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Placing the forgotten: on the positions of *Euenchytraeus* and *Chamaedrilus* in an updated enchytraeid phylogeny (Clitellata : Enchytraeidae)

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Abstract. The phylogeny of Enchytraeidae was re-estimated to establish the relationships of the now resurrected *Chamaedrilus* and *Euenchytraeus* and to confirm their status as separate taxa. The former *Cognettia* (Enchytraeidae) was recently revised and split into its two senior synonyms, *Chamaedrilus* and *Euenchytraeus*, with the majority of the species transferred to *Chamaedrilus*. *Euenchytraeus* was re-established for three species sharing a unique anatomical trait, but has never before been represented in any phylogenetic study. We included representatives from 21 (of 33) valid enchytraeid genera and used three mitochondrial and four nuclear genes. The dataset (4164 base pairs) was analysed using multi-species coalescent (MSC) and maximum likelihood (ML) methods. *Chamaedrilus* (represented by eight species) and *Euenchytraeus* (represented by *Eu. clarae*) were found in a clade together with the monotypic *Stercutus*. *Chamaedrilus* was found to be monophyletic with maximum support in both analyses. The ML tree supported *Euenchytraeus* and *Chamaedrilus* as sister groups, whereas the MSC tree placed *Euenchytraeus* together with *Stercutus*, both with low support. A Bayes factor test weakly supported *Euenchytraeus* and *Chamaedrilus* as sister groups over *Euenchytraeus* + *Stercutus*. Possible morphological synapomorphies for these genera are discussed, and we conclude that *Chamaedrilus* and *Euenchytraeus* are closely related, but their status as separate genera is justified.

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Introduction

Enchytraeidae is a family of oligochaetous clitellates (Annelida) with ~710 described species placed in 33 genera (Schmelz and Collado 2015). They are found in a broad range of aquatic habitats, but most typically they populate terrestrial soils and seashore sands.

Ecologically, the most studied enchytraeids are litter-dwelling species previously placed in the genus *Cognettia* Nielsen & Christensen, 1959, in particular *C. sphagnetorum s.l.*, which has long been used as a model in soil biology (see Martinsson and Erséus 2014 and references within). However, genetic data (Martinsson and Erséus 2014) provide firm evidence that this and the other common member of the group (*C. glandulosa*) are in fact complexes of cryptic species, and that *C. sphagnetorum s.l.* is not even monophyletic, which prompted the need for a revision of the genus.

Cognettia has two senior synonyms, *Euenchytraeus* Bretscher, 1906 and *Chamaedrilus* Friend, 1913, as pointed out by Schmelz and Collado (2010). These had been overlooked by Nielsen and Christensen (1959) when establishing *Cognettia*, and were thereafter forgotten. Consequently, Martinsson *et al*. (2015*a*, 2015*b*) carried out a formal revision of the group, proposing that *Cognettia* be treated as a junior synonym to *Chamaedrilus* and the latter be comprised of the majority of species, including the type species of *Cognettia*, *Pachydrilus sphagnetorum* Vejdovský, 1878. Moreover, *Euenchytraeus* was re-established and considered the valid name for its type species and two other, apparently aberrant, members of *Cognettia* (Martinsson *et al*. 2015*a*). Subsequently, a case has been submitted to the International Commission on Zoological Nomenclature proposing that *Cognettia* be given precedence over its senior synonyms *Euenchytraeus* and *Chamaedrilus* (Schmelz *et al*. 2015), and a comment on this has been published (Rota *et al*. 2015). Until the commission has ruled in this case, we continue to use *Chamaedrilus* as a senior synonym of *Cognettia*.

Originally, *Euenchytraeus* was established for *Eu. bisetosus* Bretscher, 1906, a species described from immature specimens collected at 2300 m in the Swiss Grison Alps and possessing nephridia at all intersegments starting from 2/3. Černosvitov (1937) synonymised *Euenchytraeus* with *Marionina* and regarded *Eu. bisetosus* as a *species dubia*, because he doubted the presence of a septum and nephridia at 2/3. Later, however, two other species with 'head nephridia' at 2/3 were described, namely, *Cognettia clarae* Bauer, 1993, from a spruce forest in Austria, and *C. piperi* Christensen & Dózsa-Farkas, 1999 from the Siberian tundra. The head nephridia are a unique feature within Enchytraeidae (Dózsa-Farkas 2010), thus both these species were transferred to *Euenchytraeus* by Martinsson *et al*. (2015*a*).

Species now placed in *Chamaedrilus* have already been included (as representatives of *Cognettia*) in DNA-based phylogenetic studies (Christensen and Glenner 2010; Erséus *et al*. 2010; Martinsson and Erséus 2014). Christensen and Glenner (2010) found *Cognettia* to be sister group to *Henlea*, but they included only nine ingroup taxa. Erséus *et al*. (2010) studied 87 ingroup species representing 18 genera; *Cognettia* was found to be sister group to *Stercutus*, and all genera except *Marionina* and *Lumbricillus* were found to be monophyletic. Martinsson and Erséus (2014), focusing on the northern European species of *Cognettia*, presented a phylogeny in which this genus was monophyletic and sister to *Stercutus*, but they used a limited sample of (intrafamily) outgroups. All the *Cognettia* species genetically examined by Martinsson and Erséus (2014) were later transferred to *Chamaedrilus*, and several cryptic lineages were revised and described as separate species (Martinsson *et al*. 2015*a*, 2015*b*). No molecular study has so far included species of *Euenchytraeus*, and its phylogenetic position is therefore basically unknown.

The present study was made possible when material of *Euenchytraeus clarae* newly collected by K. Dózsa-Farkas from Austria and Hungary (a new country record) became available for DNA-extraction. To justify the re-establishment of both *Chamaedrilus* and *Euenchytraeus*, they should be reciprocally monophyletic and not nested within any other genus (Rota *et al*. 2015). The aims of this study were to test the hypothesis of Martinsson *et al*. (2015*a*) that *Euenchytraeus* and *Chamaedrilus* are separate lineages, and therefore should be treated as different genera, and to find the phylogenetic position of *Euenchytraeus*. To do so we will re-estimate a multilocus molecular phylogeny of the family Enchytraeidae.

Materials and methods

Taxon sampling

This study includes 47 specimens representing 38 species and 21 genera of Enchytraeidae, and three outgroup taxa from Lumbriculidae, Naididae and Propappidae (see Table S1 for details). Many of the ingroup species were also assessed by Erséus *et al*. (2010), but among the additional taxa herein are *Euenchytraeus clarae* and six species of *Chamaedrilus* (former *Cognettia*). We also tried to include two additional species of *Chamaedrilus*, namely, *Ch. bisetosus* (Christensen & Dózsa-Farkas, 1999) and *Ch. quadrosetosus* (Christensen & Dózsa-Farkas, 1999), as well as *Eu. piperi*, but failed to extract and amplify DNA from the specimens, probably due to age and improper storage of the available material. Enchytraeid taxa were selected to maximise the number of distinct lineages that could be treated as genera, rather than maximising the number of included species, as most genera were found to be monophyletic with good support by Erséus *et al*. (2010).

DNA-extraction, amplification and sequencing

We selected three mitochondrial markers, parts of 16S rRNA (16S), 12S rRNA (12S) and cytochrome oxidase *c* subunit I (COI), and four nuclear markers, parts of 28S rRNA (28S), histone 3 (H3) and the complete 18S rRNA (18S) and U2 small nuclear RNA (U2). About half of the sequences were newly generated, the others were taken from published studies and downloaded from GenBank (see Table S1). DNA extraction has varied between specimens and over time, but standard methods and protocols have been used, also for the primers and programs used for the newly generated sequences (see Table S2). Sequencing was performed by either Macrogen (Geumcheon-Gu, Seoul, Korea) or Eurofins MWG Operon (Ebersberg, Germany). Sequences were assembled and aligned in Geneious pro v. 7.1. The alignments were created using the Geneious alignment algorithm with default settings, and edited by eye. All newly generated sequences are deposited on GenBank (accession numbers in Table S1).

Phylogenetic analyses

The phylogeny was estimated using both the multi-species coalescent (MSC) model as implemented through the *BEAST module in BEAST 1.8 (Drummond *et al*. 2012), and maximum likelihood (ML) using PhyML 3.0 (Guindon *et al*. 2010*a*). For any specimen missing genes, a dummy sequence consisting of only Ns was added to the data matrix.

For the MSC analysis, an XML input file was created in BEAUti 1.8. Markers that are genetically linked, i.e. the mitochondrial genes and the nuclear ribosomal genes, are assumed to share gene trees, and therefore the tree models were linked within these two groups, giving a total of four tree models, including separate ones for H3 and U2. The substitution models were unlinked and all genes were given their own HKY + Γ substitution model with empirical base frequencies. The model was selected as a compromise between the number of parameters needed to be estimated (thereby reducing computation time) and fit to the data. Clock models were also unlinked across genes, as it was assumed that the mutation rates differed between the genes, and uncorrelated lognormal relaxed clocks with mean rate estimated were used for all genes. The birth-death process speciation prior and the piecewise linear with constant root population size prior were used, and the effective population size of the mitochondrial markers was set to half that of the nuclear markers by changing the ploidy level, as the mitochondrial genome is haploid and clitellates are hermaphrodites (i.e. there should be only one allele for mitochondrial genes), but all individuals can contribute mitochondrial DNA to the next generation. The root height for the species tree was arbitrarily set to 1 using a strong normally distributed prior (mean 1, s.d. 0.01) for the tmrca (time to most recent common ancestor) for all taxa, combined with weak normally distributed priors for the relax clock rates (ucld.mean). For 16S and 12S the prior had a mean of 0.15 and s.d. 0.1; for COI mean 0.25 and s.d. 0.1; for 18S and 28S mean 0.05 and s.d. 0.1; and for H3 and U2 mean 0.1 and s.d. 0.1. These priors were based on previous knowledge on relative substitution rates between genes, combined with information about the genetic distances within the markers. For species population mean and mean growth rate priors, an exponential distribution with mean 1 was used. For all other priors, default settings were used. The analysis was run twice for 500 million generations, sampling every 50 000 generations. Tracer v1.6 was used to examine effective sample size (ESS) for parameters and determine burn-in. The runs were combined using LogCombiner v1.8.2, discarding the first 10% as burn-in, and trees were summarised using TreeAnnotator v1.8, using the maximum clade credibility tree.

For the ML analysis, a concatenated matrix consisting of the same genes and specimens as the MSC analysis was used. The analysis was performed with PhyML 3.0 (Guindon *et al*. 2010*a*) as implemented at the Montpellier Bioinformatics platform (http://www.atgc-montpellier.fr/). The smart model selection with Bayesian information criterion was used for automatic model selection; SPR+NNI were used for tree improvement. Branch support was calculated with the SH-like (Shimodaira–Hasegawa test-like) approximative likelihood ratio test (aLRT)(Anisimova and Gascuel 2006; Guindon *et al*. 2010*b*), which is in line with the SH tree selection method (Shimodaira and Hasegawa 1999) and compares the most likely topology T1 with the second most likely topology T2. The main difference from the standard SH test is that the support is calculated for each branch, and not for the entire tree; the support is expressed as $P = 1 - \text{SH}$ support for T2.

The trees were drawn with Fig Tree v. 1.4.1 (Rambaut 2014) and further edited in Adobe Illustrator CS5.

Testing alternative topologies

To test whether a model in which *Euenchytraeus* and *Stercutus* are sister groups, as in the MSC tree (see 'Results'), or a model in which *Euenchytraeus* and *Chamaedrilus* form a monophyletic group better fits the data, we performed a Bayes factor (BF) test. The marginal likelihoods (M) were estimated using stepping stone sampling (Xie *et al*. 2011) in BEAST, the analyses were run on a reduced dataset, including only the specimens of *Euenchytraeus*, *Chamaedrilus*, *Stercutus* and as outgroup *Mesenchytraeus pelicensis*. The same settings as for the MSC analysis were used, with the exception of the addition of a monophyly constraint, in one analysis forcing *Euenchytraeus* and *Chamaedrilus* to form a monophyletic group, and in the other forcing *Euenchytraeus* and *Stercutus* to form a monophyletic group. The analyses were run for 200 million generations, sampling every 10 000 generations. The stepping stone sampling was performed for both analyses, with 100 path steps, each with a chain length of 100 000 generations, with the likelihood logged every 100 generations. The Bayes factor was calculated as $2ln\text{Bfs} = 2(\ln M_0 - \ln M_1)$ and evaluated using the suggestions given by Kass and Raftery (1995).

Results

After trimming, the 12S alignment was 433 base pairs (bp) long (44 sequences), 16S 519 bp long (47 sequences), COI 652 bp (45 sequences), 18S 1750 bp (46 sequences), 28S 350 bp (47 sequences), H3 328 bp long (40 sequences) and U2 132 bp (41 sequences); in total, 4164 bp.

Genetic variation in Euenchytraeus clarae

Only one (Hungarian) of the three specimens of *Eu. clarae* could be amplified for COI, but the variation in the other mitochondrial genes (12S, 16S) was extremely low, with only the insertion of 1 bp in 12S of one of the Hungarian specimens. The amplification of H3 failed altogether, while 16S, 18S, 28S and U2 showed no intra-specific variation at all among the three individuals.

Phylogenetic analysis

The MSC analysis had high ESS values for all parameters. In the maximum clade credibility tree (Fig. 1), many well-supported clades were found. However, several internal branches were short and unsupported. A monophyletic Enchytraeidae was recovered but unsupported (PP 0.62). All genera represented by more than one species, except *Marionina* and *Lumbricillus*, were also found to be monophyletic, and all except *Henlea* with good support (PP > 0.95). A clade consisting of *Stercutus*, *Euenchytraeus* and *Chamaedrilus* received good support (PP 0.99) and *Chamaedrilus* was monophyletic with maximum support (PP 1), but the relationships between the three genera were not resolved: *Euenchytraeus* was found as sister to *Stercutus*, but without support (PP 0.68). *Mesenchytraeus* and *Cernosvitoviella* were recovered as sister groups with low support (PP 0.77). A clade consisting of *Lumbricillus lineatus*, *L. arenarius* and *Grania* was recovered (PP 0.94), and *Grania* was found as sister group to *L. arenarius*, but without support (PP 0.64), whereas *L. semifuscus* was well separated from the other two *Lumbricillus* spp. and sister to *Globulidrilus* with low support (PP 0.77). *Globulidrilus* and *L. semifuscus* form a well-supported clade (PP 0.99) with *Bryodrilus*, *Marionina communis*, *Oconnorella* and *Henlea*; but as already mentioned, the monophyly of *Henlea* is unsupported. Within this group there is low support for a clade consisting of *Henlea*, *Oconnorella* and *Marionina communis* (PP 0.84). The second species of *Marionina*, *M. spicula*, was recovered as sister group to *Enchytronia*, but with no support. *Stephensoniella* was recovered as sister group to *Enchytraeus* (PP 0.99). A clade consisting of *Achaeta*, *Guaranidrilus* and *Hemienchytraeus* was recovered with good support (PP 0.97).

For the ML analysis, the GTR + Γ + I substitution model with 6 Γ shape parameters was selected. The ML tree (Fig. S1) is highly congruent with the MSC tree, but generally with higher nodal support. However, *Chamaedrilus* and *Euenchytraeus* were found as sister groups, but with low support ($P = 0.80$), and together as sister group to *Stercutus*.

Testing alternative topologies

The *ln*M for the model forcing *Euenchytraeus* and *Chamaedrilus* to form a monophyly was –13192.75, and the *ln*M for the model forcing *Euenchytraeus* and *Stercutus* into monophyly was –13194.37, resulting in $2ln\text{Bfs} = 3.22$, which constitutes weak positive support for *Euenchytraeus* + *Chamaedrilus* (i.e. the topology found also in the ML tree).

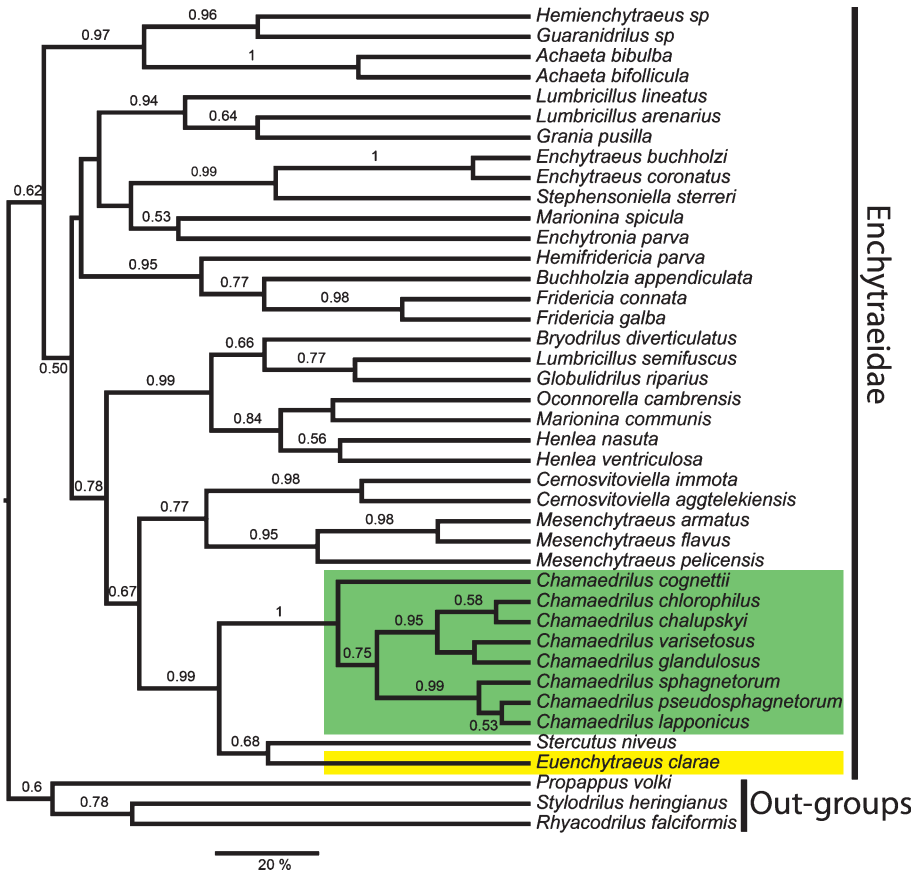

Fig. 1. Maximum clade credibility tree from multi-species coalescence analysis performed in *BEAST. The positions of *Euenchytraeus clarae* (yellow marking) and *Chamaedrilus* (green marking) are highlighted. Values at branches correspond to posterior probabilities; only values above 0.5 are shown. Scale bar represents percentage of tree length.

Discussion

We here present a phylogeny of the family Enchytraeidae that is based on more genetic markers, and including more genera, than in previous studies. The multi-gene phylogeny shows that *Euenchytraeus* and *Chamaedrilus* are closely related. We again confirm the non-monophyly of the genera *Lumbricillus* and *Marionina* (e.g. Rota *et al*. 2008; Erséus *et al*. 2010; Klinth *et al*. 2016), and find that *Guaranidrilus* (a genus not considered by Erséus *et al*. 2010) seems to be sister to *Hemienchytraeus*, and thus part of a clade that may correspond to the subfamily Achaetinae (e.g. Černosvitov 1937; Rota *et al*. 2008; Erséus *et al*. 2010; Schmelz *et al*. 2011), but with only single representatives of these genera we could not test for monophyly. All other well-supported relationships recovered in the MSC tree, and most of the ones in the ML tree, were also found by Erséus *et al*. (2010), who included fewer markers and genera, but sampled more species.

We find a well-supported clade consisting of the three genera *Chamaedrilus*, *Euenchytraeus* and *Stercutus*, with maximum support for the monophyly of *Chamaedrilus*. In the MSC tree *Euenchytraeus* and *Stercutus* form a clade, but with low support, whereas in the ML tree *Euenchytraeus* and *Chamaedrilus* are sisters, although with low support, and the Bayes factor test weakly supports the latter. As we failed to produce any sequences for *Eu. piperi*, we were unable to test the monophyly of *Euenchytraeus*. The MSC and ML trees are mainly congruent, but the overall higher support in the ML tree may be due to the assumption, in a concatenated analysis such as the ML analysis here, that all markers share the same history, even if we know that this is often not the case, which may lead to overconfident support for incorrect species trees (Degnan and Rosenberg 2009). A concatenated analysis is also likely to be misled by fast-evolving sites (Xi *et al*. 2014) (e.g. mitochondrial markers).

The close relationship between *Chamaedrilus* (as *Cognettia*) and *Stercutus* was also found by Erséus *et al*. (2010) and previously suggested by Dózsa-Farkas (1973) based on the following shared morphological characters: antero-ventral origin of nephridial duct, posteriorly incised brain, free spermathecae, and absence of oesophageal appendages and intestinal diverticula – all are features also found in *Euenchytraeus. Stercutus* differs from the other two genera by having oversized chloragogen cells, which fill the entire coelomic cavity (except in juveniles and after egg laying; see Dózsa-Farkas 1973), and a preclitellar origin of the dorsal blood vessel, as well as a weak nodulus on the chaetae. The first trait is probably an autapomorphy for the genus, whereas the origin of the dorsal blood vessel varies widely across the enchytraeid tree. The head nephridia are likely an autapomorphy for *Euenchytraeus*. Piper *et al*. (1982) described the north-eastern Siberian species, later named *Cognettia piperi* by Christensen and Dózsa-Farkas (1999), as a '*species incertae sedis*' and discussed its morphology and placement based on a combination of the sigmoid chaetae lacking nodulus, the postclitellar origin of the dorsal blood vessel, the absence of oesophageal diverticula and the free spermathecae, and Piper *et al*. (1982) concluded that the species was probably closest to *Cognettia*. The two other species in the genus are from mountain areas in central Europe: the type species *Eu. bisetosus* from the Swiss Alps, *Eu. clarae* from Austria. According to the original descriptions (Bretscher 1906; Bauer 1993), these two species differ in size, *E. bisetosus* (as immature) being longer and thicker than *Eu. clarae*, but having the same number of segments. In addition, the dorsal blood vessel seems to originate more posteriorly (in XVIII–XXI) in *Eu. clarae* than in *Eu. bisetosus* (XIV–XVI). It seems likely that these differences may be due to variations in maturity or to artefacts from fixation and mounting, and thus the two names may be synonyms. However, until we have material from the type localities for DNA analysis, we cannot test whether they are the same species or not.

Conclusions

The aim of this paper was to test the hypothesis of Martinsson *et al*. (2015*a*) that *Euenchytraeus* and *Chamaedrilus* are reciprocally monophyletic, separate lineages. We found that the two genera are closely related, and that *Chamaedrilus* is monophyletic, and *Euenchytraeus* is not nested in any other genus, but it is indeed not far from *Chamaedrilus*. Despite the lack of molecular data for any other species of *Euenchytraeus*, the head nephridia provide unique evidence supporting the status of this genus as a separate lineage. Based on the results, the split of *Cognettia* into *Euenchytraeus* and *Chamaedrilus* is supported, and if one would merge these two genera into one, it is possible that also *Stercutus* should be included. As *Stercutus* is the oldest name, it would be the valid name for such a group. For now, we suggest that these three genera are kept separate.

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