

1 **Species boundaries among extremely diverse and sexually dimorphic *Arrenurus* water**  
2 **mites (Acariformes: Hydrachnidia: Arrenuridae)**

3

4 **Mariusz Więcek<sup>a\*</sup>, Łukasz Broda<sup>a</sup>, Heather Proctor<sup>b</sup>, Mirosława Dabert<sup>c</sup>, Bruce P.**  
5 **Smith<sup>d</sup>, Jacek Dabert<sup>a</sup>**

6 <sup>a</sup> Department of Animal Morphology, Faculty of Biology, Adam Mickiewicz University,  
7 Uniwersytetu Poznańskiego 6, PL-61-614 Poznań, Poland

8 <sup>b</sup> Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada T6G 2E9

9 <sup>c</sup> Molecular Biology Techniques Laboratory, Faculty of Biology, Adam Mickiewicz  
10 University, Uniwersytetu Poznańskiego 6, PL-61-614 Poznań, Poland

11 <sup>d</sup> Department of Biology, Ithaca College, 953 Danby Road, Ithaca, NY, U.S.A. 14850

12 \* Corresponding author

13 E-mail addresses: [roztoc@wp.pl](mailto:roztoc@wp.pl) (Mariusz Więcek, corresponding author),

14 [l.t.broda@gmail.com](mailto:l.t.broda@gmail.com) (Łukasz Broda) [hproctor@ualberta.ca](mailto:hproctor@ualberta.ca) (Heather Proctor),

15 [mirkad@amu.edu.pl](mailto:mirkad@amu.edu.pl) (Mirosława Dabert), [parasitengona@gmail.com](mailto:parasitengona@gmail.com) (Bruce P. Smith),

16 [dabert@amu.edu.pl](mailto:dabert@amu.edu.pl) (Jacek Dabert).

17

18 **Abstract** *Arrenurus* (Arrenuridae) is the most species-rich genus of mites with about 950  
19 named species that inhabit standing, and to a lesser extent, running water habitats around the  
20 world. To date, distinguishing species of *Arrenurus* has been based on male reproductive  
21 morphology. Here, we use morphological and molecular approaches to examine species  
22 boundaries among 42 named species of *Arrenurus*, including four named species that have  
23 colour variants (red and green *A. americanus*, and red and blue *A. intermedius*, *A.*  
24 *manubriator* and *A. apetirolatus*), and two unnamed morphospecies. In this study, we examine  
25 male genital structures with the use of SEM techniques, and apply mitochondrial (COI  
26 barcode region) and nuclear (28S rRNA) gene fragments to test whether male morphology  
27 reflects species boundaries in *Arrenurus* assessed by molecular analyses. Our results reveal  
28 that male reproductive morphology parallels species boundaries as judged by molecular data.  
29 We discuss the cases of genetically poorly diversified, yet morphologically clearly defined  
30 named species. Moreover, we show that based on the species we examined, colour morphs  
31 within otherwise morphologically similar specimens represent within-species variation and, in  
32 the absence of other diagnostic traits, colour itself can be misleading in distinguishing species.

33 Our outcomes on molecular taxonomy of *Arrenurus* provide a background for testing  
34 hypotheses about speciation rate in water mites.

35

36 *Keywords:* 28S rRNA; Arachnida; COI; barcoding; species delimitation; genitalia;  
37 reproductive morphology

38

## 39 **1. Introduction**

40 Water mites (Actinotrichida: Parasitengonina: Hydrachnidia) are one of the most species-  
41 rich groups of arthropods in fresh water (Di Sabatino et al., 2008); however, despite being  
42 widespread and taxonomically diverse, they are poorly studied compared to freshwater insects  
43 and crustaceans (Martin et al., 2010; Proctor et al., 2015). The number of studies of water  
44 mites that incorporate molecular data is growing, and previously unrecognized diversity has  
45 been frequently revealed (e.g. Stålstedt et al., 2013; Pešić et al., 2017; Pešić & Smit, 2017;  
46 García-Jiménez et al., 2017). However, considering more than 6,000 described species found  
47 on all continents except Antarctica, placed into 57 families, 81 subfamilies and over 400  
48 genera (Di Sabatino et al., 2008), only small number of water mite taxa have been involved in  
49 those studies.

50 Based on molecular clock estimates, the highly successful and cosmopolitan genus  
51 *Arrenurus* Dugès (Dugès, 1834) began to diversify 15 MYA (Dabert et al., 2016), and clearly  
52 underwent an explosive speciation that made it the most species-rich genus of arachnids that  
53 comprises approximately 950 named species worldwide (Cook, 1974; Smit, 2012; Gerecke et  
54 al., 2016). Depending on the species, larvae parasitize hosts from the insect orders Odonata,  
55 Diptera, and, more rarely, Coleoptera (Cook, 1974; Böttger & Martin, 2003). Deutonymphs  
56 and adults are predators of ostracods, cladocerans and to a lesser extent small insect larvae  
57 (Proctor & Prichard, 1989).

58 Sperm transfer behaviour in *Arrenurus* is often complex and species-specific (Proctor,  
59 1992). It involves close pairing between males and females, and is correlated with  
60 modifications of the male's hindbody (the 'cauda') and hind legs (presence or absence of a  
61 spur-like extension of the genu designed to clasp the female's legs), and presence or absence  
62 of the petiole, a structure involved in transferring sperm (Proctor & Wilkinson, 2001). In  
63 those *Arrenurus* for which sperm transfer behaviour has been described, species whose males  
64 have well-developed petioles use them as intromittent structures to introduce sperm into the  
65 genital tract of females, whereas those that do not have well-developed petioles deposit

66 spermatophores on the substrate and then manoeuvre the genital opening of the female  
67 overtop the sperm packet (e.g. Proctor, 1992; Proctor & Smith, 1994; Proctor & Wilkinson,  
68 2001). Females of petiolate species may have less control over whether they take in sperm  
69 from a particular male. Because uptake of sperm seems to be more under female control in  
70 species lacking a well developed intromittent organ, female choice is supposed to be the  
71 dominant force of selection in these species, whereas sexual conflict is assumed to play a  
72 bigger role in species with males equipped with elaborate intromittent organs (Proctor &  
73 Smith, 1994; Proctor & Wilkinson, 2001). Female *Arrenurus* show relatively little variation in  
74 body shape, and species-level taxonomy of *Arrenurus* is based on the external reproductive  
75 morphology of males (Smit, 2012; Gerecke et al., 2016).

76 In nature, species arise in many ways including geographical isolation through to  
77 diversification of ecological niches and ending with rarely examined sexual selection (De  
78 Queiroz, 2007). The last scenario is still to a great extent a riddle, because it is especially  
79 difficult to test experimentally (Mendelson & Shaw, 2005). Theory predicts that genitalia  
80 (Eberhardt, 1985), phomonal communication (Lassance et al., 2019) and courtship  
81 behaviour (Mendelson & Shaw, 2005) can evolve rapidly, increase sexual isolation and  
82 accelerate speciation in animals (Arnqvist et al., 2000; Janicke et al., 2018). While in some  
83 species of *Arrenurus* males differ little from females, most of the diversity in the genus  
84 composes of species with males that have extravagant dimorphism. Hence, subgenus  
85 *Arrenurus* s. str. with males characterized by hindbody and hind legs modifications and  
86 presence of the elaborated intromittent organ groups about 300 species. Further 300 species  
87 belong to the subgenus *Megaluracarus* that composes of males with exaggerated, very  
88 elongated and modified hindbody and hind legs designed to grasp and hold females during  
89 copulation. However, the least modified male phenotype, with males that does not differ very  
90 much from females is the least frequent (54 species (subgenus *Truncaturus*)); data found at  
91 website: <https://bugguide.net/node/view/428959>).

92 Here, we test species boundaries among *Arrenurus* water mites based on  
93 morphological investigation and molecular analysis of DNA barcode sequences, i.e. the  
94 mitochondrial cytochrome *c* oxidase subunit I (COI) and the hypervariable D2 region of 28S  
95 rRNA gene (28S rDNA). In order to evaluate species borders among *Arrenurus* using  
96 phenotypic characters we examined the variation of morphological secondary sexual traits in  
97 males, which is the basis for the traditional classification at the subgenus and species level  
98 (Cook, 1974). In addition, we examined what appeared to be intraspecific colour phenotypes

99 of some of the examined species to ask whether distinctive colour forms among otherwise  
100 morphologically similar specimens might represent cryptic species.

101

## 102 **2. Materials and methods**

### 103 **2.1. Water mite collecting and morphological analyses**

104 In total, 262 *Arrenurus* mite specimens were collected in North America and Europe in  
105 various types of freshwater habitats including springs, streams, rivers, lakes, ponds, and  
106 temporary water bodies (Table 1). Most of the North American species came from water  
107 bodies located around the Queen's University Biological Station (Ontario, Canada;  
108 44°34'03.6"N 76°19'26.6"W), with one species collected on a private property near Elk Island  
109 National Park (Alberta, Canada; 53°39'23.7"N 112°45'37.0"W). Specimens of *Arrenurus*  
110 (*Megaluracarus*) *manubriator* Marshall that were originally from San Marcos River (Texas,  
111 US) and Lake Opinicon (Ontario) were taken from separate laboratory cultures maintained by  
112 B.P.S. in the Department of Biology, Ithaca College, New York, U.S.A. The collection sites  
113 in Europe were located in the Netherlands, Germany, Austria, Poland, and Italy (Table 1).

114 Most samples were collected using a net with mesh size 250 µm, but some were  
115 collected using underwater light traps. In the laboratory, live water mites were sorted under a  
116 stereoscope microscope and preserved in 96% ethyl alcohol. European water mites were  
117 identified to species using key of Viets (1936), whereas North American ones were identified  
118 with keys by Cook (1954a, 1954b, 1955). A number of North American individuals included  
119 in the molecular analyses were determined with the assistance of Dr. Ian Smith from the  
120 Canadian National Collection of Insects, Arachnids and Nematodes in Ottawa. Only males  
121 were identified to species based on morphology; species represented only by female  
122 specimens were identified by matching COI and D2 28S rDNA sequences with those from  
123 conspecific males. When possible, representatives of named and putative species were  
124 examined using scanning electron microscopy. After dehydration through an alcohol-HMDS  
125 (hexamethyldisilazane) series, these mites were mounted on stubs using double-sided tape,  
126 sputter coated with gold, and examined using a JEOL 630 I field emission scanning electron  
127 microscope (SEM) in the Department of Earth and Atmospheric Sciences, University of  
128 Alberta. The layout of SEM images was prepared using Photoshop 6.0. Specimen and DNA  
129 vouchers from this study are deposited in the Department of Animal Morphology, Adam  
130 Mickiewicz University in Poznań, Poland.

131

## 132 **2.2. DNA amplification and sequencing**

133 Total genomic DNA was isolated from individual mites using the nondestructive method  
134 described by Dabert et al. (2008). The COI gene fragment was amplified using bcdF01 (5'-  
135 CATTTCCHACTAAYCATAARGATATTGG-3') and bcdR04 (5'-  
136 TATAAACYTCDGGATGNCCAAAAA-3') primers (Dabert et al., 2010). The D2 region of  
137 the 28S rDNA was amplified with 28SF0001 (5'-ACCCVCYNAATTTAAGCATAT-3') and  
138 28SR0990 (5'-CCTTGGTCCGTGTTTCAAGAC-3') primers (Mironov et al., 2012). PCR  
139 amplifications were carried out in 10 µl reaction volumes containing 5 µl Type-it  
140 Microsatellite PCR Kit (Qiagen, Hilden, Germany), 0.5 µM of each primer, and 4 µl (1-5 ng)  
141 of DNA template using a thermocycling profile of one cycle of 5 min at 95 °C followed by 35  
142 steps of 30 sec at 95°C, 1 min at 50°C, 1 min at 72°C, with a final step of 5 min at 72°C. After  
143 amplification, the PCR reactions were diluted with 10 µl of water and 5 µl was analysed by  
144 agarose electrophoresis. Samples containing visible bands were purified with exonuclease I  
145 and FastAP Alkaline Phosphatase (Thermo Scientific) and sequenced using a BigDye version  
146 3.1 kit and ABI Prism 3130XL Genetic Analyzer (Applied Biosystems), following the  
147 manufacturer's instructions. Trace files were checked for accuracy and edited with  
148 ChromasPro v. 1.32 (Technelysium Pty Ltd.). The sequences generated in this study have  
149 been published in GenBank under accession numbers listed in Table 1.

150

## 151 **2.3. Sequence analyses and species delimitation**

152 From a total number of 262 *Arrenurus* specimens collected in this study, 129 were  
153 successfully sequenced with respect either to mitochondrial or nuclear marker (Table 1). The  
154 COI dataset obtained based on specimens collected in this study consisted of 123 sequences  
155 belonging to 38 named *Arrenurus* spp. (including four species possessing colour variants) and  
156 two unnamed morphospecies, and had length of 537 bp with 241 variable nps. Furthermore,  
157 we compiled a joint dataset consisting of 123 sequences obtained in this study and 54  
158 haplotypes (from the total 196 downloaded sequences representing *Arrenurus* species)  
159 gathered from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and BOLD System (<http://www.boldsystems.org/>) (Table 1). The haplotype sequences were selected with the use of  
160 ALTER software (<http://www.sing-group.org/ALTER/>). The final alignment length and also  
161 overlapping homologous region in the joint dataset was 471 bp (221 variable nps). Since four  
162 sequence haplotypes of *A. planus* Marshall (MG312595, MG320457, MG312775,  
163 HQ924310) that were gathered from BOLD did not match conspecific sequences from this  
164

165 study, which could suggest possible identification errors, we excluded these sequences from  
166 the final alignment and further analyses. The D2 28S rDNA alignment comprised 68  
167 sequences representing 41 named *Arrenurus* spp. and two morphospecies and gave 660 bp  
168 and had 241 polymorphic characters, including 29 indels. Sequences were aligned with  
169 Clustal X 2.0.10 (Larkin et al., 2007) and trimmed in GeneDoc v. 2.7.0 (Nicholas & Nicholas,  
170 1997).

171 NJ tree for species-delimitation was calculated based on 177 sequences (471 bp)  
172 obtained in this study (123 sequences) and gathered from GenBank and BOLD (54 sequences)  
173 with the Kimura 2-parameter model (Kimura, 1980) in MEGA X (Kumar et al. 2018).  
174 Support for tree branches was calculated by the nonparametric bootstrap method (Felsenstein,  
175 1985) with 1000 replicates. The critical value of bootstrap support  $\geq 70\%$  was considered to  
176 support monophyly (Douady et al., 2003). We included (*Arrenuroidea* Bogatiidae:  
177 *Horreolanus orphanus* Mitchell) as an outgroup species. COI (K2P) distances were  
178 reconstructed on COI alignment comprising 123 sequences belonging to specimens collected  
179 only in this study with the length of 537 nucleotide positions (nps) using MEGA X (Kumar et  
180 al., 2018). K2P distances for 68 D2 28S rDNA sequences (660 nps) from this study were  
181 computed in MEGA X (Kumar et al., 2018). The Automatic Barcode Gap Discovery (ABGD)  
182 method was applied to detect a barcode gap in the pairwise distance distribution and to sort  
183 the 123 COI sequences into hypothetical species (Puillandre et al., 2011) with the use of web-  
184 based program (<https://bioinfo.mnhn.fr/abi/public/abgd/>) with default settings except X  
185 (relative gap width) set to 1, because higher values failed to detect more than one group. Gene  
186 genealogies were estimated on the basis of 123 COI sequences from this project in TCS 1.21  
187 (Clement et al., 2000) using statistical parsimony networks (SP) (Templeton et al., 1992). The  
188 95% connection limit for species boundaries was applied in searching for putative species  
189 (Hart & Sunday, 2007). Additionally, the probability of species distinctiveness was estimated  
190 for closely related species by two measures estimating probability that the observed branching  
191 structure of the haplotypes originated due to random coalescence processes and not speciation  
192 events: reciprocal monophyly  $P_{AB}$  (Rosenberg, 2007) and Randomly Distinct  $P_{RD}$  (Rodrigo et  
193 al., 2008) as implemented in Geneious 9.1.5 species delimitation plugin (Masters et al., 2011).  
194 Editing of trees was performed using MEGA X (Kumar et al., 2018) editing tools and  
195 Inkscape 0.48.4-1 (Harrington, 2004-2005). Box plots were calculated in order to obtain  
196 medians and quartiles and to get a visual interpretation of genetic distances using the  
197 statistical software PAST 4.03 (Hammer et. Al, 2001).

198

### 199 3. Results

#### 200 3.1. Male sexual morphology and colour variants

201 Examination of morphological structures, in particular structures used by males for the  
202 process of sperm transfer, allowed us to assign 129 individual mites (only specimens from  
203 which DNA barcode sequences were obtained were analysed for morphology) to 42 named  
204 species of *Arrenurus*, which included colour variants of four described species that could  
205 potentially represent unnamed species: red and blue *A. (Megaluracarus) apetirolatus* Piersig,  
206 red and green *Arrenurus (Arrenurus) americanus* Marshall, red and blue *Arrenurus*  
207 *(Megaluracarus) intermedius* Marshall, and red and blue *A. (Megaluracarus) manubriator*.  
208 We also found two morphospecies that did not key to known species, which we refer to as  
209 *Arrenurus* sp. 1 represented only by female specimens which was not sufficient to identify  
210 them at a species level, and *Arrenurus* sp. 2 consisted of female and male specimens with  
211 males possessing unmodified hindbody not clearly demarcated from the body proper and  
212 therefore resembling representatives of subgenus *Truncaturus* (Fig. 2C). In the examined  
213 species four main male phenotypes were identified: a) males with short and least modified  
214 cauda (e.g. *A. (Truncaturus) stecki* Koenike, *A. (Truncaturus) fontinalis* Viets), b) males with  
215 complex, but short cauda with medial cleft and petiole (sometimes with functionally not  
216 defined membranous structure) (e.g. *A. (Micruracarus) perforatus* George, *A. (Micruracarus)*  
217 *biscissus* Lebert, Fig. 2B), c) males equipped with very elongated and exaggerated hindbody  
218 without medial cleft and pygal lobes (e.g. *A. (Megaluracarus) buccinator* (Müller), *A.*  
219 *(Megaluracarus) globator* (Müller) (Fig. 1A, Fig. 2F), d) males that possess elaborated cauda  
220 with humps, clearly marked pygal lobes and complex petiole (subgenus *Arrenurus* e.g. *A.*  
221 *bleptopetirolatus* Cook, *A. magnicaudatus* Marshall, Fig. 2D, E, G). Moreover, we found  
222 species with males that deviate from above mentioned phenotypes and have short cauda with  
223 pygal lobes and membranous sub-petiolar cavity (*A. (Micrarrenurus) albator* (Müller) (Fig.  
224 2A), *A. (Micrarrenurus) crassicaudatus* Kramer). Sexual dimorphism and subset of the  
225 diversity of male reproductive structures are presented in Figs. 1 and 2.

226

#### 227 3.2. Molecular species delimitation and morphospecies with high genetic distance

228 The examined 42 named species formed distinct and well supported clusters in the NJ tree  
229 inferred based on the COI sequences obtained in this study and gathered from GenBank and  
230 BOLD (Fig. 3). Levels of interspecific COI genetic differentiation based on K2P distances  
231 were in most cases well above 11% (Table S1) and had an average value of  $26.06\% \pm 2.06\%$

232 (mean  $\pm$  standard error) and median value was 24.65%. The intraspecific distances ranged  
233 from 0.0% (e.g. *Arrenurus (Megaluracarus) globator*) to 2.09% (*Arrenurus (Arrenurus)*  
234 *bruzelii* Koenike). The interspecific D2 28S rDNA distances had an average value 14%  $\pm$  0.98  
235 (mean  $\pm$  standard error, Table S2) and median value of 13.34%. However, genetic  
236 differentiation expressed in genetic distances varied within different subgenera (Fig. S2). The  
237 ABGD method was applied in order to estimate a barcode gap between K2P distances  
238 (Puillandre et al., 2011), which was identified between 3 and 7% (Fig. 4A, B). The analysis of  
239 123 sequences from this study revealed 38 *Arrenurus* species in all initial partitions (Fig. S5)  
240 and 39-55 species in recursive partitions. Furthermore, in the network analysis the 123  
241 sequences displayed 81 unique haplotypes that formed 21 distinct networks of which 17  
242 corresponded with single named species and one unidentified morphospecies (Fig. S3), one  
243 network contained haplotypes from more than one species and two represented split networks  
244 of single species (Fig. S4). In conclusion, all applied methods confirmed species status of 35  
245 out of 42 named species, and unnamed *Arrenurus* sp. 1, *Arrenurus* sp. 2 were identified as  
246 genetically differentiated at the species level.

247

### 3.3. Morphospecies and colour variants with low genetic divergence

248 In the present study, except for genetically well separated species, pairs of named species  
249 characterized by low genetic distances were identified. Hence, relatively low diversification  
250 of COI sequences was found in closely related *Arrenurus (Arrenurus) mucronatus* Levers and  
251 *A. (Arrenurus) americanus* (5.1%). This species pair showed a very low level of 28S  
252 differentiation (0.15%). Similarly, low 28S distances were observed among following  
253 *Arrenurus* s.str. species pairs: *A. gennadus* Cook - *A. mucronatus* (0.00%, consistently  
254 separated as distinct species based on the COI fragment), *A. americanus* - *A. gennadus*  
255 (0.15%, distinct species based on the COI ) and *A. maryellenae* Cook - *A. magnicaudatus*  
256 (0.31%; recognized as separate species in COI based analyses). The ABGD initial partitions  
257 did not separate *A. mucronatus* from *A. americanus* (Fig. S5). Furthermore, the red and green  
258 *A. americanus* showed intraspecific genetic variation. In the network analysis haplotypes *A.*  
259 *americanus* (green) and *A. americanus* (red) formed a single network, but *A. mucronatus*  
260 remained unconnected (Fig. S3). Rodrigos's P(RD) suggested that both colour forms of *A.*  
261 *americanus* were one single species (Table 2).

262 In the species pair *Arrenurus (Megaluracarus) megalurus* Marshall and *A.*  
263 *(Megaluracarus) intermedius* (red and blue) COI distances ranged from 0.95% - 1.58%



264 (Table S1). The ABGD initial partitions did not separate *A. megalurus* from *A. intermedius*  
265 (both red and blue) (Fig. S5), and statistical parsimony networks grouped together haplotypes  
266 of *A. megalurus* and *A. intermedius* (Fig. S4). Similarly, Rosenberg's  $P_{AB}$  suggested that these  
267 species are conspecific (Table 2). In addition, a low 28S distance value occurred between *A.*  
268 *intermedius* and *A. megalurus* (0.46%). Moreover, the blue and red *A. (Megaluracarus)*  
269 *apetiolutus* were separated by distance of 0.31% (28S), which could suggest that two separate  
270 species are present. However, the result remains inconclusive, because COI sequences were  
271 not successfully obtained for this species. In the colour-polymorphic *A. (Megaluracarus)*  
272 *manubriator* red specimens originated from Texas and blue ones from Ontario were separated  
273 by 0.99% (COI) and 2.18% (28S). Rosenberg's  $P_{AB}$  and Rodrigos's P(RD) indicated that red  
274 and blue *A. manubriator* should be considered as separate species (Table 2). Nevertheless, the  
275 contradictory results were obtained after inclusion in the analysis sequences gathered from  
276 BOLD (Fig. S1). Results of species delimitation in species that were not concordantly  
277 recovered as conspecific are summarized in Table 2.

278

#### 279 4. Discussion

280 Our data show that male reproductive morphology, which provides the rationale for most  
281 taxonomic decisions in *Arrenurus* tends to parallel species boundaries as judged by molecular  
282 data. The ABGD analysis identified barcode gap between 3-7%, which is lower than threshold  
283 obtained for spring-dwelling water mites by Blattner et al. (2019) (6-9%). Interspecific COI  
284 barcode distances have been estimated for congeneric vs. congeneric water mite species most  
285 frequently for > 10% (Pešić & Smit, 2017; Pešić et al., 2017; Więcek et al., 2020), and  
286 correspond to the values obtained in this study. However, noteworthy, among closely related  
287 *Atractides* species distances had values of about 5% (Pešić et al., 2020). García-Jiménez et al.  
288 (2017) obtained a statistical support for separating endemic island *Lebertia* water mites that  
289 exhibited distances of about 2% and shared the common ancestor about 4.6–5.2 Mya. The  
290 simulation study found bias against discovering young species in taxa undergoing adaptive  
291 radiation and demonstrated that single-gene thresholds can consistently discover new species  
292 with error rates of <10% if isolation was >4 million generations ago (Hickerson et al., 2006).  
293 Therefore, we suppose that low sequence divergence in species groups examined in this study  
294 may indicate young, but reproductively isolated species. In addition, the mean distance value  
295 for 28S sequences obtained in this study (14%) is in agreement with sequence diversification  
296 observed for a broad set of water mites inhabiting springs (15%K2P ± SD: 0.10%) found by  
297 Blattner et al. (2019), and in most cases paralleled results obtained with mitochondrial

298 sequences. Interestingly, we observed low differentiation at nuclear loci among members of  
299 subgenus *Arrenurus* s. str. as compared to representatives of other examined subgenera. The  
300 subgenus *Arrenurus* str. is considered as monophyletic (when excluding members of the  
301 subgenus *Micrarrenurus* proposed later by Cassagne-Méjean, 1966) and molecular dating  
302 analysis suggested its recent origin in relation to other examined *Arrenurus* taxa (5–10 Mya  
303 Dabert et al., 2016). Similar low distance values were also obtained for young eriophyoid mite  
304 species (Skoracka & Dabert 2010). To date, there are very few studies targeting certain  
305 aspects of taxonomical assessments of *Arrenurus*, where the validity of morphospecies is  
306 tested with the application of molecular markers (e.g. Blattner et al., 2019; Alarcón-Elbal et  
307 al., 2020). However, in the recent published article of Alarcón-Elbal et al. (2020) phylogenetic  
308 relationships among *Arrenurus* species were missinterpreted. Whereas the authors stated that  
309 subgenera *Arrenurus*, *Megaluracarus* and *Micruracarus* are “natural” and only subgenus  
310 *Truncaturus* is an arbitrary assemblage of species, in fact non of the examined by the authors  
311 subgenera is monophyletic when interpreting results of the phylogenetic tree presented by the  
312 authors (Alarcón-Elbal et al., 2020).

313 We obtained equivocal results for closely related *A. (Megaluracarus) intermedius* and  
314 *A. (Megaluracarus) megalurus*, which show very low genetic differentiation. Moreover, other  
315 than colour, there is no morphological evidence for separating the blue and red *A.*  
316 *intermedius*. It is noteworthy that *A. intermedius*, *A. megalurus* and *A. (Megaluracarus)*  
317 *marshallae* Piersig have been considered in literature as closely related, morphologically  
318 similar species that exhibit subtle differences in male structures associated with hindbody and  
319 that tend to occur in the same habitats at the same time (Cook, 1954b; Mitchell, 1964).  
320 Furthermore, the two colour forms of *A. (Megaluracarus) apetiolutus* are probably recently-  
321 diverged species, however we have insufficient evidence given that we were unable to obtain  
322 mitochondrial sequences. Moreover, we obtained ambiguous evidence for distinguishing  
323 species in closely related *A. (Arrenurus) mucronatus* and *A. (Arrenurus) americanus* (both red  
324 and green individuals). *Arrenurus americanus* is highly variable in colour: they are  
325 predominantly either dark green or brick red, but various other colours also occur (grey, tan,  
326 etc.) with more or less a continuum of variation (B.P.S., pers. obs.). In this study, we observed  
327 intraspecific sequence differences between both colour variants of *A. americanus*. However,  
328 we found initial stages of sequence diversification between *A. americanus* and *A. mucronatus*,  
329 which may indicate the presence of a young and potentially hybridising species. Given that *A.*  
330 *mucronatus* has consistent differences relating to size differences and structure of hindbody  
331 and intromittent organ when compared to *A. americanus*, the low differentiation in barcode

332 sequences could indicate that morphology evolves more rapidly than mitochondrial  
333 sequences, as would be expected under continuous directional sexual selection (Wojcieszek &  
334 Simmons, 2011). Nevertheless, other forces as stabilizing natural selection could be  
335 potentially responsible for divergence of male genitalia. In view of the fact, that male  
336 genitalia in males in *Arrenurus* are highly complex and include presumably functionally  
337 different components, it is likely that different sections of male genitalia in *Arrenurus* may be  
338 subject of different evolutionary processes as it was suggested for grasshopper species (Song  
339 & Wenzel, 2008).

340 We observed a clear pattern within the geographically widespread and color-  
341 polymorphic *A. (Megaluracarus) manubriator*, from which we had representatives from  
342 distinct habitats and distant regions represented by laboratory colonies established from two  
343 populations located approx. 2,500 km apart (red mites from a river in Texas vs. blue mites  
344 from a lake in Ontario). The genetic differentiation of these populations is probably the result  
345 of processes associated with speciation, as random coalescence was rejected as explanation of  
346 this divergence. However, only limited conclusions can be drawn with regard to *A.*  
347 *manubriator*, because split into two distinct clades observed based on specimens from this  
348 study is not retained after including sequences of specimens collected in a wider range of  
349 habitats (data from BOLD databasis). In the apparently closely related *Arrenurus* s.str. species  
350 group *A. fissicornis* Marshall - *A. reflexus* Marshall - *A. bleptopetirolatus* all applied methods  
351 were consistent and confirmed genetic separation at the species-level. Interestingly, we found  
352 well supported clade structure of coalescent origin within *A. fissicornis*, which was also  
353 present after including data from BOLD. Overall, the comparison of our data with sequences  
354 deposited in BOLD databasis was, however, limited to a few species, because only a small  
355 part of sequences was publically available.

356 Its clear from our study that colour is a questionable character to use when separating  
357 species: sometimes it is informative, other times it is a false lead. There are species that are  
358 highly variable, some are remarkably consistent and colour is a useful cue for identification  
359 (esp. within site). Furthermore, we observed that body colour can be either consistent within  
360 population but varying among populations (e.g., *A. intermedius*), or in other cases also highly  
361 variable within population (e.g., *A. americanus*). The occurrence of colour variants that  
362 showed within-species sequence divergence was also found in melon aphids (Lokeshwari et.  
363 al., 2014), pabble crabs (Prakash & Kumar, 2020) and sea cucumbers (Jo et. al., 2016).  
364 However, Soto-Adames (2002) revealed that most populations of springtails differing only in  
365 color pattern showed significant genetic divergence and thus were recognized as distinct

366 species. We suggest that in the absence of other morphological differences, body colour itself  
367 is not a good diagnostic trait for species separation in the genus *Arrenurus*. However, in a few  
368 cases it may be a clue that there is underlying genetic differentiation (*A. manubriator*,  
369 laboratory colonies, B.P.S., pers. obs.). Although it's possible that variation in color in some  
370 cases may be caused by local water chemistry, body colour may have adaptive value for  
371 instance as photoprotectants (red and orange carotenoid pigments) (Proctor & Garga, 2002).  
372 Although our outcomes suggest that certain pairs of named species could be conspecific, we  
373 believe that the patterns found in this study should be further tested on larger numbers of  
374 individuals from broader geographic ranges.

375

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386

### 387 **Conflict of interest**

388 The authors have no conflict of interest to declare.

389

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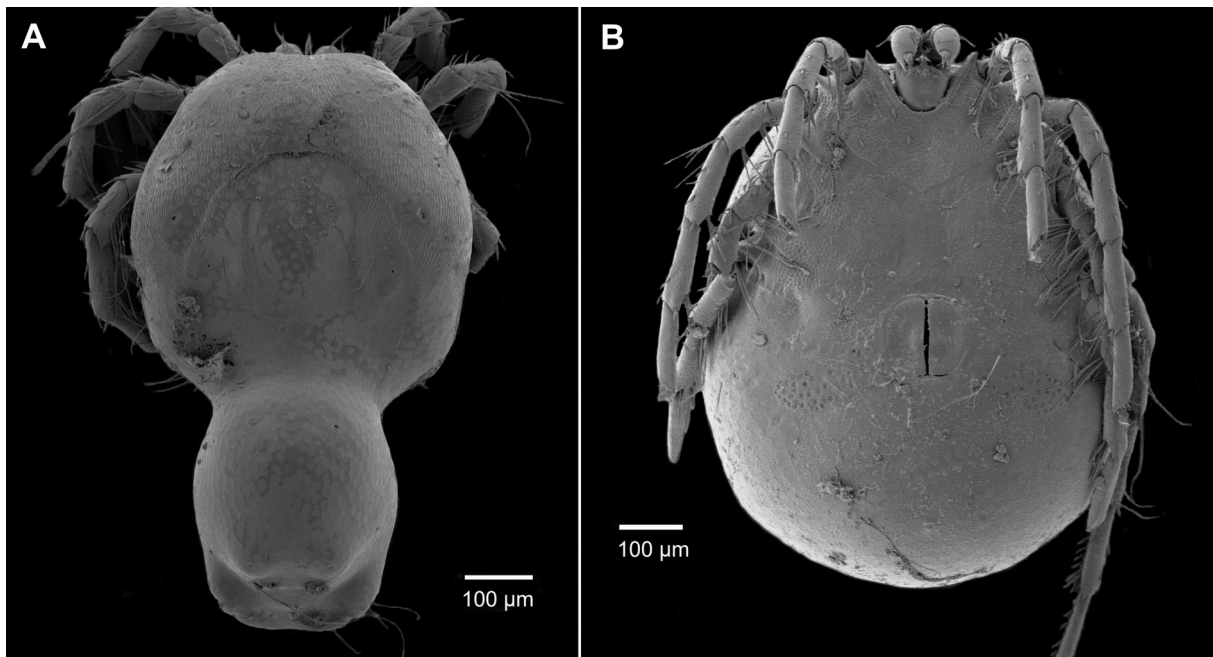


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568 Figure 1. Sexual dimorphism in *Arrenurus (Megaluracarus) globator*: A. male with elongated and  
569 modified hindbody (“spur” on IVth leg not visible), B. female possesses very little diversified body  
570 and lacks cauda.

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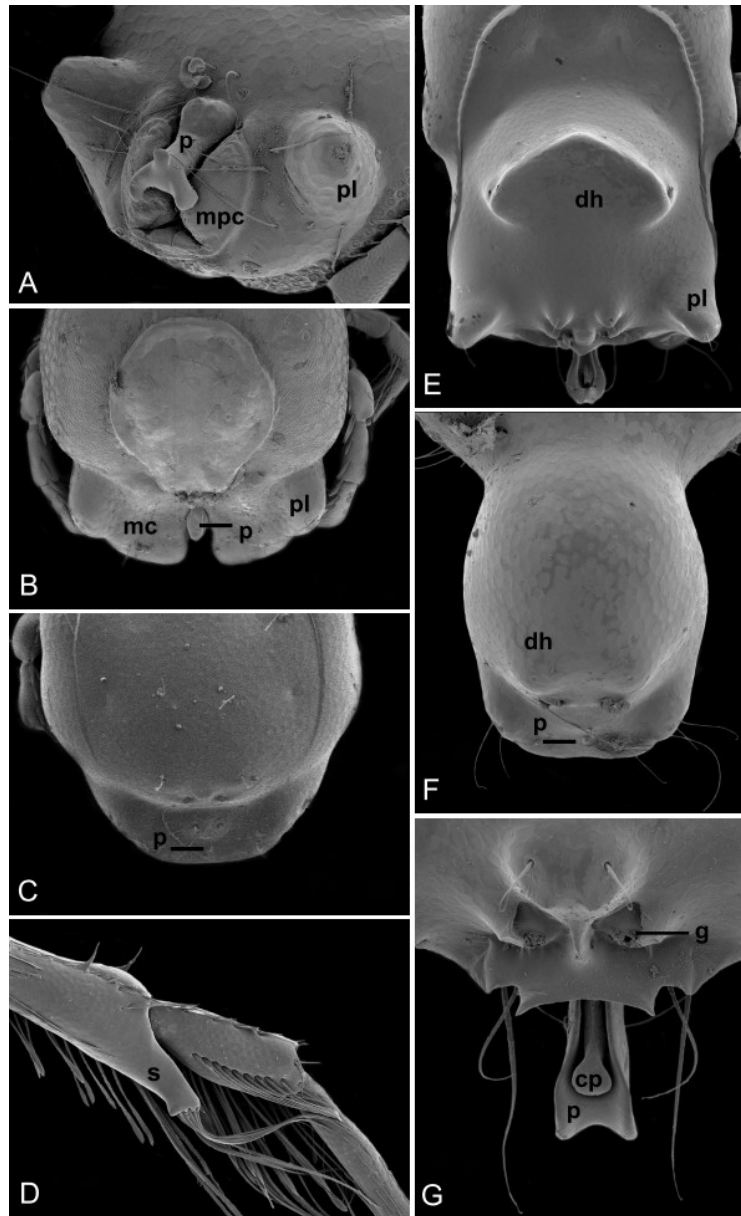
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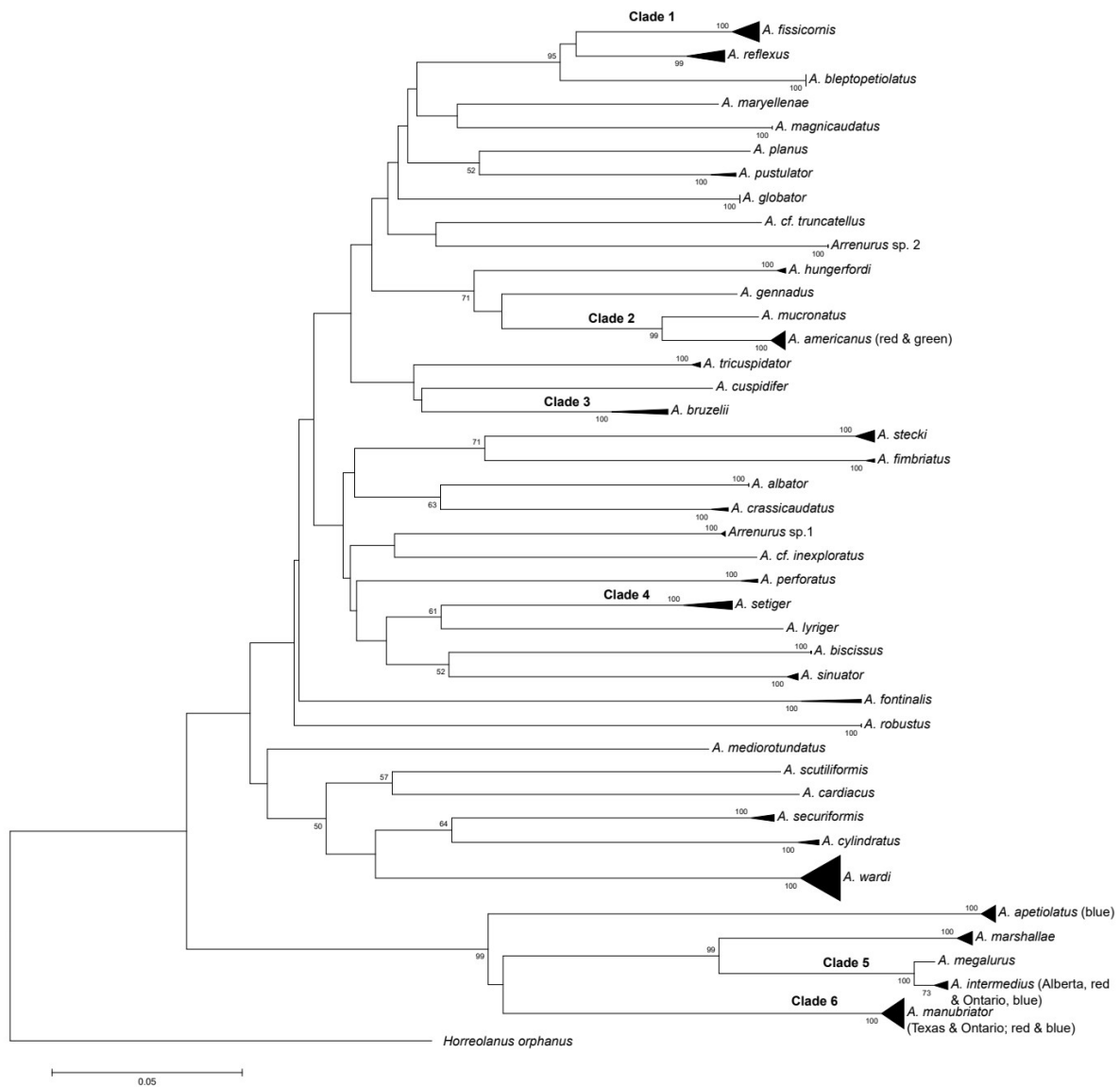
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593 Figure 2. Male hindbody (cauda), intromittent, and grasping organs in *Arrenurus* spp.; A. hindbody of  
594 *A. (Micrarrenurus) albator* with membranous sub-petiolar cavity, petiole without central piece, B.  
595 cauda of *A. (Micruracarus) biscissus* equipped with small and partly membranous petiole located in  
596 elaborated medial cleft, C. slightly elongated hindbody of *Arrenurus (Truncaturus)* sp. 2 with peg-like  
597 petiole, D. IVth leg of *A. (Arrenurus) bicuspidator* with spur (clasper organ), E. elaborate cauda with  
598 pygal lobes and petiole, *A. (Arrenurus) magnicaudatus*, F. very elongated and tubular cauda with peg-  
599 like petiole of *A. (Megaluracarus) globator*, G. intromittent organ with central piece, *A. (Arrenurus)*  
600 *bicuspidator*; abbreviations: cp – central piece of petiole, dh – dorsal hump, g - glandularium, mc –  
601 medial cleft, mpc - membranous sub-petiolar cavity, p – petiole, pl – pygal lobe, spur (grasping  
602 structure).

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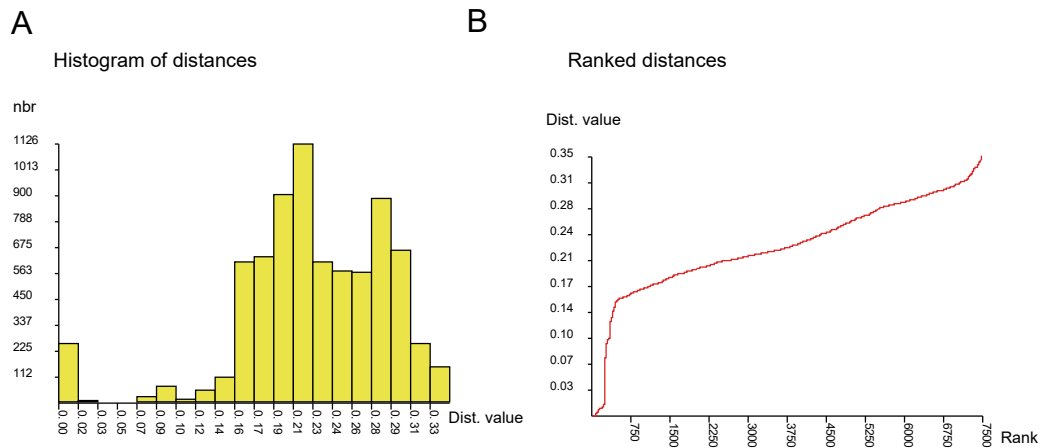
604 Figure 3. Kimura 2-parameter neighbor-joining tree of *Arrenurus* spp. based on 177 COI sequences  
605 from this study and data downloaded from GenBank and BOLD; bootstrap supports are given next to  
606 branches (only bootstrap values > 50 are shown). Clades consisted of more than one specimen are  
607 condensed; clades 1 - 6 represent closely related lineages – species delimitation analyses are  
608 summarized in Table 2. The NJ tree with expanded clades is shown in Supplementary Material, Fig.  
609 S1. *Horreolanus orphanus* is an outgroup species. See Table 1 for sequence codes and accession  
610 numbers.

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623 Figure 4. Results of ABGD species delimitation for 123 COI sequences from this study that belong to  
624 *Arrenurus* spp.; A - frequency histogram of K2P pairwise distances, B – ranked distances.

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626 Table 1. Sampling of *Arrenurus* spp. used in DNA barcoding. ‘Species’ is the *a priori* assignment that is based  
627 on morphology. Voucher information and accession numbers of sequences obtained in this study and  
628 downloaded from GenBank and BOLD are given.

Subgenus	Species	DNA voucher	28S rDNA	COI	Lat/Long	Locality	
<i>Arrenurus</i> s. str.	<i>A. gennadus</i> Cook, 1954	AMUmw255	KP836122	KP836187	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada	
	<i>A. tricuspikator</i> (O. F. Müller, 1776)	AMUmw164	KP836133	KP836199	53°06'52.6"N 08°47'45.3"E	ditches, Bremen, Germany	
	<i>A. tricuspikator</i> (O. F. Müller, 1776)	AMUmw167	MT895115	KP836200	53°06'52.6"N 08°47'45.3"E	ditches, Bremen, Germany	
	<i>A. tricuspikator</i> (O. F. Müller, 1776)	AMUmw168	-	MT891216	53°06'52.6"N 08°47'45.3"E	ditches, Bremen, Germany	
	<i>A. bruzelii</i> Koenike, 1885	AMUmw103	-	MT891199	53°06'52.6"N 08°47'45.3"E	ditches, Bremen, Germany	
	<i>A. bruzelii</i> Koenike, 1885	AMUmw104	-	KP836177	53°06'52.6"N 08°47'45.3"E	ditches, Bremen, Germany	
	<i>A. bruzelii</i> Koenike, 1885	AMUmw105	-	KP836178	48°32'31.42"N 09°09'03.38" E	Baggersee, Kirchentellinsfurt, Baden-Württemberg, Germany	
	<i>A. robustus</i> Koenike, 1894	AMUmw143	-	KP836131	-	Schleswig-Holstein, Germany	
	<i>A. robustus</i> Koenike, 1894	AMUmw132	-	MT891215	-	Schleswig-Holstein, Germany	
	<i>A. cuspidifer</i> Piersig, 1896	AMUmw161	-	KP836116	-	Italy	
	<i>A. maculator</i> (O. F. Müller, 1776)	AMUmw120	-	KP836120	-	peatland near Borne Sulnowo, Poland	
	<i>A. pustulator</i> (O. F. Müller, 1776)	AMUmw152	-	KP836194	53°29'56.5"N 16°28'38.6"E	Bagnisko Lake, Borne Sulnowo, Poland	
	<i>A. pustulator</i> (O. F. Müller, 1776)	AMUmw153	-	MT891211	53°29'56.5"N 16°28'38.6"E	Bagnisko Lake, Borne Sulnowo, Poland	
	<i>A. americanus</i> (red) Marshall, 1908	AMUmw258	-	MT895111	KP836171	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
	<i>A. americanus</i> (red) Marshall, 1908	AMUmw051	-	MT895112	KP836173	44°34'03.6"N 76°19'26.6"W	Lake Opinicon, QUBS, Canada

<i>A. americanus</i> (green) Marshall, 1908	AMUmw052	-	MT891191	44°34'03.6"N 76°19'26.6"W	Lake Opinicon, QUBS, Ontario, Canada
<i>A. americanus</i> (red) Marshall, 1908	AMUmw060	-	MT891192	44°34'03.6"N 76°19'26.6"W	Lake Opinicon, QUBS, Ontario, Canada
<i>A. americanus</i> (red) Marshall, 1908	AMUmw079	-	MT891193	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. americanus</i> Marshall, 1908	UAWM095-14	-	MG313272	53°39'23.7"N 112°45'37.0"W	private property near Elk Island National Park, Alberta, Canada
<i>A. americanus</i> Marshall, 1908	UAWM102-14	-	MG319546	53°39'23.7"N 112°45'37.0"W	private property near Elk Island National Park, Alberta, Canada
<i>A. americanus</i> Marshall, 1908	UAWM131-14	-	MG317722	53°33'10.8"N 114°29'45.6"W	Lake Wabamun, Alberta, Canada
<i>A. americanus</i> Marshall, 1908	UAWM100-14	-	MG320543	53°39'23.7"N 112°45'37.0"W	private property near Elk Island National Park, Alberta, Canada
<i>A. americanus</i> Marshall, 1908	UAWM098-14	-	MG315444	53°39'23.7"N 112°45'37.0"W	private property near Elk Island National Park, Alberta, Canada
<i>A. americanus</i> Marshall, 1908	UAWM096-14	-	MG320266	53°39'23.7"N 112°45'37.0"W	private property near Elk Island National Park, Alberta, Canada
<i>A. hungerfordi</i> Cook, 1954	AMUmw093	-	KP836185	-	Dancing Elephant Lake, east of Elk Island National Park, Alberta, Canada
<i>A. hungerfordi</i> Cook, 1954	AMUmw094	-	MT891202	53°39'23.7"N 112°45'37.0"W	private property near Elk Island National Park, Alberta, Canada
<i>A. hungerfordi</i> Cook, 1954	AMUmw096	-	MT891203	53°39'23.7"N 112°45'37.0"W	private property near Elk Island National Park, Alberta, Canada
<i>A. reflexus</i> Marshall, 1908	AMUmw013	KP836129	KP836195	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. reflexus</i> Marshall, 1908	AMUmw017	KP836130	KP836196	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. reflexus</i> Marshall, 1908	AMUmw014	-	MT891212	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. reflexus</i> Marshall, 1908	AMUmw016	-	MT891213	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. reflexus</i> Marshall, 1908	AMUmw091	-	MT891214	-	Le Roi swamp near QUBS, Ontario, Canada
<i>A. reflexus</i> Marshall, 1908	RRMFE2487-15	-	KT604056	43°22'25.0"N 80°21'54.7"W	Charitable Research Reserve, Ontario, Canada
<i>A. reflexus</i> Marshall, 1908	RRMFE2272-15	-	KT605287	43°22'25.0"N 80°21'54.7"W	Charitable Research Reserve, Ontario, Canada
<i>A. bleptepetiolatus</i> Cook, 1954	AMUmw001	MT895113	KP836175	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. bleptepetiolatus</i> Cook, 1954	AMUmw002	-	MT891194	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. bleptepetiolatus</i> Cook, 1954	AMUmw003	-	MT891195	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. bleptepetiolatus</i> Cook, 1954	AMUmw004	-	MT891196	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. bleptepetiolatus</i> Cook, 1954	AMUmw005	-	MT891197	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. bleptepetiolatus</i> Cook, 1954	AMUmw006	-	MT891198	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. bleptepetiolatus</i> Cook, 1954	AMUmw007	KP836112	KP836176	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. magnicaudatus</i> Marshall, 1908	AMUmw031	KP836121	KP836186	-	Stonehouse Creek near QUBS, Ontario, Canada
<i>A. magnicaudatus</i> Marshall, 1908	AMUmw245	-	MT891204	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. maryellenae</i> Cook, 1954	AMUmw250	KP836123	KP836188	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. planus</i> Marshall, 1908	AMUmwpla1	-	KP836193	-	pond on Indian Lake Road nr Chaffey's Lock, Ontario, Canada
<i>A. mucronatus</i> Levers, 1945	AMUmw048	KP836124	KP836189	-	Le Roi swamp near QUBS, Ontario, Canada
<i>A. fissicornis</i> Marshall, 1908	AMUmw008	KP836117	KP836182	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. fissicornis</i> Marshall, 1908	AMUmw009	-	MT891200	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada

<i>A. fissicornis</i> Marshall, 1908	AMUmw010	-	MT891201	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. fissicornis</i> Marshall, 1908	AMUmw011	KP836118	KP836183	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. fissicornis</i> Marshall, 1908	AMUmw012	KP836119	KP836184	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. fissicornis</i> Marshall, 1908	SWCHL2372-17	-	BOLD:ACL 1937 (SWCHL237 2-17)	44°30'56.2"N 76°01'28.9"W	Charleston Lake Provincial Park, Ontario, Canada
<i>A. fissicornis</i> Marshall, 1908	SWCHL2344-17	-	BOLD:ACL 1937 (SWCHL234 4-17)	44°30'56.2"N 76°01'28.9"W	Charleston Lake Provincial Park, Ontario, Canada
<i>A. fissicornis</i> Marshall, 1908	SWCHL2350-17	-	BOLD:ACL 1937 (SWCHL235 0-17)	44°30'56.2"N 76°01'28.9"W	Charleston Lake Provincial Park, Ontario, Canada
<i>A. fissicornis</i> Marshall, 1908	SWCHL2343-17	-	BOLD:ACL 1937 (SWCHL234 3-17)	44°30'56.2"N 76°01'28.9"W	Charleston Lake Provincial Park, Ontario, Canada
<i>A. fissicornis</i> Marshall, 1908	SWCHL2351-17	-	BOLD:ACL 1937 (SWCHL235 1-17)	44°30'56.2"N 76°01'28.9"W	Charleston Lake Provincial Park, Ontario, Canada
<i>A. fissicornis</i> Marshall, 1908	SWCHL2384-17	-	BOLD:ACL 1937 (SWCHL238 4-17)	44°30'56.2"N 76°01'28.9"W	Charleston Lake Provincial Park, Ontario, Canada
<i>A. fissicornis</i> Marshall, 1908	SWCHL2366-17	-	BOLD:ACL 1937 (SWCHL236 6-17)	44°30'56.2"N 76°01'28.9"W	Charleston Lake Provincial Park, Ontario, Canada
<i>A. claviger</i> Koenike 1885	AMUmwcla	MT895124		52°27'59.1"N 16°55'58.8"E	Morasko Campus, ponds, Poznań, Poland
<i>Micrarrenurus</i> <i>A. crassicaudatus</i> Kramer 1875	AMUmw235	KP836156	KP836225	52°27'59.1"N 16°55'58.8"E	Morasko Campus, ponds, Poznań, Poland
<i>A. crassicaudatus</i> Kramer 1875	AMUmw236	-	MT891240	52°27'59.1"N 16°55'58.8"E	Morasko Campus, ponds, Poznań, Poland
<i>A. albator</i> (O.F. Müller, 1776)	AMUmw098_100	KP836155	KP836224	53°06'30.4"N 08°49'27.9"E	ditches, Bremen, Germany
<i>A. fimbriatus</i> Koenike, 1885	AMUmw225	KP836157	KP836226	53°32'43.4"N 16°27'55.0"E	peatland Celnikowo near Borne Sulinowo, Poland
<i>A. fimbriatus</i> Koenike, 1885	AMUmw300	-	MT891241	53°06'30.4"N 08°49'27.9"E	ditches, Bremen, Germany
<i>Micruracarus</i> <i>A. biscissus</i> Lebert, 1879	AMUmw140	KP836158	KP836227	-	Schleswig-Holstein, Germany
<i>A. biscissus</i> Lebert, 1879	AMUmw102	-	MT891242	53°06'32.0"N 08°49'26.9"E	ditches, Bremen, Germany
<i>A. sinuator</i> (O. F. Müller, 1776)	AMUmw169	-	MT891244	53°07'37.6"N 08°47'31.1"E	ditches, Bremen, Germany
<i>A. sinuator</i> (O. F. Müller, 1776)	AMUmw171	MT895121	KP836232	53°07'37.6"N 08°47'31.1"E	ditches, Bremen, Germany
<i>A. sinuator</i> (O. F. Müller, 1776)	AMUmw234	KP836164	KP836233	52°27'59.1"N 16°55'58.8"E	Morasko Campus, ponds, Poznań, Poland
<i>A. sinuator</i> (O. F. Müller, 1776)	AMUmw159	KP836163	KP836231	53°07'39.8"N 08°47'30.1"E	ditches, Bremen, Germany
<i>A. perforatus</i> George, 1881	AMUmw157	KP836162	KP836230	53°06'52.6"N 08°47'45.3"E	ditches, Bremen, Germany
<i>A. perforatus</i> George, 1881	AMUmw158	-	MT891243	-	Stadtwaldsee, Bremen, Germany
<i>Arrenurus</i> sp1	AMUmw237	KP836165	KP836234	52°27'59.1"N 16°55'58.8"E	Morasko Campus, ponds, Poznań, Poland
<i>Arrenurus</i> sp1	AMUmw238	MT895122	KP836235	52°27'59.1"N 16°55'58.8"E	Morasko Campus, ponds, Poznań, Poland
<i>Arrenurus</i> sp1	AMUmw240	-	MT891245	52°27'59.1"N 16°55'58.8"E	Morasko Campus, ponds, Poznań, Poland
<i>A. cf. inexploratus</i> Viets, 1930	AMUmw232	KP836159	KP836228	52°27'59.1"N 16°55'58.8"E	Morasko Campus, ponds, Poznań, Poland

	<i>A. lyriger</i> Marshall, 1908	AMUmw046	KP836161	KP836229	44°34'03.6"N 76°19'26.6"W	Lake Opinicon, QUBS, Ontario, Canada
	<i>A. setiger</i> Koenike, 1895	AMUmw039	KP836166	KP836236	44°34'03.6"N 76°19'26.6"W	Lake Opinicon, QUBS, Ontario, Canada
	<i>A. setiger</i> Koenike, 1895	AMUmw040	MT895120	KP836237	44°34'03.6"N 76°19'26.6"W	Lake Opinicon, QUBS, Ontario, Canada
	<i>A. setiger</i> Koenike, 1895	AMUmw042	-	KP836238	44°34'03.6"N 76°19'26.6"W	Lake Opinicon, QUBS, Ontario, Canada
	<i>A. setiger</i> Koenike, 1895	AMUmw043	-	MT891246	44°34'03.6"N 76°19'26.6"W	Lake Opinicon, Ontario, QUBS
	<i>A. setiger</i> Koenike, 1895	AMUmw080	-	MT891247	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
	<i>A. infundibularis</i> (Marshall, 1908)	AMUmw251	KP836160	-	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>Truncaturus</i>	<i>A. stecki</i> Koenike, 1894	AMUmw223	KP836170	KP836242	53°32'43.4"N 16°27'55.0"E	peatland Celnikowo near Borne Sulinowo, Poland
	<i>A. stecki</i> Koenike, 1894	AMUmw200	MT895123	KP836241	53°32'43.4"N 16°27'55.0"E	peatland Celnikowo near Borne Sulinowo, Poland
	<i>A. stecki</i> Koenike, 1894	AMUmw199	-	MT891249	53°32'43.4"N 16°27'55.0"E	peatland Celnikowo near Borne Sulinowo, Poland
	<i>A. stecki</i> Koenike, 1894	AMUmw215	-	MT891250	53°32'43.4"N 16°27'55.0"E	peatland Celnikowo near Borne Sulinowo, Poland
	<i>A. stecki</i> Koenike, 1894	AMUmw216	-	MT891251	53°32'43.4"N 16°27'55.0"E	peatland Celnikowo near Borne Sulinowo, Poland
	<i>A. stecki</i> Koenike, 1894	AMUmw217	-	MT891252	53°32'43.4"N 16°27'55.0"E	peatland Celnikowo near Borne Sulinowo, Poland
	<i>A. stecki</i> Koenike, 1894	AMUmw219	-	MT891253	53°32'43.4"N 16°27'55.0"E	peatland Celnikowo near Borne Sulinowo, Poland
	<i>A. fontinalis</i> Viets, 1920	AMUmw141	KP836168	-	-	Schleswig-Holstein, Germany
	<i>A. fontinalis</i> Viets, 1920	NMB-TROM-10237	-	MK889748	-	Oberallgäu, Imberg, Strausbergmoos, Germany
	<i>A. fontinalis</i> Viets, 1920	NMB-TROM-10236	-	MK889747	-	Oberallgäu, Imberg, Strausbergmoos, Germany
	<i>A. cf. truncatellus</i> (O. F. Müller, 1776)	AMUmw201	KP836167	KP836239	53°32'43.4"N 16°27'55.0"E	peatland Celnikowo near Borne Sulinowo, Poland
	<i>Arrenurus</i> sp2	AMUmw303	KP836169	KP836240	44°34'03.6"N 76°19'26.6"W	Lake Opinicon, QUBS, Ontario, Canada
	<i>Arrenurus</i> sp2	AMUmw304	-	MT891248	44°34'03.6"N 76°19'26.6"W	Lake Opinicon, QUBS, Ontario, Canada
<i>Megaluracarus</i>	<i>A. cylindricus</i> Piersig, 1896	AMUmw165	KP836138	KP836206	47°37'12.7"N 9°56'07.5"E	Limnocene spring, Bayern, Germany
	<i>A. cylindricus</i> Piersig, 1896	AMUmw127	-	MT891222	-	Schleswig-Holstein, Germany
	<i>A. cylindricus</i> Piersig, 1896	AMUmw175	-	MT891223	47°37'12.7"N 9°56'07.5"E	Limnokrene, Bayern, Germany
	<i>A. securiformis</i> Piersig, 1894	AMUmw124	MT895119	KP836218	-	Schleswig-Holstein, Germany
	<i>A. securiformis</i> Piersig, 1894	AMUmw139	KP836150	KP836219	-	Schleswig-Holstein, Germany
	<i>A. securiformis</i> Piersig, 1894	AMUmw156	KP836151	KP836220	53°06'30.4"N 08°49'27.9"E	ditches, Bremen, Germany
	<i>A. securiformis</i> Piersig, 1894	AMUmw137	-	MT891238	-	Schleswig-Holstein, Germany
	<i>A. mediorotundatus</i> Thor, 1898	AMUmw142	KP836146	KP836215	-	Schleswig-Holstein, Germany
	<i>A. scutiformis</i> Garms, 1961	AMUmw256	KP836149	-	44°34'03.6"N 76°19'26.6"W	Lake Opinicon, QUBS, Ontario, Canada
	<i>A. cardiacus</i> Marshall, 1903	AMUmw259	KP836137	KP836205	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
	<i>A. globator</i> (O. F. Müller, 1776)	AMUmw211	KP836139	KP836207	52°27'59.1"N 16°55'58.8"E	Morasko Campus, ponds, Poznań, Poland
	<i>A. globator</i> (O. F. Müller, 1776)	AMUmw112	-	MT891224	52°27'59.1"N 16°55'58.8"E	Morasko Campus, ponds, Poznań, Poland
	<i>A. globator</i> (O. F. Müller, 1776)	AMUmw113	-	MT891225	-	peatland near Borne Sulinowo, Poland
	<i>A. globator</i> (O. F. Müller, 1776)	AMUmw118	-	MT891226	-	peatland near Borne Sulinowo, Poland
	<i>A. globator</i> (O. F. Müller, 1776)	AMUmw119	-	MT891227	-	peatland near Borne Sulinowo, Poland
	<i>A. buccinator</i> (O.F. Müller, 1776)	AMUmw106	KP836136	-	53°06'52.6"N 08°47'45.3"E	ditches, Bremen, Germany
	<i>A. apetirolatus</i> (blue) Piersig, 1904	AMUmw034	-	KP836201	-	Stonehouse Creek near QUBS, Canada
	<i>A. apetirolatus</i> (blue) Piersig, 1904	AMUmw036	KP836135	KP836202	44°34'03.6"N 76°19'26.6"W	Lake Opinicon, QUBS, Ontario, Canada
	<i>A. apetirolatus</i> (blue) Piersig, 1904	AMUmw074	MT895116	KP836203	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada



<i>A. apetirolatus</i> (blue) Piersig, 1904	AMUmw082	MT895117	KP836204	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. apetirolatus</i> (red) Piersig, 1904	AMUmw248	KP836134	-	-	Hebert's Bog, QUBS, Ontario, Canada
<i>A. apetirolatus</i> (blue) Piersig, 1904	AMUmw083	-	MT891219	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. apetirolatus</i> (blue) Piersig, 1904	AMUmw086	-	MT891220	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. apetirolatus</i> (blue) Piersig, 1904	AMUmw075	-	MT891218	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. apetirolatus</i> (blue) Piersig, 1904	AMUmw087	-	MT891221	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. apetirolatus</i> (blue) Piersig, 1904	AMUmw038	-	MT891217	44°34'03.6"N 76°19'26.6"W	Lake Opinicon, QUBS, Ontario, Canada
<i>A. apetirolatus</i> Piersig, 1904	BACZP1279-16	-	MG449549	44°33'47.2"N 76°33'12.6"W	Eel Lake, Ontario, Canada
<i>A. marshallae</i> Piersig, 1904	AMUmw247	KP836148	KP836217	44°34'03.6"N 76°19'26.6"W	Lake Opinicon, QUBS, Ontario, Canada
<i>A. marshallae</i> Piersig, 1904	GMOEF2171-15	-	MG314930	42°52'58.8"N 82°11'02.4"W	L.C. Henderson Conservation Area, Ontario, Canada
<i>A. marshallae</i> Piersig, 1904	GMOEF2175-15	-	MG314834	42°52'58.8"N 82°11'02.4"W	Petrolia - LC Henderson CA, Ontario, Canada
<i>A. marshallae</i> Piersig, 1904	GENWM167-16	-	MG313280	43°32'54.2"N 80°13'22.4"W	Eramosa River, Ontario, Canada
<i>A. marshallae</i> Piersig, 1904	SWTHI1242-17	-	BOLD:ACL 2521 (SWTHI124 2-17)	44°29'49.6"N 75°49'32.2"W	Thousand Island NP, Ontario, Canada
<i>A. marshallae</i> Piersig, 1904	GMOEF2170-15	-	MG317870	42°52'58.8"N 82°11'02.4"W	Petrolia - LC Henderson Conservation Area, Ontario, Canada
<i>A. marshallae</i> Piersig, 1904	SWCHL654-15	-	MG314823	44°30'17.3"N 76°02'26.9"W	Charleston Lake Provincial Park, Ontario, Canada
<i>A. marshallae</i> Piersig, 1904	GMOEF2173-15	-	MG318581	42°52'58.8"N 82°11'02.4"W	Petrolia - LC Henderson Conservation Area, Ontario, Canada
<i>A. intermedius</i> (blue) Marshall, 1940	AMUmw306	KP836140	KP836208	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. intermedius</i> (blue) Marshall, 1940	AMUmw307	KP836141	KP836209	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. intermedius</i> (blue) Marshall, 1940	AMUmw308	KP836142	KP836210	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. intermedius</i> (blue) Marshall, 1940	AMUmw089	-	MT891228	-	marshy stream on Indian Lake Road, near QUBS, Ontario, Canada
<i>A. intermedius</i> (red) Marshall, 1940	AMUmw263	KP836152	KP836221	-	East Pitlk. Nr Wabamoun Village, Alberta, Canada
<i>A. megalurus</i> Marshall, 1903	AMUmw249	KP836147	KP836216	44°32'15.8"N 76°23'27.1"W	Lindsey Lake, QUBS, Ontario, Canada
<i>A. manubriator</i> (blue) Marshall, 1903	AMUmw028	KP836143	KP836211	44°34'03.6"N 76°19'26.6"W	originally from Lake Opinicon, QUBS, Ontario, Canada
<i>A. manubriator</i> (blue) Marshall, 1903	AMUmw030	MT895118	KP836212	44°34'03.6"N 76°19'26.6"W	originally from Lake Opinicon, QUBS, Ontario, Canada
<i>A. manubriator</i> (red) Marshall, 1903	AMUmw020	KP836144	KP836213	-	originally from San Marcos River, San Marcos, Texas, USA
<i>A. manubriator</i> (red) Marshall, 1903	AMUmw023	KP836145	KP836214	-	originally from San Marcos River, San Marcos, Texas, USA
<i>A. manubriator</i> (red) Marshall, 1903	AMUmw018	-	MT891233	-	originally from San Marcos River, San Marcos, Texas, USA
<i>A. manubriator</i> (red) Marshall, 1903	AMUmw019	-	MT891234	-	originally from San Marcos River, San Marcos, Texas, USA
<i>A. manubriator</i> (red) Marshall, 1903	AMUmw021	-	MT891235	-	originally from San Marcos River, San Marcos, Texas, USA
<i>A. manubriator</i> (red) Marshall, 1903	AMUmw022	-	MT891236	-	originally from San Marcos River, San

1903						Marcos, Texas, USA
<i>A. manubriator</i> (red) Marshall, 1903	AMUmw024	-	MT891237	-		originally from San Marcos River, San Marcos, Texas, USA
<i>A. manubriator</i> (blue) Marshall, 1903	AMUmw025	-	MT891229	44°34'03.6"N 76°19'26.6"W		originally from Lake Opinicon, QUBS, Ontario, Canada
<i>A. manubriator</i> (blue) Marshall, 1903	AMUmw026	-	MT891230	44°34'03.6"N 76°19'26.6"W		originally from Lake Opinicon, QUBS, Ontario, Canada
<i>A. manubriator</i> (blue) Marshall, 1903	AMUmw027	-	MT891231	44°34'03.6"N 76°19'26.6"W		originally from Lake Opinicon, QUBS, Ontario, Canada
<i>A. manubriator</i> (blue) Marshall, 1903	AMUmw028	KP836143	KP836211	44°34'03.6"N 76°19'26.6"W		originally from Lake Opinicon, QUBS, Ontario, Canada
<i>A. manubriator</i> (blue) Marshall, 1903	AMUmw029	-	MT891232	44°34'03.6"N 76°19'26.6"W		originally from Lake Opinicon, QUBS, Ontario, Canada
<i>A. manubriator</i> Marshall, 1903	GENWM151-16	-	MG313573	44°33'47.2"N 76°33'12.6"W		Eel Lake Cottage, Ontario, Canada
<i>A. manubriator</i> Marshall, 1903	SWTHI1056-17	-	BOLD:ACL 2991 (SWTHI1056-17)	44°29'49.6"N 75°49'32.2"W		Thousand Island NP, Ontario, Canada
<i>A. manubriator</i> Marshall, 1903	GENWM152-16	-	MG317929	44°33'47.2"N 76°33'12.6"W		Eel Lake Cottage, Ontario, Canada
<i>A. manubriator</i> Marshall, 1903	PPGB134-12	-	MN359717	45°37'19.2"N 80°25'08.4"W		Georgian Bay, Ontario, Canada
<i>A. manubriator</i> Marshall, 1903	SWTHI1006-17	-	BOLD:ACL 2991 (SWTHI1006-17)	44°29'49.6"N 75°49'32.2"W		Thousand Island NP, Ontario, Canada
<i>A. wardi</i> Marshall, 1940	AMUmw078	-	MT891239	44°32'15.8"N 76°23'27.1"W		Lindsey Lake, QUBS, Ontario, Canada
<i>A. wardi</i> Marshall, 1940	AMUmw301	KP836153	KP836222	44°32'15.8"N 76°23'27.1"W		Lindsey Lake, QUBS, Ontario, Canada
<i>A. wardi</i> Marshall, 1940	AMUmw309	KP836154	KP836223	44°32'15.8"N 76°23'27.1"W		Lindsey Lake, QUBS, Ontario, Canada
<i>A. wardi</i> Marshall, 1940	SSPAA2249-13	-	KM839514	53°54'19.8"N 106°01'30.0"W		Prince Albert National Park, Saskatchewan, Canada
<i>A. wardi</i> Marshall, 1940	SSPAA2246-13	-	KM833758	53°54'19.8"N 106°01'30.0"W		Prince Albert National Park, Saskatchewan, Canada
<i>A. wardi</i> Marshall, 1940	CNGBG1826-14	-	KR096679	44°51'05.4"N 79°52'27.8"W		Georgian Bay Islands NP, Ontario, Canada
<i>A. wardi</i> Marshall, 1940	SSPAA2294-13	-	KM828158	53°54'19.8"N 106°01'30.0"W		Prince Albert National Park, Saskatchewan, Canada
<i>A. wardi</i> Marshall, 1940	CNGBF1016-14	-	KR096062	44°51'05.4"N 79°52'27.8"W		Georgian Bay Islands NP, Ontario, Canada
<i>A. wardi</i> Marshall, 1940	CNGBF1072-1	-	KR098901	44°51'05.4"N 79°52'27.8"W		Georgian Bay Islands NP, Ontario, Canada
<i>A. wardi</i> Marshall, 1940	CNGBF1646-14	-	KR100183	44°51'05.4"N 79°52'27.8"W		Georgian Bay Islands NP, Ontario, Canada
<i>A. wardi</i> Marshall, 1940	CNGBE870-14	-	KR104611	44°51'05.4"N 79°52'27.8"W		Georgian Bay Islands NP, Ontario, Canada
<i>A. wardi</i> Marshall, 1940	CNGBH715-14	-	KR102634	44°51'05.4"N 79°52'27.8"W		Georgian Bay Islands NP, Ontario, Canada
<i>A. wardi</i> Marshall, 1940	CNGBG1910-14	-	KR098840	44°51'05.4"N 79°52'27.8"W		Georgian Bay Islands NP, Ontario, Canada
<i>A. wardi</i> Marshall, 1940	CNGBH769-14	-	KR100298	44°51'05.4"N 79°52'27.8"W		Georgian Bay Islands NP, Ontario, Canada
<i>A. wardi</i> Marshall, 1940	SSPAA2265-13	-	KM830004	53°54'19.8"N 106°01'30.0"W		Prince Albert National Park, Saskatchewan, Canada
<i>A. wardi</i> Marshall, 1940	SIOCA017-10	-	JN309443	53°54'21.6"N 106°01'48.0"W		Prince Albert National Park, Saskatchewan, Canada
<i>A. wardi</i> Marshall, 1940	SIOCA018-10	-	JN309444	53°54'21.6"N 106°01'48.0"W		Prince Albert National Park, Saskatchewan, Canada

<i>A. wardi</i> Marshall, 1940	SSPAA2302-13	-	KM824466	53°54'19.8"N 106°01'30.0"W	Prince Albert National Park, Saskatchewan, Canada	
<i>A. wardi</i> Marshall, 1940	SSPAA2274-13	-	KM838807	53°54'19.8"N 106°01'30.0"W	Prince Albert National Park, Saskatchewan, Canada	
<i>A. wardi</i> Marshall, 1940	CNGBG1877-14	-	KR102519	44°51'05.4"N 79°52'27.8"W	Georgian Bay Islands NP, Ontario, Canada	
<i>A. wardi</i> Marshall, 1940	CNGBO974-14	-	KR099957	44°51'05.4"N 79°52'27.8"W	Georgian Bay Islands NP, Ontario, Canada	
<i>A. wardi</i> Marshall, 1940	CNGBG1957-14	-	KR105143	44°51'05.4"N 79°52'27.8"W	Georgian Bay Islands NP, Ontario, Canada	
<i>A. wardi</i> Marshall, 1940	SSPAA2281-13	-	KM827656	53°54'19.8"N 106°01'30.0"W	Prince Albert National Park, Saskatchewan, Canada	
<i>A. wardi</i> Marshall, 1940	SSPAA2296-13	-	KM836945	53°54'19.8"N 106°01'30.0"W	Prince Albert National Park, Saskatchewan, Canada	
<i>A. wardi</i> Marshall, 1940	SSPAA2256-13	-	KM831767	53°54'19.8"N 106°01'30.0"W	Prince Albert National Park, Saskatchewan, Canada	
<i>A. wardi</i> Marshall, 1940	SSPAA2264-13	-	KM826410	53°54'19.8"N 106°01'30.0"W	Prince Albert National Park, Saskatchewan, Canada	
<i>A. wardi</i> Marshall, 1940	CNGBG1913-14	-	KR102059	44°51'05.4"N 79°52'27.8"W	Georgian Bay Islands NP, Ontario, Canada	
<i>Horreolanus orphanus</i> Mitchell, 1955	-	-	KM100946	KM101004	-	USA

Table 2. Results of species delimitation analyses based on COI DNA sequences obtained in this study. Remaining well-supported clades within species were concordantly recovered as conspecific by all methods. Abbreviations used (+): separate species, (-): the same species, (?): ambiguous result.

Clade number	Distance comparison					P <sub>(AB)</sub>	Conclusion
	Distance between groups (%)	Inter /Intra-group distance ratio	ABGD	TCS	P <sub>(RD)</sub>		
1 <i>A. fissicornis</i>	1.4 (-)	11.1 (+)	(-)	(-)	<0.05 (+)	0.05 (+)	Conspecific, two well-supported clades of coalescent origin yet genetically poorly diversified
2 <i>A. americanus</i> (red and green), <i>A. mucronatus</i>	4.9-5.1 (?)	12.5 (+)	(-)	(+)	<0.05 (+)	0.07 (-)	Speciation or hybridization of <i>A. americanus</i> and <i>A. mucronatus</i> is not finished
3 <i>A. bruzelii</i>	2.7-3.0 (-)	6.25 (-)	(-)	(+)	NA	0.33 (-)	Conspecific, well genetically separated clades of coalescent origin
4 <i>A. setiger</i>	2.4-2.8 (-)	7.69 (-)	(-)	(+)	NA	0.1 (-)	Conspecific, well genetically separated clades of coalescent origin
5 <i>A. megalurus</i> , <i>A. intermedius</i> (red and blue)	1.3-1.7 (-)	2.38 (-)	(-)	(-)	NA	0.07 (-)	<i>A. megalurus</i> and <i>A. intermedius</i> are conspecific
6 <i>A. manubriator</i> Ontario (blue) and Texas (red)	2.18 (-)	9 (+)	(-)	(-)	<0.05 (+)	<<0.001 (+)	Conspecific, but well separated groups being formed by speciation mechanism rather than coalescence

630

### 631 Supplementary material

632 Figure S1. Kimura 2-parameter neighbor-joining tree of *Arrenurus* spp. based on 177 COI sequences  
633 from this study and data downloaded from GenBank and BOLD (bootstrap supports next to branches,  
634 only values > 50 are shown). There are included GenBank sequences of *Arrenurus fontinalis* since we  
635 have failed to obtain COI sequences from our samples. *Horreolanus orphanus* is an outgroup species.  
636 Clades 1 - 6 are closely related lineages and relate to results of species delimitation analyses shown in  
637 Table 2. Table 1 includes sequence codes and accession numbers.

638

639 Figure S2. The box plots show medians, quartiles and standard errors of genetic distances within the  
640 genus *Arrenurus* and within subgenera *Arrenurus* s.str., *Megaluracarus*, *Micruracarus*, *Truncaturus*  
641 and *Micrarrenurus*; species with exceptionally low distance values and colour variants were excluded  
642 from statistical analysis; A. COI (K2P) distances - *A. intermedius* vs. *A. megalurus* were excluded  
643 from statistical analysis; B. 28S (K2P) distances - *A. intermedius* vs. *A. megalurus* and *A. gennadus*

644 vs. *A. mucronatus* were not included; subgenera *Truncaturus* and *Micrarrenurus* were represented  
645 only by 6 (3 for COI) and 3 observations, respectively.

646

647 Figure S3. Haplotype networks of the COI gene under the 95% parsimony criterion. Networks  
648 corresponding with named species and one unnamed morphospecies are shown. The size of ovals  
649 reflects haplotype frequencies. Dots on lines indicate one missing unsampled haplotype.

650

651 Figure S4. Haplotype networks of the COI gene under the 95% parsimony criterion. Networks  
652 consisted of several named species or splitted networks are presented. The size of ovals reflects  
653 haplotype frequencies. Each bar indicates one missing unsampled haplotype.

654

655 Figure S5. ABGD output tree based on 123 COI sequences of *Arrenurus* spp. from this study. The  
656 number of groups in initial partitions is presented. See Table 1 for sequence codes and accession  
657 numbers.

658

659 Table S1. Kimura 2-parameter (K2P) distances (and standard errors) for COI sequences obtained in  
660 this study calculated within (in grey) and between species.

661

662 Table S2. Kimura 2-parameter (K2P) distances (and standard errors) for 28S sequences obtained in  
663 this study calculated within (in grey) and between species.