Investigating the Clitellata (Annelida) of Icelandic springs with alternative barcodes

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DNA barcoding is an invaluable tool to identify clitellates, regardless of life stage or cryptic morphology. However, as COI (the standard barcode for animals) is relatively long (658 bp), sequencing it requires DNA of high quality. When DNA is fragmented due to degradation, alternative barcodes of shorter length present an option to obtain genetic material. We attempted to sequence 187 clitellates sampled from springs in Iceland. However, the material had been stored at room temperature for two years, and DNA of the worms had degraded, and only three COI sequences were produced (i.e., <2% success rate). Using two alternative barcodes of 16S (one ca. 320 bp, the other ca. 70 bp long) we increased the number of sequenced specimens to 51. Comparisons of the 16S sequences showed that even the short 70 bp fragment contained enough genetic variation to separate all clitellate species in the material. Combined with morphological examinations we recognized a total of 23 species, where at least 8 are new records for Iceland, some belonging to genera new for Iceland: Cernosvitoviella and Pristina. All the new taxa are included in an updated species list of Icelandic Clitellata. The material revealed some stygophilic species previously known to inhabit springs, but true stygobionts, which are restricted to groundwater habitats, were not found. Our study shows that short 16S fragments can be obtained from DNA too degraded to be used in traditional COI barcoding, and contain enough genetic variation to separate closely related clitellate species.

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INTRODUCTION

The clitellate fauna ("oligochaetes" and leeches) of Iceland was early on studied by Černosvitov (1929, 1931, 1936) and Nielsen & Christensen (1959), but also more specifically reviewed by Bruun (1938a, 1938b) for Hirudinea, Backlund (1949) for Lumbricidae, Hrabé (1952) for Lumbriculidae and Naididae (including the former Tubificidae), Christensen (1962) and Nurminen (1973) for Enchytraeidae, and Erséus (1976) for marine Enchytraeidae and Naididae. Since then, a few additional species have been reported in scattered publications on either pure taxonomy or more general biological issues, such as parasitology (for marine leeches) or ecology; for references, see updated species list for Iceland below. The previous studies report clitellates mainly from soils, lakes, rivers and seashores, but not from freshwater springs, which are the focus of this study.

Springs represent ecotones between groundwater and surface water and give rise to specialized invertebrate communities. On the European mainland, groundwater
clitellates are rather well known, with many species endemic to various regions (e.g., Sambugar et al. 1999; Giani et al. 2001, 2011; Achurra & Rodriguez 2008; Bojková et al. 2011; Martin et al. 2015). Groundwater and spring invertebrate communities in Iceland were recently investigated by Govoni et al. (2018) and Kreiling et al. (2018), but these studies focused on insects and crustaceans and the clitellate diversity in Icelandic springs has until now been largely unknown.

In the present study, we examined clitellates collected as a part of a survey on invertebrate fauna in freshwater springs around Iceland. With the intent to save time, we decided to identify the material primarily using molecular data rather than by traditional morphological examination; and to our knowledge, there have not been any published studies of clitellates from Iceland containing genetic sequences, to this date. DNA barcoding (e.g., Hebert et al. 2003, 2004) would allow us to identify juvenile specimens and possible cryptic species. We would then corroborate the identity of the successfully sequenced specimens also by morphological observations. Although this procedure did not work exactly as first intended, the aim to present all the identified species from the springs will still be achieved in this paper. We will also provide an updated list of all species of Clitellata known from Iceland.

**Figure 1.** Map of the 19 freshwater springs in Iceland from which clitellata have been collected and successfully sequenced in this study.

**MATERIAL AND METHODS**

Worms were collected from 31 springs during the summer of 2015 as a part of a broader study on spring invertebrates in Iceland (Kreiling et al. in prep.). A Surber sampler (0.093 m$^2$) with 63 µm mesh size was used for collection of clitellates in the benthic substrate of the spring, and electrobugging (Lento & Morin 2014, Kreiling et al. 2018) was used for collection of invertebrates in the spring source. The clitellates were stored in 96 % ethanol at room temperature (~20°C) for about two years before further processing. As described below, identification to species level was unsuccessful for a part of the collection, and in the end, the results of the study were based on material from only 19 of the freshwater springs (Figure 1; Table 1).

The clitellate specimens were first examined under a stereomicroscope and the amputated posterior ends of 187 specimens were used for DNA extraction using the QuickExtract DNA Extraction Solution 1.0 (Epicentre, Madison, WI, USA), following the manufacturer’s instructions.

The original idea was to barcode all selected specimens using the standard animal barcode COI (cytochrome c oxidase subunit I). However, the DNA proved to have deteriorated considerably (probably due to prolonged storage at room temperature, possibly in too low alcohol concentration), as we were unable to obtain COI sequences for a vast majority of the
Table 1. List of the sampling locations with habitat description and some abiotic measurements. Spring names refer either to the name of the water body (stream or lake), or to the surrounding area, or the closest farm. Limnocrene (L) springs form pools of standing water, whereas rheocrene (R) springs originate streams. Elev. = Elevation, O.S. = Oxygen Saturation.

| Spring (type)     | Habitat                                                                 | Latitude Longitude (WGS84) | Elev. [m] | Sampling date | Water temp. [°C] | pH   | O.S. [%] |
|-------------------|--------------------------------------------------------------------------|----------------------------|-----------|---------------|------------------|------|----------|
| Botnar I (R) SE Iceland | Gushing, shallow spring at the edge of lava field; fine sand               | 63°38.707' N 018°14.749' W | 10 July 2015 | 5.6           | 8.0             | 74.2 |
| Botnar II (L) SE Iceland | Spring emerging from lava field; low primary production; fine sand           | 63°39.275' N 018°15.142' W | 10 July 2015 | 7.4           | 7.9             | 78.2 |
| Enni (R) NW Iceland | Spring forming a small stream on a grassy hillslope; sand                   | 65°53.371 N 019°19.755' W | 19 September 2015 | 4.5          | 7.3             | 75.9 |
| Galtalækur (R) S Iceland | Spring forming a small stream in wooded area; high density of surrounding vegetation (grasses, shrubs and trees); gravel | 64°00.453' N 019°55.148' W | 8 July 2015 | 5.1           | 7.9             | 72.3 |
| Granavatn Norður (L) NE Iceland | Spring on lake shore; gravel and mud                                        | 65°32.905' N 016°58.908' W | 22 July 2015 | 6.5           | 8.9             | 60.6 |
| Granavatn Suður (L) NE Iceland | Spring on lake shore; high primary production; lava rock                      | 65°32.205' N 017°00.477' W | 22 July 2015 | 4.5           | 9.0             | 63.3 |
| Hengill IS8 (R) S Iceland | Hot spring forming a stream in geothermal area; high primary production; rock | 64°03.414' N 021°18.439' W | 13 July 2015 | 16.6          | 7.5             | 66.1 |
| Herðubreiðarlindir (L) Central Highlands | Big spring in the Central Highlands, forming a deep stream; high density of surrounding vegetation (grasses and shrubs); fine sand   | 65°11.548' N 016°13.508' W | 16 August 2015 | 5.9           | 6.8             | 65.6 |
| Hruni (L) SE Iceland | Spring in a garden pond; mud and gravel                                        | 63°51.547' N 017°44.486' W | 11 July 2015 | 3.5           | 7.9             | 75.8 |
| Kálfaströnd (L) NE Iceland | Spring on lake shore; lava rock and sand                                     | 65°33.759' N 016°56.710' W | 21 July 2015 | 5.1           | 9.2             | 54.0 |
| Krákárbotnar (R) Central Highlands | Small, isolated spring in the Central Highlands with almost no surrounding vegetation; sand | 65° 19.852 N 017°04.654' W | 26 July 2015 | 8.6           | 8.8             | 69.5 |
| Lón (L) NE Iceland | Hot spring at lake shore; high primary production; lava rock                   | 66°05.785' N 016°55.514' W | 24 July 2015 | 4.9           | 8.0             | 77.8 |
| Lækjarbotnar Hol (R) S Iceland | Spring forming a small stream on meadow; high density of surrounding vegetation (grasses); lava rock and sand | 63°57.422' N 020°15.892' W | 8 July 2015 | 5.5           | 7.9             | 75.6 |
| Miðhússaskógur (L) S Iceland | Spring at the shore of shallow pond; low primary production; fine sand and lava rock | 64°17.373' N 019°30.706' W | 8 July 2015 | 2.4           | 9.3             | 78.1 |
| Staðarhraun Bær (R) W Iceland | Spring at the edge of lava field, forming a small stream; lava rock and gravel | 64°44.610' N 020°05.647' W | 28 July 2015 | 5.1           | 5.3             | 79.0 |
| Staðarhraun Kirkja (R) W Iceland | Spring at the edge of lava field; sand and lava rock                           | 64°44.855' N 020°05.812' W | 28 July 2015 | 4.6           | 5.3             | 79.2 |
| Steinshastir (R) NW Iceland | Hot spring forming a small stream; high primary production; sand and mud       | 65°28.162' N 019°21.390' W | 4 August 2015 | 40.24         | 8.47            | 86.3 |
| Iverá (L) SE Iceland | Spring in shallow pond; sand                                                  | 63°52.396' N 017°49.199' W | 11 July 2015 | 5.1           | 7.5             | 76.1 |
PCR programs

95°C 5 min, (35 cycles of 95°C 40 s, 50°C 45 s 72°C 1 min), 72°C 8 min

95°C 5 min, (35 cycles of 95°C 30 s, 50°C 30 s 72°C 1 min), 72°C 8 min

95°C 5 min, (35 cycles of 95°C 30 s, 58°C 30 s 72°C 10 s), 72°C 5 min

RESULTS

It soon became apparent that the DNA of the samples had degraded substantially, as we obtained successful COI sequences from only three of the 187 selected worms. They were genetically identified as *Bimastos rubidus* (Savigny, 1826) *sensu lato*, *Cernosvitoviella pusilla* Nurminen, 1973, and *Chaetogaster cf. diastrophus* (Gruithuisen, 1828), respectively (Table 3).

The longer (320 bp) of the two 16S barcodes was more successful than the COI barcode, but we still only got results for 54 specimens. Moreover, after examination of the microscope slides, the morphology did not agree with the DNA results for eight of these 54 worms, most likely due to DNA contamination, leaving only 46 individuals confidently identified by both DNA and morphology (Table 3 specifies how each specimen was identified).

Table 2. Primers and PCR programs used to sequence COI and 16S.

| Target | Primers | PCR program | Reference |
|--------|---------|-------------|-----------|
| COI    | LCO1490 (forward) | 95°C 5 min, (35 cycles of 95°C 40 s, 45°C 45 s 72°C 1 min), 72°C 8 min | (Folmer et al. 1994) |
| 658 bp | GGTCAACAAATCATAAAGATATTGG HCO2198 (reverse) TAAACCTTCAAGGTGACCAAAAAATCA |
|       | Ann16SF (forward) | 95°C 5 min, (35 cycles of 95°C 30 s, 50°C 30 s 72°C 1 min), 72°C 8 min | (Sjölin et al. 2005) |
| 16S    | GCGGTATCCTGACCGTCAAGGTGACCAAAAAATCA |
| ca. 320 bp | Ann16SR (reverse) | TCTAGGTCAGGATACGATGTGACCA |
|       | ewD (forward) | 95°C 5 min, (35 cycles of 95°C 30 s, 58°C 30 s 72°C 10 s), 72°C 5 min | (Bienert et al. 2012) |
| 16S    | ATTCGGTGTGGGCGACC ewE (reverse) CTGTTATCCTAAGGTAGCTT |
Table 3. List of Icelandic specimens used in this study with specimen ID's (identification numbers), identification method (B = BOLD; G = Genbank; M = morphology; R = based on match to other non-Icelandic reference material, presented at the end of the table, and with sampling sites specified in material and methods), the spring in which they were collected, Genbank accession numbers and museum voucher ID's. We only deposited the longer 16S fragment. Sequences from the shorter 16S fragment are presented in the text of the Results. More detailed description of the springs in Table 1.

| Taxon (Identification method) | Specimen ID | Spring locality | Genbank acc. no. | Voucher ID |
|------------------------------|-------------|-----------------|------------------|------------|
|                              |             | COI 16S (320 bp) | 16S (70 bp)      |            |
| **Enchytraeidae**            |             |                 |                  |            |
| Cernosvitoviella aggtelekniensis Dózsa-Farkas, 1970 (GM) | CE30974 | Hruni | MK837025 | SMNH 176517 |
| Cernosvitoviella cf. minor Dózsa-Farkas, 1990 (GM) | CE31592 | Hruni | Sequence in Results | SMNH 176518 |
| Cernosvitoviella pusilla Nurminen, 1973 (M) | CE30979 | Botnar II | MK837026 | SMNH 176519 |
| Cernosvitoviella pusilla Nurminen, 1973 (B) | CE31607 | Staðarhraun Bær | MK837024 | SMNH 176520 |
| Cognettia varisetosa (Martinsson, Rota & Erséus, 2015) (M) | CE30958 | Hruni | Sequence in Results | SMNH 176522 |
| Enchytraeus buchholzi 1 Vejdovský, 1879 (M) | CE30973 | Hruni | MK837028 | SMNH 176523 |
| Enchytraeus buchholzi 1 Vejdovský, 1879 | CE31564 | Botnar I | Sequence in Results | SMNH 176524 |
| Enchytraeus buchholzi 2 Vejdovský, 1879 (M) | CE31504 | Langivogur | MK837029 | SMNH 176525 |
| Fridericia dura (Eisen, 1879) (R) | CE30963 | Krákárbotnar | MK837030 | SMNH 176526 |
| Henlea perpusilla Friend, 1911 (G) | CE30978 | Botnar II | MK837031 | SMNH 176527 |
| Lumbricillus arenarius (Michaelsen, 1889) (GM) | CE31573 | Herðubreiðarlindir | MK837032 | SMNH 176528 |
| Lumbricillus arenarius (Michaelsen, 1889) (GM) | CE31575 | Herðubreiðarlindir | MK837033 | SMNH 176529 |
| Lumbricillus arenarius (Michaelsen, 1889) (GM) | CE31577 | Herðubreiðarlindir | MK837034 | SMNH 176530 |
| Marionina cf. argentea (Michaelsen, 1889) (R) | CE31590 | Þverá | MK837035 | SMNH 176531 |
| Marionina sp. | CE31579 | Herðubreiðarlindir | MK837036 | SMNH 176532 |
| Marionina sp. | CE31580 | Herðubreiðarlindir | MK837037 | SMNH 176533 |
| Marionina sp. | CE31583 | Þverá | MK837038 | SMNH 176534 |
| Marionina sp. | CE31587 | Þverá | MK837039 | SMNH 176535 |
| Marionina sp. | CE31589 | Þverá | MK837040 | SMNH 176536 |
| Marionina sp. | CE31603 | Grænavatn Norður | MK837041 | SMNH 176537 |
| Mesenchytraeus cf. armatus (Levinsen, 1884) (GM) | CE30954 | Miðhúsaskógur | MK837042 | SMNH 176538 |
| Mesenchytraeus cf. armatus (Levinsen, 1884) (GM) | CE30968 | Grænavatn Suður | Sequence in Results | SMNH 176539 |
| Mesenchytraeus cf. armatus (Levinsen, 1884) (GM) | CE30972 | Hruni | MK837043 | SMNH 176540 |
| Taxon (Identification method) | Specimen ID | Spring locality | Genbank acc. no. | Voucher ID |
|-------------------------------|-------------|----------------|-----------------|------------|
| *Mesenchytraeus cf. armatus* (Levinsen, 1884) (GM) | CE30986     | Botnar II      | MK837045        | SMNH 176542 |
| Lumbricidae                   |             |                |                  |            |
| *Aporrectodea caliginosa* (Savigny, 1826) (G) | CE30987     | Staðarhraun Bær | MK837046        | SMNH 176543 |
| *Bimastos rubidus s. lat.* (Savigny, 1826) (GB) | CE30982     | Botnar II      | MK837022        | SMNH 176544 |
| *Dendrobaena octaedra* (Savigny, 1826) (G) | CE30975     | Steinsstaðir   | Sequence in Results | SMNH 176545 |
| *Dendrobaena octaedra* (Savigny, 1826) (G) | CE31506     | Galtalækur     | MK837047        | SMNH 176546 |
| *Eiseniella tetraedra* (Savigny, 1826) (G) | CE30950     | Langivogur     | MK837048        | SMNH 176547 |
| Naididae                      |             |                |                  |            |
| *Chaetogaster cf. diastrophus* (Gruithuisen, 1828) (G) | CE31491     | Staðarhraun Kirkja | MK837023 | SMNH 176548 |
| *Chaetogaster sp. = langi?* (M) | CE31604     | Grænavatn Norður | MK837050      | SMNH 176549 |
| *Nais communis/variabilis* spe- cies complex, morphotype A3 (Envall et al. 2012) (G) | CE30951     | Langivogur     | MK837051        | SMNH 176550 |
| *Nais elinguis* Müller, 1773 (GM) | CE30948     | Lón            | MK837052        | SMNH 176551 |
| *Nais elinguis* Müller, 1773 (GM) | CE30949     | Lón            | MK837053        | SMNH 176552 |
| *Nais elinguis* Müller, 1773 (GM) | CE30967     | Lækjarbotnar Hol | MK837054      | SMNH 176553 |
| *Nais elinguis* Müller, 1773 (GM) | CE30971     | Þverá           | MK837055        | SMNH 176554 |
| *Nais elinguis* Müller, 1773 (GM) | CE30980     | Botnar II      | MK837056        | SMNH 176555 |
| *Nais elinguis* Müller, 1773 (GM) | CE30981     | Botnar II      | MK837057        | SMNH 176556 |
| *Nais elinguis* Müller, 1773 (GM) | CE30983     | Botnar II      | MK837058        | SMNH 176557 |
| *Nais elinguis* Müller, 1773 (GM) | CE30984     | Botnar II      | MK837059        | SMNH 176558 |
| *Nais elinguis* Müller, 1773 (GM) | CE30985     | Botnar II      | MK837060        | SMNH 176559 |
| *Nais elinguis* Müller, 1773 (GM) | CE31480     | Kálfaströnd     | MK837061        | SMNH 176560 |
| *Nais elinguis* Müller, 1773 (GM) | CE31493     | Staðarhraun Kirkja | MK837062      | SMNH 176561 |
| *Nais elinguis* Müller, 1773 (GM) | CE31581     | Lækjarbotnar Hol | MK837063      | SMNH 176562 |
| *Nais elinguis* Müller, 1773 (GM) | CE31582     | Grænavatn Suður | MK837064        | SMNH 176563 |
| *Nais elinguis* Müller, 1773 (GM) | CE31605     | Staðarhraun Bær | MK837065        | SMNH 176564 |
| *Nais elinguis* Müller, 1773 (GM) | CE31606     | Staðarhraun Bær | MK837066        | SMNH 176565 |
| *Nais elinguis* Müller, 1773 (GM) | CE31619     | Staðarhraun Bær | MK837068        | SMNH 176567 |
### Table 3. Continued.

| Taxon (Identification method) | Specimen ID | Spring locality | Genbank acc. no. (COI) | Genbank acc. no. (16S 320 bp) | Genbank acc. no. (16S 70 bp) | Voucher ID |
|-------------------------------|-------------|-----------------|------------------------|-------------------------------|-----------------------------|------------|
| *Nais elinguis* Müller, 1773 (GM) | CE31620 | Staðarhraun Bær | MK837069 | SMNH 176568 |  |
| *Pristina foreli* (Piguet, 1907) (M) | CE30943-45 | Hengill IS8 | SMNH 176569 |  |  |
| *Tubifex cf. tubifex* (Müller, 1774) (G) | CE31560 | Botnar 1 | SMNH 176570 |  |  |
| *Uncinais uncinata* (Orsted, 1842) (M) | CE31593 | Hrungi | SMNH 176571 |  |  |

**Non-Icelandic reference material**

| Taxon | Specimen ID | Spring locality | Genbank acc. no. (COI) | Genbank acc. no. (16S 320 bp) | Genbank acc. no. (16S 70 bp) | Voucher ID |
|-------|-------------|-----------------|------------------------|-------------------------------|-----------------------------|------------|
| *Fridericia dura* (Eisen, 1879) (M) | CE19501 | Norway | MN395701 | MN394410 (478 bp) | ZMBN 110172 |  |
| *Marionina cf. argentea* (Michaelis, 1889) (M) | CE22027 | Norway | MN395702 | MN394411 (474 bp) | ZMBN 110740 |  |

The shorter 16S barcode (70 bp) only produced 17 successful sequences (Table 3), mostly from specimens already successfully barcoded with the longer 16S fragment, increasing the total number of DNA-barcoded (but non-contaminated) specimens to 51 (27 % of the original 187 specimens). The sequences of the five specimens that were successfully sequenced only for the shorter 16S fragment (i.e., five sequences not overlapping with our longer 16S uploaded on Genbank) are presented here (note that the sequence for CE31592 is incomplete):

CE31592 *Cernosvitoviella cf. minor*
TTGGGGGCACCAAGGAAATAATCATCCTTAATAAAAA AGACATAC;
CE31564 *Enchytraeus buchholzi* 1
ATTCGGTTGGGGCGACCATGGATAAATATCCTCAGTAGTAA AAAAAATAGACAATAATATGCAACCATAGTAACCTAGT AATGCACAGATCAGCTACTCTTAGGGAATAACAGA;
CE30968 Mesenchytraeus cf. armatus
TATTCGGTTGGGGCGACCATGGATAAATATCCTCAGTAGTAA AAAAAATAGACAATAATATGCAACCATAGTAACCTAGT AATGCACAGATCAGCTACTCTTAGGGAATAACAGA;
CE30982 *Dendrodrilus rubidus* 
ATTCGGTTGGGGCGACCATGGATAAATATCCTCAGTAGTAA AAAAAATAGACAATAATATGCAACCATAGTAACCTAGT AATGCACAGATCAGCTACTCTTAGGGAATAACAGA;
CE30975 *Dendrobaena octaedra* 
ATTCGGTTGGGGCGACCATGGATAAATATCCTCAGTAGTAA AAAAAATAGACAATAATATGCAACCATAGTAACCTAGT AATGCACAGATCAGCTACTCTTAGGGAATAACAGA;

Some worms were thus successfully sequenced only for one or two of the three barcode markers.

In the NJ analyses, both the 320 bp (Figure 2) and 70 bp (Figure 3) 16S barcodes clustered specimens of the same species, and clearly separated the recognized species from each other.

Among the 51 DNA-barcoded individuals we identified 20 different species, at least six of which are new records for Iceland (Table 4): *Cernosvitoviella agttelekiensis* Dózsa-Farkas, 1970, *C*. *cf. minor* Dózsa-Farkas, 1990, *C*. *pusilla*, *Fridericia dura* (Eisen, 1879), *Mesenchytraeus cf. armatus* (Levinsen, 1884), and *Chaetogaster cf. diastrophus*. Among the barcoded worms, we also found a small specimen of *Chaetogaster*, which is possibly *C*. *langi* Bretscher, 1896 (previously known from Iceland; Hrabě 1952), but not yet confidently identified. Its 16S barcode (320 bp) matches a species also found in Sweden, Norway and the Azores (Klinth & Erséus, unpublished data). The species referred to as *Marionina* sp. could also potentially be new to Iceland.

In the barcoded material, most species were represented by a single or a few specimens only, except *Nais elinguis* Müller, 1773, for which we obtained 16S (320 bp) sequences from 18 individuals (Table 3). Some of the other species reported here (Table 3) belong to complexes of closely related, possibly cryptic, species: For the time being, they are identified as the closest name-bearing morpho-species, but may in the future be recognized and described as separate taxa. These taxa are: *Enchytraeus buchholzi* Vejdovský, 1879 (for which we found two separate species matching the general *E. buchholzi* morphology, “*buchholzi* 1” and “2”), *Ch*. *cf. diastrophus*, *Marionina cf. argentea* (Michaelis, 1889), *Mesenchytraeus cf. armatus*, and *Nais communis/variabilis* (Piguet, 1906; i.e., we found here morphotype A3 sensu Envall et al. 2012). Moreover, there is still some uncertainty whether the earthworm *Bimastos rubidus* (Savigny, 1826) should include *B. subrubicundus* (Eisen, 1874), and *B. tenuis* (Eisen, 1874), all three of which have been reported from Iceland.

The material that did not produce any molecular data was examined based on morphology and could in most cases only be determined to genus level; such specimens will not be further
Cognettia varisetosa (Martinsson, Rota & Erséus, 2015), earlier regarded as C. glandulosa (previously recorded from Iceland); C. varisetosa is thus, at least nominally, a new record for Iceland. We also found specimens of Pristina foreli (Piguet, 1907), which represents a genus (Pristina Ehrenberg, 1828) never recorded in Iceland before. Finally, we found Uncinais uncinata (Ørsted, 1842), a taxon already known from the country (see Table 4).

In total, we identified 23 species, of which at least 8 are new records to Iceland. These identified species were collected from 19 of the 31 sites sampled.

DISCUSSION

Clitellates of the Icelandic springs

The species found in the Icelandic springs are a mixture of Lumbricidae, Enchytraeidae and Naididae. Earthworms (Lumbricidae) are mostly terrestrial, but among our four species found, Eiseniella tetraedra is a characteristic inhabitant of running water or wet soils, and common also in caves and springs, in the Western Palaearctic (Sims & Gerard 1985). The other three are terrestrial worms "accidently found in water" (Timm 2009, p. 188).

All Enchytraeidae (13 species) and Naididae (7 species) in the studied springs are known also from continental Europe. This conclusion is largely based on molecular data, as we were able to compare the 16S barcodes of the Icelandic specimens with the corresponding barcodes of <400 species of Enchytraeidae and Naididae from Sweden and Norway (Erséus and Klinth, unpubl.). This enabled us to identify certain (cryptic) forms within the species complexes of some traditional morphospecies (i.e., Cernosvitoviella minor s. lat., Enchytraeus buchholzi s. lat., Marionina argentea s. lat., Chaetogaster diastrophus s. lat., Tubifex tubifex s. lat.) and one small, yet unidentified Chaetogaster species. However, proper binominal names of these cryptic species are not yet established.

Fridericia dura (Enchytraeidae) is typically terrestrial (Dózsa-Farkas 2019), but was found outside its normal habitat in this study. The remaining enchytraeids and all naidids are normally restricted to aquatic or semi-aquatic habitats (Timm 2009; Schmelz & Collado 2010; Klinth et al. 2017b), and they appear as a somewhat impoverished assemblage of the clitellates typical of streams, rivers, lakes and ponds in other parts of Northern Europe.

Springs are windows into the stygofauna, i.e., stygofaunal species are categorized as those restricted to groundwater (stygobites), those inhabiting both surface and ground waters, or preferring a transition zone of these habitats (stygophiles), and those accidentally or occasionally present in groundwater (stygoxenes). No Icelandic clitellates so far known are treated here (this is why not all originally sampled springs are shown in Figure 1 and Table 1). However, we did identify a few additional species in our spring material based on morphology alone. One being Cognettia varisetosa (Martinsson, Rota & Erséus, 2015), earlier regarded as C. glandulosa (previously recorded from Iceland); C. varisetosa is thus, at least nominally, a new record for Iceland. We also found specimens of Pristina foreli (Piguet, 1907), which represents a genus (Pristina Ehrenberg, 1828) never recorded in Iceland before. Finally, we found Uncinais uncinata (Ørsted, 1842), a taxon already known from the country (see Table 4).

In total, we identified 23 species, of which at least 8 are new records to Iceland. These identified species were collected from 19 of the 31 sites sampled.

DISCUSSION

Clitellates of the Icelandic springs

The species found in the Icelandic springs are a mixture of Lumbricidae, Enchytraeidae and Naididae. Earthworms (Lumbricidae) are mostly terrestrial, but among our four species found, Eiseniella tetraedra is a characteristic inhabitant of running water or wet soils, and common also in caves and springs, in the Western Palaearctic (Sims & Gerard 1985). The other three are terrestrial worms “accidently found in water” (Timm 2009, p. 188).

All Enchytraeidae (13 species) and Naididae (7 species) in the studied springs are known also from continental Europe. This conclusion is largely based on molecular data, as we were able to compare the 16S barcodes of the Icelandic specimens with the corresponding barcodes of <400 species of Enchytraeidae and Naididae from Sweden and Norway (Erséus and Klinth, unpubl.). This enabled us to identify certain (cryptic) forms within the species complexes of some traditional morphospecies (i.e., Cernosvitoviella minor s. lat., Enchytraeus buchholzi s. lat., Marionina argentea s. lat., Chaetogaster diastrophus s. lat., Tubifex tubifex s. lat.) and one small, yet unidentified Chaetogaster species. However, proper binominal names of these cryptic species are not yet established.

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Clitellata of Iceland, an updated species list

In Table 4, >90 taxa of Clitellata reported from Iceland to date are listed. The exact number of species is not yet known, considering that several taxa are species complexes. The present study has added eight binominal species new for Iceland, plus one unidentified Marionina sp. that may be new to science, and the small unidentified Chaetogaster sp., which if not a new species is possibly C. langi. Cernosvitoviella and Pristina are genera that have not been reported from Iceland before.

Barcodes and species identification

This study shows that when traditional (COI) barcoding fails due to DNA degradation, at least part of the material may be identified by targeting a shorter gene fragment (i.e., another barcode). The problem is to decide how short a barcode can be and still be species-specific enough for secure species identification. In theory, when degraded DNA is fragmented into ever-smaller pieces, the smaller the target sequence selected the higher the yield of successful sequences, but at the cost of less genetic information for distinguishing species. In our case, the longer of the two 16S fragments (320 bp), produced significantly more sequences than COI (46 compared to 3), and revealed enough genetic variation to separate closely related species (Figure 2). For some of our taxa, however, species separation was based on only one or a few substitutions in the 16S fragment. It is therefore important to note that we refer to these similar 16S sequences as belonging to separate species, on the basis of other genetic information of other individuals of the same species, mainly from the more variable markers ITS (Internal Transcribed Spacer region) and COI (Erséus and Klinth, unpubl.). To be able to use a short gene fragment such as our 320-bp 16S to identify species it is clear that a large library with multiple sequences from all potential species, representing both inter- and intraspecific variability, is required.

Concerning the 70-bp 16S barcode, we surprisingly found that it did not produce more sequences than the 320-bp one, given the degraded DNA. Instead, it produced fewer successful barcodes. A likely explanation for this is sub-optimal binding of the primers, either due to the annealing temperature, or nucleotide variations in the primer-binding site (also indicated by a lack of bands in the post-PCR electrophoresis gel). The primers were originally designed for earthworms (Bienert et al. 2012), and in the present study they generally worked better for Lumbricidae than for the other families (Table 3), for which modified primers may be needed. It is possible that this very short 16S partition does not contain enough variation to delimit all closely related species of Clitellata, and yet it proved variable enough to distinguish all the 17 successfully sequenced specimens in our current material from each other (Figure 3).

We had problems with contamination in eight of our specimens; their 16S sequences (320 bp) did not match the species revealed by the morphology of the vouchers. In most cases we could attribute this to cross-contamination between samples, or possibly from the extraction lab. There were also some cases where the resulting sequences were those of human or bacterial DNA, but they were directly excluded from the counts of barcoded worms.

The integration of molecular and morphological data is particularly important in the delimitation of clitellate species (e.g., Martinsson et al. 2013; Klinth et al. 2017a). However, using DNA barcoding alone as a reliable shortcut to actual species identification has its pros and cons. In theory, clitellate barcoding is near to perfect when all species have been properly delimited. Moreover, it has the advantages of handling all life stages and even extra-organismal DNA (e.g., DNA from mucus left behind by tunnelling earthworms), and it separates cryptic species. On the other hand, this study has shown that problems occur in practice. We studied samples that suffered from

stygobites. For instance, there are no records of species of the genera typically containing stygobitic (often endemic) taxa in continental Europe, such as Trichodrilus Claparède, 1862 (Lumbriculidae), Aberrantiyadris Martin, 2015, Aktridris Knölker, 1935, Gianius Erséus, 1992, Protuberodrilus Giann & Martinez-Ansemil, 1979, Rhyacodrilus Bretscher, 1901, and Troglodrilus Juget et al., 2006 (all Naididae). However, five meiofaunal species found in the present study (Cernosvitoviella aggetelekiensis, C. pusilla, C. cf. minor, Marionina cf. argentea and Pristina foreli) are associated with surface waters as well as springs and groundwater in Norway and Sweden, and (when in springs) often in various combinations with each other (Erséus & Klinth, unpubl.). These taxa may be regarded as stygophiles, and their small size may be advantageous in springs, where nutrient levels are often low. Moreover, three other taxa (Lumbricillus arenarius, Marionina sp. and Nais elinguis) are normally associated with marine, intertidal habitats. Lumbricillus arenarius is also known from a spring in Northern Svalbard (Klinth et al. 2017b), and Nais elinguis is well known from both springs and coastal streams (e.g., Timm 2009), but the unidentified, possibly new species of Marionina was earlier collected only in marine habitats in Norway and Sweden (Erséus & Klinth, unpubl.).

Enchytraeus buchholzi s. lat., a species complex generally associated with “not too acidic” soils (Schmelz and Collado, 2010), sometimes occurs in freshwater (Timm 2009). The two genetically distinct forms of E. buchholzi found in our study are common in wet soils, including springs, in mainland Scandinavia (Erséus & Klinth, unpubl.). They thus appear to be more aquatic than other members of the complex. As for Tubifex tubifex s. lat., most of the cryptic species studied by us (Erséus & Klinth, including the one from the (Icelandic) Botnar I spring, are occasionally found in springs of other parts of Northern Europe. To conclude, we consider our recorded Lumbricidae spp. (possibly excepting the somewhat “stygophilic” Eiseniella tetraedra), Enchytraeus buchholzi 1 & 2, Henlea perpusilla, Mesenchytraeus cf. armatus, T. cf. tubifex, Cognettia variseta, Chaetogaster spp. and Uncinains uncinata as stygoxenes.

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Table 4. Updated checklist of clitellate species from Iceland. Previously recorded species from Iceland are presented together with the reference paper.

| Species sorted by family | References |
|--------------------------|------------|
| **Enchytraeidae**        |            |
| *Achaeta unibulba* Graefe, Dózsa-Farkas & Christensen 2005 | Graefe et al. 2005 |
| *Bryodrilus parvus* Nurminen, 1970 | Nurminen 1973 |
| *Buchholzia appendiculata* (Buchholz, 1862) | Christensen 1962; Nurminen 1973 |
| *Cernosvitoviella aggelekiensis* Dózsa-Farkas, 1970 | This study (new record) |
| *Cernosvitoviella cf. minor* Dózsa-Farkas, 1990 (one in a species complex) | This study (new record) |
| *Cernosvitoviella pusilla* Nurminen, 1973 | This study (new record) |
| *Claparedrilus semifuscoide* Klinth, Rota & Erséus, 2017 (previously reported as *L. semifuscoide*) | Christensen 1962; Erséus 1976 |
| *Cognettia glandulosa* (Michaelson, 1888) previous records could have been *C. glandulosa* or *C. varisetosa* (see Martinsson, Rota & Erséus, 2015a) | Christensen 1962; Nurminen 1973 |
| *Cognettia sphagnetorum* (Vejdovský, 1877) previous records could have been *C. chalupskyi*, *C. chlorophila*, *C. pseudosphagnetorum* or *C. sphagnetorum* (see Martinson, Rota & Erséus, 2015b) | Christensen 1962; Nurminen 1973 |
| *Cognettia varisetosa* (Martinsson, Rota & Erséus, 2015a) (previously a part of *C. glandulosa*) | This study (new record) |
| *Enchytraeus albidus* Henle, 1837 | Christensen 1962; Erséus 1976 |
| *Enchytraeus buchholzi* Vejdovský, 1879 (species complex) | Christensen 1962; Nurminen 1973; two species found in this study |
| *Enchytraeus coronatus* Nielsen & Christensen, 1959 | Christensen 1962 |
| *Enchytraeus minutus* Nielsen & Christensen, 1961 | Nurminen 1973 |
| *Enchytraeus norvegicus* Abrahamsen, 1969 | Nurminen 1973 |
| *Fridericia bisetosa* (Levinsen, 1884) | Christensen 1962; Nurminen 1973 |
| *Fridericia bulboides* Nielsen & Christensen, 1959 | Christensen 1962; Nurminen 1973 |
| *Fridericia bulbosa* (Rosa, 1887) | Christensen 1962; Nurminen 1973 |
| *Fridericia callosa* (Eisen, 1878) | Christensen 1962 |
| *Fridericia dura* (Eisen, 1879) | This study (new record) |
| *Fridericia galba* (Hoffmeister, 1843) | Christensen 1962; Nurminen 1973 |
| *Fridericia leydigii* (Vejdovský, 1877) | Nurminen 1973 |
| *Fridericia maculata* Issel, 1905 | Christensen 1962 |
| *Fridericia perrieri* (Vejdovský) | Christensen 1962 |
| *Fridericia ratzei* (Eisen, 1872) | Christensen 1962; Nurminen 1973 |
| *Fridericia striata* (Levinsen, 1884) | Christensen 1962 |
| *Grania postclitellochaeta* (Knöllner, 1935) | Rota & Erséus 2003 |
| *Henlea glandulifera* Nurminen, 1970 | Nurminen 1973 |
| *Henlea nasuta* (Eisen, 1878) | Christensen 1962 |
| *Henlea perpusilla* Friend, 1911 | Christensen 1962; Nurminen 1973; this study |
| *Henlea ventriculosa* (Udekem, 1854) | Christensen 1962; Nurminen 1973 |
| *Lumbricillus arenarius* (Michaelson, 1889) | Christensen 1962, this study |
| *Lumbricillus lineatus* (Müller, 1774) | Christensen 1962; Erséus 1976 |
| *Lumbricillus macrosthecatus* Erséus, 1976 | Erséus 1976 |
| *Lumbricillus pagenstecheri* (Ratzel, 1869) | Christensen 1962; Erséus 1976 |
| *Lumbricillus pumilio* Stephenson, 1932 | Erséus 1976 |
| *Lumbricillus reynoldsoni* Backlund, 1948 | Christensen 1962 |
| *Lumbricillus rivalis* Levinsen, 1883 emend. Ditlevsen, 1904 | Christensen 1962 |
Table 4. Continued.

| Species sorted by family | References |
|--------------------------|------------|
| *Lumbricillus scoticus* Elmhirst & Stephenson, 1926 | Christensen 1962; Erséus 1976 |
| *Lumbricillus viridis* (Stephenson, 1911) | Christensen 1962; Erséus 1976 |
| *Marionina argentea* (Michaelsen, 1889) (species complex) | Nurminen 1973; one species found in this study |
| *Marionina communis* Nielsen & Christensen, 1959 | Christensen 1962; Nurminen 1973 |
| *Marionina spicula* (Leuckart, 1847) | Christensen 1962; Erséus 1976 |
| *Marionina sp.* This study (unidentified/new species?) | |
| *Mesenchytraeus cf. armatus* (Levinsen, 1884) (one in a species complex) | This study (unidentified/new species?) |
| *Mesenchytraeus flavus* (Levinsen, 1884) | Christensen 1962; Nurminen 1973 |

**Hirudinea**

| Species | References |
|---------|------------|
| *Callobdella nodulifera* Malm, 1863 | Bruun 1938a |
| *Glossiphonia complanata* (Linnaeus, 1758) | Bruun 1938b; Lindegaard 1979 |
| *Helobdella stagnalis* (Linnaeus, 1758) | Bruun 1938b; Lindegaard 1979 |
| *Heptacyclus scorpii* (Malm, 1863) | Bruun 1938a |
| *Johanssonia arctica* (Johansson, 1899) | Perdiguero-Alonso *et al.* 2008 |
| *Oceanobdella microstoma* (Johansson, 1896) | Bruun 1938a |
| *Oxytonostoma typica* Malm, 1863 | Bruun 1938a |
| *Platybdera anarrhicae* (Diesing, 1859) | Bruun 1938a |
| *Pontobdella muricata* (Linnaeus, 1758) | Bruun 1938a |
| *Theromyzon garjaewi* (Livanow, 1903) valid species? | Bruun 1938b |
| *Theromyzon maculosum* (Rathke, 1862) valid species? | Fjeldså & Raddum 1973 |
| *Theromyzon tessulatum* (Müller, 1774) | Bruun 1938b; Lindegaard 1979 |

**Lumbricidae**

| Species | References |
|---------|------------|
| *Aporrectodea caliginosa* (Savigny, 1826) | Backlund 1949; Lindroth *et al.* 1973; this study |
| *Aporrectodae rosea* (Savigny, 1826) | Backlund 1949; Lindroth *et al.* 1973 |
| *Bimastos rubidus* s. lat. (reported as *Dendrodrilus rubidus* (Savigny, 1826), *Dendrodrilus subrubicundus* (Eisen, 1874), and *Dendrodrilus tenuis* (Eisen, 1874)) | Backlund 1949; Lindroth *et al.* 1973; this study |
| *Dendrobaena octaedra* (Savigny, 1826) | Backlund 1949; Lindroth *et al.* 1973; this study |
| *Eisenia foetida* (Savigny, 1826) | Backlund 1949 |
| *Eiseniella tetraedra* (Savigny, 1826) | Backlund 1949; Lindroth *et al.* 1973; Lindegaard 1979; this study |
| *Lumbricus castaneus* (Savigny, 1826) | Backlund 1949; Lindroth *et al.* 1973 |
| *Lumbricus rubellus* Hoffmeister, 1843 | Backlund 1949; Lindroth *et al.* 1973 |
| *Lumbricus terrestris* Linnaeus, 1758 | Backlund 1949; Lindroth *et al.* 1973 |
| *Octolasium cyaneum* (Savigny, 1826) | Backlund 1949 |

**Lumbriculidae**

| Species | References |
|---------|------------|
| *Lumbricus variegatus* (Müller, 1774) | Hrabé 1952; Lindegaard 1979 |
| *Stylodrilus heringianus* Claparède, 1862 | Hrabé 1952; Lindegaard 1979 |

**Naididae**

| Species | References |
|---------|------------|
| *Aktoedrus arcticus* (Erséus, 1978) | Erséus 1978 |
| *Aulodrilus limnobius* Bretcher, 1899 | Hrabé 1952 |
| *Aulodrilus plurisetata* (Piguet, 1906) | Hrabé 1952 |
DNA deterioration, which considerably reduced the number of identified specimens. We also found evidence of DNA contamination, which would have led to the wrong conclusions, had we not compared the morphology of a specimen with the barcode sequence obtained. The conclusion is that any samples to be used for DNA analysis must be handled properly, e.g., kept at low temperature, conserved in high concentration of ethanol or DNA preserving buffers, and minimizing storage time. By doing so, the risk of low sequencing success as well as obtaining erroneous identifications due to contamination will be considerably reduced.

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