New specific primers for amplification of the Internal Transcribed Spacer region in Clitellata (Annelida)

Yingkui Liu | Christer Erséus

Department of Biological and Environmental Sciences, University of Gothenburg, Göteborg, Sweden

Correspondence
Christer Erséus, Department of Biological and Environmental Sciences, University of Gothenburg, Göteborg, Sweden. Email: christer.erseus@bioenv.gu.se

Funding information
Vetenskapsrådet, Grant/Award Number: 621-2010-5325; China Scholarship Council; Royal Society of Arts and Sciences in Gothenburg

Abstract
Nuclear molecular evidence, for example, the rapidly evolving Internal Transcribed Spacer region (ITS), integrated with maternally inherited (mitochondrial) COI barcodes, has provided new insights into the diversity of clitellate annelids. PCR amplification and sequencing of ITS, however, are often hampered by poor specificity of primers used. Therefore, new clitellate-specific primers for amplifying the whole ITS region (ITS: 29F/1084R) and a part of it (ITS2: 606F/1082R) were developed on the basis of a collection of previously published ITS sequences with flanking rDNA coding regions. The specificity of these and other ITS primers used for clitellates were then tested in silico by evaluating their mismatches with all assembled and annotated sequences (STD, version r127) from EMBL, and the new primers were also tested in vitro for a taxonomically broad sample of clitellate species (71 specimens representing 11 families). The in silico analyses showed that the newly designed primers have a better performance than the universal ones when amplifying clitellate ITS sequences. In vitro PCR and sequencing using the new primers were successful, in particular, for the 606F/1082R pair, which worked well for 65 of the 71 specimens. Thus, using this pair for amplifying the ITS2 will facilitate further molecular systematic investigation of various clitellates. The other pair (29F/1084R), will be a useful complement to existing ITS primers, when amplifying ITS as a whole.

KEYWORDS
Hirudinida, Internal Transcribed Spacer region, Oligochaeta, polymerase chain reactions, primers

1 | INTRODUCTION

In molecular systematics, multilocus sequence data, both from mitochondrial and nuclear genomes, provide a better understanding of speciation than any single-locus data (typically maternally inherited mitochondrial ones) (Dupuis, Roe, & Sperling, 2012; Mallo & Posada, 2016). As the analysis of a single-locus data produces a gene tree rather than a species tree, such data should be integrated with nuclear evidence to establish species boundaries more accurately (Dasmahapatra, Elias, Hill, Hoffman, & Mallet, 2010; Kodandaramaiah, Simonsen, Bromilow, Wahlberg, & Sperling, 2013). This has been performed for many species of Clitellata (see Figure 1; they are segmented hermaphroditic annelid worms, bearing a unique clitellum ("girdle") during sexual maturity, and many of them (earthworms, sludge worms, leeches) are important in agriculture, industry, environmental monitoring, and medicine (Elissen, Hendrickx, Temmink, & Buisman, 2006; Martin, Martinez-Ansemil, Pinder, Timm, & Wetzel, 2008; Rodriguez & Reynoldson, 2011; Sket & Trontelj, 2008). Closely
related clitellates are often difficult to distinguish morphologically, but molecular studies have shown that several well-known morphotaxa, even those used as model organisms, are complexes of cryptic species (Erséus & Gustafsson, 2009; James et al., 2010; Römcke et al., 2016; Siddall, Trontelj, Utevsky, Nkamany, & Macdonald, 2007).

Using mitochondrial COI barcodes suggested for animals (Hebert, Ratnasingham, & de Waard, 2003) to validate and identify the currently >5,000 described species of Clitellata (Erséus, 2005), however, is still far from satisfactory (Trebitz, Hoffman, Grant, Billehus, & Pilgrim, 2015; Vivien, Wyler, Lafont, & Pawlowski, 2015). Such single-locus data only reflect the history of one gene; however, they may still give hints of cryptic speciation by showing “barcoding gaps.” Therefore, in more comprehensive studies of species delimitation, COI data have been used to produce primary species hypotheses only, and the final species hypotheses have then been formulated based on congruence with hypotheses derived from independent nuclear markers (Kvist, Sarkar, & Erséus, 2010; Liu, Fend, Martinsson, & Erséus, 2017; Martinsson & Erséus, 2014; Martinsson, Rhodén, & Erséus, 2017; Vivien et al., 2015).

One of these nuclear markers, the Internal Transcribed Spacer (ITS) region, has been commonly used in combination with COI in taxonomic works (Bucklin, Steinke, & Blanco-Bercial, 2011; Coissac, Hollingsworth, Lavergne, & Taberlet, 2016; Raupach et al., 2010), as well as in studies of phylogeny, biogeography, and population genetics (De Wit & Erséus, 2010; Hallett, Atkinson, & Bartholomew, 2005; Trontelj & Sket, 2000; Trontelj & Utevsky, 2012; Villalobos et al., 2014). This region, which comprises two fast-evolving spacers (ITS1 and ITS2) flanking the conserved 5.8S rDNA, has indeed been suggested as a universal DNA barcode marker for Fungi (Schoch et al., 2012), and a supplementary barcode for plants (Li et al., 2015; Pecník & Buzan, 2014). However, there has been a long debate about the relative value of ITS1 and ITS2 (Bazzicalupi, Balint, & Schmitt, 2013; Blažič et al., 2013; Wang et al., 2015; Yao et al., 2010).

The ITS1 spacer seems to be more variable than ITS2, due to the frequent occurrence of indels (Edger et al., 2014; Martin & Rygiewicz, 2005; Nilsson, Kristiansson, Ryberg, Hallenberg, & Larsson, 2008; Rampersad, 2014). ITS1 is used in molecular identification of fungi in the publicly available databases UNITE (Koljalg et al., 2013) and ITSoneDB (Fosso et al., 2012), but the annotation and analyses of ITS1 of other taxonomic groups may be challenging. Because annotation is commonly performed by directly comparing new amplicons with those published sequences, however, the coverage of both the ITS1 and ITS2 regions in GenBank is often incomplete or incorrectly annotated. On the other hand, a comprehensive ITS2 database (Schultz et al., 2006) has facilitated the annotation of ITS2 sequences across many groups of organisms, by predicting their 5.8S-28S interactions in a homology-based structure modeling approach (Selig, Wolf, Müller, Dandekar, & Schultz, 2008). In particular, throughout the eukaryotes, the four helices in the secondary structure of ITS2 are consistent (Coleman, 2007; Gottschling & Pioter, 2004; Hausner & Wang, 2005; Schultz, Maisel, Gerlach, Müller, & Wolf, 2005), which is essential for successful excision of ITS2 from the precursor rDNA (Henras, Plisson-Chastang, O’Donohue, Chakraborty, & Gleizes, 2015; Mullineux & Lafontaine, 2012). The rather conservative secondary structure of ITS2 makes it realistically suitable also for higher level systematics (Caisova, Marin, & Melkonian, 2011; Coleman, 2003; Marinho et al., 2012; Porras-Alfaro, Liu, Kuske, & Xie, 2014; Salvi & Mariottini, 2017; Schultz et al., 2006). Knowledge of ITS2 secondary structure can improve the quality of an alignment using other carefully annotated sequences as a backbone (Katoh & Standley, 2013; Keller et al., 2010), which makes it possible to identify the consensuses motifs universally shared by closely related species (Pepato & Klimov, 2015). Thus, ITS2 may also provide sufficient information for cryptic species and young radiations (Bertrand et al., 2014; Coleman, 2009; Martinsson et al., 2017; Ruhl, Wolf, & Jenkins, 2010; Schill, Forster, Dandekar, & Wolf, 2010; Wiemmers, Keller, & Wolf, 2009), and estimation of gene flow within panmictic populations of deeply divergent mitochondrial lineages (Martinsson et al., 2017). Yao et al. (2010) even suggested that ITS2 should be used as a complementary locus for the identification of animals along with COI barcodes. Considering the general annotation and structure prediction tools provided by the ITS2 database (Schultz et al., 2006), it seems that ITS2, at present, is a more suitable nuclear marker than ITS1 for nonfungal groups such as clitellates.

Various universal primer pairs (Figure 2 and Table 1) have been used for amplification of the entire or parts of the ITS region in
clitellate studies. However, universal primers sometimes have low success rate in the polymerase chain reactions (PCR) (Oceguera-Figueroa, 2012; Shekhovtsov, Golovanova, & Peltek, 2013; Trontelj & Utevsky, 2012; Vivien et al., 2015), due to poor specificity of these primers (Bellemain et al., 2010; Sipos et al., 2007). Furthermore, mismatches between primer and DNA templates might also introduce biases in PCR-based high-throughput Next Generation Sequencing (Aird et al., 2011; Deakin et al., 2014; Schirmer et al., 2015).

Universal primers thus often have to be modified to make them suitable for amplification of specific organisms (Bellemain et al., 2010; Cheng et al., 2016; Kohout et al., 2014; Toju, Tanabe, Yamamoto, & Sato, 2012). For example, Källersjö, Von Proschwitz, Lundberg, Eldenäs, and Erseus (2005) amplified ITS sequences of freshwater bivalves using the more bivalve-specific forward primer MITS1F together with the universal primer ITS4, instead of using the primer pair ITS5/ITS4 (White, Bruns, Lee, & Taylor, 1990), which were originally developed for Fungi but are now used as a universal primer (see https://unite.ut.ee/primers.php). PCR failure may also be caused by intra-individual polymorphism (Kook et al., 2015), which has been found, for example, in the European earthworm Aporrectodea longa (Martinsson et al., 2017).

As yet, no clitellate-specific ITS primers have been formally proposed. In this paper, two new pairs of primers specifically designed to amplify the whole ITS region and ITS2 spacer in clitellates are proposed. One of them (606F/1082R for ITS2) was successfully tested also by Martinsson et al. (2017), and Liu et al. (2017).
2 | MATERIAL AND METHODS

2.1 | Primer design

In contrast to the fast-evolving ITS1 and ITS2 spacers, the flanking 18S and 28S rDNA, as well as 5.8S rRNA between the two spacers, are more conserved and thus suitable as annealing regions for primers. An alignment was generated from a collection of 742 ITS sequences referred to Clitellata, that is, all those publicly available in GenBank (NCBI), and which include at least a part of 5.8S rDNA; several of them also include parts of 18S and/or 28S rDNA. Annotation and separation of ITS1, ITS2 and 5.8S rDNA are crucial for proper alignment, but aligning ITS sequences from divergent taxa may be problematic due to length variations (Alvarez & Wendel, 2003; Simmons & Freudenstein, 2003). Therefore, the three partitions of each downloaded ITS sequence were first identified using ITStx (Bengtsson-Palme et al., 2013). In addition, boundaries of rDNAs were tested against the Rfam databases (Nawrocki et al., 2015), and the annotations of ITS2 were also checked using the Hidden Markov model (HMM) in the ITS2 database (E-value < .001, metazoan) (Keller et al., 2009). Alignments of each ITS partition were conducted using the MAFFT V 7.017 plugin with default settings as implemented in Geneious 6.1.8. Based on the consensus sequence of this alignment, primer candidates were identified within the retained series of multiple conservative sites (each >14 nucleotides long), and two primer pairs with the highest possible scores, for ITS as a whole and ITS2, respectively, were identified using the software Oligo 7 (Rychlik, 2007). Heterozygosity within PCR primer binding sites do have negative effects for amplification, but in most cases, heterozygosity is more commonly found in ITS spacer sequences than in the short flanking rDNA sequences (see Martinsson et al., 2017).

2.2 | Experimental verification of new primers

The universality of the new primers among clitellates was tested by PCR, amplifying specific fragments from 71 genomic DNA samples (47 genera, 11 families; Table 2); for extraction protocols, see Liu, Fend, et al. (2017). The samples were chosen to represent as many available families as possible, but also to cover several genera in the highly diverse family Naididae and to include some samples of very closely related species; three nominal naidids (Doliodrilus tener, Limnodrilus grandisetosus, and L. rubripennis) were even each represented by two specimens that are likely to be different (cryptic) species. A typical naidid, Limnodrilus hoffmeisteri, is shown in Figure 1. This mixture was chosen to obtain general information about ITS variability within both higher and lower taxa, which will facilitate a better annotation of new clitellate amplicons (as future reference sequences, for example, in secondary structure-based analyses of ITS). In addition, samples that did not successfully amplify with the new primers were also tested using the universal primer pair ITS5/ITS4 without additional primers (see Table 1).

The entire ITS and the ITS2 sequences were amplified, each with its new primer pair. The PCR reaction mixtures consisted of 15 μl of VWR red Taq Master Mix kit (We Enable Science, Denmark), 1 μl of primer (10 mmol/l), 2 μl of DNA template, and 6 μl distilled water. The PCR protocol for both pairs was as follows: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 60 s and elongation at 72°C for 90 s, followed by a final extension at 72°C for 8 min. Gel electrophoresis (1% agarose in 10 × TAE buffer) was carried out to check the quality of PCR products, which were then were purified using 5 μl ExoTAP (Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase). Amplicons were sequenced by Eurofins (Germany). For both of the new primer pairs, amplicons at least 200 bp long were regarded as successful. The amplified sequences were then checked for adherence to clitellates by blasting them against the NCBI database.

2.3 | Primer evaluation in silico

The specificity of the new primers to clitellates (relative to other organisms) was evaluated in silico by the number of mismatches between DNA templates and primers, and the results of this were also compared with the specificity of primers previously used in clitellate studies (Figure 2). These analyses were performed using ecoPCR (Ficetola et al., 2010) against assembled and annotated sequences (STD, version r127) in EMBL. To achieve simulation under realistic PCR conditions, up to three mismatches between a primer and its annealing sequence were allowed. The complete length of clitellate ITS sequences at NCBI normally varies between 500 and 900 bp; however, members of Branchiobdellida have a rather long (about 1200 bp) ITS1 spacer (Williams, Gelder, Proctor, & Colman, 2013). Thus, in the simulations, sizes of ITS (as a whole) between 400 and 2500 bp were allowed, and the minimum and maximum amplified ITS2 lengths were set as 200 and 1250 bp long, respectively.

3 | RESULTS

3.1 | Annotation of ITS sequences and primer design

As mentioned above, 742 GenBank sequences, representing a total of at least 46 genera belonging to 14 clitellate families (Table S1), were obtained, annotated, and aligned. As expected, in this alignment, sequence variation is much greater in the ITS spacers than in the 18S, 5.8S, and 28S rDNA partitions. The majority of the published complete 5.8S sequences contain 153 ± 1 nucleotides. Figure 1 shows the variations in a part of 5.8S among the 27 haplotypes found in our newly amplified complete ITS sequences, with taxa ranked by number of mismatches. Neither the first nor the third of the three conserved 5.8S motifs proposed by Harpke and Peterson (2008) are identical with our current clitellate ones (Figure 2: CM1 and CM3), but in most cases the second motif (Figure 2: CM2) is the same as the conserved motif in vertebrates (Harpke & Peterson, 2008). The complete ITS2 spacer, recognized by the 5.8S–28S rDNA interaction (Keller et al., 2009), varied from 174 to 503 bp in the current clitellate sample. The motif CATTA was identified as the end of 18S by the software
ITSx, and this ending motif was found in eukaryote sequences from the Rfam database. In addition, it also has been found that, in some fungi, the ITS1 spacer starts after this motif CATT (Nagy et al., 2012; Schoch et al., 2014). The complete ITS1 sequences, which begin after the conserved motif CATTA, ranged from 314 to 1117 bp in the published clitellate sequences.

Two new primer pairs suggested by Oligo 7, and now referred to as 29F/1084R and 606F/1082R, were found to be suitable for amplifications of the whole ITS region, and the ITS2 subregion, respectively, of Clitellata. The forward primer 29F (AAAGTCGTAACAGGTTTCCGTA) matches the terminal end of 18S but after E18S-2, with its anchoring sites partly overlapping with those of the old primers ITS5 and ETTS2, and the reverse primer 1084R (YGTTAGTTTCTTTTCCTCCGCTT) partly overlaps with ITS4 but is separated from ETTS1 and E28S-2 (Figure 2 and Figure S1). The new forward primer for ITS2, 606F (GTCGATGAAGAGCGCAGCCA), partly overlaps with ITS3 and 5.8SF but was designed to fully match the motif CM1 (Figure 1), and the corresponding reverse primer, 1082R (TTAGTTTCTTTTCCTCCGCTT), is almost identical to 1084R (Figure 2 and Figure S1), but two nucleotides shorter at the 5’ end, which makes its melting properties similar to those of 606F.

3.2 | Experimental verification of new primers

From our 71 genomic samples, 52 (73%) ITS amplicons were successfully amplified using the primer pair 29F/1084R, and 65 (91.5%) ITS2 amplicons were successfully amplified using 606F/1082R. Sequences are deposited in the NCBI database (for more details see Table 2). All samples that gave no amplifications, and those that yielded amplicons <200 bp long, also failed in PCR reactions using only one universal primer pair IT5S/ITS4. Successfully amplified ITS and ITS2 sequences from the same individual were identical in their overlapping parts, after trimming. The average GC content of the successfully amplified ITS and ITS2 sequences was around 59%, but amplicon lengths varied significantly across taxa. After trimming, the completely amplified ITS sequences using 29F/1084R spanned from 844 to 1439. But in one case, KY982581 (a branchiobdellidan Xironigton victoriensis CE18252), the length was 2,060 bp, and yet this ITS region was not completely amplified. ITS2 amplicons using 606F/1082R ranged from 329 to 912 bp, and they often include parts of 5.8S and 28S sequences. The complete 5.8S for CE1790 (a naidid, Aulodrilus acutus, KY637027) was 154 bp, while all other complete sequences of 5.8S were 153 bp; all new 5.8S are consistent with the published 5.8S sequences in length. The completely amplified ITS1 spacer ranged from 351 to 733 bp, whereas the ITS2 spacer varied from 247 bp to 747 bp. The amplified ITS1, even incomplete ones, was generally longer than ITS2 of the same individual or a closely related species (see Table 2).

Interestingly, our attempt to amplify ITS of Chamaedrilus spagheetorum (CE11317) using the new primer pair 29F/1084R failed, while a 909-bp-long ITS sequence (KF672519) was successfully amplified from the same individual using two pairs of primers (Martinsson & Erséus, 2014). Nevertheless, our new ITS2 amplicon (primers 606F/1082R) of this worm is identical to the corresponding part in KF672519.

The mismatches between the primers and their targeting 5.8S were investigated (see Figure 2 and Figure S1). The primers 5.8SF, 5.8SR, ITS3, and ITS1B often had more than one mismatch against the amplified DNA sequences, while 606F showed only one mismatch with the sequences from Haplotaxidae (CE5731, Haplotaxis gordioides) and Haemopodidae (CE18378, Haemopsis sanguisuga). For all other sequences of our samples of clitellates, 606F showed a 100% match with its annealing region.

3.3 | Primer evaluation in silico

The in silico results varied considerably across simulations with different primer pairs (Figure 3 and Table S2). Generally, only a few ITS sequences of clitellates were successfully (in silico) amplified due to the limited number of full-length ITS sequences available. A much larger number of nonclitellate amplicons come from fungal groups, in particular, followed by, for example, chlorophytes (green algae) and some of the more species-rich invertebrate groups, such as Cnidaria, Nematoda, Arthropoda, and Platyhelminthes (Table S2). Under strict PCR conditions (0–1 mismatch for each primer), about 70 clitellate sequences of the complete ITS region were amplified in silico with ETTS2/ETTS1, ITS5/ITS4, and the new primer pair 29F/1084R (Figure 3). On the other hand, even under more relaxed PCR conditions (up to three mismatches per primer), the number of nonclitellate amplicons was dramatically decreased when using 29F/1084R instead of ETTS2/ETTS1 and ITS5/ITS4. For the evaluation of ITS2 primers and their specificity for clitellates, 606F/1082R and 5.8SF/ITS4 did better than ITS3/ITS4 and E58S-F1/E28S-2 under the strict conditions (0–1 mismatch). Under relaxed PCR conditions (2–3 mismatches), a higher number (131) of clitellate ITS2 sequences were amplified with 5.8FS/ITS4, and a similar number of ITS2 amplicons for the primer pairs 606F/1082R and ITS3/ITS4. The amplified nonclitellate sequences using 5.8FS/ITS4 were also fewer than those using 606F/1082R, and even fewer than those using ITS3/ITS4.

In addition, the possible mismatches between each primer and the haplotypes of the corresponding template regions in the newly amplified (Figure 2) and previously published clitellate ITS sequences were estimated, and differences in all these mismatches (number and position) are summarized in Figure S1.

4 | DISCUSSION

4.1 | Annotation of ITS

When using ITS for phylogenetic analysis, verification and annotation of amplicons are critical. Nonfunctional pseudogenes or chimeric sequences are readily recognizable by irregularities in the 5.8S rDNA and/or by the absence of some or all of the conserved regions of the ITS spacers (Freire et al., 2012; Harpke & Peterson, 2008; Hřibová et al., 2011; Rampersad, 2014). Only the GenBank clitellate sequences
### TABLE 2  
Taxonomic sampling, collection sites and GenBank accession numbers of specimens used in this study. DNA sequences were derived from tissue samples from the posterior part of the worms.

| Specimen ID | Family name | Species | $606F / 1082R$ | $29F / 1084R$ | ITS1 (bp) | ITS2 (bp) | GenBank | Voucher ID | Location and habits | Latitude | Longitude | Date | Collector |
|-------------|-------------|---------|--------------|--------------|-----------|-----------|---------|-----------|---------------------|-----------|-----------|------|-----------|
| CE18252     | Branchiobdellidae | Xironogton victoriensis (Gelder & Hall, 1990) | + | + | >1.097 | 153 | >810 | KY982581 | No voucher | Luxembourg, near Welscheid, Wark Brook, from a crayfish (Pacificaostaus leniusculus) | 49.880 N | 6.044 E | 15-May-2013 | David Templeman |
| CE14346     | Capilloventridae | Capilloventer australis (Erséus, 1993) | + | - | - | >92 | 747 | KY982554 | No voucher | Australia, Victoria, Acheron River (NE of Melbourne), gravel and sand | 37.3526 S | 145.7066 E | 12-April-2012 | C. Erséus & Richard Marchant |
| CE13745     | Enchytraeidae | Achaeta aberrans (Nielsen & Christensen 1961) | + | + | >439 | 153 | 330 | KY982545 | SMNH 162129 | Sweden, Västergötland, Vårgårda, Bergstena near Lundagården Spring | 58.069 N | 12.689 E | 28-November-2011 | Christer Erséus, N. Bekkouche & Marcus Svensson |
| CE11317     | Enchytraeidae | Chamaedrilus sphagnetorum (Vejdovský, 1878) (s.str.) | + | - | - | >70 | 248 | KY982555 | SMNH 133623 | KF672519 | Sweden, Närke, Hallsberg, Östansjö, Ögonkällan Spring | 59.0389 N | 15.0186 E | 7-April-2011 | Ainara Achurra & Christer Erséus |
| CE19554     | Enchytraeidae | Fridericia magna (Friend, 1899) | + | + | >436 | 153 | 287 | KY982559 | ZMBN 110195 | Norway, Møre og Romsdal, Tingvoll, Kanestraum, at ferry terminal (ferry across Halsfjorden) | 63.0531 N | 8.1233 E | 13-August-2013 | Christer Erséus |
| CE19299     | Enchytraeidae | Lumbricillus lineatus (Müller, 1774) | + | + | >464 | 153 | 289 | KY982569 | ZMBN 107874 | Norway, Sogn og Fjordane, Luster, Nes, seashore | 61.3864 N | 7.3691 E | 12-August-2013 | Christer Erséus |
| CE5731      | Haplotaxidae | Haplotaxis gordoi (Hartmann, 1821) | + | + | >472 | 153 | 302 | KY982561 | SMNH 162130 | Sweden, Västergötland, Göteborg, Vitsippadal (at Botanical Garden), wet soil | 57.6752 N | 11.9644 E | 8-April-2009 | Christer Erséus |
| CE18378     | Hirudinidae | Haemopis sanguisuga (Linnaeus, 1758) | + | + | >322 | 153 | 381 | KY982560 | No voucher | Sweden, Västergötland, Vårgårda, Lången Lake, shallow water | 58.0111 N | 12.582 E | 27-July-2013 | Christer Erséus |

(Continues)
| Specimen ID | Family name | Species | GenBank | Voucher ID | Location and habits | Latitude | Longitude | Date       | Collector                  |
|-------------|-------------|---------|---------|------------|---------------------|----------|-----------|------------|---------------------------|
| CE12000     | Lumbricidae | Allolobophora caliginosa (Savigny, 1826) | KY982547 | ZMBN 108456 | Norway, Telemark, Porsgrunn, Eidanger, Langansvegen | 59.1162 N | 9.7216 E  | 16-June-2011 | Christer Erséus             |
| CE16075     | Lumbricidae | Allolobophora caliginosa (Savigny, 1826) | KY982549 | ZMBN 108577 | Norway, Nordland, Fauske, E of Tøresvik, at Rd 80 | 67.2656 N | 15.2939 E | 17-August-2012 | Endre Willassen & Christer Erséus |
| CE10969     | Lumbriculidae | Dorydilus michaelseni Pignut, 1913 | KY982565 | No voucher | England, Devon, Ivybridge, Higher Ludbrook Farm, spring | 50.37 N  | 3.89 W    | 18-March-2010 | Tim Jones                  |
| CE14379     | Lumbriculidae | Kincaidiana hexatheca Altman, 1936 | KY982565 | No voucher | USA, Oregon, Rock Creek (Portland) | 45.5 N   | 122.9 E   | 27-March-2012 | Sam James                  |
| CE19888     | Lumbriculidae | Lumbriculus variagatus (Müller, 1774) | KY982570 | No voucher | Norway, Oslo, Majorstua, Vigelandsparken, stream near swimming pools | 59.9281 N | 10.7059 E | 10-October-2012 | Christer Erséus, Svante Martinsson & Yingkui Liu |
| CE17795     | Lumbriculidae | Stylodrilus heringianus Claparède, 1862 | KY982578 | No voucher | Sweden, Södermanland, Vingäker, Läppe, Hjälmaren Lake, sand and gravel | 59.13 N  | 15.81 E   | 27-July-2012 | Christer Erséus             |
| CE2048      | Megascolecidae | Dichogaster bolaui (Michaelsen, 1891) | KY982556 | SMNH 162132 | Sweden, Västergötland, Göteborg, Tynnered, bathroom (apartment building) | 57.64 N  | 11.89 E   | 27-September-2006 | Daniel Gustafsson            |
| CE713_1     | Naididae | Branchiura sowerbyi Beddard, 1892 | KY982556 | SMNH 160320 | Sweden, Västmanland, Västerås, Mälaren Lake, Västeråsfjärden, Djuphammen, | 59.589 N | 16.527 E | 17-September-2003 | Tommy Odelström             |
| Specimen ID | Family name | Species | 606F/1082R | 29F/1084R | ITS1 (bp) | 5.8S (bp) | ITS2 (bp) | GenBank | Voucher ID | Location and habits | Latitude | Longitude | Date | Collector |
|-------------|-------------|---------|-------------|----------|-----------|-----------|-----------|---------|------------|---------------------|----------|-----------|------|-----------|
| CE10030     | Naididae    | Adelodrilus pusillus Erséus, 1978 | + | + | >490 | 153 | 385 | KY982546 SMNH 162133 | Sweden, Bohuslän, Strömstad, Brattebergsund (strait between Öddö and Tjärnö Islands), 8 m | 58.894 N | 011.163 E | 14-September-2010 | Christer Erséus |
| CE37        | Naididae    | Aktedrilus arcticus (Erséus, 1978) | + | + | >459 | 153 | 258 | KY637025 No voucher | Sweden, Bohuslän, Strömstad, Tjärnö, beach in front of Research Station, intertidal sand | 58.8755 N | 11.1458 E | 1-August-1997 | Christer Erséus |
| CE1790      | Naididae    | Aulodrilus acutus Ohtaka & Usman, 1997 | + | + | >426 | 153 | 377 | KY637027 SMNH 160319 | Cambodia, Kampong Chnhang, Lake Tonle Sap | 12.261 N | 104.681 E | 21-May-2005 | Akifumi Ohtaka |
| CE14362     | Naididae    | Aulodrilus japonicus Yamaguchi, 1953 | + | + | >515 | 153 | 389 | KY982550 No voucher | Australia, Victoria, Acheron River (NE of Melbourne), gravel and sand | 37.3526 S | 145.7066 E | 12-April-2012 | C. Erséus & Richard Marchant |
| CE281       | Naididae    | Aulodrilus pluriseta Piguet, 1906 | + | − | − | >64 | 288 | KY637028 No voucher | Estonia, Rannu, Vörtsjärv Limnological Station, lab culture kept by Tarmo Timm | 58.212 N | 26.110 E | 1-December-2000 | Timm Tarmo |
| CE196_2     | Naididae    | Baltidrilus costatus (Claparède, 1863) | + | + | >715 | 153 | 481 | KY637029 No voucher | Sweden, Bohuslän, Strömstad, Koster archipelago, subtidal sand, | 58.875 N | 11.080 E | 1-September-2000 | Christer Erséus |
| CE17439     | Naididae    | Bathyrhilus formosus Erséus, 1986 | + | + | >591 | 153 | 357 | KY982551 SMNH 162134 | Bahamas, Exuma, cut between Darby Island and Little Darby Island, 6 m, coarse sand | 23.8559 N | 76.2248 W | 1-April-2013 | Christer Erséus |
| CE17759     | Naididae    | Bothrioneurum vejdoovskyanum Stoic, 1886 | + | + | 351 | 153 | 251 | KY982552 SMNH 162135 | Sweden, Södermanland, Vingåker, Läppe, Hjälmaren Lake, sand and gravel | 59.13 N | 15.81 E | 27-July-2012 | Christer Erséus |

(Continues)
| Specimen ID | Family name | Species                  | GenBank     | Location and habits                                                                 | Latitude  | Longitude  | Date             | Collector          |
|-------------|-------------|--------------------------|-------------|----------------------------------------------------------------------------------|-----------|------------|------------------|--------------------|
| CE2213      | Naididae    | Branchiodrilus hortensis | KY982553    | Netherlands, Utrecht, Overvecht, city canal along Moldaudef                     | 52.1156   | 5.1261 E   | 4-September-2006 | M. Vilhelm        |
| CE12487     | Naididae    | Branchura sp             | No voucher  | China, Hubei, Wuhan, Donghu Lake                                                | 30.55 N   | 114.358 E  | 15-June-2011     | Hong-zhu Wang      |
| CE112       | Naididae    | Clitellio arenarius      | KY637031    | Sweden, Bohuslän, Strömstad, Tjärnö, Tjärnäviken, subtidal sand                | 58.876 N  | 11.145 E   | 1-November-1998  | Christer Erséus   |
| CE138       | Naididae    | Doliodrilus tener        | KY637032    | China, Hainan, E of Sanya City, fish pond at road to Teng Hai, brackish water, coarse sand with black mud | 18.28 N   | 109.73 E   | 16-March-2000    | Christer Erséus   |
| CE14133     | Naididae    | Doliodrilus tener        | SMNH 162136 | Hong Kong, New territories, Mai Po marshes                                     | 22.49 N   | 114.03 E   | 1-December-2011  | Qiu Jian-wen       |
| CE754       | Naididae    | Epirodrilus pygmaeus     | SMNH 82594  | Czech Republic, about 60 km W of Brno, Rokytňá village, Rokytňá River (Thay River basin) | 49.17 N   | 15.79 E    | 1-May-2004       | Jana Schenkova     |
| CE236       | Naididae    | Heronidrilus fastigatus  | SMNH 160321 | New Caledonia, Loyalty Islands, Lifou, Baie de Chateaubriand, Wé, 0.5 m, marine, medium sand; | 20.55 S   | 167.17 E   | 21-November-2000 | Christer Erséus   |
| CE18212     | Naididae    | Heronidrilus gravidus    | SMNH 162137 | Belize, off Dangriga, sand bores area between Carrie Bow Cay and Wee Wee Cay, 2 m | 16.7589 N | 88.1127 W  | 13-April-2013    | Judith Zimmermann, Cecilia Wentrup & Christer Erséus |
| Specimen ID | Family name | Species | 606F/1082R | 29F/1084R | ITS1 (bp) | ITS2 (bp) | GenBank | Voucher ID | Location and habits | Latitude | Longitude | Date | Collector |
|------------|-------------|---------|-------------|-----------|-----------|-----------|----------|-----------|----------------------|-----------|-----------|------|----------|
| CE17490    | Naididae    | Heterodrilus ersei | +         | +         | >377      | 153       | 296      | KY982563 | SMNH 162138 Bahamas, Exuma, Norman’s Pond Cay, lagoon outlet channel, coarse sand, | 23.7681 N | 76.1313 W | 2-April-2013 | Christer Erséus |
| CE18015    | Naididae    | Inaniidrilus leukodermatus | +         | +         | 416       | 153       | 258      | KY982564 | SMNH 162139 Belize, off Dangriga, Carrie Bow Cay, seagrass bed, shallow subtidal, fine sand | 16.8030 N | 88.0812 W | 11-April-2013 | Judith Zimmermann, Cecilia Wentrup & Christer Erséus |
| CE131      | Naididae    | Limnodriloides anxius | +         | +         | >880      | 153       | 353      | KY637034 | No voucher Bahamas, Exuma, Lee Stocking Island, subtidal sand | 23.77 N | 76.10 W | 20-April-1999 | Christer Erséus |
| CE16954    | Naididae    | Limnodriloides australis | +         | +         | >876      | 153       | 312      | KY982566 | SMNH 162140 Australia, Queensland, Heron Island | 23.44528 S | 151.91316 E | 31-August-2012 | Cecilia Wentrup, Manuel Kleiner & C. Erséus |
| CE2730     | Naididae    | Limnodrilus cf. cervix | +         | +         | >480      | 153       | 408      | KY982567 | SMNH 162141 Sweden, Västergötland, Ålängsås, Anten Lake, shallow water, sand | 57.9911 N | 12.4072 E | 4-August-2007 | Christer Erséus |
| CE2128     | Naididae    | Limnodrilus claparedianus/cervix (see Liu, et al., 2017) | +         | +         | 378       | 153       | 346      | KY369387 | SMNH 159226 Germany, Osnabrück, lab culture at Zool Dep, Univ Osnabrück | 52.283 N | 8.033 E | 16-November-2006 | Annette Bergter |
| CE1785     | Naididae    | Limnodrilus grandisetosus | +         | +         | 471       | 153       | 515      | KY637016 | SMNH 160311 Indonesia, Central Kalimantan, Tehang Lake | 2.029 S | 113.934 E | 21-March-2005 | Akifumi Ohtaka |
| CE1786     | Naididae    | Limnodrilus grandisetosus | +         | -         | >107      | 359       | 397      | KY637017 | SMNH 160312 Japan, Shimosakamoto, south basin of Biwa Lake | 35.053 N | 135.891 E | 13-February-2003 | Akifumi Ohtaka |
| Specimen ID | Family name | Species | 606F/1082R | 29F/1084R | ITS1 (bp) | ITS2 (bp) | GenBank | Voucher ID | Location and habits | Latitude | Longitude | Date | Collector |
|-------------|-------------|---------|-------------|------------|------------|-----------|---------|------------|---------------------|-----------|-----------|------|-----------|
| CE1784      | Naididae    | *Limnodrilus hoffmeisteri* Claparède, 1862 (s.str., IX) (See Liu, et al., 2017) | +          | +          | 341       | 153       | KY369406 | SMNH 159141 | Japan, Akita-ken, Minamakita-gun, Gojôme-machi, Akita Prefecture, Lake Hachiro-gata | 39.933 N | 140.082 E | 9-July-2005 | Akifumi Ohtaka |
| CE22814     | Naididae    | *Limnodrilus hoffmeisteri* II (See Liu, et al., 2017) | +          | -          | -         | >70       | KY652931 | SMNH 158977 | Switzerland, Chêne-Bougeries, Chemin de la Montagne 22C, Seymaz River, organic (mostly leaf) matter (10-25 cm) | 46.199 N | 6.194 E | 24-August-2014 | Yingkui Liu |
| CE2740      | Naididae    | *Limnodrilus hoffmeisteri* VIII (See Liu, et al., 2017) | +          | +          | 349       | 153       | KY369440 | SMNH 159126 | Sweden, Västergötland, Vårgårda, Lången Lake, 0.5-1 m, sand | 57.997 N | 12.587 E | 9-August-2007 | Christer Erséus |
| CE1991      | Naididae    | *Limnodrilus hoffmeisteri* X (see Liu, et al., 2017) | +          | +          | 338       | 153       | KY369446 | SMNH 159181 | Sweden, Västergötland, Vårgårda, Lången Lake, shallow water | 58.011 N | 12.582 E | 7-August-2006 | Christer Erséus |
| CE10781     | Naididae    | *Limnodrilus rubripennis* Loden, 1977 | +          | +          | 547       | 153       | KY637018 | SMNH 160313 | USA, Louisiana, Tangipahoa Co, Tangipahoa River at bridge on Road 10, near Arcola, sandy river bank | 30.777 N | 90.498 W | 16-January-2011 | Christer Erséus |
| CE10853     | Naididae    | *Limnodrilus rubripennis* Loden, 1977 | +          | +          | 550       | 153       | KY637020 | SMNH 160315 | USA, Louisiana, Washington Co., Silver Creek, at bridge near Mount Hermon, muddy sand on banks and in water | 30.971 N | 90.289 W | 17-January-2011 | Christer Erséus |
| CE10482     | Naididae    | *Limnodrilus sulphurensis* Fend, Liu & Erséus, 2016 | +          | +          | >589      | 153       | KY637022 | DMNS ZEA.6275 | USA, Colorado, Routt Co, City of Steamboat Springs, Sulfur Cave, high H2S stream in dark zone | 40.48 N | 106.75 W | 11-April-2010 | David Steinmann & Fred Lüüszer |

(Continues)
| Specimen ID | Family name | Species | $606F$/1082R | $29F$/1084R | ITS1 (bp) | 5.8S (bp) | ITS2 (bp) | GenBank | Voucher ID | Location and habits | Latitude | Longitude | Date | Collector |
|-------------|-------------|---------|---------------|-------------|-----------|-----------|-----------|---------|-----------|---------------------|----------|-----------|------|-----------|
| CE1839      | Naididae    | *Limnodrillus udekemianus* Claparède, 1862 | +          | +          | >524      | 153       | 392       | KY982568 | SMNH 162142 | Sweden, Småland, Jönköping, Strömsbergsbäcken Stream | 57.753 N | 14.182 E | 21-May-2006 | Daniel Gustafsson |
| CE211       | Naididae    | *Lophochaeta ignota* Štolc, 1886 | +          | +/-        | >188      | 153       | 439       | KY637036 | No voucher | Sweden, Västergötland, Vårgårda, Längen Lake | 57.997 N | 12.887 E | 1-October-2000 | Christer Erséus |
| CE20081     | Naididae    | *Monopylephorus irroratus* (Verrill, 1873) | +          | +          | >370      | 153       | 330       | KY982571 | No voucher | Norway, Östfold, Fredrikstad, Öyenkilen, marina at Öyenkilveien, seashore | 59.1733 N | 10.8485 E | 23-September-2013 | Christer Erséus |
| CE50        | Naididae    | *Monopylephorus rubroniveus* Levinsen, 1884 | +          | +          | >435      | 153       | 370       | KY637037 | No voucher | Norway, Sogn og Fjordane, Luster, Nes, seashore | 58.84 N | 17.87 E | 1-September-1998 | Michael Norén |
| CE19318     | Naididae    | *Nais elinguis* Müller, 1774 | +          | +          | >421      | 153       | 292       | KY982572 | No voucher | Norway, Sogn og Fjordane, Luster, Nes, seashore | 61.3864 N | 7.3691 E | 12-August-2013 | Christer Erséus |
| CE16885     | Naididae    | *Olavius albidus* (Jamieson, 1977) | +          | +          | >400      | 153       | 274       | KY982573 | SMNH 162143 | Australia, Queensland, Heron Island | 23.4434 S | 151.9131 E | 30-August-2012 | Cecilia Wentrup, Manuel Kleiner & C. Erséus |
| CE17410     | Naididae    | *Potamothrix bavaricus* (Oschmann, 1913) | +          | +          | >479      | 153       | 383       | KY982574 | No voucher | Australia, Western Australia, S of Dunsborough, about 20 km S of Yallingup, near Woodlands, Willyabrup Brook at Caves Road, stream | 33.7948 S | 115.0313 E | 17-September-2012 | Christer Erséus, Adrian Rinder & Yongde Cui |
| CE283       | Naididae    | *Potamothrix moldavensis* Vejdovský & Mrázek, 1903 | +          | +          | >466      | 153       | 396       | KY637042 | No voucher | Estonia, Ramu, Võrtsjärv Limnological Station, lab culture kept by Tarmo Timm | 58.212 N | 26.110 E | 1-December-2000 | Timm Tarmo |

(Continues)
| Specimen ID | Family name | Species                        | 606F/1082R | 29F/1084R | ITS1 (bp) | 5.8S (bp) | ITS2 (bp) | GenBank     | Voucher ID | Location and habits                                                                 | Latitude | Longitude | Date       | Collector                  |
|-------------|-------------|--------------------------------|------------|-----------|-----------|-----------|-----------|-------------|------------|---------------------------------------------------------------|-----------|------------|------------|---------------------------|
| CE2883      | Naididae    | Psammoryctides albicola        | +          | +         | >487      | 153       | 518       | KY637043    | SMNH 160323 | Sweden, Södermanland, Österåker, Vingåker, Låttären Lake, sand near shore | 59.0854  | 16.0426    | 30-July-2007 | Christer Erséus |
| CE289       | Naididae    | Psammoryctides barbatus        | +          | +         | 391       | 153       | 373       | KY637044    | No voucher | Estonia, Rannu, Vörtsjärv Limnological Station, lab culture kept by Tarmo Timm | 58.212   | 26.110     | 1-December-2000 | Tarmo Timm   |
| CE623       | Naididae    | Rhyacodrilus coccineus         | +          | +         | 340       | 153       | 308       | KF267996    | No voucher | Sweden, Västergötland, Vårgårda, stream between Iglåsjön and Långens Lakes, sand | 58.0103  | 012.5836   | 6-July-2003  | Christer Erséus |
| CE17550     | Naididae    | Smithsonidrilus hummelinioki   | +          | +/-       | >69       | 153       | 697       | KY982576    | SMNH 162144 | Bahamas, Exuma, Little Darby Island, in front of Research Station, intertidal sand | 23.8558  | 76.2248    | 4-April-2013 | Christer Erséus |
| CE1984      | Naididae    | Spirosperma ferox              | +          | -         | -         | >69       | >306      | KY982577    | SMNH 162145 | Sweden, Västergötland, Vårgårda, Långens Lake, shallow water | 58.0111  | 12.582     | 6-August-2006 | Christer Erséus |
| CE18140     | Naididae    | Thalassodrilides bruneti       | +          | +         | >514      | 153       | 281       | KY982579    | SMNH 153613 | Belize, off Dangriga, Carrie Bow Cay, shallow substratal, 0.7 m | 16.803   | 88.082     | 12-August-2013 | Judith Zimmermann, Cecília Wentrup & Christer Erséus |
| CE2038      | Naididae    | Trieminentia corderoi          | +/-        | -         | -         | >63       | >161      | KY982580    | SMNH 104788 | Argentina, Entre Ríos, 31.665 S NW of Paraná City, floodplain lake connected to Middle Paraná River | 60.590   | 60.590     | 18-August-2006 | Mercedes Marchese |
| CE2044      | Naididae    | Tubifex blanchardi             | +          | +         | >604      | 153       | 446       | KY637046    | SMNH 160324 | Belgium, Oost-Vlaanderen, near Schoonaarde, Paddebeek River | 51.02    | 4.05       | 7-September-2006 | Jan Soors |
| Specimen ID | Family name | Species | 606F/1082R | 29F/1084R | ITS1 (bp) | ITS2 (bp) | GenBank | Voucher ID | Location and habits | Latitude | Longitude | Date | Collector |
|-------------|-------------|---------|------------|------------|-----------|-----------|---------|-----------|-------------------|----------|-----------|------|----------|
| CE272       | Naididae    | Tubifex newaensis (Michaelsen, 1903) | + | + | >490 | 153 | 336 | KY637047 | No voucher | Estonia, Rannu, Vörtsjärv Limnological Station, lab culture kept by Tarmo Timm | 58.212 N | 26.110 E | 1-December-2000 | Timm Tarmo |
| CE212       | Naididae    | Tubifex smirnowi Lastockin, 1927 | + | + | 447 | 153 | 321 | KY637048 | No voucher | Sweden, Västerås, Värgårda, Lången Lake | 57.997 N | 12.887 E | 13-July-2002 | Christer Erséus |
| CE276       | Naididae    | Tubifex tubifex (Müller, 1774) | + | + | >515 | 153 | 393 | KY637049 | No voucher | Originally from Kyrgyzstan Republic, Frunze (Bishkek); kept in Timm’s lab culture | 42.85 N | 74.37 E | 1-December-2000 | Timm Tarmo |
| CE186       | Naididae    | Tubificoides benedii (Udekom, 1855) | + | + | >464 | 153 | 332 | KY637050 | No voucher | Sweden, Bohuslän, Strömstad, Tjärnö, at Research Station, intertidal sand | 58.876 N | 11.146 E | 1-September-2000 | Christer Erséus |
| CE3600      | Naididae    | Varichaetadrilus cf. angustipenis (Brinkhurst and Cook, 1966) | - | - | - | - | - | SMNH 160325 | USA, Alabama, Madison County, Huntsville, WEUP Radio Station Pond | 34.7603 N | 86.6431 W | 17-March-2008 | Christer Erséus & Mark Wetzel |
| CE3621      | Naididae    | Varichaetadrilus sp (see Liu, et al., 2017) | +/- | - | >105 | 494 | KY637051 | SMNH 160326 | USA, Alabama, Madison County, Huntsville, WEUP Radio Station Pond | 34.7603 N | 86.6431 W | 17-March-2008 | Christer Erséus & Mark Wetzel |
| CE14357     | Phreodrilidae | Antarctodrilus proboscidea (Brinkhurst & Fulton, 1979) | + | - | >85 | 747 | KY982548 | No voucher | Australia, Victoria, Acheron River (NE of Melbourne), gravel and sand | 37.3526 S | 145.7066 E | 12-April-2012 | C. Erséus & Richard Marchant |
| CE14476     | Randiellidae | Randiella sp (undescribed) | + | + | >548 | 153 | 319 | KY982575 | No voucher | Australia, Queensland, Lizard Island, Watson’s Bay, Ferrier’s Creek, brackish water | 14.666 S | 145.451 E | 20-April-2012 | Christer Erséus |
with recognizable 5.8S region were selected for primer design. Many such published ITS sequences are commonly co-amplified with some rDNA residues, but the various parts of the (18S)-ITS1-5.8S-ITS2-(28S) sequences are neither properly annotated nor partitioned. It is widely accepted that an accurate alignment of positional homologies is highly important for the final phylogenetic reconstruction (Katoh & Standley, 2013; Ogden & Rosenberg, 2006). However, indel events make multiple alignment of divergent ITS sequences challenging, due to a high risk of inferring false-positive positional homologies and increasing artefactual support for incorrect relationships (Nagy et al., 2012). In particular, when incomplete ITS sequences are included in an alignment, short unannotated 18S and 28S residues are prone to misalign with highly variable ITS spacer sequences. Moreover, if residues are <25 nucleotides long, annotation of ITS sequences with short mismatches with highly variable ITS spacer sequences.

Palme et al., 2013; Nagy et al., 2012). Our new ITS sequences, amplified from 11 clitellate families, are meant to be used as references to improve annotation of similar amplicons in the future.

4.2 Limitations of universal ITS primers

Universal ITS primers do not perfectly match their annealing template sequences of all organisms (see https://unite.ut.ee/primers.php). Even for the well-studied Kingdom Fungi, it is difficult to amplify the whole ITS region of all groups using a single universal primer pair (Konieczny, Roterman-Konieczna, & Spólnik, 2014). The in silico analyses of published data showed that the ITS primers traditionally used for clitellates are neither universal nor efficient enough for this group; for example, the primer 5.8SF may have up to five mismatches with its template DNA (Figure 2). Although this result may have been biased by the limited number of clitellate sequences (and lacking representation of some families) in the EMBL database, we also observed notable mismatches (Figure S1) between the newly amplified complete ITS sequences (using 29F/1084R) and primers targeting 5.8S rDNA: E58S-F1, ITS3, 5.8SF, 5.8SR, ITS1B, and E58S-R1 (see also Figure 2). Unfortunately, there is not much information about the flanking 18S rDNA (Figure S1) to optimize the specific clitellate primers for amplification of the whole ITS region. Still, however, as noted above, Martinsson and Erséus (2014) obtained a 909-bp ITS sequence (KF672519) from the DNA extract of an enchytraeid (CE11317) using the universal primer pair ITS5/ITS4, but for which we failed when using 29F/1084R. This may be explained by the former authors’ use also of 5.8SF/5.8SR, which in this case only show a few mismatches with KF672519.

For primers, in general, even one or a few mismatches between primer and DNA template may jeopardize amplification (Bellemain et al., 2010; Bru, Martin-Laurent, & Philipott, 2008; Huang, Arnheim, & Goodman, 1992; Ihrmark et al., 2012; Wright et al., 2014; Wu, Hong, & Liu, 2009). In addition, especially for clitellates feeding on plant material and fungi (Bonkowski, Griffiths, & Ritz, 2000; Curry & Schmidt, 2006; Uchida et al., 2004), it could be hypothesized that universal primers may amplify fragments of contaminating plant or fungal sequences instead of sequences of clitellates. However, it is likely to avoid, or at least minimize, contamination, and also amplification of pseudogene sequences, using the new primer 606F, which targets a specific conservative motif in the clitellate 5.8S.

The sensitivity of PCR success rate to primer mismatches probably needs further investigation, but amplification of GC-rich ITS sequences may be improved by following a combination strategy of adding enhancers and modifying the PCR cycle conditions (Mamedov et al., 2008; Saheb, Saini, Tiwari, Saxena, & Singh Saini, 2007). In our case, however, the GC contents of the whole ITS and its partial ITS2 sequence are almost equal. It seems that the length of target loci is more critical for successful amplification and sequencing than any of the other factors mentioned above. To use a single primer pair to amplify ITS sequences longer than about 1,500 bp is challenging. Thus, to choose one of the generally much shorter ITS amplicons (with flanking rDNAs providing reliable primer templates) may be the optimal option for broad samples of clitellate taxa.

4.3 Choosing primers

Although only two-thirds of the citellate samples were successfully amplified using the primer pair 29F/1084R, the in silico test showed that the specificity of this primer pair is better than that of IT5S/ITS4 and ETTS1/ETTS2 (Figure 3). Therefore, when this pair proves to work for some clitellate taxa, it is likely to be a good option for sequencing the ITS region as a whole; that is, if it is <about 1,500 bp long.

The in silico results not only give a hint about the relative performance of commonly used and new ITS primer pairs, but they also predict potential nontarget amplicons and length of amplicons before selecting a primer pair for studies of a specific clitellate group. In the in silico test of different ITS2 primers, 5.8SF/ITS4 theoretically performed better than 606F/1082R, that is, the former pair amplified more clitellate sequences and less nonclitellate sequences than the latter (Figure 3). However, this was only under rather relaxed conditions (2-3 mismatches allowed). Moreover, poor specificity of the 5.8SF (as shown in the Figure S1), originally designed for bivalves (Källersjö et al., 2005), limits the potential number of ITS2 amplicons. Because of this, while ITS5/ITS4 produced almost 70,000 nonclitellate ITS2 amplicons, 5.8SF/ITS4 could only generate a very low number of ITS2 amplicons (Figure 3). On the other hand, 606F, targeting a conservative and unique 5.8S motif of clitellates, was much more specific than any of the older primers for clitellates (Figure 2; Figure S1). The pair 606F/1082R also had a lower success rate in silico amplifications of nonclitellate groups (Figure 3). Therefore, this new primer pair is more suitable than other published primers to amplify the ITS2 regions from a taxonomically broad range of clitellates.

The primer with a 3'-terminal "A" nucleotide, that is, our new primers 29F, 606F, and 1082R, may be less efficient in amplifications using Taq DNA polymerase, regardless of the corresponding nucleotide in the template strand (Arezi, Xing, Sorge, & Hogrefe, 2003; Ayyadevara, Thaden, & Shmookler Reis, 2000). Therefore, alternative polymerases may help to increase the success rate for some clitellate specimens.
For some polyploid clitellates (e.g., within Lumbricidae, Enchytraeidae, and Naididae (see Casellato, 1984; Gregory & Hebert, 2002) with multiple copies of the ITS region, however, sequencing using our new primer may still be challenging. This is because the Sanger sequencing method can only be performed on a single pure amplicon. Using a particular PCR primer pair to amplify multiple copies of a gene may lead to double peaks in the chromatograms at sites that differ between the copies. The PCR may even fail completely because all sites after indels (introns leading to sequence length differences) will produce seemingly undecipherable double peaks (Griffin, Robin, & Hoffmann, 2011). In such cases, the software Champuru (http://seqphase.mpg.de/champuru/), which is able to detect and separate the gene copies, may be useful for diploids (Flot, 2007), while cloning or Next Generation Sequencing may be more practical tools for polyploids (Aversano et al., 2012; Brassac & Blattner, 2015; Griffin et al., 2011).

5 | CONCLUSION

This study has shown that the new primer pair 606F/1082R has great specificity in amplification of the ITS2 of Clitellata, at least for the 18 families investigated by either in vitro or in silico analyses: Bdellodrilidae, Branchiobdellidae, Cambarincola, Capillventridae, Enchytraeidae, Erpobdellidae, Glossiphoniidae, Glossoscolecidae, Haemadipsidae, Haemoporidae, Haplotaxidae, Hirudinidae, Lumbricidae, Lumbriculidae, Megascolecidae, Naididae, Phreodrilidae, and Randiellidae. This will facilitate many kinds of molecular systematic studies of this common and ecologically important group of worms. The other pair, 29F/1084R amplifying the whole ITS, will be a useful complement to existing ITS primers.

ACKNOWLEDGMENTS

The first author was sponsored by a PhD student fellowship from the China Scholarship Council. The second author was supported by the Swedish Research Council, and the Royal Society of Arts and Sciences in Gothenburg. We are grateful to Per Alström, Svante Martinsson, and two anonymous reviewers, for constructive criticism on the manuscript.

CONFLICT OF INTEREST

None declared.

REFERENCES

Aird, D., Ross, M. G., Chen, W. S., Danielsson, M., Fennell, T., Russ, C., ... Gniirke, A. (2011). Analyzing and minimizing PCR amplification bias in illumina sequencing libraries. Genome Biology, 12, R18.
Alvarez, I., & Wendel, J. F. (2003). Ribosomal ITS sequences and plant phylogenetic inference. Molecular Phylogenetics and Evolution, 29, 417–434.
Arezi, B., Xing, W., Sorge, J. A., & Hogrefe, H. H. (2003). Amplification efficiency of thermostable DNA polymerases. Analytical Biochemistry, 321, 226–235.
Aversano, R., Ercolano, M. R., Caruso, I., Fasano, C., Rosellini, D., & Carputo, D. (2012). Molecular tools for exploring polyploid genomes in plants. International Journal of Molecular Sciences, 13, 10316.
Ayyadevara, S., Thaden, J. J., & Shmoolker Reis, R. J. (2000). Discrimination of primer 3′-nucleotide mismatch by taq DNA polymerase during polymerase chain reaction. Analytical Biochemistry, 284, 11–18.
Bazzicalupo, A. L., Balint, M., & Schmitt, I. (2013). Comparison of ITS1 and ITS2 RNA in 454 sequencing of hyperdiverse fungal communities. Fungal Ecology, 6, 102–109.
Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P., & Kauwserud, H. (2010). ITS as an environmental DNA barcode for fungi: An in silico approach reveals potential PCR biases. BMC Microbiology, 10, 189.
Bengtsson-Palme, J., Ryberg, M., Hartmann, M., Branco, S., Wang, Z., Godhe, A., ... Nilsson, R. H. (2013). Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. Methods in Ecology and Evolution, 4, 914–919.
Bertrand, C., Janzen, D. H., Hallwachs, W., Burns, J. M., Gibson, J. F., Shokralla, S., & Hajibabaei, M. (2014). Mitochondrial and nuclear phylogenetic
Katoh, K., & Standley, D. M. (2013). MAFFT Multiple Sequence Alignment Software Version 7: Improvements in performance and usability. *Molecular Biology and Evolution, 30*, 772–780.

Keller, A., Forster, F., Muller, T., Dandekar, T., Schultz, J., & Wolf, M. (2010). Including RNA secondary structures improves accuracy and robustness in reconstruction of phylogenetic trees. *Biology Direct, 5*, 4.

Keller, A., Schleicher, T., Schultz, J., Muller, T., Dandekar, T., & Wolf, M. (2009). 5.8S-28S rRNA interaction and HMM-based ITS2 annotation. *Gene, 430*, 50–57.

Kerans, B. L., Rasmussen, C., Stevens, R., Colwell, A. E., & Winton, J. R. (2004). Differential propagation of the metazoan parasite Myxobolus cerebralis by Limnodrilus hoffmeisteri, Ilyodrillus templetoni, and genetically distinct strains of Tubifex tubifex. *Journal of Parasitology, 90*, 1366–1373.

Kodandaramaiah, U., Simonsen, T. J., Bromilow, S., Wahlberg, N., & Sperling, M. H., & Azeredo-Espin, A. M. L. (2012). Molecular phylogenetics of the oysters’ subfamily Saccostreinae and the genus *Ostrea*. *Molecular Phylogenetics and Evolution, 65*, 112–127.

Kowata, K., & Matsumoto, S. (2006). Cryptic diversity in the well-studied terrestrial worm *Cognettia* sphagnetorum (Clitellata: Enchytraeidae). *Biology Direct, 1*, 27–35.

Kowata, K., & Ryséus, C. (2017). Barcoding gap, but no support for cryptic speciation in the earthworm *Aporrectodea longa* (Clitellata: Lumbricidae). *Mitochondrial DNA, 28*, 147–155.

Kownacka, M., & Sagorski, J. (2015). Mapping the cleavage sites on mammalian pre-rRNAs: Where do we stand? *Biochimie, 94*, 1521–1532.

Liu, Y. K., Ogden, T. H., & Rosenberg, M. S. (2006). Multiple sequence alignment accuracy and phylogenetic inference. *Systematic Biology, 55*, 314–328.

Mamedov, T. G., Pienaar, E., Whitney, S. E., & Winton, J. R., Pienaar, E., Whitney, S. E., & Winton, J. R. (2012). Deceptive single-focus taxonomy and phylogeny: Wolbachia-associated divergence in mitochondrial DNA is not reflected in morphology and nuclear markers in a butterfly species. *Ecology and Evolution, 3*, 5167–5176.

Kohout, P., Sudoval, R., Janoušková, M., Čtvrtilková, M., Hejda, M., Pánková, H., Sykorová, Z. (2014). Comparison of commonly used primer sets for evaluating arbuscular mycorrhizal fungal communities: Is there a universal solution? *Soil Biology and Biochemistry, 68*, 482–493.

Koljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., & Larsson, K. H. (2013). Towards a unified paradigm for sequence-based identification of fungi. *Molecular Ecology, 22*, 5271–5277.

Komenclzy, L., Roterman-Konieczna, I., & Spólnik, P. (2014). Systems biology functional strategies of living organisms. Dordrecht: Springer Netherlands.

Kook, E., Vedler, E., Püssa, K., Kaleamares, R., Reier, U., & Pihu, S. (2015). Intra-individual ITS polymorphism and hybridization in *Limnodrilus obscura* Dumort. and *Limnodrilus angustifolia* L. (Boraginaceae). *Plant Systematics and Evolution, 301*, 893–910.

Kvit, S., Sarkar, I. N., & Eréus, C. (2010). Genetic variation and phylogeny of the cosmopolitan marine genus *Tubificoides* (Annelida: Clitellata: Naididae: Tubificinae). *Molecular Phylogenetics and Evolution, 57*, 687–702.

Li, J., Yang, Y., Henry, R. J., Rossetto, M., Wang, Y., & Chen, S. (2015). Plant DNA barcoding: From gene to genome. *Biological Reviews, 90*, 157–166.

Liu, Y. K., Fend, S. V., Martinsson, S., & Eréus, C. (2017a). Extensive cryptic diversity in the cosmopolitan sludge worm *Limnodrilus hoffmeisteri* (Clitellata: Naididae). *Organisms Diversity & Evolution, 17*, 477–495.

Liu, Y. K., Fend, S. V., Martinsson, S., Luo, X., Ohtaka, A., & Eréus, C. (2017b). Multi-locus phylogenetic analysis of the genus *Limnodrilus* (Annelida: Clitellata: Naididae). *Molecular Phylogenetics and Evolution, 112*, 244–257.

Malo, D., & Posada, D. (2016). Multilocus inference of species trees and DNA barcoding. *Philosophical Transactions of the Royal Society B: Biological Sciences, 371*, 20150335.

Mamedov, T. G., Pienaar, E., Whitney, S. E., TerMaat, J. R., Carvill, G., Goliath, R., ... Vlijgen, H. (2008). A fundamental study of the PCR amplification of GC-rich DNA templates. *Computational Biology and Chemistry, 32*, 452–457.

Marinho, M. A. T., Junqueira, A. C. M., Paulo, D. F., Espósito, M. C., Villet, M. H., & Azevedo-Espín, A. M. L. (2012). Molecular phylogenetics of *Oestroidea* (Diptera: Calyptratae) with emphasis on *Calliphoridae*: Insights into the inter-familial relationships and additional evidence for paraphyly among blowflies. *Molecular Phylogenetics and Evolution, 65*, 840–854.

Martin, P., Martinez-Ansemili, E., Pinder, A., Tinn, T., & Wetzel, M. (2008). Global diversity of oligochaetous clitellates (“Oligochaeta”; Clitellata) in freshwater. *Hydrobiologia, 595*, 117–127.

Martin, P. J., & Rygiewicz, P. T. (2005). Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microbiology, 5*, 28.
structures to identify three new species in Paramacrobiotus (Tardigrada), Organisms Diversity & Evolution, 10, 287–296.

Schirmer, M., Ijaz, U. Z., D’Amore, R., Hall, N., Sloan, W. T., & Quince, C. (2015). Insight into biases and sequencing errors for amplicon sequencing with the Illumina MiSeq platform. Nucleic Acids Research, 43, e37.

Schoch, C. L., Robbertse, B., Robert, V., Vu, D., Cardinali, G., Irinyi, L., … Federhen, S. (2014). Finding needles in haystacks: Linking scientific names, reference specimens and molecular data for Fungi. Database, 2014, 1–21.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., … Consortium, F. B. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences of the United States of America, 109, 6241–6246.

Schultz, J., Maisel, S., Gerlach, D., Muller, T., & Wolf, M. (2005). A common core of secondary structure of the internal transcribed spacer 2 (ITS2) throughout the Eukaryota. RNA, 11, 361–364.

Schultz, J., Muller, T., Achtziger, M., Seibel, P. N., Dandekar, T., & Wolf, M. (2006). The internal transcribed spacer 2 database—a web server for (not only) low level phylogenetic analyses. Nucleic Acids Research, 34, W704–W707.

Selig, C., Wolf, M., Muller, T., Dandekar, T., & Schultz, J. (2008). The ITS2 Database II: Homology modelling RNA structure for molecular systems. Nucleic Acids Research, 36, D377–D380.

Shekhovtsov, S. V., Golovanova, E. V., & Peiltel, S. E. (2013). Cryptic diversity within the Nordenskiold’s earthworm, Eisenia nordenskioldi subsp. nordenskioldi (Lumbricidae, Annelida). European Journal of Soil Biology, 58, 13–18.

Siddall, M. E., Trontelj, P., Utevsky, S. Y., Nkamany, M., & Macdonald, K. S. (2007). Diverse molecular data demonstrate that commercially available medicinal leeches are not Hirudo medicinalis. Proceedings of the Royal Society of London B: Biological Sciences, 274, 1481–1487.

Simmons, M. P., & Freudenstein, J. V. (2003). The effects of increasing genetic distance on alignment of, and tree construction from, rDNA internal transcribed spacer sequences. Molecular Phylogenetics and Evolution, 26, 444–451.

Sipos, R., Szekely, A. J., Palatinsky, M., Revesz, S., Marialigeti, K., & Nikolausz, M. (2007). Effect of primer mismatch, annealing temperature and PCR cycle number on 16S rRNA gene-targeting bacterial community analysis. FEMS Microbiology Ecology, 60, 341–350.

Siqueira, F. F., Sandes, S. H. C., Drumond, M. A., Campos, S. H., Martins, R. P., da Fonseca, C. G., & Carvalho, M. R. S. (2013). Genetic diversity and population genetic structure in giant earthworm Rhinodrilus alatus (Annelida: Clitellata: Glossoscolecidae). Pedobiologia, 56, 15–21.

Sket, B., & Trontelj, P. (2008). Global diversity of leeches (Hirudinea) in freshwater. Hydrobiologia, 595, 129–137.

Toju, H., Tanabe, A. S., Yamamoto, S., & Sato, H. (2012). High-Coverage ITS Primers for the DNA-Based Identification of Ascomycetes and Basidiomycetes in Environmental Samples. PLoS ONE, 7, e40863.

Trebitz, A. S., Hoffman, J. C., Grant, G. W., Billehus, T. M., & Pilgrim, E. M. (2015). Potential for DNA-based identification of Great Lakes fauna: Match and mismatch between taxa inventories and DNA barcode libraries. Scientific Reports, 5, 12162.

Trontelj, P., & Sket, B. (2000). Molecular re-assessment of some phylogenetic, taxonomic and biogeographic relationships between the leech genera Dina and Trocheta (Hirudinea: Erpobdellidae). Hydrobiologia, 438, 227–235.

Trontelj, P., & Utevsky, S. Y. (2005). Celebrity with a neglected taxonomy: Molecular systematics of the medicinal leech (genus Hirudo). Molecular Phylogenetics and Evolution, 34, 616–624.

Trontelj, P., & Utevsky, S. Y. (2012). Phylogeny and phylogeography of medicinal leeches (genus Hirudo): Fast dispersal and shallow genetic structure. Molecular Phylogenetics and Evolution, 63, 475–485.

Uchida, T., Kaneko, N., Ito, M. T., Futagami, K., Sasaki, T., & Sugimoto, A. (2004). Analysis of the feeding ecology of earthworms (Megascolecidae) in Japanese forests using gut content fractionation and delta N-15 and delta C-13 stable isotope natural abundances. Applied Soil Ecology, 27, 153–163.

Villalobos, G., Orozco-Mosqueda, G. E., Lopez-Perez, M., Lopez-Escamilla, E., Cordoba-Aguilar, A., Rangel-Gamboa, L., … Martinez-Hernandez, F. (2014). Suitability of internal transcribed spacers (ITS) as markers for the population genetic structure of Blastocystis spp. Parasites & Vectors, 7, 461.

Vivien, R., Wyler, S., Lafont, M., & Pawlowski, J. (2015). Molecular barcoding of aquatic oligochaetes: Implications for biomonitoring. PLoS ONE, 10, e0125485.

Wang, X.-C., Liu, C., Huang, L., Bengtsson-Palme, J., Chen, H., Zhang, J. H., … Li, J. Q. (2015). ITS1: A DNA barcode better than ITS2 in eukaryotes? Molecular Ecology Resources, 15, 573–586.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In D. H. G. Michael. A. Innis, J. J. Sninsky & T. J. White (Eds.), PCR protocols: A guide to methods and applications (pp. 315–322). New York, NY: Academic Press.

Wiemmers, M., Keller, A., & Wolf, M. (2009). ITS2 secondary structure improves phylogeny estimation in a radiation of blue butterflies of the subgenus Agroaetis (Lepidoptera: Lycaenidae: Polyommatinae). BMC Evolutionary Biology, 9, 300.

Williams, B. W., Gelder, S. R., Proctor, H. C., & Coltman, D. W. (2013). Molecular phylogeny of North American Branchiobdellida (Annelida: Clitellata). Molecular Phylogenetics and Evolution, 66, 30–42.

Wright, E. S., Yilmaz, L. S., Ram, S., Gasser, J. M., Harrington, G. W., & Noguera, D. R. (2014). Exploiting extension bias in polymerase chain reaction to improve primer specificity in ensembles of nearly identical DNA templates. Environmental Microbiology, 16, 1354–1365.

Wu, J. H., Hong, P. Y., & Liu, W. T. (2009). Quantitative effects of position and type of single mismatch on single base primer extension. Journal of Microbiological Methods, 77, 267–275.

Yao, H., Song, J. Y., Liu, C., Luo, K., Han, J. P., Li, Y., … Chen, S. L. (2010). Use of ITS2 region as the universal DNA barcode for plants and animals. PLoS ONE, 5, e13102.

SUPPORTING INFORMATION
Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Liu Y, Erséus C. New specific primers for amplification of the Internal Transcribed Spacer region in Clitellata (Annelida). Ecol Evol. 2017;7:10421–10439. https://doi.org/10.1002/eece3.3212