, and has occurred at least three times independently within the family.

**Keywords:** Galea, molecular systematics, trichobothriotaxy, Pseudoscorpiones

Pseudoscorpion adults and nymphs spin silken chambers using their galeae, or spinnerets, present on the movable finger of each chelicera, which connect to silk glands in the cephalothorax (Chamberlin 1931). Most pseudoscorpions spin silken chambers for molting or brooding, and some species also build them for periods of quiescence, such as hibernation or aestivation (Kew 1914). Other species have more specialized uses for silken chambers. For example, *Lasiochernes pilosus* (Ellingsen, 1910) uses them as retreats to which they return after foraging, and therefore leave an opening through which they can enter and exit (Weygoldt 1969). *Halobisium occidentale* Beier, 1931 lives in the mud of marshes and estuaries and uses the silken chambers to create a dry space to live in (Lee 2007). Regardless of the function, the general shape of silken chambers appears to be very similar throughout the order, but there is a great diversity in the shape of the galeae among lineages (Kew 1914).

Differences in galeae shape comprise one of only two characters currently used to distinguish between members of two neobisiid subfamilies, the Neobisiinae Chamberlin, 1930 and Microcreagrinae Balzan, 1892. Members of the Microcreagrinae usually have elongate galeae, whereas members of the Neobisiinae have reduced galeae (Chamberlin 1930; Beier 1932; Harvey 1992). However, the stability and taxonomic utility of this character has been questioned (Murienne et al. 2008; Zaragoza 2008) as there are several microcreagrines that have neobisiine-like, reduced galeae (e.g., *Roncocreagris iglesiasae* Zaragoza, 2003; *R. murphyorum* Judson, 1992; *R. galeonuda* (Beier, 1955); *R. clavata* (Beier, 1955); and *R. robustior* (Beier, 1959)).

The only other character used to distinguish between these two subfamilies is a difference in the position of the trichobothrium *ist*, on the prolateral surface of the fixed finger of the pedipalp chelae. Harvey & Volschenk (2007) summarized the position of trichobothrium *ist* across the genera of Neobisiidae. Their work reveals that, in general, most microcreagrines have *ist* subbasal (Fig. 1c), whereas most neobisiines have *ist* subdistal (Fig. 1a). However, there are also exceptions for this character. For example, *Acanthocreagris* Mahnert, 1974 and *Insulocreagris* Ćurčić, 1987 are microcreagrines with *ist* subdistal, and *Parobisium* Chamberlin, 1930, *Occitanobisium* Heurtault, 1977, and *Trisetobisium* Ćurčić, 1982 are neobisiines with *ist* subbasal.

Not surprisingly for groups that lack defining apomorphies, the monophyly of both subfamilies have long been questioned (Vachon 1946; Mahnert 1974; Zaragoza 2008; Judson 2013). A molecular phylogenetic analysis of the entire order Pseudoscorpiones (Murienne et al. 2008) provided further indication that the Microcreagrinae may not be monophyletic, although only a few neobisiid genera were included. Here, we build upon this work to test whether the subfamilies are monophyletic. In particular, we explore the placement of the genus *Parobisium*, currently assigned to the subfamily Neobisiinae, since species in this genus have reduced galea but also have trichobothrium *ist* placed in a subbasal position (Chamberlin 1930).

## METHODS

Neobisiid specimens were freshly collected by GBH, donated by other collectors, or obtained from the collection at the Museum of Comparative Zoology (MCZ) at Harvard University. Sequences of non-neobisiid taxa for the molecular phylogeny were primarily obtained from the NCBI GenBank database. In total, we analyzed COI and 28S sequences from 56 specimens representing 42 species of neobisiids and 9 outgroup taxa (Table 1).

DNA extractions were performed with the Qiagen DNEasy Blood & Tissue kit following the standard ATL buffer protocol for extraction from tissues. For each specimen,

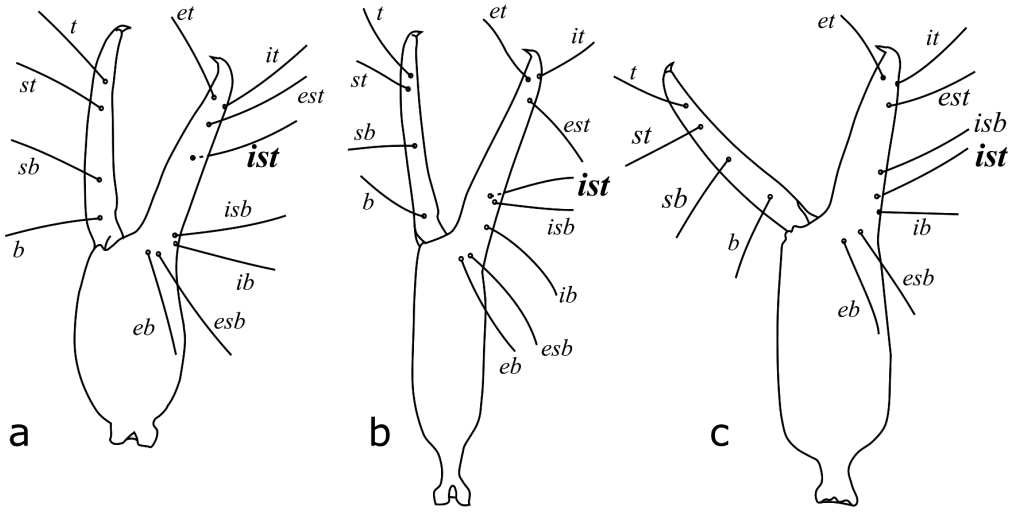


Figure 1.—External aspect of pedipalp chelae, illustrating the different positions of trichobothrium *ist*: The *ist* is subdistal and nearest to trichobothrium *est* (a) or subbasal and nearest trichobothrium *isb* (b and c). a. *Novobisium carolinense*, b. *Parobisium charlotteae*, c. *Americocreagris columbiana*. Illustrations adapted from Chamberlin (1962).

DNA was extracted either from the chelae or from the whole body. For extractions from the chelae, the chelae were dissected from the rest of the body and placed directly in a Proteinase K solution. For the whole body extractions before placing specimens in a Proteinase K solution, we removed the pedipalps and punctured the pleural membrane of the abdomen to facilitate access of the protein-degrading solution to the internal soft tissues. Voucher specimens for this project are deposited in the MCZ, the University of Arizona Insect Collection (UAIC), and the Muséum National d'Histoire Naturelle, Paris (MNHN) as indicated in Table 1.

DNA was amplified using Eppendorf Mastercycler model 5333 or Eppendorf Mastercycler gradient model 5331 (Eppendorf, Hamburg, Germany). Primers used for cytochrome oxidase subunit 1 (COI) were the forward primer LCO1490 (GCATAGTTCACCATCTTTC) and the reverse primer HCO2198 (TAAACTTCAGGGTGACCAAAAAATCA). Primers for 28S ribosomal DNA (28S) included the forward primers LS30F (ACCCCCTRAATTTAAGCATAT) and LS58F (GGGAGGAAAAGAAACTAAC), and the reverse primers and LS1066R (CGACCGATTTGCACGTCAG) and LS1126R (TCGGAAGGAACCAGCTACTA). For all genes, the PCR protocol included an initial temperature of 94°C for 2 minutes, followed by 35 cycles of denaturing at 94°C for 30 seconds, annealing at 50–55°C for 30 seconds, and extension at 72°C for 60–95 seconds. PCR products were cleaned, quantified, normalized and sequenced in both directions at the University of Arizona's Genomic and Technology Core Facility using a 3730 or 3730XL Applied Biosystems automatic sequencer. Chromatograms were assembled and initial base calls were made for each gene with Phred (Green & Ewing 2002) and Phrap (Green 1999) as orchestrated by Mesquite Ver. 3.4 (Maddison & Maddison 2018) and Chromaseq vers. 1.3 (Maddison & Maddison 2017). Final base calls were made in Mesquite and ambiguous bases were designated by a standard ambiguity code.

Sequences were aligned with MAFFT v.7.310 (Katoh & Standley 2013) within Mesquite using the E-INS-I setting for the 28S sequences and default settings for COI. The aligned matrix was partitioned by gene and by codon position, with each partition allowed to have independent parameter values for the model of evolution. Maximum likelihood (ML) inference was conducted using 500 heuristic searches RAxML 8.0.9 (Stamatakis 2014) under the GTR+gamma model of evolution on CIPRES Science Gateway portal (Miller et al. 2010). Clade support was conducted using rapid bootstrapping with a subsequent ML search and letting RAxML halt bootstrapping automatically (using MRE-based bootstopping criterion).

## RESULTS

The optimal tree resulting from maximum likelihood analysis of the concatenated matrix is presented in Fig. 2b and the majority-rule consensus trees for the bootstrap analyses are presented for the concatenated matrix (Fig. 2a), the COI matrix (Fig. 3a), and the 28S matrix (Fig. 3b). In all analyses, Microcreagrinae was recovered as paraphyletic and Neobisiinae (minus *Parobisium*) formed a well-supported clade.

Our phylogeny implies three independent reductions of the galeae within the family (see black dots on Fig. 2b) and that trichobothrium *ist* is subdistal only in the Neobisiinae.

## DISCUSSION

That the Microcreagrinae was recovered as paraphyletic in our analysis is not surprising given the number of authors who have commented on the heterogeneity of this subfamily and the potential for paraphyly (e.g., Vachon 1946; Mahnert 1974; Murienne et al. 2008; Zaragoza 2008). Several North American genera erected in the 1980s remain poorly diagnosed, making placement of new species into those genera problematic (Harvey & Muchmore 2010).

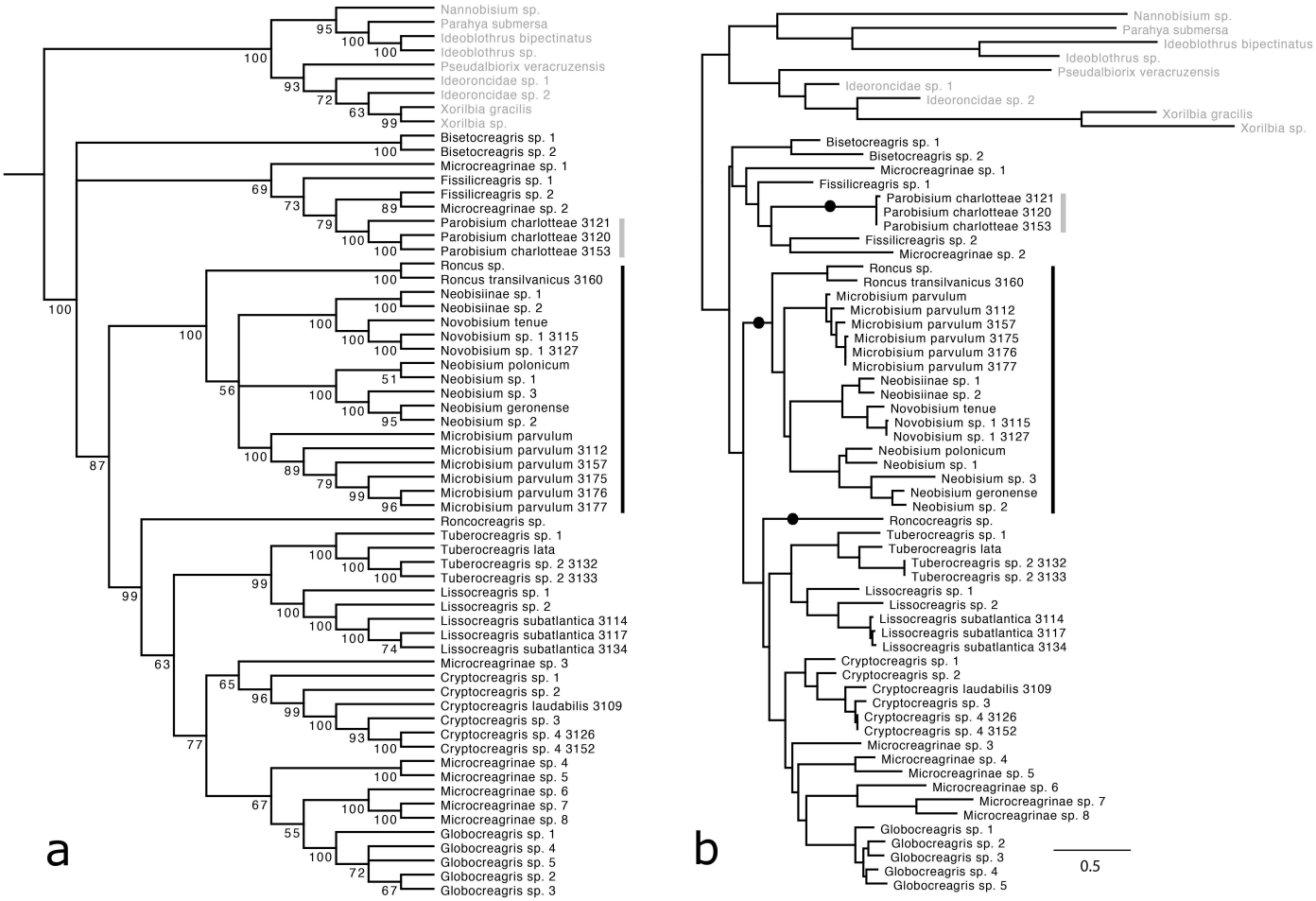


Figure 2.—Maximum likelihood trees of the concatenated dataset. The subfamily Neobisiinae is indicated with a black vertical line to the right of the terminals. *Parobisium charlotteae* is indicated with a light grey vertical line to the right of the terminals. a. Majority-rule consensus tree of 150 maximum likelihood bootstrap replicates. Bootstrap support values are shown below the branches. b. Maximum likelihood tree based on 500 search replicates. Branches are shown proportional to lengths. Black dots indicate the three independent reductions of the galeae within the Neobisiidae.

Neobisiinae was recovered as polyphyletic in our analysis, due solely to the placement of *Parobisium charlotteae* outside of an otherwise well-supported clade containing all other members of this subfamily (Fig. 2). Although we have molecular data for only one of the 16 described *Parobisium* species, striking morphological similarity among all congeners gives us confidence in moving the entire genus out of Neobisiinae. Therefore, we transfer the genus *Parobisium* to the subfamily Microcreagrinae to simultaneously obtain a monophyletic Neobisiinae and resolve character inconsistency for the position of trichobothrium *ist,* which is subdistal in all Neobisiinae taxa included in our study.

Future in-depth molecular and morphological investigations of the Neobisiidae (with more complete taxonomic sampling than in the present study) will be necessary to detect all of the natural subfamily groups. This work should include members of four genera in particular: *Trisetobisium* Ćurčić, 1982; *Occitanobisium* Heurtault, 1977; *Acanthocreagris* Mahnert, 1974; *Insulocreagris* Ćurčić, 1987. *Trisetobisium* and *Occitanobisium* are currently assigned to the Neobisiinae; however, they have a subbasal trichobothrium *ist*. *Trisetobisium* is similar to *Parobisium* in having a reduced galea, and we suspect that it also needs to be removed from the Neobisiinae. *Occitanobisium* is more of a mystery. While members of this genus have reduced galeae, the enlarged trichobothrial areolae and the small size of the chelae makes categorizing the relative position of *ist* subjective. While Harvey & Volschenk (2007) reported that it has a subbasal *ist*, Heurtault (1977) considered *ist* to be subdistal in this genus. *Acanthocreagris* and *Insulocreagris* are currently classified as microcreagrines but they have a subdistal *ist*. *Insulocreagris* also has a reduced galea, which leads us to suspect this genus actually belongs in Neobisiinae. However, *Acanthocreagris* has elongate galeae, leading us to predict that it might not belong to the Neobisiinae, in which case, there could be two lineages with a subdistal *ist,* the Neobisiinae and *Acanthocreagris*.

Results of our molecular phylogenetic analyses indicate that there have been at least three independent reductions of the galea within Neobisiidae (Fig. 2b). These reductions occurred in the most recent common ancestor of the Neobisiinae, the

Table 1.—List of specimens included in this study, indicating locality information for newly sequenced specimens, DNA extraction numbers, body part extracted (wb=whole body, c=chelae), taxonomic identification, and voucher numbers. MCZ voucher numbers are lot numbers, all others are unique specimen numbers. GenBank accession numbers are provided for each sequence. All new COI sequences were amplified with HCO1490 and HCO2198. All new 28S sequences were amplified with LS30F and LS1126R unless otherwise noted by a superscript letter after the GenBank accession number ([a] indicates the sequence was amplified with LS58F and [b] indicates the sequence was amplified with LS1066R).

| Taxon/geographic origin | Moore lab DNA Number (body part) | Specimen/lot number | GenBank accession number COI | GenBank accession number 28S |
|---|---|---|---|---|
| **Ideoroncidae** | | | | |
| Ideoroncidae sp. 1 | | MNHN-JAD70 | JN018183 | NA |
| Ideoroncidae sp. 2 USA: AZ | 3194 (wb) | UAIC1113067 | MF124552 | NA |
| *Pseudalbiorix veracruzensis* Hoff, 1945 | 3155 (c) | MCZ 130499 | EU559567 | MF124381 |
| *Xorilbia gracilis* | 3171 (c) | MCZ 36800 | MF124532 | MF124395 |
| *Xorilbia* sp. | 3170 (c) | MCZ 36676 | NA | MF124394 |
| **Syarinidae** | | | | |
| *Ideoblothrus bipectinatus* Daday, 1897 | 3179 (c) | MCZ 45816 | MF124537 | MF124401 |
| *Ideoblothrus* sp. | 3166 (c) | MCZ 130524 | MF124390 | MF124528 |
| *Nannobisium* sp. | 3167 (c) | MCZ 130525 | MF124529 | MF124391 |
| **Parahyidae** | | | | |
| *Parahya submersa* Bristowe, 1931 | | MCZ 130517 | EU559548 | EU559478 |
| **Neobisiidae** | | | | |
| *Bisetocreagris* sp. 1 | | MNHN-JAC35 | JN018181 | JN018395 |
| *Bisetocreagris* sp. 2 | | MNHN-JAD69 | JN018182 | NA |
| *Cryptocreagris laudabilis* Hoff, 1956 USA: NM | 3109 (wb) | UAIC1113005 | MF124479 | NA |
| *Cryptocreagris* sp. 1 USA: CA | 3128 (wb) | UAIC1113024 | MF124497 | MF124358 |
| *Cryptocreagris* sp. 2 USA: NV | 3149 (wb) | UAIC1113045 | MF124513 | MF124375 |
| *Cryptocreagris* sp. 3 USA: NM | 3135 (wb) | UAIC1113031 | MF124504 | NA |
| *Cryptocreagris* sp. 4 USA: NM | 3126 (wb) | UAIC1113022 | MF124495 | MF124356 |
| *Cryptocreagris* sp. 4 USA: NM | 3152 (wb) | UAIC1113048 | MF124516 | MF124378 |
| *Fissilicreagris* sp. 1 USA: CA | 3110 (wb) | UAIC1113006 | MF124480 | MF124341 [b] |
| *Fissilicreagris* sp. 2 USA: CA | 3129 (wb) | UAIC1113025 | MF124498 | MF124359 [b] |
| *Globocreagris* sp. 1 USA: AZ, Pinaleño Mts. | 3108 (wb) | UAIC1113004 | MF124478 | MF124340 |
| *Globocreagris* sp. 2 USA: AZ, Santa Catalina Mts. | 3104 (wb) | UAIC1113000 | MF124474 | MF124336 |
| *Globocreagris* sp. 3 USA: AZ, Santa Catalina Mts. | 3106 (wb) | UAIC1113002 | MF124476 | MF124338 |
| *Globocreagris* sp. 4 USA: AZ, Rincon Mts. | 3105 (wb) | UAIC1113001 | MF124475 | MF124337 |
| *Globocreagris* sp. 5 USA: AZ, Santa Rita Mts. | 3107 (wb) | UAIC1113003 | MF124477 | MF124339 |
| *Lissocreagris* sp. 1 | | MCZ 130501 | EU559555 | EU559450 |
| *Lissocreagris subatlantica* Chamberlin, 1962 USA: TN | 3117 (wb) | UAIC1113013 | MF124486 | MF124347 |
| *Lissocreagris subatlantica* Chamberlin, 1962 USA: NC | 3134 (wb) | UAIC1113030 | MF124503 | MF124364 |
| *Lissocreagris subatlantica* Chamberlin, 1962 USA: TN | 3114 (wb) | UAIC1113010 | MF124484 | MF124345 |
| *Lissocreagris* sp. 2 USA: TN | 3124 (wb) | UAIC1113020 | MF124493 | MF124354 |
| *Microbisium parvulum* Banks, 1895 | | MCZ 130502 | EU559558 | EU559476 |
| *Microbisium parvulum* Banks, 1895 Mexico | 3175 (wb) | MCZ 37879 | MF124533 | MF124398 |
| *Microbisium parvulum* Banks, 1895 Mexico | 3176 (c) | MCZ 37883 | MF124534 | MF124399 |
| *Microbisium parvulum* Banks, 1895 Mexico | 3177 (wb) | MCZ 37886 | MF124535 | MF124400 |
| *Microbisium parvulum* Banks, 1895 USA: MD | 3157 (c) | MCZ 130502 | MF124520 | MF124383 |
| *Microbisium parvulum* Banks, 1895 USA: UT | 3112 (wb) | UAIC1113008 | MF124482 | MF124343 |
| Microcreagrinae sp. 1 USA: CA | 3130 (wb) | UAIC1113026 | MF124499 | MF124360 |
| Microcreagrinae sp. 2 USA: CA | 3119 (wb) | UAIC1113015 | MF124488 | MF124349 [b] |
| Microcreagrinae sp. 3 USA: OR | 3123 (wb) | UAIC1113019 | MF124492 | MF124353 |
| Microcreagrinae sp. 4 USA: CA | 3118 (wb) | UAIC1113014 | MF124487 | MF124348 |
| Microcreagrinae sp. 5 USA: CA | 3641 (wb) | UAIC1113114 | MF124591 | MF124462 |
| Microcreagrinae sp. 6 USA: AZ | 3600 (wb) | UAIC1113073 | MF124558 | MF124421 |
| Microcreagrinae sp. 7 USA: AZ | 3604 (wb) | UAIC1113077 | MF124562 | MF124425 |
| Microcreagrinae sp. 8 USA: AZ | 3637 (wb) | UAIC1113110 | MF124587 | MF124458 |
| Neobisiinae sp. 1 | 3122 (wb) | UAIC1113018 | MF124491 | MF124352 |
| Neobisiinae sp. 2 | 3125 (wb) | UAIC1113021 | MF124494 | MF124355 |
| *Neobisium geronense* Beier, 1939 | | MNHN-JAC22 | JN018184 | JN018398 |
| *Neobisium polonicum* Rafalski, 1936 | | MCZ 130503 | EU559556 | EU559457 |
| *Neobisium* sp. 1 | | MNHN-JAA9 | JN018185 | JN018399 |
| *Neobisium* sp. 2 | | MNHN-JAC14 | JN018208 | NA |
| *Neobisium* sp. 3 | | MNHN-JAC15 | JN018209 | NA |

Table 1.—Continued.

| Taxon/geographic origin | Moore lab DNA Number (body part) | Specimen/lot number | GenBank accession number COI | GenBank accession number 28S |
|---|---|---|---|---|
| *Novobisium tenue* | | MCZ 130504 | EU559559 | EU559452 |
| *Novobisium* sp. 1 USA: TN | 3127 (wb) | UAIC1113023 | MF124496 | MF124357 |
| *Novobisium* sp. 1 USA: TN | 3115 (wb) | UAIC1113011 | MF124485 | MF124346 |
| *Parobisium charlotteae* Chamberlin, 1962 USA: OR | 3120 (wb) | UAIC1113016 | MF124489 | MF124350 |
| *Parobisium charlotteae* Chamberlin, 1962 USA: OR | 3121 (wb) | UAIC1113017 | MF124490 | MF124351 |
| *Parobisium charlotteae* Chamberlin, 1962 USA: OR | 3153 (wb) | UAIC1113049 | MF124517 | MF124379 |
| *Roncocreagris* sp. Spain | 3169 (c) | MCZ 130546 | MF124531 | MF124393 |
| *Roncus* sp. | | MNHN-JAC28 | JN018186 | JN018400 |
| *Roncus transsilvanicus* Beier, 1928 Slovakia | 3160 (c) | MCZ 130505 | MF124523 | MF124385 |
| *Tuberocreagris lata* Hoff, 1945 | | MCZ 130508 | EU559552 | EU559451 |
| *Tuberocreagris* sp. 1 USA: VA | 3131 (wb) | UAIC1113027 | MF124500 | MF124361 |
| *Tuberocreagris* sp. 2 USA: VA | 3132 (wb) | UAIC1113028 | MF124501 | MF124362 |
| *Tuberocreagris* sp. 2 USA: VA | 3133 (wb) | UAIC1113029 | MF124502 | MF124363 |

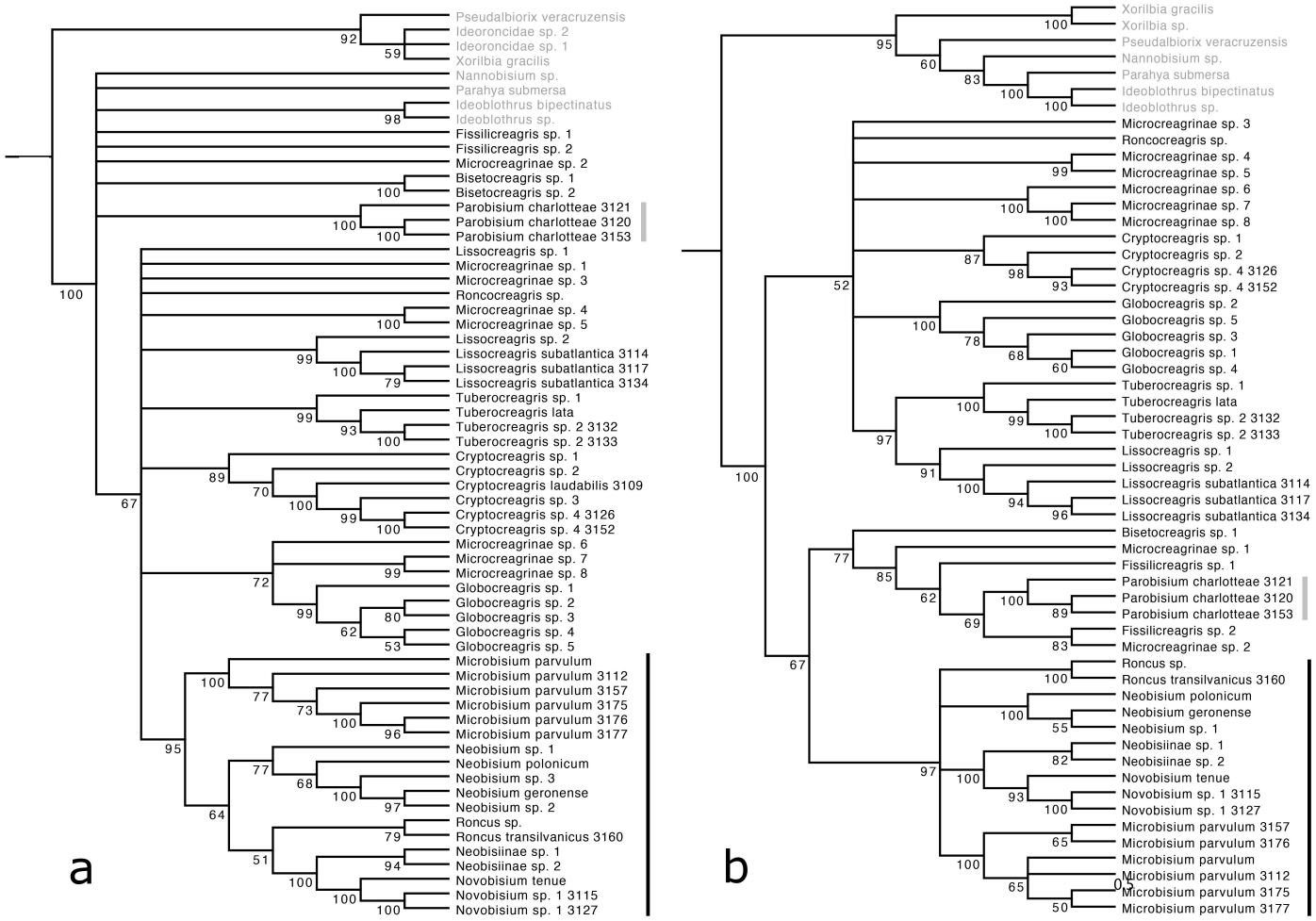


Figure 3.—Majority-rule consensus trees of bootstrap runs based on the single gene matrices. The subfamily Neobisiinae is indicated with a black vertical line to the right of the terminals. *Parobisium charlotteae* indicated with a light grey vertical line to the right of the terminals. a. Consensus tree of 354 maximum likelihood bootstrap replicates for the COI dataset. b. Consensus tree of 252 maximum likelihood bootstrap replicates for the 28S dataset.

*Roncocreagris galeonuda* species group, and the genus *Parobisium*. We caution future neobisiid researchers that it is sometimes difficult to distinguish between naturally reduced galeae, from those in species that once had elongate galeae but are broken. Cokendolpher & Krejca (2010) used scanning electron microscopy to reveal the evenly rounded microstructure of the galea of *Parobisium*. Indeed, three species previously belonging to *Parobisium* were recently moved to the microcreagrine genus *Bisetocreagris* when it was discovered that the galeae were not reduced but had merely broken off (Mahnert & Li 2016).

Although Kew (1914) stated that the behaviors used to build silken chambers are universally the same across different forms of galea, we suspect that the reduction of the galeae is, in fact, associated with a difference in the way these species spin their chambers as compared with species that have long, branched galeae. It is widely known that in some species adult males have slightly smaller galea with less prominent branches than females (e.g., Kew 1914; Harvey 1995). This sexual dimorphism is particularly associated with species in which only juveniles and females make silken chambers (for molting and brooding respectively). This dimorphism is not present in species in which the both sexes build silken chambers for quiescent periods (aestivation or hibernation). Since such differences in behavior result in minor differences in galea shape between sexes of a single species, we expect there to be a significant behavioral and/or ecological explanation for the drastic reduction of the galea seen in the Neobisiinae, the *Roncocreagris galeonuda* species group, and *Parobisium*. To date, however, such explanations remain elusive. Much work remains to be done on these small and elusive arthropods from ecological, behavioral, and taxonomic perspectives.

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