



# The First Phylogenetic Analysis of Palpigradi (Arachnida)—The Most Enigmatic Arthropod Order

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1 **The first phylogenetic analysis of Palpigradi (Arachnida)—the most**  
2 **enigmatic arthropod order**

3 *Gonzalo Giribet<sup>A,K</sup>, Erin McIntyre<sup>A</sup>, Erhard Christian<sup>B</sup>, Luis Espinosa<sup>C</sup>, Rodrigo L. Ferreira<sup>D</sup>, Óscar F.*  
4 *Francke<sup>E</sup>, Mark S. Harvey<sup>F</sup>, Marco Isaia<sup>G</sup>, Ľubomír Kováč<sup>H</sup>, Lynn McCutchen<sup>I</sup>, Maysa F. V. R.*  
5 *Souza<sup>D</sup> and Maja Zagamajster<sup>J</sup>*

6

7 <sup>A</sup>Museum of Comparative Zoology, Department of Organismic and Evolutionary Biology, Harvard  
8 University, 26 Oxford Street, Cambridge, MA 02138, USA.

9 <sup>B</sup>Institut für Zoologie, Universität für Bodenkultur, Gregor-Mendel-Straße 33, 1180 Wien, Austria.

10 <sup>C</sup>School of Science, Marist College, 3399 North Road, Poughkeepsie, New York, USA.

11 <sup>D</sup> Centro de Estudos em Biologia Subterrânea, Departamento de Biologia, Universidade Federal  
12 de Lavras, Lavras, MG. CEP 37200-000, Brazil.

13 <sup>E</sup>Colección Nacional de Arácnidos, Instituto de Biología, UNAM, Apartado Postal 70-153, C. P.  
14 04510, Mexico, D. F., Mexico.

15 <sup>F</sup>Department of Terrestrial Zoology, Western Australian Museum, Locked Bag 49, Welshpool DC,  
16 WA 6986, Australia.

17 <sup>G</sup>Dipartimento di Scienze della Vita e Biologia dei Sistemi, Università di Torino, Via Accademia  
18 Albertina 13, 10123 Torino, Italy.

19 <sup>H</sup>Department of Zoology, Institute of Biology and Ecology, Faculty of Science, P. J. Šafárik  
20 University, Moyzesova 11, 040 01 Košice, Slovakia.

21 <sup>I</sup>Department of Biology, Kilgore College, 1100 Broadway, Kilgore, TX 75662, USA.

22 <sup>J</sup>SubBioLab, Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111,  
23 SI-1000 Ljubljana, Slovenia.

24 <sup>K</sup>Corresponding author. Email: ggiribet@g.harvard.edu

25 **Abstract.** Palpigradi are a poorly understood group of delicate arachnids, often found in  
26 caves or other subterranean habitats. Concomitantly, they have been neglected from a  
27 phylogenetic point of view. Here we present the first molecular phylogeny of palpigrades based  
28 on specimens collected in different subterranean habitats, both endogean (soil) and hypogean  
29 (caves), from Australia, Africa, Europe, South America and North America. Analyses of two  
30 nuclear ribosomal genes and COI under an array of methods and homology schemes found  
31 monophyly of Palpigradi, Eukoeneriidae, and a division of Eukoeneriidae into four main clades,  
32 three of which include samples from multiple continents. This supports either ancient vicariance  
33 or long-range dispersal, two alternatives we cannot distinguish with the data at hand. In  
34 addition, we show that our results are robust to homology scheme and analytical method,  
35 encouraging further use of the markers employed in this study to continue drawing a broader  
36 picture of palpigrade relationships.

37

38

39 **Additional keywords:** Arachnida, micro-whip scorpions, palpigrades, speleobiology,  
40 biogeography.

## 41 Introduction

42 The arachnid order Palpigradi (micro-whip scorpions or palpigrades) is one of the smallest,  
43 rarest and most neglected groups of terrestrial arthropods, and one of the last arachnid orders  
44 to be discovered—it was first reported only in 1885 (Grassi and Calandruccio 1885). The first  
45 photographs of living palpigrades did not appear published until the first decade of the 21st  
46 century (Kováč *et al.* 2002; Beccaloni 2009). Additionally, only a handful of DNA sequence data  
47 are available in GenBank; with only 64 sequences, 56 are for *Prokoenenia wheeleri* (Rucker,  
48 1901), a species that was part of a multi-gene phylogeny of arthropods (Regier *et al.* 2010),  
49 while the remaining eight sequences are unidentified specimens from three studies on  
50 chelicerate phylogenetics (Giribet *et al.* 2002; Pepato *et al.* 2010; Arabi *et al.* 2012). Contrary to  
51 this, one can find more DNA sequences for other small arachnid orders in GenBank: 105 for  
52 Uropygi, 200 for Schizomida, 200 for Ricinulei, 251 for Amblypygi, and 502 for Pseudoscorpiones,  
53 [checked on October 25th, 2013]. In addition, there are only 2 sequences available in the  
54 Barcode of Life website (<http://www.barcodinglife.org>).

55 Palpigrades are delicate animals that walk sensing the substrate with what seems a nervous  
56 behaviour of the first pair of walking legs, and use their unmodified palps for walking, unlike all  
57 other arachnids (Fig. 1). While moving, most palpigrades keep the flagellum upward, moving it  
58 laterally. Accordingly, it is possible that the uplifted flagellum is associated with perception of  
59 the environment (Ferreira and Souza 2012). These small, depigmented and highly translucent  
60 arachnids range in size from 0.65 mm in *Eukoenenia grassii* (Hansen, 1901) to 2.4 mm in the  
61 “giant” *E. draco* (Peyerimhoff, 1906) from caves on the island of Majorca (Mayoral and Barranco  
62 2013). *Eukoenenia spelaea* (Peyerimhoff, 1902) from Slovakia has recently been reported to  
63 feed on heterotrophic Cyanobacteria (Smrž *et al.* 2013). The mode of sperm transfer in these  
64 arachnids remains unknown.

65 The living members of the order are currently divided in two families, Eukoeneniidae  
66 Petrunkevitch, 1955, with 4 genera and 85 named species, and Prokoeneniidae Condé, 1996,  
67 with 2 genera and 7 named species (Harvey 2002; Prendini 2011; Souza and Ferreira 2013).  
68 Eukoeneniidae includes the genera *Allokoenenia* Silvestri, 1913 (1 sp. from West Africa),  
69 *Eukoenenia* Börner, 1901 (71 spp., on all continents under tropical and subtropical climate; in  
70 temperate regions predominantly in caves), *Koeneriodes* Silvestri, 1913 (8 Palaeotropical spp.)  
71 and *Leptokoenenia* Condé, 1965 (5 spp. in the Afrotropical, Neotropical and Palearctic regions).

72 Prokoeneniidae includes the genera *Prokoenenia* Börner, 1901 (6 spp. in the Nearctic,  
73 Neotropical and Oriental regions) and *Triadokoenenia* Condé, 1991 (1 sp. from Madagascar).  
74 Further unnamed new species are known to us from various parts of the world.

75 The position of Palpigradi among the arachnid orders remains highly debated. The largest set of  
76 data analysed to date places them as the sister group to Acariformes mites in a basal position  
77 within arachnids, although without support (Regier *et al.* 2010). The most recent morphological  
78 cladistic analysis of arachnid relationships leaves them mostly unresolved among the clades  
79 Stomothecata, Haplocnemata, Pantetrapulmonata, and Acaromorpha (Shultz 2007). Earlier  
80 studies combining morphology and a small set of molecular data placed Palpigradi as the sister  
81 group of Ricinulei + Tetrapulmonata or as sister to Pycnogonida when fossils were considered,  
82 although again, without significant clade support (Giribet *et al.* 2002); as sister to a clade  
83 including Acari and Solifugae, based on the same two markers used in earlier studies (Pepato *et*  
84 *al.* 2010); or in an unresolved position within arachnids (Arabi *et al.* 2012). Even less is known  
85 about the internal relationships of the group, since no published study—molecular or  
86 morphological—has yet incorporated information for more than one palpigrade species, and  
87 only one unpublished masters thesis has explored palpigrade relationships cladistically, using  
88 morphology (Montaño Moreno 2008).

89 To bridge this important gap in the knowledge of this arachnid order, although acknowledging  
90 the difficulties in sampling and identification of these elusive animals, we obtained samples for  
91 as many species of palpigrades as possible and from as many localities as possible with the aim  
92 to obtain molecular DNA sequence data to generate a first hypothesis of internal palpigrade  
93 relationships.

94

## 95 **Materials and Methods**

### 96 *Taxon sampling*

97 Palpigrades are difficult to obtain and identify, and success of field sampling differed among  
98 regions included in the study. In Western Australia, many samples were collected indirectly in  
99 caves and bore holes. In Brazil and Europe, they can be abundant in caves, where fresh  
100 specimens have recently become available for inclusion in molecular studies. Additional samples

101 were from soil samples in Australia, Italy and the USA. In addition to fresh material collected for  
102 this study, older specimens were used, especially from the diverse cave systems in Brazil, where  
103 several new species have been recently described (Souza and Ferreira 2010; Ferreira *et al.* 2011;  
104 Souza and Ferreira 2011a; Souza and Ferreira 2011b; Souza and Ferreira 2012a; Souza and  
105 Ferreira 2012b). While a recently collected specimen of *Eukoenenia ferratilis* Souza & Ferreira,  
106 2011 amplified well for some of the studied markers, none of the six specimens of *Allokoenenia*  
107 spp. and the two specimens of *Leptokoenenia* sp. collected from the caves yielded workable  
108 DNA. We also obtained a relatively large collection of specimens from the Western Australian  
109 bore holes from Barrow Island and the Pilbara, but these were collected from litter traps and  
110 many specimens did not amplify or only yielded some amplicons. Some of these specimens are  
111 probably related to the Western Australian endemic *E. guzikae* Barranco & Harvey, 2008, but  
112 unrelated to the more widespread species *E. mirabilis* (Grassi & Calandruccio, 1885), also found  
113 in Western Australia (Harvey *et al.* 2006; Barranco and Harvey 2008). A single specimen of  
114 *Prokoenenia wheeleri* was obtained from the Austin area (Texas, USA), but amplified well for all  
115 fragments attempted. In addition, we obtained samples of *Eukoenenia mirabilis* from Italy  
116 (Christian *et al.* 2010) and Australia (Harvey *et al.* 2006), *E. spelaea* (Peyerimhoff, 1902) from  
117 multiple localities in Slovenia and Slovakia (Kováč *et al.* 2002; Zagamajster and Kováč 2006; Král *et*  
118 *al.* 2008). Italian samples also include *E. bonadonai* Condé, 1979 and *E. strinatii* Condé, 1977,  
119 collected in caves. We also included specimens from multiple localities from the hanseni-  
120 chilanga group of *Eukoenenia* from Mexico and the USA (Montaño-Moreno 2012). Additional  
121 specimens come from Mexican caves and South Africa. Details on collecting localities are  
122 available in Table 1 and in MCZBASE (<http://mczbase.mcz.harvard.edu/SpecimenSearch.cfm>).  
123 Vouchers or additional specimens are deposited in the Museum of Comparative Zoology,  
124 Harvard University (MCZ), and in the Western Australian Museum (WAM).

125 We included three species available in GenBank, one from South Africa sequenced by  
126 Giribet *et al.* (2002), one from Brazil from Pepato *et al.* (2010), and one of unknown origin  
127 published by Arabi *et al.* (2012). Here we added sequences from an additional South African  
128 specimen from the same collection of that from Giribet *et al.* (2002), and a specimen of *E.*  
129 *ferratilis* from Brazil, which was identical to the specimen reported by Pepato *et al.* (2010) as  
130 *Eukoenenia* sp., and to which we refer to as *E. cf. ferratilis* in the present study. Outgroup taxa  
131 were selected from GenBank (Table 2), mostly from previous studies on arthropod or arachnid  
132 phylogeny using nuclear ribosomal genes (Giribet *et al.* 2002; Mallatt and Giribet 2006).

133

134 *Molecular methods*

135 Although we attempted to amplify and sequence five molecular markers typically used in other  
136 analyses of arachnid systematics (e.g., Dimitrov *et al.* 2012; Giribet *et al.* 2012), the  
137 mitochondrial 16S rRNA gene only amplified for *Prokoenenia wheeleri* and the nuclear protein-  
138 encoding gene histone H3, although amplified for several samples, did not produce clean reads.  
139 We thus restricted our study to the two broadly available nuclear ribosomal genes, the  
140 complete 18S rRNA and ca. 2.2 Kb of 28S rRNA, and the mitochondrial protein-encoding  
141 cytochrome *c* oxidase subunit I (COI hereafter) (as in Murienne *et al.* 2008), although the latter  
142 gene only amplified for about a third of the specimens (Table 1). For two of the bore-hole  
143 Western Australian specimens, poorly preserved, only the middle amplicon of 28S rRNA worked.

144 Total DNA was extracted from whole specimens or from the opisthosomal region using  
145 Qiagen's DNEasy® tissue kit (Valencia, CA, USA). Although we were aiming to preserve the  
146 digested carcass as a morphological voucher, it was completely digested and not recoverable.  
147 Purified genomic DNA was used as a template for Polymerase chain reactions (PCR)  
148 amplification. PCR, visualization by agarose gel electrophoresis, and direct sequencing were  
149 conducted for most specimens as described in earlier work, e.g., Edgecombe and Giribet (2009).  
150 Chromatograms obtained from the automatic sequencer were read and sequences assembled  
151 using the sequence editing software Sequencher™ (Gene Codes Corporation, Ann Arbor, MI,  
152 USA). Sequence data were edited in MacGDE (Linton 2005). The three genes were analysed as  
153 follows:

154 18S rRNA: This marker was amplified in three amplicons (*a*, *b*, *c*), as in previous studies  
155 (Edgecombe and Giribet 2009; Giribet *et al.* 2010; Giribet *et al.* 2012). In the present study we  
156 include 27 palpigrade specimens plus 8 outgroups, for a total of 1760-1771 bp per complete  
157 sequence (up to 1805 bp for one of the outgroups). From the 27 palpigrade sequences all but  
158 three were complete; *E. spelaea* is missing fragment *a* and the sample of *Eukoenenia* from South  
159 Africa (DNA100456.2) is missing fragment *b*. For the direct optimization analyses the three  
160 amplicons were treated as a single input file, containing 23 sequences, and divided into six  
161 fragments. The three amplicons were concatenated for the static alignment analyses.

162 28S rRNA: This nuclear gene was amplified in three amplicons (*a*, *b*, *c*), as described in  
163 Giribet and Shear (2010). The data set includes 29 palpigrade specimens plus 8 outgroups, for a  
164 total of 2,150 to 2,204 bp, with some length variation among species. These three fragments  
165 correspond to primer pairs 28S rd1a—28D rd4b, 28Sa—28S rd5b, and 28S rd4.8a—28S rd7b1.  
166 Some of the published sequences were amplified with a shorter fragment *b*, generated with  
167 primers 28Sa—28Sb (Whiting *et al.* 1997), and therefore fragment *b* was divided into fragments  
168 *b1* and *b2* to accommodate these two amplicons. Fragment *a* was available for 22 palpigrades  
169 and divided into three fragments, fragment *b* for 29 palpigrades and three fragments, and  
170 fragment *c* for 25 palpigrades and analysed as a single fragment. These were treated as three  
171 different amplicons for the dynamic homology analyses, but aligned together for the static  
172 homology approaches.

173 COI: This widely used mitochondrial marker amplified for ten palpigrade terminals in a  
174 single amplicon using primers LCO—HCO, showing no length variation (654 bp analysed), plus  
175 one was available in GenBank. COI did not amplify for many individuals, perhaps due to major  
176 changes in this marker, as evidenced by the deletion of one amino acid with respect to the  
177 outgroups. Five outgroup sequences were obtained from GenBank, but these were 3 bp longer  
178 in all cases except for the pseudoscorpion. It was analysed as a single fragment; not pre-aligned  
179 due to the length difference with some outgroups.

180

### 181 *Phylogenetic analyses*

182 Parsimony analyses were based on a direct optimization (DO) approach (Wheeler 1996) using  
183 POY v. 5.0 (Varón *et al.* 2012). Tree searches were performed using the timed search function in  
184 POY, i.e., multiple cycles of (a) building Wagner trees, (b) subtree pruning and regrafting (SPR),  
185 and (c) tree bisection and reconnection (TBR), (d) ratcheting (Nixon 1999), and (e) tree-fusing  
186 (Goloboff 1999, 2002) [command: search (max\_time:00:01:00, min\_time:00:00:10,  
187 hits:20, memory:gb:2)]. For the individual partitions, timed searches of 1 hour were run on  
188 4 processors under six parameter sets, as in Giribet *et al.* (2012) (see Table 3). For the combined  
189 analysis of the three markers we started with the same search strategy, giving the 28S rRNA  
190 trees as input—as these contained all the taxa in the combined data set—, and the resulting  
191 trees were given as input for a second round of analyses (sensitivity analysis tree fusing; SATF),



192 as described by Giribet (2007), and continued until the tree lengths stabilised (Giribet *et al.*  
193 2012). The optimal parameter set was estimated using the modified  $w$ ILD metrics (Wheeler  
194 1995; Sharma *et al.* 2011), as a proxy for the parameter set that minimizes overall incongruence  
195 among data partitions (Table 4). Nodal support for the optimal parameter set was estimated via  
196 jackknifing (250 replicates) with a probability of deletion of  $e^{-1}$  (Farris *et al.* 1996) using  
197 `auto_sequence_partition`, as discussed in earlier work (Giribet *et al.* 2012).

198 Maximum likelihood (ML) analyses were conducted on static multiple sequence  
199 alignments (MSA) inferred in MUSCLE v. 3.6 (Edgar 2004) through the EMBL-EBI server  
200 (<http://www.ebi.ac.uk/Tools/msa/muscle/>). We also used an implied alignment (IA) generated  
201 in POY (Wheeler 2003; Giribet 2005) for subsequent analyses based on static alignments, as  
202 recently explored by Giribet and Edgecombe (2013b) for a centipede data set. The MUSCLE  
203 alignments were conducted for each gene independently. The IA and MSA therefore were based  
204 on the same data (see length for each gene in Table 5). In order to evaluate the impact of the  
205 hypervariable regions in the data set, MSAs and IAs were subsequently trimmed with Gblocks v.  
206 0.91b (Castresana 2000; Talavera and Castresana 2007) to cull positions of ambiguous homology  
207 (see length for each trimmed gene in Table 5). In the case of 28S, fragments *a* and *bc* were  
208 Gblocked separately, due to the larger proportion of missing data in the *a* fragment, which  
209 otherwise would be deleted from the final 28S alignment. These data sets are thus based on  
210 different data from their original sources and from each other, but the remaining data use the  
211 same homology scheme as the source. Data sets were concatenated with SequenceMatrix  
212 (Vaidya *et al.* 2011).

213 Maximum likelihood analyses were conducted using RAxML ver. 7.2.7 (Stamatakis *et al.*  
214 2008b) in the CIPRES server (Miller *et al.* 2010). For the searches, a unique General Time  
215 Reversible (GTR) model of sequence evolution with corrections for a discrete gamma  
216 distribution (GTR +  $\Gamma$ ) was specified for each data partition, and 100 independent searches were  
217 conducted. Nodal support was estimated via the rapid bootstrap algorithm (1000 replicates)  
218 using the GTR-CAT model (Stamatakis *et al.* 2008a). Bootstrap resampling frequencies were  
219 thereafter mapped onto the optimal tree from the independent searches.

220 In total we analysed five data sets accounting for different optimality criteria, homology  
221 schemes, and/or amount of data, as follows:

- 222 • Analysis 1. Direct optimization/dynamic homology under parsimony (full sensitivity  
223 analysis of 6 parameter sets) analysed in POY
- 224 • Analysis 2. Static homology from the implied alignment for the optimal parameter  
225 set under ML (analysed in RAxML)
- 226 • Analysis 3. Static homology from the implied alignment for the optimal parameter  
227 set trimmed with Gblocks under ML (analysed in RAxML)
- 228 • Analysis 4. Static homology based on MUSCLE multiple sequence alignment  
229 (analysed in RAxML)
- 230 • Analysis 5. Static homology based on MUSCLE/Gblocks (analysed in RAxML)

231

## 232 **Results and Discussion**

233 All phylogenetic analyses yielded very similar results with respect to the ingroup relationships,  
234 while the outgroup relationships were incongruent from analysis to analysis and unsupported  
235 for the most part (Figs. 2 and 3). The latter was expected given the small amount of data and  
236 outgroup taxa and the poor resolution in deep arachnid relationships in other studies (e.g.,  
237 Wheeler and Hayashi 1998; Giribet *et al.* 2002; Pepato *et al.* 2010; Regier *et al.* 2010). The  
238 optimal parameter set under parsimony direct optimization was 3211 (where indel opening  
239 costs 3, indel extension 1, transversions cost 2 and transitions cost 1;  $wILD = 0.00913$ ), with a  
240 cost of 10,408 weighted steps (Fig. 2). Nearly all examined parameter sets concurred on the  
241 topology of the optimal parameter set, with the exception of *Eukoenenia spelaea* IZ-19346 from  
242 Slovenia, and the resolution of one of the *Eukoenenia* clades (see below). Likewise, the analyses  
243 of the four data sets analysed under maximum likelihood were nearly identical, except for some  
244 of the shallowest relationships. One of these trees, the one for the multiple sequence alignment  
245 trimmed with Gblocks—the one that could be potentially the most different from the POY  
246 analysis—is presented in Fig. 3, and it is virtually identical to the direct optimization tree. From  
247 the 10 nodes depicted in Fig. 2 summarizing the six direct optimization and the four maximum  
248 likelihood analyses, 5 were recovered in all analyses. Support values for these five nodes is high  
249 for most analyses (jackknife values are lower by definition), with the exception of clades III and  
250 IV in the DO analysis. Basically, nearly all analyses concur on the overall topology of the  
251 palpigrade tree.

252 All analyses show a basal dichotomy between *Prokoenenia wheeleri* (the only  
253 Prokoeneniidae represented in our analyses) and the remaining samples, which we consider as  
254 *Eukoenenia* for further discussion—even if some samples from GenBank or from the Australian  
255 boreholes were not identified. *Eukoenenia* is divided into four main clades, indicated in Figures 2  
256 and 3. Clade I includes *E. florenciae* from Slovakia, Brazil, and unidentified specimens probably  
257 belonging to the same species from the USA and Mexico, and another species from a cave in  
258 Guerrero, Mexico (IZ-128499). Clade II includes *E. spelaea* and *E. s. hauseri* Condé, 1974 from  
259 Slovenia and Slovakia, and several additional samples from Slovenia and Italy, including *E.*  
260 *strinatii*, *E. bonadonai* and *E. austriaca* (Hansen, 1926); *E. spelaea* IZ-19346 from Slovenia  
261 clusters with these species in some analyses, but not all (Fig. 2). Clade III includes *E. ferratilis*  
262 from Brazil, the specimens from the Australian bore holes, and an undescribed species from  
263 Brazil (IZ-19345). Clade IV includes *E. mirabilis* from Australia and Italy, and unidentified  
264 specimens from South Africa, plus a specimen from a cave in Chiapas, Mexico (IZ-136274) and a  
265 GenBank specimen (JA-2011) of unknown origin. Clades I and II are supported in all analyses;  
266 Clade III is supported in all analyses except for the DO analysis under parameter set 211; Clade  
267 IV is unsupported in the ML analysis of the trimmed MSA. *Eukoenenia spelaea* IZ-19346 appears  
268 as the sister group to Clade II under 4 analytical parameter sets in DO and in the untrimmed ML  
269 analyses, both for the IA and for the MSA. The *E. florenciae* clade (Clade I) always forms the  
270 sister group of the *E. spelaea* clade (Clade II), although *E. spelaea* IZ-19346 sometimes forms the  
271 sister group of the *E. florenciae* clade. While the *E. ferratilis* clade (Clade III) often forms the  
272 sister group to the *E. mirabilis* clade (Clade IV) (Figs. 2, 3), and is well supported in the  
273 probabilistic analyses (97 to 100% bootstrap support, depending on the analysis), under some  
274 parameter sets Clade III is sister to the *E. spelaea*—*E. florenciae* clade (parameter sets 111, 211,  
275 221, 3221).

276 Irrespective of these small differences, our analyses show high congruence between  
277 alternative methods (parsimony and maximum likelihood) based on identical raw data with  
278 different homology schemes (implied alignments versus multiple sequence alignments), or  
279 different data sets (trimmed implied alignments and trimmed multiple sequence alignments).  
280 There are very few cases with such consistency across weighting schemes, homology schemes,  
281 and methodologies, but a recent case was documented for scutigeromorph centipedes (Giribet  
282 and Edgecombe 2013b). In that case, the fossil record and denser sampling allowed for accurate  
283 molecular dating and analyses of diversification of lineages through time, and it was suggested

284 that the congruence across analyses was due to constant rates of diversification through more  
285 than 400 million years of evolution in the group. We can only guess this for palpigrades, as the  
286 fossil record for this group is rare, and a single Pliocene specimen is known (Rowland and Sissom  
287 1980; Delclòs *et al.* 2008; Dunlop 2010), although the group must be much older in origin (see  
288 for example Giribet and Edgecombe 2013a).

289           Phylogenetic analysis of the three molecular markers combined and for all analyses  
290 performed resolves into Prokoeneniidae (although represented by a single species) and  
291 Eukoeneniidae, supporting the monophyly of Eukoeneniidae—palpigrades without sternal  
292 opisthosomal vesicles (Condé 1996). We were, however, unable to obtain samples of  
293 *Triadokoenia* or of additional *Prokoenia* species, thus not being able to test the taxon  
294 Prokoeneniidae. Within Eukoeneniidae, the four main clades discussed above are supported in  
295 nearly all analyses. But species identifications in palpigrades do not seem straightforward.  
296 Within Clade I, the specimens of *Eukoenia* from Texas (USA), the Mexican state of Yucatán, *E.*  
297 *cf. florenciae* from Brazil and *E. florenciae* from Slovakia show nearly identical COI sequences  
298 and identical nuclear ribosomal RNA sequences, suggesting that they may be conspecific (see  
299 Edgecombe and Giribet 2008; Vélez *et al.* 2012). In contrast, Clade II includes three lineages of  
300 the morphospecies *E. spelaea*. From these, two samples identified as *E. spelaea* and *E. spelaea*  
301 *hauseri* from Slovenia appear identical for the nuclear ribosomal genes (but did not amplify for  
302 COI).

303           Clade III includes the Western Australian samples and *Eukoenia ferratilis* from the Iron  
304 caves of Minas Gerais (Brazil). Difficulties in amplifying the Australian samples and the lack of  
305 COI information for any of the members of the clade precludes us from understanding genetic  
306 variability within this clade of geographically distant species (both between the continents, but  
307 also among the Western Australian localities), although most analyses consistently resolve this  
308 clade of six individuals with reciprocal monophyly of the two geographic regions.

309           Clade IV, although with less support than the other three clades, includes the sample of  
310 unknown provenance sequenced by Arabi *et al.* (2012), a specimen from caves in Chiapas, and  
311 the cosmopolitan *E. mirabilis*, including two specimens from Italy (identical for all markers) and  
312 two putative members of this species from South Africa plus a sample of *E. mirabilis* from  
313 Australia. While *E. mirabilis* has been suggested to be a synanthropic species originating in the  
314 Mediterranean region with recent introductions to South Africa, Australia, Chile and

315 Madagascar (Harvey *et al.* 2006), our limited data suggest a close relationship between one of  
316 the South African samples and the Australian specimen, even in the absence of COI data, and  
317 therefore suggesting changes in the nuclear ribosomal genes with respect to the Italian sample.  
318 Further study of Gondwanan *E. mirabilis* and addition of circum-Mediterranean samples should  
319 be undertaken to bring this matter to conclusion.

320           Given the sampling of this study it is still early to make any firm conclusions about  
321 palpigrade relationships. We were not able to test for the monophyly of Prokoeneniidae, and  
322 monophyly of *Eukoenenia* is not thoroughly tested either. Attempts to sequence *Allokoenenia*  
323 and *Leptokoenenia* were unsuccessful, and we were unable to obtain specimens of the  
324 Palaeotropical *Koeneniodes* and *Triadokoenenia*. Few studies have looked at variation among  
325 palpigrade species, but Král *et al.* (2008) investigated the karyotypes of *E. spelaea* from Slovakia  
326 and *E. mirabilis*, which appear in different clades in our study (Clades II and IV, respectively).  
327 However, the karyotypes of both species showed no variation, both consisting of a low number  
328 of tiny chromosomes that decrease gradually in size and a lack of morphologically differentiated  
329 sex chromosomes, suggesting that molecular data may be more informative than karyotypic  
330 data for separating species.

331           Morphologically, the characters used to differentiate *Eukoenenia* species are mostly  
332 restricted to the number of lobules in the lateral organs or the number of setae in different body  
333 regions, but the significance of these characters has not been tested phylogenetically—for  
334 example, *E. mirabilis* and *E. ferratilis* are very similar morphologically with many somatic traits,  
335 considered important for taxonomy, virtually identical (Souza and Ferreira 2011a). However,  
336 these two species belong to different clades, reflecting that their differences in genital  
337 morphology and chaetotaxy may be better systematic characters than the ones outlined above.  
338 Our study thus provides a new framework for adding new sequences and testing the significance  
339 of these characters. Additional samples and especially more genera must however be added  
340 before we can attempt a taxonomic revision of the higher taxa in Palpigradi.

341

## 342 **Conclusions**

343 Palpigrades are a poorly understood group of tiny soil arthropods, often found exclusively in  
344 caves, and have received little attention from a phylogenetic point of view. Here we were able

345 to amass specimens from different environments (caves and soil) from Australia, Africa, Europe,  
346 South America and North America with the aim of generating a molecular phylogenetic  
347 hypothesis for the group. The difficulty in obtaining well-preserved material for molecular work  
348 is reflected in the large number of specimens that did not yield DNA of enough quality for  
349 sequencing, but we were able to propose the first phylogenetic hypothesis of the group based  
350 on molecular data to find monophyly of Eukoeneriidae and its division into four main clades,  
351 three of these including samples from multiple continents. Given the absence of denser  
352 sampling and proper clock calibrations, our data cannot discern whether palpigrades are a very  
353 old group that diversified prior to the breakup of Pangaea, or a group of animals that disperses  
354 across large geographic distances, as suggested by some widespread species. Long-range  
355 dispersal is however difficult to reconcile with the narrow ecological conditions and the facility  
356 with which these animals desiccate once removed from their environments.

357

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- 543

544 **Fig. 1.** Photographs of (A) *Eukoenenia spelaea*, Ardovská Cave (Slovak Karst, Slovakia),  
545 photographed by Ľ. Kováč & V. Kóňa; (B) *Prokoenenia wheeleri*, Austin (Texas, USA),  
546 photographed by L. McCutchen; (C) *Eukoenenia mirabilis*, flagellum, segments 1 to 10; (D)  
547 *Eukoenenia bonadonai*, male genital lobes; (E) *E. bonadonai*, female genital lobes; (F) *E.*  
548 *bonadonai*, mouth cone and chelicerae (C-D photographed by E. Christian).

549

550 **Fig. 2.** Left: Optimal tree at 10,408 weighted steps obtained from the direct optimization  
551 analysis under parameter set 3211 of the combined analysis of the three genes. Numbers on  
552 branches indicate jackknife support values. Navajo rugs are shown in selected nodes; Black  
553 square indicates monophyly, white square non-monophyly. Specific parameter sets or analyses  
554 indicated in the figure. Numerals indicate parameter set under parsimony direct optimization; IA  
555 (ML analysis using implied alignment under parameter set 3211); IAg (Idem, Gblocked); MSA  
556 (ML analysis of the MUSCLE multiple sequence alignment); MSAg (Idem, Gblocked). Clades I to  
557 IV are indicated.

558

559 **Fig. 3.** Optimal maximum likelihood tree ( $-\ln L = -24955.690470$ ) of the combined data set using  
560 the MUSCLE multiple sequence alignment trimmed with Gblocks. Numbers on nodes indicate  
561 bootstrap support values.

**Table 1. Palpigrade specimens, accession numbers, collecting information and amplified loci with GenBank accession numbers**

IZ: Department of Invertebrate Zoology, Museum of Comparative Zoology, Cambridge; DNA: MCZ DNA collection; WAM: Western Australian Museum, Perth; MNHN: Muséum national d'histoire Naturelle, Paris. A dash (-) indicates a missing amplicon. New sequences are KF823823 to KF823883

				18S rRNA			28S rRNA			COI
	<b>MCZ No.</b>		<b>Country</b>	<b>a</b>	<b>b</b>	<b>c</b>	<b>a</b>	<b>b</b>	<b>c</b>	
<i>Prokoenenia wheeleri</i>	IZ-134477	DNA107078	Texas, USA	KF823823	KF823823	KF823823	KF823848	KF823848	KF823848	KF823874
<i>Eukoenenia austriaca</i>	IZ-19349	-	Slovenia	KF823824	KF823824	KF823824	KF823849	KF823849	KF823849	-
<i>Eukoenenia bonadonai</i>	IZ-19340	-	Italy	KF823825	KF823825	KF823825	KF823850	KF823850	KF823850	-
<i>Eukoenenia ferratilis</i>	IZ-127609	-	Brazil	KF823826	KF823826	KF823826	KF823851	KF823851	KF823851	-
<i>Eukoenenia cf. ferratilis</i>	-	GenBank		HM070336	HM070336	HM070336	HM070299	HM070299	HM070299	-
<i>Eukoenenia florenciae</i>	IZ-19351	-	Slovakia	KF823827	KF823827	KF823827	KF823852	KF823852	KF823852	KF823875
<i>Eukoenenia cf. florenciae</i>	IZ-19343	-	Brazil	KF823828	KF823828	KF823828	KF823853	KF823853	KF823853	-
<i>Eukoenenia mirabilis</i>	IZ-127901	-	Italy	KF823829	KF823829	KF823829	KF823854	KF823854	KF823854	KF823876
<i>Eukoenenia mirabilis</i>	IZ-127902	-	Italy	KF823830	KF823830	KF823830	KF823855	KF823855	KF823855	KF823877
<i>Eukoenenia mirabilis</i>	IZ-16117	-	Australia	KF823831	KF823831	KF823831	KF823856	KF823856	KF823856	-
<i>Eukoenenia spelaea</i>	IZ-135126	DNA106786	Slovakia	-	KF823832	KF823832	KF823857	KF823857	KF823857	-
<i>Eukoenenia spelaea</i>	IZ-19346	-	Slovenia	KF823833	KF823833	KF823833	KF823858	KF823858	KF823858	KF823878
<i>Eukoenenia spelaea</i>	IZ-19347	-	Slovenia	KF823834	KF823834	KF823834	KF823859	KF823859	KF823859	-
<i>Eukoenenia spelaea hauseri</i>	IZ-19348	-	Slovenia	KF823835	KF823835	KF823835	KF823860	KF823860	KF823860	-
<i>Eukoenenia strinatii</i>	IZ-19341	-	Italy	KF823836	KF823836	KF823836	KF823861	KF823861	KF823861	-
<i>Eukoenenia sp.</i>	IZ-19350	-	Slovenia	KF823837	KF823837	KF823837	KF823862	KF823862	KF823862	KF823879

<i>Eukoeneria</i> sp.	-	DNA100456.1	South Africa	AF207648	AF207648	AF207648	-	AF207653	-	-
<i>Eukoeneria</i> sp.	-	DNA100456.2	South Africa	KF823838	-	KF823839	-	KF823863	-	-
<i>Eukoeneria</i> sp.	IZ-134549	DNA107079	USA	KF823840	KF823840	KF823840	KF823864	KF823864	KF823864	KF823880
<i>Eukoeneria</i> sp.	IZ-127598.1	-	Mexico	KF823841	KF823841	KF823841	KF823865	KF823865	KF823865	KF823881
<i>Eukoeneria</i> sp.	IZ-127598.2	-	Mexico	KF823842	KF823842	KF823842	KF823866	KF823866	KF823866	KF823882
<i>Eukoeneria</i> sp.	IZ-128499	-	Mexico	KF823843	KF823843	KF823843	KF823867	KF823867	KF823867	KF823883
<i>Eukoeneria</i> sp.	IZ-136274	-	Mexico	KF823844	-	KF823844	KF823868	KF823868	KF823868	-
<i>Eukoeneria</i> sp.	IZ-127636	WAM T81111	Australia	-	-	-	-	KF823869	-	-
<i>Eukoeneria</i> sp.	IZ-127639	WAM T116012	Australia	KF823845	KF823845	KF823845	-	KF823870	KF823870	-
<i>Eukoeneria</i> sp.	IZ-127640	WAM T111422	Australia	-	-	-	-	KF823871	-	-
<i>Eukoeneria</i> sp.	IZ-127643	-	Australia	KF823846	KF823846	KF823846	-	KF823872	KF823872	-
<i>Eukoeneria</i> sp.n.	IZ-19345	-	Brazil	KF823847	KF823847	KF823847	-	KF823873	KF823873	-
Palpigradi sp.	-	MNHN-JAA76		JN018286.1	JN018286.1	JN018286.1	JN018383.1	JN018383.1	JN018383.1	JN018169.1



**Table 2. Outgroup sampling with GenBank accession numbers**

		<b>18S rRNA</b>	<b>28S rRNA</b>	<b>COI</b>
<i>Anoplodactylus portus</i>	Pycnogonida	AY859551	AY859550	GQ912859
<i>Limulus polyphemus</i>	Xiphosura	U91490	AF212167	AF216203
<i>Pandinus imperator</i>	Scorpiones	AY210831	AY210830	AY156582
<i>Metasiro americanus</i>	Opiliones	DQ825542	DQ825595	DQ825645
<i>Calocheiridius termitophilus</i>	Pseudoscorpiones	AY859559	AY859558	EU559544
<i>Dermacentor</i> sp.	Acari	Z74480	AY859582	-
<i>Eremobates</i> sp.	Solifugae	AY859573	AY859572	-
<i>Mastigoproctus giganteus</i>	Uropygi	AF005446	AY859587	JN018215

**Table 3. Result of the POY timed searches (search) and improvement after each round of SATF for the six explored parameter sets**

	<b>1</b>	<b>SATF2</b>	<b>SATF3</b>
<b>111</b>	6520	6520	6520
<b>121</b>	10076	10076	10076
<b>211</b>	7543	7543	7543
<b>221</b>	11851	11851	11851
<b>3211</b>	10408	10408	10408
<b>3221</b>	13526	13526	13526

**Table 4. Number of weighted steps for each data partition, the combination of them (MOL) and  $wILD$  value**

The optimal parameter set is indicated in italics

	<b>18S</b>	<b>28S</b>	<b>COI</b>	<b>MOL</b>	<b>wILD</b>
<b>111</b>	1125	3967	1354	6520	0.01135
<b>121</b>	1655	6272	2051	10076	0.00973
<b>211</b>	1246	4840	1381	7543	0.01008
<b>221</b>	1867	7780	2080	11851	0.01046
<b><i>3211</i></b>	<i>1704</i>	<i>6535</i>	<i>2074</i>	<i>10408</i>	<i>0.00913</i>
<b>3221</b>	2314	8305	2777	13526	0.00961

**Table 5. Length of each data partition (28S rRNA is divided into three amplicons) and total length of alignment**

IA (121) is for implied alignment under parameter set 121; IA+Gb is for implied alignment trimmed with Gblocks; Muscle is for MUSCLE multiple sequence alignment; Muscle+Gb is for multiple sequence alignment trimmed with Gblocks

	<b>18S</b>	<b>28Sa</b>	<b>28Sbc</b>	<b>COI</b>	<b>TOTAL</b>
<b>Unaligned</b>	1760-1805	832-873	1265-1347	654-657	
<b>IA (3211)</b>	1860	1323	1555	669	5407
<b>IA+Gb</b>	1676	378	1162	626	3842
<b>Muscle</b>	1818	1046	1409	663	4936
<b>Muscle+Gb</b>	1695	609	1212	636	4152