



## A new Scandinavian *Chamaedrillus* species (Clitellata: Enchytraeidae), with additional notes on others

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### Abstract

*Chamaedrillus* (earlier referred to as *Cognettia*) is a well-known genus of terrestrial and limnic enchytraeids, currently with 19 known species in the world. Some of its species are morphologically cryptic and can only be identified using genetic (DNA) information. Many of them reproduce asexually, and the prevalence of sexual mature individuals is generally low in the populations. *Chamaedrillus asloae* **sp. nov.** (Clitellata: Enchytraeidae) is described based on material from two rivers in Norway, one in Sweden, and from a wet deciduous forest in Denmark. With the material at hand, no morphological characters completely separate *C. asloae* from *C. chalupskyi*; none of the available specimens of the new species are sexually mature. However, four molecular markers (two mitochondrial, two nuclear) support that *C. asloae* is a distinct, separately evolved lineage, which is sister to a clade consisting of *C. glandulosus* and *C. varisetosus*. In this study, too, the fully developed sexual organs of *C. chalupskyi* and *C. varisetosus* are described and illustrated.

**Key words:** *Cognettia*, cryptic species, DNA-barcoding

### Introduction

*Chamaedrillus* Friend, 1913 [*Cognettia* sensu Nielsen & Christensen (1959), see Martinsson *et al.* (2015a)] is a genus in the family Enchytraeidae (Annelida: Clitellata) with 19 described species worldwide (Martinsson *et al.* 2015a; Schmelz & Collado 2012; Schmelz & Collado 2015; Torii 2015; Doza-Farkas *et al.* 2018), eight of which have been found in Scandinavia to date. Species of *Chamaedrillus* are found either in moist soil, or in freshwater, and they often dominate the soil fauna in coniferous forests and peat bogs (e.g. Nurminen 1967). The North European species have recently been revised using a combination of genetic and morphological data (Martinsson & Erséus 2014; Martinsson *et al.* 2015a; 2015b), and some of them are cryptic species. In this genus, reproduction is often asexual, with body fragmentation followed by regeneration of new head or tail segments. Thus, for some species, fully mature individuals with signs of sexual activity (i.e., occurrence of sperm in spermathecae) are rarely found.

During a DNA-barcoding based inventory of Scandinavian clitellates, a distinct genetic lineage morphologically similar to *Chamaedrillus chalupskyi* Martinsson, Rota & Erséus, 2015a was discovered at four sites, in Norway, Sweden, and Denmark. The aim of this paper is to describe this lineage as a new species, and to estimate its phylogenetic position in *Chamaedrillus*. We also amend the descriptions of the genital organs of *C. chalupskyi* and *C. varisetosus* Martinsson, Rota & Erséus 2015b, on the basis of fully mature, post-copulatory, specimens.

### Material and methods

In total, seven specimens of the new species, from two Norwegian and one Swedish river as well as a Danish deciduous forest (Table 1) were studied. The worms were first preserved in 80% ethanol. The posteriormost segments were then used for DNA-extraction, and the remaining anterior parts were stained using paracarmine and

**TABLE 1.** List of material included in this study, with specimen identification numbers, museum voucher numbers, collection data with GPS coordinates, and GenBank accession numbers. The letters in parentheses after the spm no. indicates type status, H = holotype, P = paratype. Accession numbers in bold are newly generated sequences. Locality data are in most cases given in the form of country, province, municipality and locality, GPS coordinates are given as decimal degrees. AT = Austria, DK = Denmark, HU = Hungary, NO = Norway, SE = Sweden.

Species	Spm. no.	Museum voucher no.	Collection locality		Coordinates		Leg.	Coll. date	GenBank accession nos.			
			N	E	N	E			16S	COI	H3	ITS2
<i>Chamaedrillus astolae</i> sp.nov	CE26608	ZMBN111448	NO, Oslo, Frognerparken, Frognerelva River	59.9280	10.7059	C. Erséus	Aug 2015	MH810209	MH810225	MH810236	MH810239	
<i>C. astolae</i> sp.nov	CE27752	SMNH170826	SE, Närke, Örebro, Svartån River	59.2642	15.1415	C. Erséus	Jul 2015	MH810210	MH810226	MH810237	MH810244	
<i>C. astolae</i> sp.nov	CE32715	ZMBN126052	NO, Akershus, Baerum, Sandvikselva River	59.8932	10.5094	C. Erséus & M. Klinth	Jun 2017	MH810211	MH810230	MH810232	MH810240	
<i>C. astolae</i> sp.nov	CE32716	ZMBN126053	NO, Akershus, Baerum, Sandvikselva River	59.8932	10.5094	C. Erséus & M. Klinth	Jun 2017	MH810212	MH810227	MH810233	MH810243	
<i>C. astolae</i> sp.nov	CE32735(P)	ZMBN126054	NO, Akershus, Baerum, Sandvikselva River	59.8933	10.5094	C. Erséus & M. Klinth	Jun 2017	MH810213	MH810228	MH810234	MH810241	
<i>C. astolae</i> sp.nov	CE32736(P)	ZMBN126055	NO, Akershus, Baerum, Sandvikselva River	59.8933	10.5094	C. Erséus & M. Klinth	Jun 2017	MH810214	MH810229	MH810235	MH810242	
<i>C. astolae</i> sp.nov	CE35144	SMNH170827	DK, Aarhus, Sydøstjurs, Mols Bjerge, Askelunden	56.2277	10.5877	M. Klinth & E. Eriksson	May 2018	MH810215	MH810224	MH810231	MH810238	
<i>C. chalupekyi</i>	CE823 (P)	MCZR 0188	SE, Västergötland, Götene, Hällekis	58.6189	13.4266	E. Rota & C. Erséus	May 2004	KF672456 <sup>2</sup>	KF672410 <sup>2</sup>	KF672493 <sup>2</sup>	KF672532 <sup>2</sup>	
<i>C. chalupekyi</i>	CE4759	-	SE, Bohuslän, Uddevalla, Bokenäs	58.3692	11.6164	C. Erséus	Jul 2008	KF672454 <sup>2</sup>	KF672408 <sup>2</sup>	KF672491 <sup>2</sup>	KF672531 <sup>2</sup>	
<i>C. chalupekyi</i>	CE2055	SMNH133648	SE, Västergötland, Göteborg, Torslanda	57.7573	11.8585	D. Gustafsson	Oct 2006	KF672452 <sup>2</sup>	KF672402 <sup>2</sup>	KF672489 <sup>2</sup>	KF672529 <sup>2</sup>	
<i>C. chalupekyi</i>	CE2211	ZMBN110765	NO, Hordaland, Kvernherad, Melselva River	60.1294	6.3153	C. Erséus	May 2014	-	MH810217	-	-	
<i>C. chalupekyi</i>	CE2212	ZMBN110766	NO, Hordaland, Kvernherad, Melselva River	60.1294	6.3153	C. Erséus	May 2014	-	MH810218	-	-	
<i>C. chalupekyi</i>	CE32524	ZMBN126056	NO, Møre og Romsdal, Rauma, Rauma River	62.4295	7.9616	C. Erséus & M. Klinth	Jun 2017	-	MH810219	-	-	
<i>C. chalupekyi</i>	CE32998	ZMBN126057	NO, Oppland, Lom, Visa River (Spiterstulen)	61.6334	8.4106	C. Erséus	Jul 2017	-	MH810220	-	-	
<i>C. chalupekyi</i>	CE32999	ZMBN126058	NO, Oppland, Lom, Visa River (Spiterstulen)	61.6334	8.4106	C. Erséus	Jul 2017	-	MH810221	-	-	
<i>C. chalupekyi</i>	CE33033	ZMBN126059	NO, Oppland, Skjåk, Vassendinjönmin Lake	61.9795	7.5121	C. Erséus	Jul 2017	-	MH810222	-	-	
<i>C. chalupekyi</i>	CE33370	SMNH170828	SE, Lappland, Kiruna, Torneträsk Lake	68.3023	19.2482	C. Erséus & M. Klinth	Sep 2017	-	MH810223	-	-	
<i>C. chlorophilus</i>	CE2334	SMNH133668	SE, Skåne, Sjöbo, Vallarum	55.7371	13.8556	A. Ansebo	May 2007	KF672459 <sup>2</sup>	KF672412 <sup>2</sup>	KF672495 <sup>2</sup>	KF672534 <sup>2</sup>	
<i>C. chlorophilus</i>	CE6627	SMNH133670	SE, Uppland, Vallentuna, Brottbö	59.5477	18.2467	C. Erséus	Jun 2009	KF672460 <sup>2</sup>	KF672413 <sup>2</sup>	KF672496 <sup>2</sup>	KF672535 <sup>2</sup>	
<i>C. chlorophilus</i>	CE1041	SMNH133667	SE, Halland, Laholm, Hallandsåsen	56.395	13.000	E. Rota, P. De Wit, L. Matamoros, A. Ansebo & C. Erséus	May 2005	KF672457 <sup>2</sup>	KF672411 <sup>2</sup>	KF672494 <sup>2</sup>	KF672533 <sup>2</sup>	

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TABLE 1. (Continued)

Species	Spm. no.	Museum voucher no.	Collection locality		Coordinates		Leg.	Coll. date	GenBank accession nos.			
			Collection locality		N	E			16S	COI	H3	ITS2
<i>C. cognettii</i>	CE1042	SMNH108410	SE. Halland, Laholm, Hallandsåsen		56.395	13.000	E. Rota, P. De Wit, L. Matamoros, A. Ansebo & C. Erséus	May 2005	GU901781 <sup>1</sup>	GU902044 <sup>1</sup>	KF672469 <sup>2</sup>	KF672508 <sup>2</sup>
<i>C. cognettii</i>	CE1043	-	SE. Halland, Laholm, Hallandsåsen		56.395	13.000	E. Rota, P. De Wit, L. Matamoros, A. Ansebo & C. Erséus	May 2005	KF672430 <sup>2</sup>	KF672366 <sup>2</sup>	KF672470 <sup>2</sup>	KF672509 <sup>2</sup>
<i>C. glandulosus</i>	CE2011	SMNH133613	SE. Västergötland, Vårgårda, Fly, Lången Lake		57.9973	12.5868	C. Erséus	Jun 2006	KF672435 <sup>2</sup>	KF672372 <sup>2</sup>	KF672475 <sup>2</sup>	KF672514 <sup>2</sup>
<i>C. glandulosus</i>	CE2890	SMNH133618	SE. Södermanland, Vingåker, Låtern Lake		59.0854	16.0426	C. Erséus	Jun 2007	KF672440 <sup>2</sup>	KF672378 <sup>2</sup>	KF672478 <sup>2</sup>	KF672517 <sup>2</sup>
<i>C. lapponicus</i>	CE13849	SMNH133621	SE. Lappland, Gällivare, 20 km WSW Gällivare		67.0793	20.3391	C. Erséus	Jun 2010	KF672441 <sup>2</sup>	KF672380 <sup>2</sup>	KF672479 <sup>2</sup>	KF672518 <sup>2</sup>
<i>C. pseudo-sphagnetorum</i>	CE4023 (P)	SMNH TYPE-8686	SE. Skåne, Vellinge, Skanörs Ljung		55.4011	12.8919	C. Erséus	May 2008	KF672465 <sup>2</sup>	KF672419 <sup>2</sup>	KF672501 <sup>2</sup>	KF672540 <sup>2</sup>
<i>C. pseudo-sphagnetorum</i>	CE4055	SMNH133692	SE. Blekinge, Olofstrom, Halen Nature Reserve		56.2842	14.4911	C. Erséus	Jun 2008	KF672467 <sup>2</sup>	KF672422 <sup>2</sup>	KF672503 <sup>2</sup>	KF672542 <sup>2</sup>
<i>C. sphagnetorum</i>	CE6672	SMNH133639	SE. Västergötland, Vårgårda, Fly		57.9972	12.5873	C. Erséus	Jun 2009	KF672448 <sup>2</sup>	KF672396 <sup>2</sup>	KF672485 <sup>2</sup>	KF672525 <sup>2</sup>
<i>C. sphagnetorum</i>	CE786	-	SE. Västergötland, Vårgårda, Fly		57.9855	12.4313	E. Rota & C. Erséus	May 2004	KF672449 <sup>2</sup>	KF672397 <sup>2</sup>	KF672486 <sup>2</sup>	KF672526 <sup>2</sup>
<i>C. sphagnetorum</i>	CE11317	SMNH133623	SE. Närke, Hallsberg, Östansjö		59.0389	15.0186	A. Achurra & C. Erséus	Apr 2011	KF672442 <sup>2</sup>	KF672381 <sup>2</sup>	KF672480 <sup>2</sup>	KF672519 <sup>2</sup>
<i>C. varisetosus</i>	CE2634	SMNH133600	SE. Öland, Borgholm, Södra Greda		56.9929	16.8765	A. Ansebo, L. Matamoros & C. Erséus	Jun 2007	KF672431 <sup>2</sup>	KF672367 <sup>2</sup>	KF672471 <sup>2</sup>	KF672510 <sup>2</sup>
<i>C. varisetosus</i>	CE4027	SMNH133602	SE. Skåne, Ystad, Nyvångsskogen Forest		55.5606	13.8239	C. Erséus	May 2008	KF672433 <sup>2</sup>	KF672369 <sup>2</sup>	KF672473 <sup>2</sup>	KF672512 <sup>2</sup>
<i>C. varisetosus</i>	CE23081	ZMBN126060	NO. Troms, Kvaenangen, Badderren, small river		69.8409	22.0121	C. Erséus	Aug 2015	MH810216	-	-	-
<i>Euenchytraeus clariae</i>	SM221	SMNH155445	HU. Vas, Kőszeg Mountains		47	16	K. Dózsa-Farkas	Apr 2015	KX618757 <sup>3</sup>	KX618736 <sup>2</sup>	-	MH810246
<i>Mesenchytraeus pelicensis</i>	CE742	-	DK. Aarhus, Mols Laboratory, Sletten		56.23	10.59	E. Rota	Jul 2001	GU901844 <sup>1</sup>	GU902101 <sup>1</sup>	KX644884 <sup>3</sup>	MH810245
<i>Stercutius niveus</i>	CE841	-	SE. Västergötland, Lerum, Kästenhof		57.779	12.286	E. Rota & C. Erséus	Jun 2004	GU901852 <sup>1</sup>	GU902112 <sup>1</sup>	KF672507 <sup>2</sup>	KF672547 <sup>2</sup>

\*Museo Civico di Zoologia di Roma <sup>1</sup>from Erséus *et al.* (2010); <sup>2</sup>from Martinsson & Erséus (2014); <sup>3</sup>from Martinsson *et al.* (2017)

slide-mounted in Canada balsam as described by Erséus (1994). All measurements and observations were made on preserved and somewhat compressed animals under a compound microscope. As the posterior parts of the specimens had been removed, the body size was arbitrarily given as the length of the 20 anteriormost segments and the width of segment XII (the latter was chosen to represent general body width). This size estimate was used also in Martinsson *et al.* (2015a; 2015b). In the descriptions, body measurements are given as a range, followed by the mean  $\pm$  1 standard deviation. Line drawings were drawn using a camera lucida and digital photographs were taken with a Nikon DXM1200 digital camera. Both drawings and photos were processed in Adobe Photoshop.

The new specimens studied are deposited in the University Museum Bergen (ZMBN), Norway, and the Swedish Museum of Natural History (SMNH), Sweden (see Table 1).

DNA was extracted using Epicentre's QuickExtract DNA Extraction Solution 1.0. Four molecular markers were amplified; the mitochondrial genes 16S ribosomal RNA (16S) and Cytochrome C Oxidase subunit I (COI), the nuclear genes Histone 3 (H3) and the Internal Transcribed Spacer 2 (ITS2), using the primers and PCR programs listed in Table 2. Sequencing was carried out by Eurofins MWG Operon (Ebersberg, Germany). Sequences were assembled into consensus sequences using Geneious v.8.1.9 (Biomatters Ltd., Auckland, New Zealand). All new sequences are deposited in GenBank (see Table 1 for accession numbers). Sequences for the other Scandinavian species of *Chamaedrillus*, and the out-groups *Mesenchytraeus pelicensis* Issel, 1905, *Stercutus niveus* Michaelsen, 1888, and *Euenchytraeus clarae* (Bauer, 1993) were either taken from previous studies (Martinsson *et al.* 2017; Martinsson & Erséus 2014), or were in a few cases newly produced (see Table 1 for details). Totally 27 specimens were included in the molecular analyses. The sequences of each marker were aligned using MAFFT v7.017 (Kato *et al.* 2002) as implemented in Geneious, using default settings.

Single gene trees were estimated for each of the four markers using Bayesian inference in MrBayes v.3.2.6 (Ronquist *et al.* 2012). The two protein coding genes COI and H3 were partitioned according to codon position, partitions were unlinked. Rate variation across sites was set to gamma distribution with a proportion of invariable sites; model jumping was implemented to integrate over substitution model space. The analyses ran for 10 million generations sampling every 10 000 generations, the first 25% were discarded as burn-in, and a majority-rule consensus tree was constructed.

Further, a species tree was estimated using the multi-species coalescent (MSC) model as implemented through the \*BEAST module in BEAST 1.8 (Drummond *et al.* 2012). For any specimen missing genes, a dummy sequence consisting of only Ns was added to the data matrix. An XML input file was created in BEAUti 1.8. The two mitochondrial genes are genetically linked and are assumed to share gene trees, therefore the tree models were linked for them. The substitution models were unlinked and all genes were given their own HKY+ G substitution model with empirical base frequencies. Clock models were also unlinked across genes, with separate strict clocks, and clock rates estimated, for each gene. The Yule 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. 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 clock rates (clock.rate) (mean 0.2, s.d. 0.1 for 16S, COI, and ITS2; mean 0.05, s.d. 0.1 for H3) for all genes. 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 ran for 100 million generations, sampling every 10 000 generations. Tracer v1.6 was used to examine effective sample size (ESS) for parameters and determine burn-in, and trees were summarised using TreeAnnotator v1.8, discarding the first 50 % as burn-in, and using the maximum clade credibility tree.

All trees were drawn in FigTree 1.4.2 (Rambaut 2014) and further edited in Adobe Illustrator.

## Results

### Taxonomy

#### *Chamaedrillus asloae* sp. nov.

Figure 1A–E

**Holotype.** . ZMBN126053, specimen CE32716, immature, anterior part on slide, COI barcode, GenBank acc. no. MH810224, leg. Christer Erséus & Mårten Klinth, 2 Jun 2017.

**Type locality.** NORWAY: Akershus, Baerum, Sandvika, Sandvikselva River, in sandy river sediment close to the river bank; N 59.8932, E 10.5094.

**Paratypes.** ZMBN126052, specimen CE32715, immature, anterior part on slide, COI barcode, GenBank acc. no. MH810230, from type locality, same collection data as for holotype; ZMBN126054, specimen CE32735, immature, anterior part on slide, COI barcode, GenBank acc. no. MH810228; NORWAY: Akershus, Baerum, Sandvika, Sandvikselva River, in sandy, gravelly river sediment in the middle of the river; N 59.8933, E 10.5094, same collection data as for holotype; ZMBN126055, specimen CE32736, immature, anterior part on slide, COI barcode acc. no. MH810229; same locality and collection data as for second paratype above.

**Other material.** Three immature specimens on slides, one from Svartån River, Örebro, Sweden (CE27752), one from Frognerelva River, Oslo, Norway (CE26608), and one from Askelunden Forest, Mols Bjerger, Denmark (CE35144), see Table 1 for more details.

**Etymology.** The species epithet *asloae* is a noun in the genitive case formed of the Latin name for the capital of Norway, Oslo; two of the four localities where the species was found are in or close to Oslo.

**Diagnosis.** The species can be separated from all other *Chamaedrillus* species, except *C. chalupskyi* Martinsson, Rota & Erséus, 2015a on the basis of the following combination of characters: 2 chaetae in lateral bundles in II, 3 in all other bundles, 4–6 pairs of pharyngeal glands, 0–2 pairs of pharyngeal glands fused dorsally; dorsal blood vessel originates posterior to segment XV.

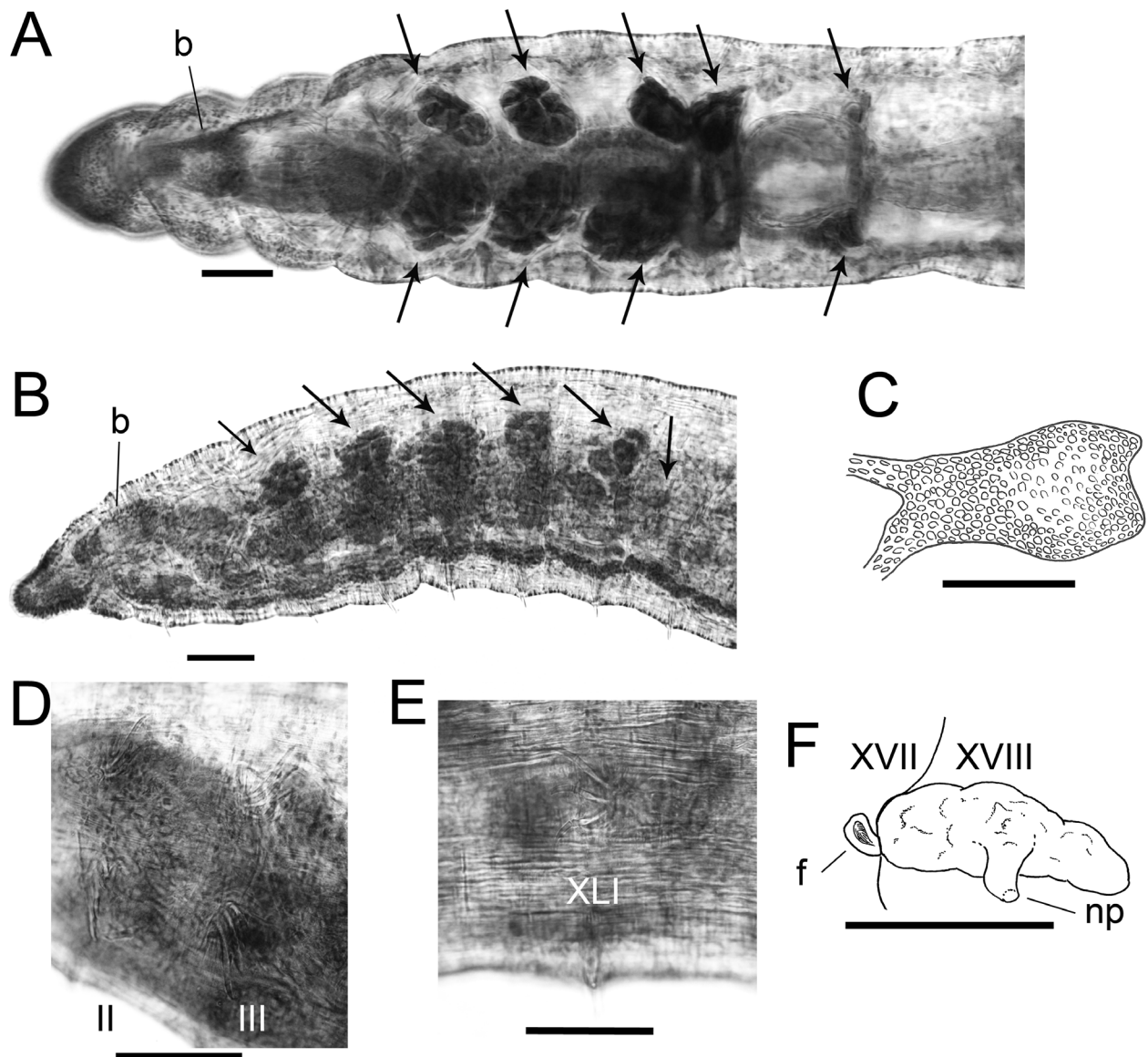
**Description.** *External characters:* Size: length of 20 anteriormost segments 3.2–4.2 mm, mean 3.8±0.42 mm (n=5); body width in XII 0.24–0.43 mm, mean 0.34±0.07 mm (n=6). Chaetae (Fig. 1D–E) sigmoid without nodulus, 55–90 µm long, chaetal formula 3–3(4):3–3, but with only 2 chaetae in the lateral bundles of II. *Internal characters:* Brain (Fig. 1C) slightly concave posteriorly, 150–240 µm long, about 1.5–2 times as long as broad. Pharyngeal glands (Fig. 1A–B, arrows) 4–6 primary pairs, sixth pair rudimentary if present, 0–2 anteriormost pairs dorsally fused, pair 4 and/or 5 with ventral lobes; development is irregular and glands are often smaller, or completely missing at one side, but present at the other. Dorsal blood vessel arising in XV–XVI. First pair of nephridia present at 8/9–9/10; nephridia (Fig. 1F) about 100–130 µm long, with efferent duct originating antero-ventrally, close to septum in preclitellar segments, in postclitellar segments duct originating in middle of postseptale; anteseptale consisting of funnel only; postseptale elongate. Chloragogen cells 30–55 µm long. Coelomocytes finely granulated, round to oval, about 15–30 µm long, concentrated to posterior part of body.

One specimen (Paratype CE32715) with developing testes and sperm funnels in VIII and developing ovaries in IX, but these organs are too undeveloped to be further described. All other specimens immature.

**Habitat and distribution.** Mainly benthic, so far found in three rivers, Frognerelva and Sandvikselva in South-East Norway, and Svartån in South-Central Sweden, as well in wet soil in a deciduous forest in Central Denmark. The specimen from Frognerelva was found in sandy soil in a pond, with slowly flowing water directly connected with the river, in Sandvikselva the worms were found in sandy sediment with some gravel, two close to the shore (CE32715, CE32716) and two in the middle of the river (CE32735, CE32736), and in both places with rapid water moment above. The Svartån individual was also found in sandy sediment in a fast-flowing stretch of the river. At the Danish site at Askelunden also the aquatic *Stylodrilus heringianus* Claparède, 1862 (Lumbriculidae) was found, which indicates groundwater seepage.

**Biology.** The main mode of reproduction is unknown. One specimen (CE27752, from Sweden) had a regenerating head, and one paratype was in early stages of sexual maturity.

**Remarks.** No morphological characters clearly separating this species from *C. chalupskyi* were found in the studied specimens, and the two taxa must be considered a pair of cryptic species. In our scrutiny of the limited material of *C. asloae*, we did note a tendency for the new species to have fewer (only 0–2) pairs of pharyngeal glands that are fused dorsally than what is the case for *C. chalupskyi* (normally with 2–4 such pairs), but the state of this character is not always easy to determine in whole-mounted worms. The new species is also similar to *C. valeriae* (Dumnicka, 2010), only known from a stream in the Italian Alps. Both species share the fused anterior pharyngeal glands (2 pairs fused in *C. valeriae*), presence of ventral lobes on some pharyngeal glands (present on 2–3 pair in *C. valeriae*), and a more posterior arising of the dorsal blood vessel (arises in XXII in *C. valeriae*), but *C. valeriae* has a mix of 2 and 3 chaetae in the preclitellar lateral bundles (Dumnicka 2010; Martinsson et al. 2015a).



**FIGURE 1.** *Chamaedrillus asloae* sp. nov. **A.** Habitus of anterior part of body in dorsal view; pharyngeal glands marked with arrows (Holotype) **B.** Habitus of anterior part of body in lateral view, showing the size, shape and number of pharyngeal glands (arrows) (specimen CE26608). **C.** Brain, dorsal view (Holotype). **D.** Chaetal bundles in segments II and III (Paratype CE32736). **E.** Lateral chaetal bundle in segment XLI (Paratype CE32736). **F.** Nephridium, at 17/18, ventral view (Paratype CE32735). Abbreviations: b = brain; f = funnel; np = nephridial pore. Scale bars 100  $\mu$ m.

***Chamedrilus chalupskyi* Martinsson, Rota & Erséus, 2015a**

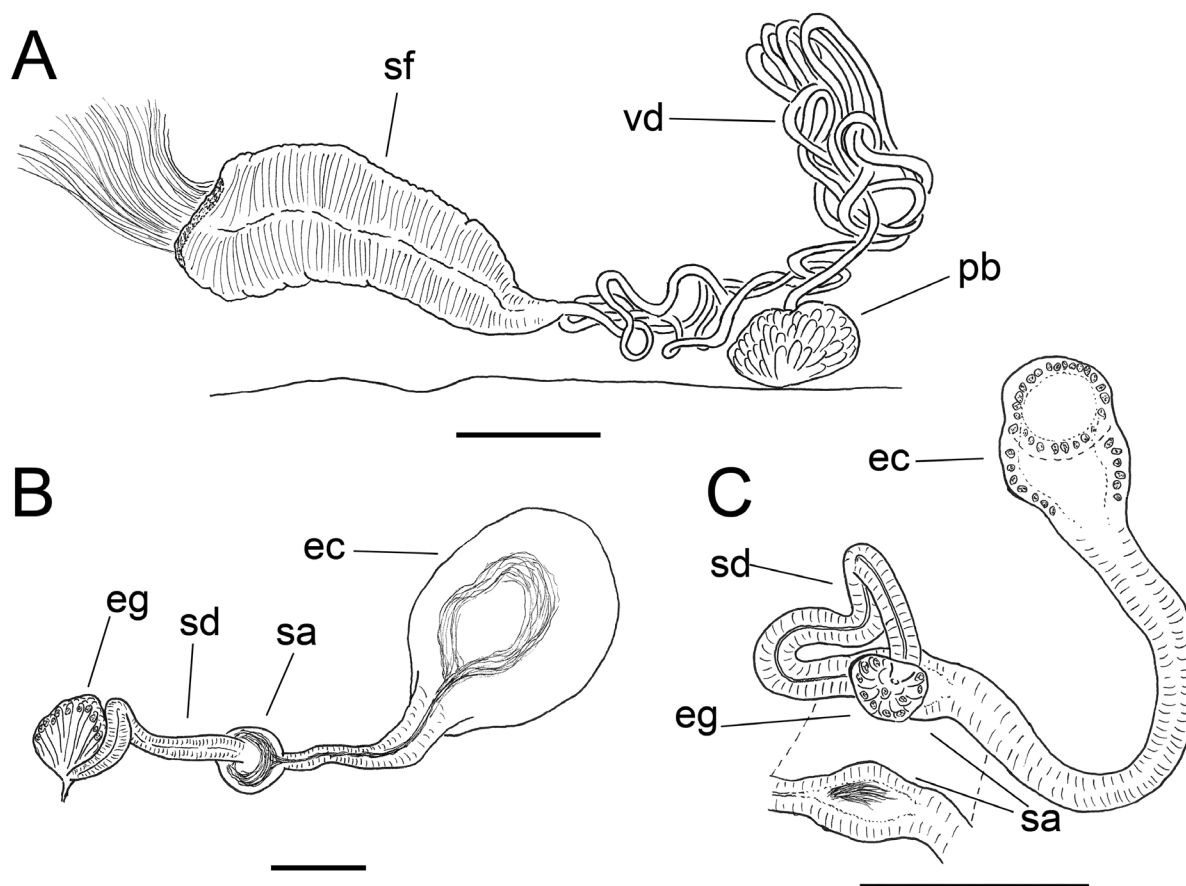
Figure 2A–B

**Material studied.** CE22111, CE22112, CE32524, CE32998, CE32999, CE33033 (all from Norway), and CE33370 (from Sweden), all specimens mature, see Table 1 for more information

**Description of genital organs.** Male genitalia (Fig. 2A) paired. Sperm funnel cylindrical, about 255–330  $\mu$ m long, 80–115  $\mu$ m wide, making them about 3 times longer than wide; collar 55–90  $\mu$ m wide. Vas deferens coiled numerous times around ovaries (Fig. 2A), at least 2000  $\mu$ m long, about 10  $\mu$ m wide. Penial bulb about 75–100  $\mu$ m long. Male pores in one of segments IX–XII, mostly X. Spermathecae (Fig. 2B) paired; pore located slightly below line of lateral chaetae; duct smooth, 155–285  $\mu$ m long, about 20–25  $\mu$ m wide; ectal gland 55–85  $\mu$ m in diameter; ampulla spherical, about 45–70  $\mu$ m wide, containing sperm arranged in circle; ampulla followed by duct

connecting to spherical ental chamber, about 60–190  $\mu\text{m}$  wide, containing loosely packed sperm; ampulla in V or VI, ental chamber of one spermatheca usually occupying VI and the other VII; spermatheca not connected to oesophagus.

**Remarks.** In the original description of this species, Martinsson *et al.* (2015a) described the sexual characters from a specimen with rudimentary male genitalia. Thus, we here amend this by describing the fully developed male genital duct and spermatheca. However, this species mainly reproduces by fragmentation, and sexually mature specimens are rare.



**FIGURE 2.** A, B. *Chamaedrillus chalupskyi*. A. Male genital duct. B. Spermatheca. C. *C. varisetosus*, spermatheca. Abbreviations: ec = ental chamber; eg = ectal gland; pb = penial bulb; sa = spermathecal ampulla; sd = spermathecal duct; sf = sperm funnel; vd = vas deferens. Scale bars: 100  $\mu\text{m}$ .

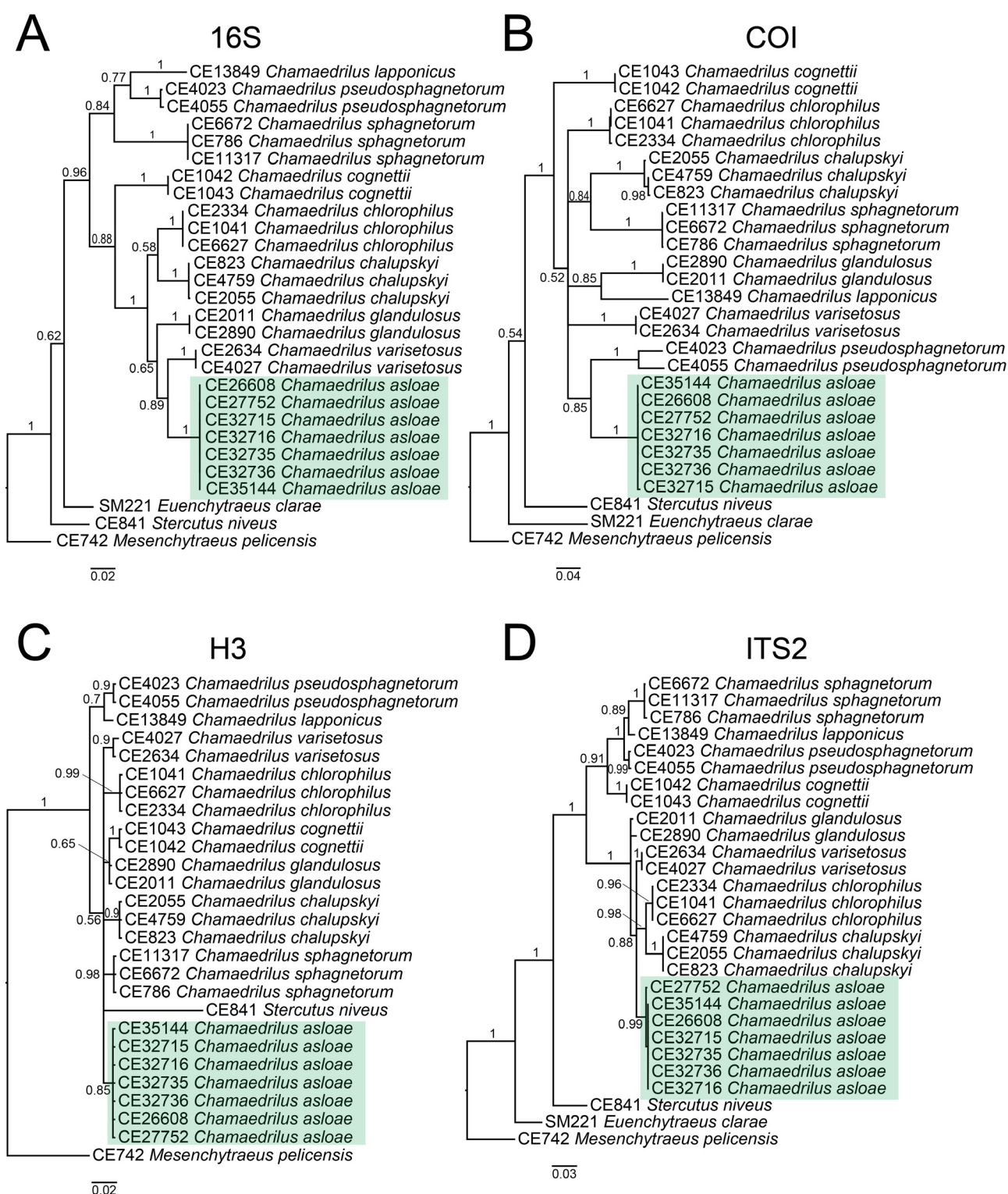
### *Chamaedrillus varisetosus* Martinsson, Rota & Erséus, 2015b

Figure 2C

**Material studied.** CE23081 (from northern Norway), mature specimen, see Table 1 for more information

**Description of spermatheca.** Spermathecae (Fig. 2C) paired; pore located slightly below line of lateral chaetae; duct smooth, 195–225  $\mu\text{m}$  long, about 15  $\mu\text{m}$  wide; ectal gland about 35  $\mu\text{m}$  in diameter; ampulla oval, about 35  $\mu\text{m}$  wide, containing loosely arranged sperm; ampulla followed by duct connecting to spherical ental chamber, about 55  $\mu\text{m}$  wide; ampulla in V or VI, ental chamber of one spermatheca usually occupying VI and the other VII; spermatheca not connected to oesophagus.

**Remarks.** In the original description, Martinsson *et al.* (2015b) illustrated a spermatheca that was not fully developed, here we describe and illustrate one that is.



**FIGURE 3.** Gene trees of *Chamaedrilus* estimated by Bayesian inference; *C. asloae* sp. nov. shaded in green. **A.** 16S **B.** COI. **C.** H3. **D.** ITS2. Posterior probabilities shown at branches. Scales show expected number of changes per site.

### Molecular analysis

*Chamaedrilus asloae* is found as a distinct lineage within *Chamaedrilus* in all gene trees (Fig. 3A–D), and all other species are also distinct lineages, with a few exceptions: *C. glandulosus* (Michaelsen, 1888) is not monophyletic in the H3 (Fig. 3C) and ITS2 (Fig. 3D) trees. That is, in the H3 tree a monophyletic *C. cognettii* (Issel, 1905) is nested



within *C. glandulosus*, but without support, and in the ITS2 tree a somewhat supported clade (PP=0.88) consisting of *C. asloae*, *C. chlorophilus* Friend, 1913, *C. chalupskyi*, and *C. varisetosus* is unresolved from *C. glandulosus*. *Chamaedrillus* is monophyletic with high support in all gene trees except the H3 tree (Fig. 3C), where *Stercutus niveus* is found nested within *Chamaedrillus*, but without support (and *Euenchytraeus clarae* is missing from the dataset). The relationships within *Chamaedrillus* vary between the gene trees. The COI (Fig. 3B) and H3 (Fig. 3C) trees are mainly unresolved within *Chamaedrillus*. In the 16S (Fig. 3A) and ITS2 (Fig. 3D) trees, *C. asloae* is found in a clade also consisting of *C. chlorophilus*, *C. chalupskyi*, *C. glandulosus* and *C. varisetosus*. Finally, in the 16S tree, *C. asloae* is sister to *C. varisetosus*, whereas the position of *C. asloae* within the clade is unresolved in the ITS2 tree.

The species tree (Fig. 4) is largely congruent with species trees in Martinsson and Erséus (2014) and Martinsson *et al.* (2017). *Chamaedrillus* is monophyletic with maximum support, and is divided into two well-supported clades, one (PP=0.98) consisting of the species with three chaetae in each bundle, i.e., *C. sphagnetorum* (Vejdovský, 1879), *C. pseudosphagnetorum* Martinsson, Rota & Erséus, 2015a, and *C. lapponicus* (Nurminen, 1965), where the latter two are sisters (PP=0.96). In the other clade (P=0.97), consisting of the species with two chaetae in some lateral bundles, *C. cognettii* is the sister to the remaining species (PP=0.93). *Chamaedrillus chalupskyi* and *C. chlorophilus* are sisters (PP=0.95), and *C. asloae*, *C. glandulosus*, and *C. varisetosus* are found together (PP=0.87), and the two latter are sisters (PP=0.80). All the out-groups form a clade separated from *Chamaedrillus* (see also end of Discussion below).

**TABLE 2.** Primers and thermal profiles used for amplification and sequencing of the mitochondrial 16S and COI and nuclear H3 and ITS2 markers.

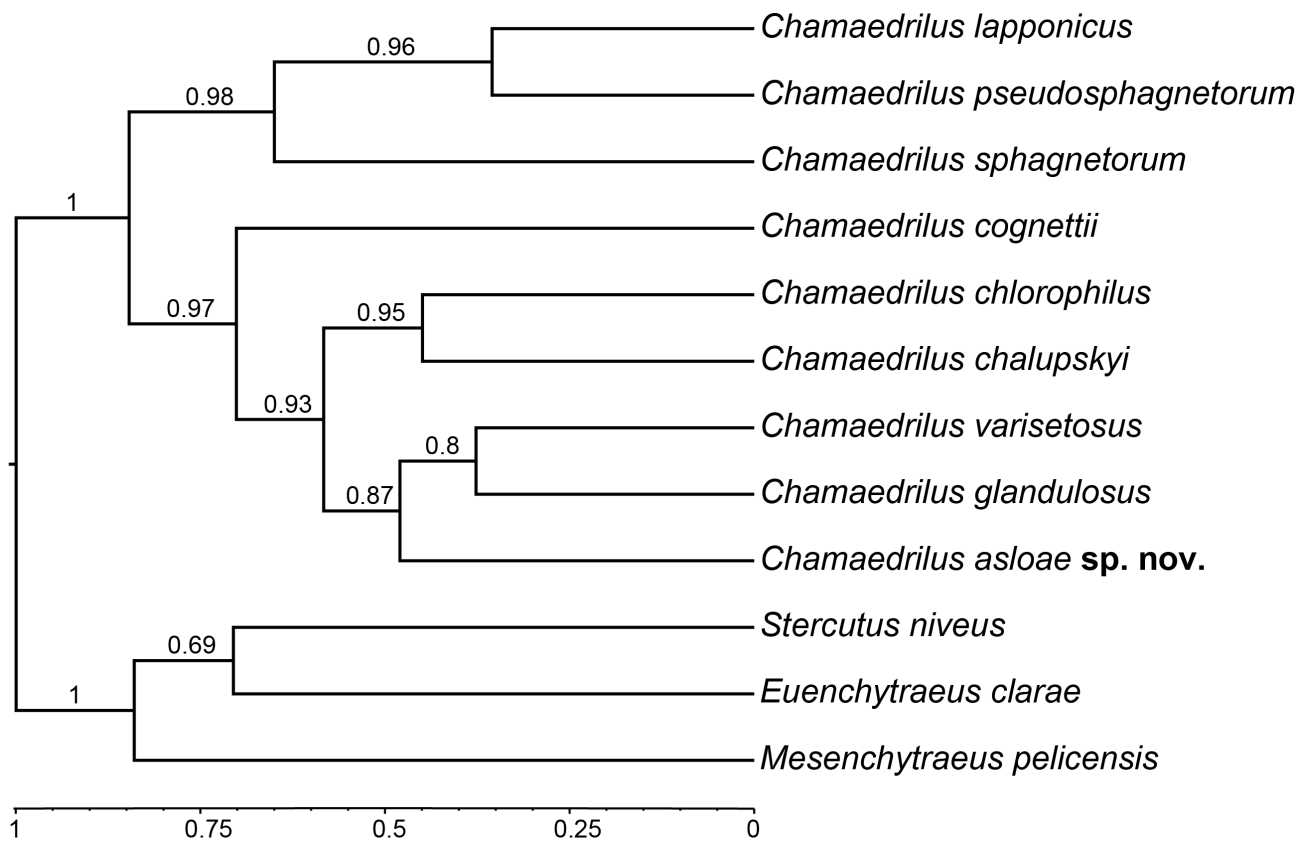
Gene	Primer	Sequence 5'-3'	Reference	Amplification program
16S	16SAR-L	CGCCTGTTTATCAAAAACAT	Palumbi <i>et al.</i> (1991)	95°C for 5 min, 35 cycles each of 95°C for 30 sec, 50°C for 30 sec and 72°C for 60 sec, finally, 72°C for 8 min.
	16SBRH	CCGGTCTGAACTCAGATCACGT	Palumbi <i>et al.</i> (1991)	
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer <i>et al.</i> (1994)	95°C for 5 min, 35 cycles each of 95°C for 40 sec, 45°C for 45 sec and 72°C for 60 sec, finally, 72°C for 8 min.
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer <i>et al.</i> (1994)	
ITS2	606F	GTCGATGAAGAGCGCAGCCA	Liu and Erséus (2017)	95°C for 5 min, 35 cycles each of 95°C for 45 sec, 55°C for 60 sec and 72°C for 90 sec, finally, 72°C for 8 min.
	1082R	TTAGTTTCTTTTCCTCCGCTT	Liu and Erséus (2017)	
H3	H3F	ATGGCTCGTACCAAGCAGACVGC	Colgan <i>et al.</i> (1998)	95°C for 5 min, 35 cycles each of 95°C for 30 sec, 50°C for 30 sec and 72°C for 90 sec, finally, 72°C for 8 min.
	H3R	ATATCCTTRGGCATKATRGTGAC	Colgan <i>et al.</i> (1998)	

## Discussion

Cryptic species, i.e., species that are morphologically indistinguishable or so similar that they have been classified under the same species name (Bickford *et al.*, 2007) are common among enchytraeids (e.g. Collado *et al.* 2012; Martinsson & Erséus 2018; Matamoros *et al.* 2012; Westheide & Graefe 1992) and other clitellates (e.g. Erséus & Gustafsson 2009; King *et al.* 2008; Liu *et al.* 2017). Recently Schmelz *et al.* (2017) published an overview of cryptic species in Enchytraeidae, with recommendation for how to describe them. In the present study we follow those recommendations as far as possible. The new species *Chamaedrillus asloae* and *C. chalupskyi* cannot be separated using morphological characters, making the two taxa a pair of morphologically cryptic species.

The description of *C. asloae* is based on a rather small number of sexually immature specimens, or in one case a specimen in early stages of sexual maturity; ideally species descriptions should be based on a larger material,

including sexually mature specimens. However, we believe it is justified to describe the new species, as it is genetically distinct, and as the somatic characters distinguish it from all except one described species in the genus. The rarity of the new species is noteworthy; we have only found six specimens despite having over 750 barcoded Scandinavian specimens of *Chamaedrilus*, from both terrestrial and aquatic samples, in the senior author's collection. It is possible that the species has a more southern distribution and that it is more common in, e.g., Continental Europe. So far, *C. asloae* has been found in fully limnic (or rather, lotic) environments, as well as in wet forest-soil with possible groundwater seepage, and thus, together with *C. glandulosus*, it appears to be among the more aquatic species of *Chamaedrilus* in Northern Europe. However, it should be noted that also *C. chalupskyi* and *C. varisetosus* are commonly found in aquatic habitats (Martinsson *et al.* 2015b; CE unpublished data).



**FIGURE 4.** Species tree for *Chamaedrilus* estimated using the Multi-Species Coalescent model. Posterior probabilities are shown at branches. Scale shows proportion of tree length.

The spermathecae and male genitalia are similar in most species of *Chamaedrilus*, and seem to be of limited use for species identification. In a majority of species the spermatheca looks like in the two species illustrated here (Fig. 2B,C), where the ampulla is divided into one ental and one ectal chamber, and these two are connected to each other via a duct. However, in six species, *C. anomalus* (Černosvitov, 1928), *C. floridae* (Healy, 1996), *C. hayachinensis* (Nakamura, 2001), *C. hibernica* (Healy, 1975), *C. ozensis* Torii, 2015, and *C. paxi* (Moszyński, 1938), the spermatheca has only one chamber in the ampulla, and in some of them the spermatheca also is missing the (ectal) gland at the pore. In *C. anomalus* and *C. paxi* the ampulla is elongated and could be an undeveloped spermatheca of the normal *Chamaedrilus* type. Unfortunately, none of the above-mentioned species has been included in phylogenies of the genus, and no sequence data are available. Several species of *Chamaedrilus* reproduce mainly by fragmentation, and for some of them sexually mature specimens are rare, further limiting the usefulness of sexual characters for species identification in this group.

The suggested monophyly of the three outgroups in our multi-species coalescent-based species tree (Fig. 4) is likely due to the poor out-group sampling. With a larger number of outgroup taxa included, Martinsson *et al.* (2017) concluded that *Euenchytraeus* and *Stercutus* are most closely related to *Chamaedrilus*.

The species tree (Fig. 4) suggests, with reasonable support, that the morphologically very similar *Chamaedrilus asloae* and *C. chalupskyi* do not form a monophyletic group. Instead, *C. asloae* is more closely

related to *C. glandulosus* and *C. varisetosus*, from which it differs morphologically, and *C. chalupskyi* is sister to *C. chlorophilus*. Another cryptic species pair in the genus, *C. sphagnetorum* and *C. pseudosphagnetorum* is similarly not monophyletic, as the latter is the sister-species to *C. lapponicus* (Fig.4). Thus, the morphological similarities within the *asloae/chalupskyi* and *sphagnetorum/pseudosphagnetorum* pairs are likely to be homoplasious traits within their respective lineages.

## Acknowledgements

We are grateful to Per Hjelmstedt for assistance in the molecular lab; Elise Eriksson for assistance in the fieldwork; and the Swedish Species Information Centre (ArtDatabanken), the Norwegian Biodiversity Information Centre (Artsdatabanken), the Adlerbert Research Foundation, and the Royal Society of Arts and Sciences in Gothenburg, for financial support.

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