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Cryptic diversity in the well-studied terrestrial worm *Cognettia sphagnetorum* (Clitellata: Enchytraeidae)

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ABSTRACT

The terrestrial worm *Cognettia sphagnetorum* has been used as a model in several studies focusing on research areas such as climate change as well as forest and soil ecology; it has also been shown to play a key role in the decomposition of organic matter and nutrient cycling. *Cognettia* is an enchytraeid genus commonly found in acidic terrestrial habitats, such as coniferous forests and bogs. In this study, the diversity of the genus, with particular focus on the morphospecies *C. sphagnetorum* in northern Europe, is assessed using four molecular markers, the mitochondrial COI (cytochrome *c* oxidase subunit I) and 16S (16S ribosomal RNA), and the nuclear H3 (Histone 3) and ITS (Internal Transcribed Spacer). The datasets were first delimited into Molecular Operational Units (MOTUs) and the existence of global barcoding-gaps was tested. Single gene-trees were then estimated for all genes using Bayesian Inference, and a species tree was estimated with all markers combined using the multi-species coalescence. The results show that in northern Europe the genus consists of at least eight MOTUs supported by all genes except H3. Four of these MOTUs were within the morphotaxon *C. sphagnetorum* and two within *Cognettia glandulosa*. *C. sphagnetorum* s.l. was found to be non-monophyletic in all gene-trees, as well as in the species tree. As the MOTUs were well separated and non-monophyly was observed within *C. sphagnetorum s.l.*, we conclude that the MOTUs are best treated as separate species. Given that cryptic diversity was found in this genus, we recommend that material of *Cognettia* used in future studies should be identified using molecular barcodes.

Introduction

In terrestrial ecology, several studies focusing on the effects of climate change (e.g. Briones et al., 1997, 1998; Haimi et al., 2005; Maraldo et al., 2008), soil pollutants (e.g. Haimi et al., 2006), forestry (Lundkvist, 1983), and soil processes, such as nutrient mineralization and availability (e.g. Standen, 1978; Abrahamsen, 1990; Haimi and Siira-Pietkäinen, 2003; Mira et al., 2002; Maraldo et al., 2011), have used *Cognettia sphagnetorum* (Vejdovský, 1878) as a model organism. This common clitellate has been shown to play a key role in the decomposition of organic matter and in nutrient cycling (Standen, 1978; Laakso and Setala, 1999). The results of these studies, however, are not always consistent. For instance, Briones et al. (1997) found a positive correlation between numbers of *C. sphagnetorum* and temperature, whereas Haimi et al. (2005) did not find such a correlation. The differences between these two studies were briefly discussed by Haimi et al. (2005) who gave several possible explanations for them, but the possibility that the animals in the two studies were different cryptic species was overlooked.

C. sphagnetorum is placed in *Cognettia* Nielsen and Christensen, 1959, a genus of Enchytraeidae (Annelida: Clitellata) commonly found in coniferous forests, bogs and similar acidic environments. The genus consists of about 15 nominal species (Nakamura, 2000; Schmelz and Collado, 2012a) of which *Cognettia cognettii* (Issel, 1905), *Cognettia glandulosa* (Michaelsen, 1888), *Cognettia lapponica* Nurminen, 1965 and *C. sphagnetorum* are recorded from the Nordic region. In addition to these species, Chalupsky (1992) described a form from Sweden as *Cognettia sp. C. sphagnetorum* is an abundant enchytraeid taxon in boreal forests and bogs (e.g. Lundkvist, 1983; Schlaghamerský, 2012).

Its primary reproductive strategy is fragmentation, while *C. glandulosa* reproduces either by fragmentation or parthenogenetically. Occasionally sexually mature specimens of these species are found (Christensen, 1959; Nielsen and Christensen, 1959), but it is uncertain whether sexual reproduction occurs in these taxa at all. In contrast, *C. cognettii* and *C. lapponica* mainly reproduce sexually (Schmelz and Collado, 2010). Sexually mature specimens of *C. glandulosa* and *C. sphagnetorum* have their genital organs displaced forward by two to four segments, with the male pores in segments viii–x (Nielsen and Christensen, 1959), similar to

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what is seen in some naidids (subfamilies Naidinae and Pristininae sensu Erséus et al. 2008). The latter mainly reproduce by budding, but often engage in seasonal sexual reproduction (Loden, 1981). The proportion of sexually reproducing individuals varies between species of *Cognettia* and perhaps also among populations (Schmelz and Collado, 2010). Moreover, sudden changes in the environment, such as clear-cutting of forests, seems to induce sexual maturity in at least *C. sphagnetorum* (Lundkvist, 1983). Two karyotypes are known to occur in *C. sphagnetorum*, one with $n = 54$ and one with $n \approx 160$. In *C. glandulosa*, only one karyotype is known ($n = 54$) (Christensen, 1961). This suggests that *C. sphagnetorum* is a complex of cryptic species. Cryptic species are species that are morphologically similar and may live sympatrically, and therefore have been classified under the same species name (Bickford et al., 2007). This phenomenon seems to be common within Clitellata (see review by Erséus and Gustafsson, 2009). Several species closely resembling *C. sphagnetorum* have been described, but sexually mature specimens (normally used for specimen identification in Enchytraeidae) are rarely found. Therefore Schmelz and Collado (2010) proposed a '*sensu lato*' approach to *C. sphagnetorum*. They included *Cognettia anomala* (Černosvitov, 1928) and *Cognettia praxi* (Moszyński, 1938) in their *C. sphagnetorum s.l.*, and later noted that *Cognettia valeriae* Dumnicka, 2010 probably to belongs to this group too (Schmelz and Collado, 2012b). The *Cognettia sp.* of Chalupsky (1992) would also fall within this definition of *C. sphagnetorum*.

Mitochondrial markers have been widely used in studies of recent divergence and species delimitation in a large number of animal groups, due to their faster lineage sorting (Neigel and Avise, 1986) and mutation rate (Brown et al., 1979, 1982) compared to the rates in nuclear markers. Several studies on annelid systematics based on mitochondrial markers have been published (e.g. Heethoff et al., 2004; James et al., 2010; Nygren and Pleijel, 2011; Dózsa-Farkas et al., 2012; Roman Dial et al., 2012). In particular the Cytochrome Oxidase C subunit I (COI) gene has been used in these studies, and a part of COI has been proposed as the standard "DNA-barcode" for identifying animal species (Hebert et al., 2003). However, using mitochondrial markers may overestimate the number of species if used alone without nuclear markers (e.g. King et al., 2008; Dasmahapatra et al., 2010; Torres-Leguizamon et al., 2012; Achurra and Erséus, 2013; Martinsson et al. 2013).

De Queiroz (2007) has proposed a *unified species concept*, whereby a separately evolving meta-population lineage is the sole requirement of a species. In this proposal most other concepts of species are incorporated to provide secondary species criteria for the assessment of lineage separation (species delimitation). The more secondary species criteria support a divergence, the stronger is the case for speciation. However, one piece of evidence (i.e. separately evolving meta-population lineages), if properly examined, may be enough to establish lineage separation. This evidence can be used for any organism, whereas, a criterion such as the biological species concept (Mayr 1942) works for sexual organisms only. Asexual organisms, such as bdelloid rotifers, form morphologically and genetically discrete clusters, where the differences between clusters are pronouncedly larger then within clusters, similar to what is found for sexually reproducing organisms (e.g. Barraclough et al. 2003; Birky et al. 2005; Fontaneto et al. 2007). These discrete clusters could be seen as species using the unified species concept, and if they are sufficiently divergent they can be delimited as such. De Queiroz's unified species concept will be used throughout this paper.

The aims of this study are (1) to assess the diversity of *Cognettia* in northern Europe, using molecular methods and focusing on *C. sphagnetorum*, and (2) to examine the possible existence of cryptic species within the genus.

Materials and methods

Specimens and sequences

Specimens collected between 2004 and 2011, mainly from Sweden and a few from Finland and Slovakia, (total 101 specimens), were included in this study. They are listed in Table 1 with information on collecting sites. Procedures of DNA extractions, PCR and sequencing differed slightly between years but have always been done using standard methods and recommended protocols. For most worms DNA was extracted from the posterior part, while the anterior part was stained with paracarmine and mounted whole in Canada balsam on slides as outlined by Erséus (1994). The mounted vouchers were examined under a compound microscope and identified to morphospecies following Schmelz and Collado (2010) and Chalupsky (1992). DNA extraction was performed using either the EZNA Tissue DNA kit (Omega Bio-Tek, Norcross, GA, USA) or the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). DNA amplification was carried out as 25 μl reactions using either PuRe-Taq Ready-To-Go (GE Healthcare, Chalfont St. Giles, UK) or Red Taq DNA Polemerase Master Mix (VWR, Haasrode, Belgium) with the primers and programs listed in Table S1; the same primers were used for the sequencing reactions. A few specimens were sequenced at the University of Gothenburg on a Beckman-Coulter CEQ 8000. DNA sequencing was, in most cases, performed by Macrogen (Geumcheon-Gu, Seoul, Korea) using the same primers as for amplification. Yet another part of the material was extracted and sequenced by the Canadian Centre for DNA Barcoding (CCDB) (Guelph, Canada) with data stored at the Barcode of Life Datasystems (BOLD). The mitochondrial gene COI was amplified and sequenced for all specimens. For a subset of 43 specimens, based on the preliminary delimitation of COI clusters (see below), also fragments of the mitochondrial 16S (16S ribosomal RNA), nuclear H3 (Histone 3) and the complete ITS region (Internal Transcribed Spacers 1, 2 and 5.8S ribosomal RNA) were sequenced.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pedobi.2013.09.006.

Stercutus niveus Michaelsen, 1888, *Mesenchytraeus flavus* (Levinsen, 1884), *Mesenchytraeus armatus* (Levinsen, 1884), *Cernosvitoviella immota* (Knöllner, 1935) and *Cernosvitoviella aggtelekiensis* Dózsa-Farkas, 1970 were used as outgroups in the gene-tree estimations; this was based on the phylogeny of Enchytraeidae presented by Erséus et al. (2010). A few in-group and out-group sequences were taken from that study, but the vast majority were newly generated (see Table 1).

The COI and H3 datasets were aligned using the Geneious Alignment with default settings in GENEIOUS PRO v5.6 (Biomatters Ltd., Auckland, New Zealand), the 16S and ITS datasets using *MAFFT v6.814b* (Katoh et al., 2002) as implemented in GENEIOUS PRO v5.6, with the auto algorithm and edited by eye.

Barcoding gap analysis and preliminary delimitation of MOTUs

The four datasets without outgroups were tested for the existence of "global barcode gaps" and assessed for a preliminary clustering of the specimens into Molecular Operational Taxonomic Units (MOTUs) using ABGD (Automatic Barcode Gap Discovery) (Puillandre et al., 2012). Both uncorrected p-distances and corrected distances were calculated in MEGA 5.1 (Tamura et al., 2011) using default settings. The models for correcting distances were chosen after conducting model testing in MEGA 5.1 using the Bayesian information criterion (BIC). The models used for COI and ITS were TN93; for 16S and H3 T92. AGBD was run using the web version (available at http://wwwabi.snv.jussieu.fr/public/abgd/)

Table 1.
List of specimens used in this study, with collection data, specimen identification numbers, voucher numbers, BOLD accession numbers and GenBank accession numbers, numbers in bold are new sequences generated in this study. Letters for *Cognettia sphagnetorum and C. glandulosa* refer to barcoding clusters. Locality data is given in the form, country, province, municipality and locality. FIN = Finland, SLO = Slovakia and SWE = Sweden.

Species	ID No.	Collection locality	BOLD Acc. No:	GenBank Accession No.				Voucher No.
				COI	16S	ITS	H3	
C. cognettii	CE1042	**SWE. Halland**, Laholm, Hallandsåsen		GU902044	GU901781	**KF672508**	**KF672469**	**SMNH 108410**
C. cognettii	CE1043	**SWE. Halland**, Laholm, Hallandsåsen		**KF672366**	**KF672430**	**KF672509**	**KF672470**	**–**
C. glandulosa A	CE2634	**SWE. Öland**, Borgholm, S Greda		**KF672367**	**KF672431**	**KF672510**	**KF672471**	**133600**
C. glandulosa A	CE2931	**Swe. Öland**, Borgholm, Egby		**KF672368**	**KF672432**	**KF672511**	**KF672472**	**133601**
C. glandulosa A	CE4027	**SWE. Skåne**, Ystad, Nyvångsskogen		**KF672369**	**KF672433**	**KF672512**	**KF672473**	**133602**
C. glandulosa A	CE4028	**SWE. Skåne**, Ystad, Nyvångsskogen		**KF672370**	–	**–**	**–**	**133603**
C. glandulosa A	CE6626	**SWE. Uppland**, Vallentuna, Brottby		**KF672371**	**KF672434**	**KF672513**	**KF672474**	**133604**
C. glandulosa A	CE9376	**SWE. Medelpad**, Timrå, Söråker	ENSWD183-11	**KF672424**	**–**	**–**	**–**	**133605**
C. glandulosa A	CE9517	**SWE. Lappland**, Kiruna, Björkliden	ENSWD240-11	**JN260194**	**–**	**–**	**–**	**133606**
C. glandulosa A	CE9524	**SWE. Lappland**, Kiruna, Björkliden	ENSWD242-11	**KF672425**	**–**	**–**	**–**	**133607**
C. glandulosa A	CE9525	**SWE. Lappland**, Kiruna, Björkliden	ENSWD243-11	**JN260195**	**–**	**–**	**–**	**133608**
C. glandulosa A	CE9526	**SWE. Lappland**, Kiruna, Björkliden	ENSWD375-11	**JN260282**	**–**	**–**	**–**	**133609**
C. glandulosa A	CE9536	**SWE. Lappland**, Kiruna, Kiruna	ENSWD247-11	**JN260198**	**–**	**–**	**–**	**133610**
C. glandulosa A	CE9581	**SWE. Lappland**, Vilhelmina, Klimpfjäll	ENSWD266-11	**JN260206**	**–**	**–**	**–**	**133611**
C. glandulosa B	CE10655	**FIN.** Jyväskylä, Lake Alvajärvi	ENSWD355-11	**JN260270**	**–**	**–**	**–**	**133612**
C. glandulosa B	CE2011	**SWE. Västergötland**, Vårgårda, Fly		**KF672372**	**KF672435**	**KF672514**	**KF672475**	**133613**
C. glandulosa B	CE2012	**SWE. Västergötland**, Vårgårda, Fly		**KF672373**	**KF672436**	**KF672515**	**KF672476**	**–**
C. glandulosa B	CE2841	**SWE. Öland**, Borgholm, Åketorp		**KF672374**	**KF672437**	**KF672516**	**–**	**133614**
C. glandulosa B	CE2887	**SWE. Södermanland**, Vingåker, Lake Låttern		**KF672375**	**KF672438**	**–**	**KF672477**	**133615**
C. glandulosa B	CE2888	**SWE. Södermanland**, Vingåker, Lake Låttern		**KF672376**	**KF672439**	**–**	**–**	**133616**
C. glandulosa B	CE2889	**SWE. Södermanland**, Vingåker, Lake Låttern		**KF672377**	**–**	**–**	**–**	**133617**
C. glandulosa B	CE2890	**SWE. Södermanland**, Vingåker, Lake Låttern		**KF672378**	**KF672440**	**KF672517**	**KF672478**	**133618**
C. glandulosa B	CE2891	**SWE. Södermanland**, Vingåker, Lake Låttern		**KF672379**	**–**	**–**	**–**	**133619**
C. glandulosa B	CE8510	**SWE. Lappland**, Kiruna, Abisko	ENSWD160-11	**JN260143**	**–**	**–**	**–**	**133620**
C. lapponica	CE13849	**SWE. Lappland**, Gällivare, 20 km WSW Gällivare		**KF672380**	**KF672441**	**KF672518**	**KF672479**	**133621**
C. lapponica	CE9552	**SWE. Lappland**, Gällivare, 20 km WSW Gällivare	ENSWD254-11	**KF672426**	**–**	**–**	**–**	**133622**
C. sphagnetorum A	CE11317	**SWE. Närke**, Hallsberg, Östansjö		**KF672381**	**KF672442**	**KF672519**	**KF672480**	**133623**
C. sphagnetorum A	CE2337	**SWE. Skåne**, Sjöbo, Vallarum		**KF672382**	**–**	**–**	**–**	**133624**
C. sphagnetorum A	CE2339	**SWE. Skåne**, Sjöbo, Vallarum	ENSWD001-11	**JN260041**	**–**	**–**	**–**	**133625**
C. sphagnetorum A	CE3890	**SWE. Västergötland**, Lerum, Aspenäs		**KF672383**	**–**	**–**	**–**	**133626**
C. sphagnetorum A	CE3891	**SWE. Västergötland**, Lerum, Aspenäs		**KF672384**	**KF672443**	**–**	**–**	**133627**
C. sphagnetorum A	CE3969	**SWE. Halland**, Kungsbacka, Särö Västerskog		**KF672385**	**–**	**KF672520**	**–**	**133628**
C. sphagnetorum A	CE3970	**SWE. Halland**, Kungsbacka, Särö Västerskog		**KF672386**	**–**	**–**	**–**	**133629**
C. sphagnetorum A	CE3971	**SWE. Halland**, Kungsbacka, Särö Västerskog		**KF672387**	**–**	**–**	**–**	**133630**
C. sphagnetorum A	CE3980	**SWE. Halland**, Kungsbacka, Särö Västerskog		**KF672388**	**–**	**KF672521**	**–**	**133631**
C. sphagnetorum A	CE3981	**SWE. Halland**, Kungsbacka, Särö Västerskog		**KF672389**	**KF672444**	**KF672522**	**KF672481**	**133632**
C. sphagnetorum A	CE4056	**SWE. Blekinge**, Olofström, Halen Nature reserve		**KF672390**	**KF672445**	**KF672523**	**KF672482**	**133633**
C. sphagnetorum A	CE4061	**SWE. Småland**, Gislaved, Bosebo		**KF672391**	**–**	**–**	**–**	**133634**
C. sphagnetorum A	CE4062	**SWE. Småland**, Gislaved, Bosebo		**KF6723912**	**KF672446**	**KF672524**	**KF672483**	**133635**
C. sphagnetorum A	CE4063	**SWE. Småland**, Gislaved, Bosebo		**KF672393**	**–**	**–**	**–**	**133636**
C. sphagnetorum A	CE6669	**SWE. Västergötland**, Vårgårda, Fly		**KF672394**	**KF672447**	**–**	**KF672483**	**133637**
C. sphagnetorum A	CE6670	**SWE. Västergötland**, Vårgårda, Fly		**KF672395**	**–**	**–**	**–**	**133638**
C. sphagnetorum A	CE6672	**SWE. Västergötland**, Vårgårda, Fly		**KF672396**	**KF672448**	**KF672525**	**KF672485**	**133639**
C. sphagnetorum A	CE786	**SWE. Västergötland**, Vårgårda, Fly		**KF672397**	**KF672449**	**KF672526**	**KF672486**	**–**
C. sphagnetorum A	CE832	**SWE. Västergötland**, Lerum, Aspenäs		GU902045	GU901782	**–**	**–**	**–**
C. sphagnetorum A	CE9482	**SWE. Norrbotten**, Överkalix, Grelsbyn		**KF672398**	**–**	**–**	**–**	**133640**
C. sphagnetorum A	CE9483	**SWE. Norrbotten**, Överkalix, Grelsbyn	ENSWD373-11	**JN260280**	**–**	**–**	**–**	**133641**
C. sphagnetorum A	CE9487	**SWE. Norrbotten**, Överkalix, Grelsbyn	ENSWD225-11	**JN260186**	**–**	**–**	**–**	**133642**
C. sphagnetorum A	CE9492	**SWE. Norrbotten**, Överkalix, Grelsbyn	ENSWD277-11	**JN260214**	**–**	**–**	**–**	**133643**
C. sphagnetorum A	CE9605	**SWE. Jämtland**, Strömsund, Lake Leipikvattnet	ENSWD276-11	**KF672427**	**–**	**–**	**–**	**133644**
*C. sphagnetorum B**	CE11325	**SWE. Närke**, Hallsberg, Östansjö		**KF672399**	**KF672450**	**KF672527**	**KF672487**	**133645**
C. sphagnetorum B	CE1719	**SWE. Västergötland**, Göteborg, Torslanda		**KF672400**	**KF672451**	**KF672528**	**KF672488**	**133646**
C. sphagnetorum B	CE1720	**SWE. Västergötland**, Göteborg, Torslanda		**KF672401**	**–**	**–**	**–**	**133647**

Table 1. (*Continued*)

Species	ID No.	Collection locality	BOLD Acc. No:	GenBank Accession No.				Voucher No.
				COI	16S	ITS	H3	
C. sphagnetorum B	CE2055	**SWE. Västergötland**, Göteborg, Torslanda		**KF672402**	**KF672452**	**KF672529**	**KF672489**	**133647**
C. sphagnetorum B	CE3860	**SWE. Västergötland**, Lerum, Aspenäs		**KF672403**	**–**	**–**	**–**	**133648**
C. sphagnetorum B	CE4026	**SWE. Skåne**, Ystad, Nyvångsskogen		**KF672404**	**–**	**–**	**–**	**133659**
C. sphagnetorum B	CE4034	**SWE. Skåne**, Ystad, Nyvångsskogen		**KF672405**	**–**	**–**	**–**	**133651**
C. sphagnetorum B	CE4035	**SWE. Skåne**, Ystad, Nyvångsskogen		**KF672406**	**KF672453**	**KF672530**	**KF672490**	**133652**
C. sphagnetorum B	CE4036	**SWE. Skåne**, Ystad, Nyvångsskogen		**KF672407**	**–**	**–**	**–**	**133653**
C. sphagnetorum B	CE4759	**SWE. Bohuslän**, Uddevalla, Bokenäs		**KF672408**	**KF672454**	**KF672531**	**KF672491**	**–**
C. sphagnetorum B	CE4760	**SWE. Bohuslän**, Uddevalla, Bokenäs		**KF672409**	**–**	**–**	**–**	**133654**
C. sphagnetorum B	CE6153	**SWE. Bohuslän**, Lysekil, Ingalsröd	ENSWD054-11	**JN260067**	**–**	**–**	**–**	**133655**
C. sphagnetorum B	CE6154	**SWE. Bohuslän**, Lysekil, Ingalsröd	ENSWD055-11	**JN260068**	**–**	**–**	**–**	**133656**
C. sphagnetorum B	CE722	**SWE. Västergötland**, Lerum, Hillefors		**–**	**KF672455**	**–**	**KF672492**	**–**
C. sphagnetorum B	CE7712	**SWE. Västergötland**, Göteborg, S. Guldheden	ENSWD129-11	**JN260116**	**–**	**–**	**–**	**133657**
C. sphagnetorum B	CE7713	**SWE. Västergötland**, Göteborg, S. Guldheden	ENSWD130-11	**JN260117**	**–**	**–**	**–**	**133658**
C. sphagnetorum B	CE7714	**SWE. Västergötland**, Göteborg, S. Guldheden	ENSWD365-11	**JN260273**	**–**	**–**	**–**	**133659**
C. sphagnetorum B	CE823	**SWE. Västergötland**, Götene, Hällekis		**KF672410**	**KF672456**	**KF672532**	**KF6724993**	**–**
C. sphagnetorum B	CE8823	**SLO. Š**tiavnik, Ráztola	ENSWD169-11	**JN260151**	**–**	**–**	**–**	**133660**
C. sphagnetorum B	CE9381	**SWE. Medelpad**, Timrå, Söråker	ENSWD368-11	**JN260276**	**–**	**–**	**–**	**133661**
C. sphagnetorum B	CE9386	**SWE. Medelpad**, Timrå, Söråker	ENSWD185-11	**JN260163**	**–**	**–**	**–**	**133662**
C. sphagnetorum B	CE9411	**SWE. Ångermanland**, Nordmaling, Långed	ENSWD199-11	**JN260170**	**–**	**–**	**–**	**133663**
C. sphagnetorum B	CE9591	**SWE. Lappland** Vilhelmina, Klimpfjäll	ENSWD268-11	**JN260208**	**–**	**–**	**–**	**133664**
C. sphagnetorum B	CE9641	**SWE. Gotland**, Gotland, Roma	ENSWD295-11	**JN260227**	**–**	**–**	**–**	**133665**
C. sphagnetorum B	CE9647	**SWE. Gotland**, Gotland, Etelhem	ENSWD298-11	**JN260230**	**–**	**–**	**–**	**133666**
C. sphagnetorum C	CE1041	**SWE. Halland**, Laholm, Hasslöv		**KF672411**	**KF672457**	**KF672533**	**KF672494**	**133667**
C. sphagnetorum C	CE1200	**SWE. Västergötland**, Göteborg, Rya skog		**–**	**KF672457**	**–**	**–**	**–**
C. sphagnetorum C	CE2334	**SWE. Skåne**, Sjöbo, Vallarum		**KF672412**	**KF672459**	**KF672534**	**KF672495**	**133668**
C. sphagnetorum C	CE6492	**SWE. Uppland**, Österåker, Stora Säby	ENSWD076-11	**JN260078**	**–**	**–**	**–**	**133669**
C. sphagnetorum C	CE6627	**SWE. Uppland**, Vallentuna, Brottby		**KF672413**	**KF672460**	**KF672535**	**KF672496**	**133670**
C. sphagnetorum C	CE6635	**SWE. Södermanland**, Nyköping, Nävekvarn		**KF672414**	**KF672461**	**KF672536**	**KF672497**	**133671**
C. sphagnetorum C	CE6636	**SWE. Södermanland**, Nyköping, Nävekvarn		**KF672415**	**–**	**–**	**–**	**133672**
C. sphagnetorum C	CE6678	**SWE. Västergötland**, Vårgårda, Fly		**KF672416**	**KF672462**	**KF672537**	**KF672498**	**133673**
C. sphagnetorum C	CE6679	**SWE. Västergötland**, Vårgårda, Fly	ENSWD097-11	**JN260092**	**–**	**–**	**–**	**133674**
C. sphagnetorum C	CE6680	**SWE. Västergötland**, Vårgårda, Fly	ENSWD098-11	**JN260093**	**–**	**–**	**–**	**133675**
C. sphagnetorum C	CE9408	**SWE. Ångermanland**, Kramfors, Bönhamn	ENSWD198-11	**KF672428**	**–**	**–**	**–**	**133676**
C. sphagnetorum C	CE9412	**SWE. Ångermanland**, Nordmaling, Långed	ENSWD200-11	**KF672429**	**–**	**–**	**–**	**133677**
C. sphagnetorum C	CE9428	**SWE. Västerbotten**, Robertsfors, Bygdeå	ENSWD205-11	**JN260174**	**–**	**–**	**–**	**133678**
C. sphagnetorum C	CE9429	**SWE. Västerbotten**, Robertsfors, Bygdeå	ENSWD369-11	**JN260277**	**–**	**–**	**–**	**133679**
C. sphagnetorum C	CE9433	**SWE. Västerbotten**, Robertsfors, Bygdeå	ENSWD206-11	**JN260175**	**–**	**–**	**–**	**133680**
C. sphagnetorum C	CE9459	**SWE. Norrbotten**, Överkalix, S Sandsjärv	ENSWD216-11	**JN260181**	**–**	**–**	**–**	**133681**
C. sphagnetorum C	CE9460	**SWE. Norrbotten**, Överkalix, S Sandsjärv	ENSWD217-11	**JN260182**	**–**	**–**	**–**	**133682**
C. sphagnetorum C	CE9490	**SWE. Norrbotten**, Överkalix, Grelsbyn	ENSWD226-11	**JN260187**	**–**	**–**	**–**	**133683**
C. sphagnetorum C	CE9555	**SWE. Lappland**, Gällivare, 20 km WSW Gällivare	ENSWD255-11	**JN260200**	**–**	**–**	**–**	**133684**
C. sphagnetorum C	CE9595	**SWE. Lappland**, Vilhelmina, Röberg	ENSWD271-11	**JN260210**	**–**	**–**	**–**	**133685**
C. sphagnetorum C	CE9599	**SWE. Lappland**, Vilhelmina, Röberg	ENSWD272-11	**JN260211**	**–**	**–**	**–**	**133686**
C. sphagnetorum D	CE3973	**SWE. Halland**, Kungsbacka, Särö Västerskog		**KF672417**	**KF672463**	**KF672538**	**KF672499**	**133687**
C. sphagnetorum D	CE3974	**SWE. Halland**, Kungsbacka, Särö Västerskog		**KF672418**	**KF672464**	**KF672539**	**KF672500**	**133688**
C. sphagnetorum D	CE4023	**SWE. Skåne**, Vellinge, Skanörs Ljung		**KF672419**	**KF672465**	**KF6725340**	**KF672501**	**133689**
C. sphagnetorum D	CE4024	**SWE. Skåne**, Vellinge, Skanörs Ljung		**KF672420**	**–**	**–**	**–**	**133690**
C. sphagnetorum D	CE4025	**SWE. Skåne**, Vellinge, Skanörs Ljung		**KF672421**	**KF672466**	**KF6725341**	**KF672502**	**133691**
C. sphagnetorum D	CE4055	**SWE. Blekinge**, Olofström, Halen NR		**KF672422**	**KF672467**	**KF6725342**	**KF672503**	**133692**
Cernosvitoviella immota	CE895	**SWE. Bohuslän**, Strömstad, S Öddö		GU902042	GU901779	**KF6725343**	**KF672504**	**–**
Cer. aggtelekiensis	CE839	**SWE. Västergötland**, Lerum, Kastenhof		GU902040	GU901777	**KF6725344**	**KF672505**	**–**
Mesenchytraeus flavus	CE847	**SWE. Västergötland**, Lerum, Aspenäs		GU902100	GU901843	**KF6725345**	**–**	**–**
Mes. armatus	CE2056	**SWE. Västergötland**, Göteborg, Torslanda		**KF672423**	**KF672468**	**KF6725346**	**KF672506**	**133693**
Stercutus niveus	CE841	**SWE. Västergötland**, Lerum, Kastenhof		GU902112	GU901852	**KF6725347**	**KF672507**	**–**

* This lineage, *Cognettia sphagnetorum* B morphologically resembles the form described by Chalupský (1992) as *Cognettia sp.*

using default settings; the initial partition was used for the preliminary delimitation of the clusters.

Estimation of gene-trees

Single gene-trees of the four sequenced markers were estimated with Bayesian Inference using the parallel version of MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Coding regions (COI and H3) were partitioned according to codon positions. Substitution models used in the analyses were selected with the Akaike Information Criterion (AIC) (Akaike, 1974) as implemented in MrMODELTEST v.2.2 (Nylander, 2004). The best fitting models for the first, second and third positions of COI were SYM + I + Γ, HKY + I and HKY + I + Γ respectively, and of H3 they were GTR + I, JC and GTR respectively. For 16S and ITS GTR + Γ was the best fitting model.

MrBayes was conducted with two independent runs with 4 MC^3 in each for 10 million generations and with sampling every 1000th generation. In analyses with more than one partition, the priors for branch length were adjusted to1/10th of the default value, to reduce the problem with unrealistic long branch-lengths that can occur in Bayesian analyses (see Marshall, 2010). To diagnose convergence and examine performance AWTY (Are We There Yet) (Nylander et al., 2008) and Tracer v1.5 (Rambaut and Drummond, 2007) were used. An initial 'burn-in phase' of 25% was discarded, and the resulting trees were rooted with the *Cernosvitoviella* and *Mesenchytraeus* spp., in accordance with the topology recovered by Erséus et al. (2010).

Estimation of species trees

In order to see the branching patterns of the delimited MOTUs, a species-tree was estimated using the multi-species coalescent as implemented in *BEAST (Heled and Drummond, 2010). The analysis included *Cognettia* specimens that had all four genes sequenced (32 specimens), and the MOTUs found in the ABGD analysis and in the gene-trees (see results) were used as species. An xml input file was created using BEAUti v1.7.5 (Drummond et al., 2011), substitution models, clock models and tree models were unlinked across genes, uncorrelated lognormal relaxed clocks were used for all genes and the mean rate of the molecular clock was fixed to 1 for COI and was estimated for the other markers. The substitution models used were GTR + I + Γ for COI and H3; and GTR + Γ for 16S and ITS. The Yule process speciation prior, the piecewise linear and constant root population size prior were used. For species population mean and birth rate priors a lognormal distribution with log (mean) = 0 and log (Stdev) = 1 was used, and for the relax clock, mean uniform priors were used, for 16S and ITS the priors were between 0 and 2 and for H3 between 0 and 1. For all other priors the default settings were used. The analysis was run in BEAST v1.7.5. (Drummond and Rambaut, 2007; Drummond et al., 2012) for 100 million generations, sampling every 1000th generation. Tracer v1.5 (Rambaut and Drummond, 2007) was used for examining effective sample size (ESS) for parameters and determining burn-in. Trees were summarized using TreeAnnotator v1.7.5, discarding the first 10% as burn-in.

Trees from all analyses were drawn with Fig Tree v1.3.1 (Rambaut, 2009) and further edited in Adobe Illustrator CS5.

Data deposition

All new sequences generated in this study were deposited in GenBank (submission numbers in Table 1); for material handled by CCDB sequence data were also stored in BOLD (accession numbers in Table 1); trees and data matrices were deposited in TreeBASE (http://treebase.org/), submission #14726. The xml file used in the *BEAST analysis, and log file from the analysis, as well as input and output files from the MrBayes runs were deposited in the Dryad Data Repository (http://www.datadryad.org/) at DOI:10.5061/dryad.6mh29. Specimen vouchers were deposited in the Swedish Museum of Natural History, Stockholm (voucher numbers in Table 1).

Results

DNA amplification and sequencing

The mitochondrial gene COI was successfully sequenced for 100 specimens. For the subset of 43 specimens that was selected for sequencing of the other three markers, 16S was successfully sequenced for 40 specimens, H3 for 35 specimens and ITS for 35 specimens. In 32 specimens all four markers were successfully sequenced. After trimming the length of the in-group sequences varied for COI between 534 and 554 bp, for 16S between 453 and 476 bp, for H3 between 326 and 328 bp and for ITS between 546 and 1028 bp. The alignments of COI, H3 and 16S were unproblematic, for ITS, especially with out-groups added, the alignment was more problematic. However, except for editing a few obviously misaligned nucleotides the alignments were used without further adjustment.

Barcoding gap analysis and preliminary delimitation of MOTUs

In the ABGD analyses of COI, 16S and ITS the datasets were divided into eight MOTUs using the initial ABGD partition, regardless of whether uncorrected or corrected distances were used. One MOTU represents *C. cognettii*, one *C. lapponica*, two *C. glandulosa* (clusters A and B) and four are *C. sphagnetorum* (clusters A–D), clusters are specified in Table 1. All clusters of *C. glandulosa* and *C. sphagnetorum* except *C. sphagnetorum D* are evidently widespread in Sweden, *C. glandulosa B* was also collected in Finland and *C. sphagnetorum B* in Slovakia, whereas all specimens of *C. sphagnetorum D* are from southern Sweden only (see Table 1). The analyses of the H3 dataset yielded four clusters, where *C. cognettii*, *C. sphagnetorum A* and the two MOTU:s of *C. glandulosa* were fused into one cluster, *C. lapponica* and *C. sphagnetorum D* into another cluster while *C. sphagnetorum B* and *C. sphagnetorum C* were separate. Clear global barcoding gaps were found in COI and 16S (Fig. 1A and B); in the COI analyses, however, intermediate distances were caused by specimen CE4055, which was rather distant to the other specimens of its cluster (*C. sphagnetorum D*). In the ITS and H3 analyses, no clear global barcoding gap was found, but a tendency for one was seen in the analyses of ITS (Fig. 1C and D).

Gene trees

In all gene-trees (Figs. 2 and 3) *Cognettia* was found to be monophyletic with maximum support, except in the 16S tree (Fig. 3A) where monophyly was only weakly supported (posterior probability, pp 0.76). All eight clusters were monophyletic with maximum support in COI (Fig. 2), 16S (Fig. 3A), and ITS (Fig. 3B) analyses. In the H3 analysis (Fig. 3C) the specimens of *C. glandulosa A* were in a basal polytomy with all other *Cognettia*, and the *C. glandulosa B* specimens were in a polytomy with *C. cognettii*. The gene-trees were not congruent; notably, the relationships of the clusters within *Cognettia* varied between the trees, but *C. sphagnetorum* s.l. was not monophyletic in any of them. In the 16S, ITS and H3 gene trees a clade consisting of *C. lapponica*, *C. sphagnetorum A* and *D* was found and in the 16S and H3 trees *C. lapponica* and *C. sphagnetorum D* were recovered as sister-groups with good support (pp 0.96 and 1, respectively), whereas in the ITS tree *C. lapponica* and *C. sphagnetorum A* were sister-groups, although with low support (pp 0.71).

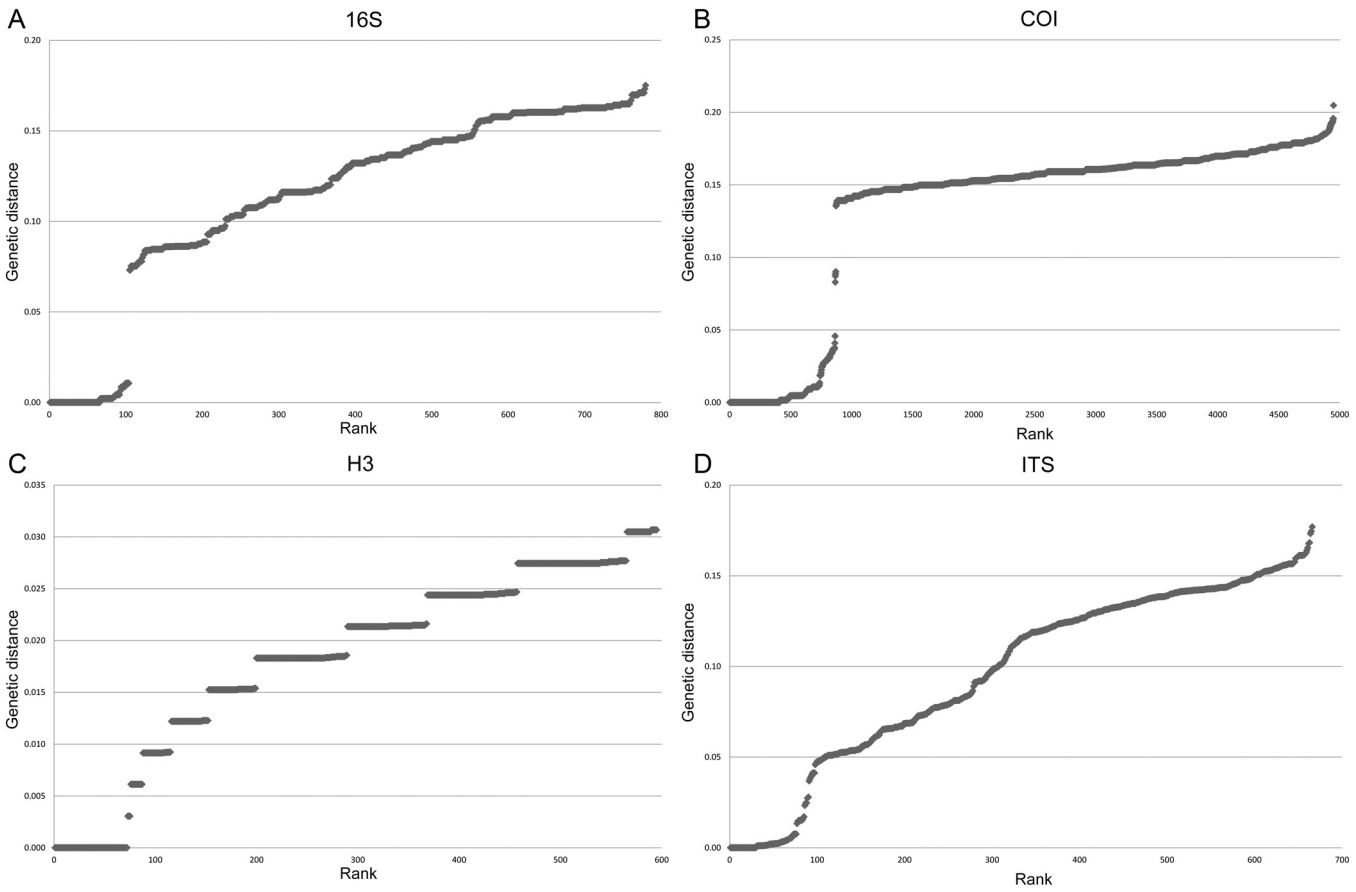

Fig. 1. Ranked pairwise genetic distances, given as uncorrected p-distances, of *Cognettia* specimens. (A) 16S. (B) COI. (C) H3. (D) ITS.

In the 16S and ITS trees *C. sphagnetorum B* and *C* and *C. glandulosa A* and *B* formed a clade with maximum support. In the ITS tree *C. cognettii* was found as the sister-group to the clade consisting of *C. lapponica*, *C. sphagnetorum A* and *D*, and in the 16S tree to the clade consisting of *C. glandulosa A* and *B* as well as *C. sphagnetorum B* and *C*.

Species tree estimation

The effective sample size (ESS) was high for most parameters, and the tree (Fig. 4) had generally good support. *C. sphagnetorum s.l.* was found not to be monophyletic. *C. sphagnetorum D* was the sister-group of *C. lapponica* (pp 1) and *C sphagnetorum A* was a sister-group to the two latter species (pp. 0.99). *C. glandulosa A* and *B* formed a monophyletic clade (pp 1), *C. sphagnetorum C* was recovered as their sister-group but without support (pp 0.45), and *C. sphagnetorum B* was sister-group to this clade (pp 1). *C. cognettii* was recovered with low support (pp 0.69) as the sister-group to the clade with *C. glandusosa A* and *B*, and *C. sphagnetorum B* and *C*.

Discussion

The phylogenetic analyses of individual markers recovered the same eight clades as those found in the ABGD analyses of COI, 16S and ITS, with the exception of the H3 analyses where the two clusters of *C. glandulosa* were not supported as monophyletic; this difference is most likely due to the much lower mutation rate in H3 compared to the other genes. The low mutation rate of H3 is also the likely cause for the difference between H3 and the other markers in the ABGD analyses. The reciprocal monophyly suggests that the clades have long separate evolutionary histories, and that the individuals of each clade have acquired molecular synapomorphies.

The MOTUs found in *Cognettia* were reciprocally monophyletic and phenetically distinguishable in three of the four markers used, and can therefore be delimited as separate species using the unified species concept (de Quiroz, 2007). Moreover, the karyotype variation (Christensen, 1961) and different responses to climatic conditions (Briones et al. 1997; Haimi et al. 2005) points toward the existence of several cryptic species at least within the morphotaxon *C. sphagnetorum*.

C. glandulosa and *C. sphagnetorum* reproduce mainly asexually, but as sexually maturity in *C. sphagnetorum* seems to be induced by stress (Lundkvist, 1983), it is possible that they occasionally shift to sexual reproduction. The change between asexual and sexual reproduction has been suggested as part of the explanation for the radiation within groups of Naididae (see *Introduction* section). In sexual reproduction advantageous combinations of alleles may be formed, asexual reproduction may then rapidly increase the proportion of such combinations in the population (Erséus, 1984). It could be hypothesized that divergent selection leading to adaptation to different environmental factors could be an explanation for the species radiation also seen in *Cognettia*.

The phylogenetic analyses also support that *C. sphagnetorum*, as currently delimited, is a non-monophyletic complex of cryptic species, and that cryptic species are found also within *C. glandulosa*. If we instead chose to treat *C. sphagnetorum s.l.* as a single variable species, we would have to lump all North European species of *Cognettia* into it to keep it monophyletic. Non-monophyly of species in single gene-trees is not uncommon (Crisp and Chandler 1996; Funk and Omland 2003), and reciprocal monophyly is not a necessity for species delimitation (see e.g. Doyle 1995; Helbig et al. 2002) except under a strict monophyletic species concept.

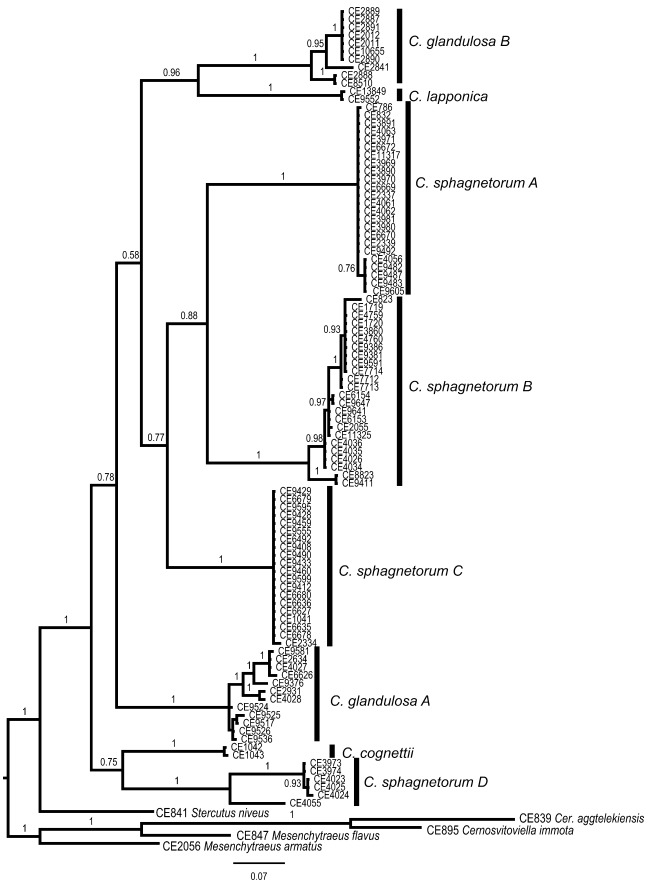

Fig. 2. COI gene tree of *Cognettia* estimated by Bayesian inference. Posterior probabilities above branch. Scale shows expected number of changes per site.

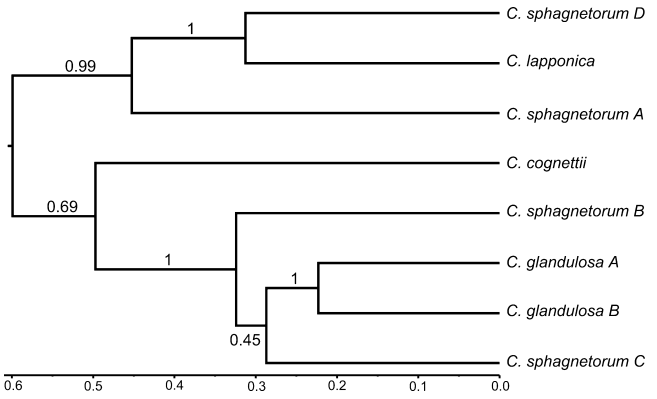

Fig. 4. Species tree of North European species of *Cognettia* estimated using multispecies coalescence in *BEAST, using the delimited MOTU:s as species. Posterior probabilities are shown at branches. Scale shows expected number of changes per site in COI.

The phylogenetic relationships of the species varied between the gene trees. Possible explanations for these discrepancies are incomplete lineage sorting, hybridization and differences in mutation rate, or combinations of these factors. For instance, hybridization events resulting in allopolyploidy are supported by the high chromosome numbers in some lineages of *Cognettia*. In *C. sphagnetorum* s.l. there is one form with $n = 54$ and another, probably hexaploid form with $n \approx 160$ (Christensen, 1961). More problematic is that the gene-trees from the two mitochondrial markers (16S and COI) differ considerably despite the fact that they are supposed to share the same evolutionary history. Unless there are cases of rare mitochondrial recombination or that one of the genes is not mitochondrial, but instead has been incorporated in the nuclear genome, these discrepancies can only be explained by phylogenetic errors, such as substitution saturation that the model did not correct for.

Evidently both *C. sphagnetorum* and *C. glandulosa* need to be taxonomically revised, but this must first involve a thorough morphological assessment of the lineages, including the recognition of those that are the most appropriate to bear these and other available names. These tasks, however, were beyond the aims of this paper.

Our sample of specimens showed that the majority of our species occur throughout Sweden, from the south to the far north, and that at least different lineages of the *C. sphagnetorum* complex often occur together at the same site (Table 1). Physiological and ecological differences between cryptic lineages have been demonstrated for other clitellate taxa (e.g. Sturmbauer et al., 1999; Beauchamp et al., 2002; Feckler et al., 2013; Kille et al., 2013). Such differences between the species within *C. sphagnetorum* is a possible explanation for why different ecological studies on this taxon have reached different conclusions (see Introduction). Thus,

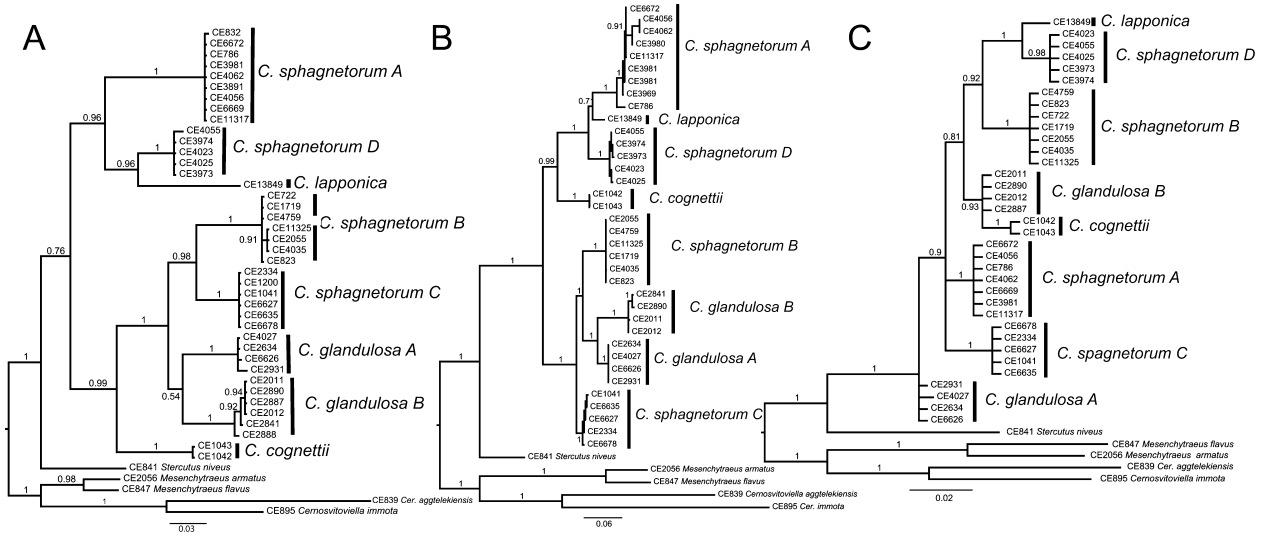

Fig. 3. Gene trees of *Cognettia* estimated by Bayesian inference. (A) 16S gene tree. (B) ITS gene tree. (C) H3 gene tree. Posterior probabilities are shown above branch. Scale shows expected number of changes per site.

investigations on the ecological and physiological responses of the different lineages to environmental variables are needed to show if this is the case in *C. sphagnetorum* as well.

We strongly recommend that material of *Cognettia* used in future studies is identified by a molecular barcode that should be publicly archived (see Whitlock, 2011).

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