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Haplotaxis gordioides (Hartmann in Oken, 1819) (Annelida, Clitellata) as a sub-cosmopolitan species: a commonly held view challenged by DNA barcoding

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Abstract

Haplotaxis Hoffmeister, 1843 is a poorly known genus: it is rarely collected because it usually inhabits groundwater, and many species are only known from immature specimens. Even the type species, *Haplotaxis gordioides* (Hartmann in Oken, 1819) remains poorly defined because of the absence of reference types. Most of the *Haplotaxis* species have been placed in synonymy with *H. gordioides* since the end of the 19th century, a situation that has remained essentially unchanged until now. As a result, the species is supposedly present on all continents, except Antarctica. This observation is all the more questionable as the aquatic subterranean environment is nowadays well known to harbour many species with restricted distribution, due to the strong hydrogeographic isolation and the low dispersal abilities of its inhabitants.

In this study we assessed the hypothesis of *H. gordioides* as a single species with wide distribution versus a complex of cryptic species with narrow distribution. We used a DNA-barcoding approach based on the COI mitochondrial marker of 46 *Haplotaxis* specimens collected in Switzerland, mostly as part of a countrywide sampling campaign to study groundwater macroinvertebrates.

Preliminary results suggested that *H. gordioides* is a complex of at least 6 cryptic species in Switzerland, which has important implications both for the knowledge of the exact identity of the type species and for the synonymy of most of the species described in the 19th century. However, as it is based on a single-locus approach, this study should be seen as the first step in an integrative taxonomic process that should include additional biological material, the study of complementary markers (especially nuclear), and the morphological study of specimens.

Key words: aquatic oligochaetes, Clitellata, *Haplotaxis gordioides*, DNA barcoding, diversity

Introduction

Among the aquatic oligochaetes, the genus *Haplotaxis* Hoffmeister, 1843 has been of constant interest since the discovery and the description of the type species, *Haplotaxis gordioides* (Hartmann in Oken, 1819) by Georg Leonhard Hartmann, in the well of his property in St. Gallen (Switzerland) (Hartmann, 1821). This interest is due to several specific features of this genus. Its constituent species are generally considered as carnivorous, a rare feeding mode within the oligochaetes, which are primarily detritivores (Brinkhurst, 1988). The genus has a cosmopolitan distribution (Timm & Martin, 2015) but remains poorly known because of its rarity related to its presence mainly in groundwater and the fact that several species are only known from immature specimens (Brinkhurst & Jamieson,

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1971; Michaelsen, 1899). Like the other members of the family Haplotaxidae, the genus also shows anatomical simplicity, especially of the reproductive organs (Brinkhurst, 1984). Because of these distinctive features, the genus, and the family Haplotaxidae in general, played a key role early in the understanding of oligochaete evolution, as a potential basal lineage or as an evolutionary lineage close to the megadriles (Crassiclitellata) (Brinkhurst, 1984, 1994). Since then, the situation has been changing and the family Haplotaxidae has been shown to be a non-monophyletic family, with some haplotaxids grouping with Crassiclitellata + Moniligastridae or sister (in part) to Lumbriculata (Erséus *et al.*, 2020).

The type species *Haplotaxis gordioides* alone illustrates the problems linked to a fundamental lack of knowledge of this family: supposedly present on most continents, it remains poorly defined because of the absence of reference types and the lack of information on the type locality.

In his revision of the genus, Michaelsen (1899) was the first to suggest that all previously described species of *Haplotaxis* from Europe and North America belong to a single, slightly variable species. This conclusion was based on the observation that many characters used to characterise the different species were the result of erroneous or incomplete observations, or that differences depended mainly on the age of the animal. Soon, however, Michaelsen (1905) questioned his earlier judgement when he realised that it was only through the study of mature specimens of *Haplotaxis ascaridoides* Michaelsen, 1905 that he was able to distinguish this new species from *Haplotaxis gordioides* s. lat. (basically, 4 vs. 3 spermathecal pairs and dorsolateral vs. lateral spermathecal pores), a distinction he would have been unable to make from immature specimens alone. Despite this, he still considered all occurrences of *Haplotaxis* from Germany and neighbouring countries to belong to a single species *Haplotaxis gordioides*, but he regarded the assignment of specimens from other continents (Asia and North America) to this species as doubtful. Since then, the situation has remained almost unchanged. In his taxonomic analysis of the Haplotaxidae, Brinkhurst (1988) retained many species as *species dubia*, admitting that "material is too scarce and fragmentary to allow for a proper revision of these species".

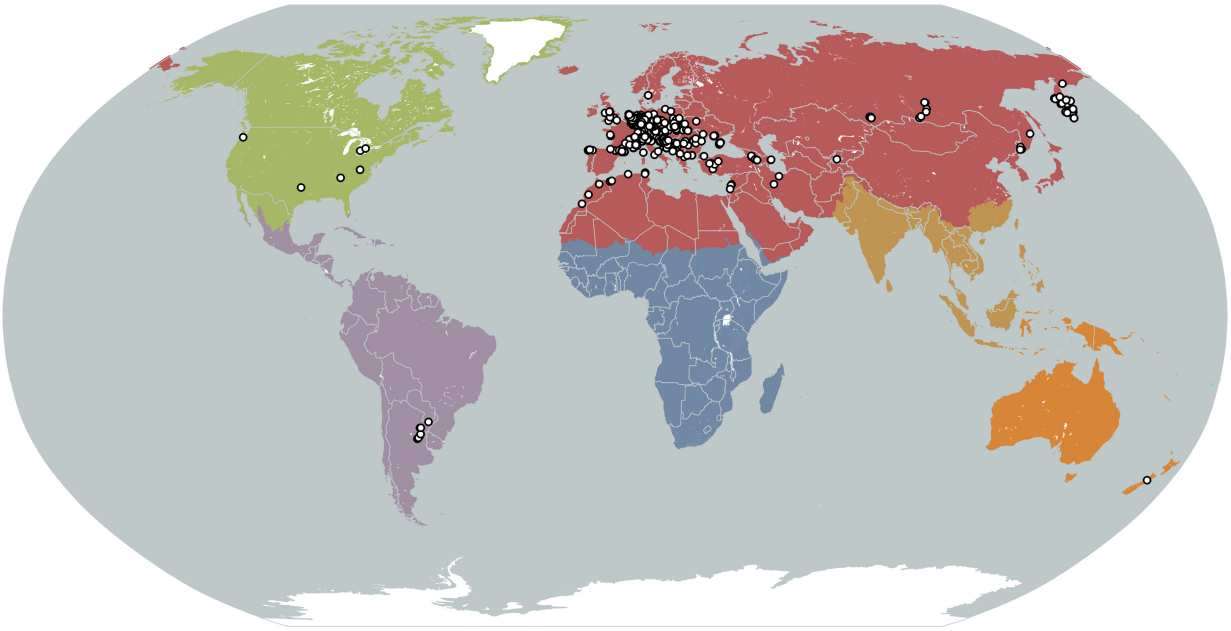

FIGURE 1. Global distribution of *Haplotaxis gordioides* according to biogeographic realms (Dinerstein *et al.*, 2017). Distribution data from GBIF.org (2022a); shapefiles of ecoregions from: https://ecoregions.appspot.com/.

To date, 80% of the 1122 occurrence records of the genus *Haplotaxis* mentioned in GBIF (Global Biodiversity Information Facility; GBIF.org, 2022b) are related to *H. gordioides* or "*Haplotaxis* cf. *gordioides*". The species is documented from all biogeographic realms, except Afrotropic, Indo-Malay, Oceania and Antarctica (Fig. 1). From an ecological point of view, *H. gordioides* is usually considered to be a stygophile (i.e., with strong hypogean affinities — Gibert, Stanford, *et al.*, 1994) being present in a diversity of habitats generally associated with groundwater (springs, caves, wells, hyporheic zone of streams) (Artheau & Giani, 2006). This observation is interesting given that

groundwater habitats are known to have exceptionally high levels of endemism caused by strong hydrogeographical isolation and low dispersal abilities of their inhabitants (Gibert *et al.*, 2009). Trontelj *et al.* (2009) even suggested that groundwater species showing range sizes over 200 km were most likely an assemblage of cryptic species with much smaller geographic ranges. Hence, the validity of a sub-cosmopolitan, or even European *H. gordioides* as a single species is questionable.

Recently, significant material of *Haplotaxis* cf. *gordioides* was collected in Switzerland as part of a countrywide sampling campaign to study groundwater macroinvertebrates (see: Alther *et al.*, 2020; Alther *et al.*, 2021). Some were also found in Switzerland in surface sediments as part of projects on the development of sediment quality indices based on oligochaete community composition, and others were collected in a cave. This Swiss material gives us the opportunity to provide a first insight into the species diversity within *H. gordioides* s. lat., using a Sanger-based DNA barcoding approach, and to test the hypothesis of *H. gordioides* as a single species with a sub-cosmopolitan distribution.

Material and methods

Specimens

Most *Haplotaxis* specimens were obtained during the above-mentioned sampling campaign. Faunistic samples were collected at spring catchment boxes (hereafter referred to as spring boxes), where groundwater is sourced passively through perforated pipes and then fed into local drinking water supply systems. Sampling was conducted directly by the local drinking water providers, with instructions and sampling material provided (same method as in Alther *et al.* 2021 and Studer *et al.* 2022). The sampling campaign was carried out between January 2021 and May 2022. Water providers were asked to apply two complementary sampling methods, depending on the accessibility of the water inside the spring box. First, the water providers could attach a filternet (mesh size 800 µm, Sefiltec AG, Höri, Switzerland) to the inlet of the drainage pipe(s). With this method, organisms were filtered directly from the passively inflowing groundwater. Water providers were instructed to attach the filternet for seven days and then to collect any organisms. As a second method, a small hand net (mesh size 350 µm, JBL GmbH & Co. KG, Neuhofen, Germany) was provided to sample the sedimentation/overflow basin inside the spring box. The collected organisms were stored in 80% ethanol at 4 °C until further processing. All organisms were identified under a stereomicroscope (Leica M205 C) based on Schminke and Gad (2007), and oligochaetes were separated from other groundwater organisms. Thirty-eight specimens of *Haplotaxis* were found among these collected oligochaetes (sites No. 1–31 and 45; 8 cantons: Thurgau, St. Gallen, Zug, Bern, Vaud, Grisons, Valais and Zurich).Beside this material, two *Haplotaxis* specimens (sites No. 36 & 37) were sampled in a cave in Klein Melchtal (Canton Obwalden) by Martin Trüssel (NeKO-Stifung, Alpnach), speleologist, and five specimens (sites No. 34, 35, and 44) were sampled in surface sediments (fine—coarse sediments) of streams, at locations where groundwater seepage is suspected (Cantons Bern, Neuchâtel and Vaud) as part of projects (OligoGen, EcoImpact) on the development of oligochaete indices (e.g. Vivien *et al.*, 2020; Vivien *et al.*, 2017; Vivien *et al.*, 2019) (Table 1).

Only one *Haplotaxis* specimen was obtained at each site, except at sites No. 5 and 29 (2 specimens per site) and at sites No. 14, 15 and 34 (3 specimens per site). All collected *Haplotaxis* specimens (45 in total, hereafter referred to by the sampling site number and a letter when there are several specimens per site) were stored in 80% or 100% ethanol at 4 °C or –20 °C.

For each *Haplotaxis* specimen, the posterior part (about 0.5 cm long) was cut and preserved in absolute ethanol at –20 °C (in Eppendorf tubes) for subsequent genetic analyses. The anterior part of these specimens was stored in absolute ethanol at –20 °C (in vials) for further morphological analysis. These specimens best fitted the morphological diagnosis of Michaelsen (1899) and Brinkhurst (1966), still used as a reference for *H. gordioides*, and were therefore tentatively identified as *H.* cf. *gordioides.*

TABLE 1. Specimens included in the study, with their place of deposit (POD—RBINS: Royal Belgian Institute of Natural Sciences), general inventory numbers (I.G.), voucher numbers (N/V: no voucher), the individual specimen numbers used in molecular studies, MOTUs identified in the ASAP analysis, collection data (country, canton/department, locality, municipality, habitat, latitude, longitude—datum WGS84, station and sampling identifiers, collection date, collector) and GenBank accession numbers. Collectors: KAA = M. Knüsel, R. Alther, F. Altermatt (AmphiWell), RV = Régis Vivien, MT = Martin Trüssel, ML = P. Martin, M. Lagnika.

POD	IG No	Voucher	Specimen No	MOTU	Country	Canton/ Department	Locality	Habitat	Long.	Lat.	Station ID	Sample ID	Collection date	Collector	COI
RBINS	IG 34562	22.241.02	1	2	Switzerland	Thurgau	Wängi, near Ragatz	groundwater: aquifer	8.9647	47.4774	1	23_K_ 2_olig	3/25/2021	KAA	OQ351740
RBINS	IG 34562	23.003.01	2	7	Switzerland	St. Gallen	Schänis, near Hüsliberg	groundwater: aquifer	9.0751	47.1921	2	106_F_ 5_olig	3/29/2021	KAA	OQ351741
RBINS	IG 34562	23.003.02	3	7	Switzerland	St. Gallen	Schänis, near Hüsliberg	groundwater: aquifer	9.0751	47.1921	3	106_F_ 12_olig	4/6/2021	KAA	OQ351742
RBINS	IG 34562	23.003.03	4	2	Switzerland	St. Gallen	Braunau, near Uerental	groundwater: aquifer	9.0698	47.4939	4	113_F_ 3_olig	3/25/2021	KAA	OQ351743
RBINS	IG 34562	23.003.04	5a	7	Switzerland	St. Gallen	Eschenbach, near Rossfallen	groundwater: aquifer	8.9878	47.2906	5	271_K_ 1_olig	3/31/2021	KAA	OQ351744
RBINS	IG 34562	23.003.05	5b	7	Switzerland	St. Gallen	Eschenbach, near Rossfallen	groundwater: aquifer	8.9878	47.2906	5	271_K_ 1_olig	3/31/2021	KAA	OQ351745
RBINS	IG 34562	23.003.06	6	7	Switzerland	Zug	Unterägeri, near Elsisried	groundwater: aquifer	8.5524	47.1223	6	58_K_ 1_olig	4/28/2021	KAA	OQ351746
RBINS	IG 34562	23.003.07	7	7	Switzerland	Zug	Unterägeri, near Elsisried	groundwater: aquifer	8.5524	47.1223	7	58_F_ 1_olig	5/5/2021	KAA	OQ351747
RBINS	IG 34562	23.003.08	8	7	Switzerland	Zug	Unterägeri, near Elsisried	groundwater: aquifer	8.5524	47.1223	8	58_F_ 7_olig	5/19/2021	KAA	OQ351748
RBINS	IG 34562	23.003.09	9	3	Switzerland	Bern	Rüeggisberg, near Beissern	groundwater: aquifer	7.4481	46.8191	9	201_K_ 1_olig	4/9/2021	KAA	OQ351749
RBINS	IG 34562	23.003.10	10	7	Switzerland	Bern	Rüeggisberg, near Kohliloch	groundwater: aquifer	7.4317	46.8284	10	201_F_ 4_olig	4/29/2021	KAA	OQ351750

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TABLE 1. (Continued)

POD	IG No	Voucher	Specimen No	MOTU	Country	Canton/ Department	Locality	Habitat	Long.	Lat.	Station ID	Sample ID	Collection date	Collector	COI
RBINS	IG 34562	23.003.11	11	7	Switzerland	Bern	Stocken-Höfen, near Baachegg	groundwater: aquifer	7.5555	46.7024	11	202_F_8_olig	6/23/2021	KAA	OQ351751
RBINS	IG 34562	23.003.12	12	7	Switzerland	Vaud	Corbeyrier, near Luan	groundwater: aquifer	6.9768	46.3582	12	282_F_7_olig	8/9/2021	KAA	OQ351752
RBINS	IG 34562	22.241.01	13	1	Switzerland	Grisons	Valsot, near Tschlin	groundwater: aquifer	10.4127	46.8836	13	523_F_7_olig	8/5/2021	KAA	OQ351753
RBINS	IG 34562	23.003.13	14a	7	Switzerland	Grisons	Laax, near Val Fraissen	groundwater: aquifer	9.2474	46.8122	14	578_K_8_olig	7/30/2021	KAA	OQ351754
RBINS	IG 34562	23.003.14	14b	7	Switzerland	Grisons	Laax, near Val Fraissen	groundwater: aquifer	9.2474	46.8122	14	578_K_8_olig	7/30/2021	KAA	OQ351755
RBINS	IG 34562	23.003.15	14c	7	Switzerland	Grisons	Laax, near Val Fraissen	groundwater: aquifer	9.2474	46.8122	14	578_K_8_olig	7/30/2021	KAA	OQ351756
RBINS	IG 34562	23.003.16	15a	7	Switzerland	Grisons	Laax, near Val Fraissen	groundwater: aquifer	9.2474	46.8122	15	578_K_9_olig	8/20/2021	KAA	OQ351757
RBINS	IG 34562	23.003.17	15b	7	Switzerland	Grisons	Laax, near Val Fraissen	groundwater: aquifer	9.2474	46.8122	15	578_K_9_olig	8/20/2021	KAA	OQ351758
RBINS	IG 34562	23.003.18	15c	7	Switzerland	Grisons	Laax, near Val Fraissen	groundwater: aquifer	9.2474	46.8122	15	578_K_9_olig	8/20/2021	KAA	OQ351759
RBINS	IG 34562	23.003.19	16	7	Switzerland	Grisons	Laax, near Val Fraissen	groundwater: aquifer	9.2472	46.8109	16	578_F_20_olig	8/18/2021	KAA	OQ351760
RBINS	IG 34562	23.003.20	17a	7	Switzerland	Vaud	Ollon, near Roc à l'Ours	groundwater: aquifer	7.0745	46.3214	17	290_K_1_olig	7/23/2021	KAA	OQ351761
RBINS	IG 34562	23.003.21	18a	7	Switzerland	Vaud	Ollon, near Roc à l'Ours	groundwater: aquifer	7.0745	46.3214	18	290_K_2_olig	7/29/2021	KAA	OQ351762
RBINS	IG 34562	23.003.22	19a	7	Switzerland	Vaud	Ollon, near Roc à l'Ours	groundwater: aquifer	7.0745	46.3214	19	290_K_3_olig	8/20/2021	KAA	OQ351763

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TABLE 1. (Continued)

POD	IG No	Voucher	Specimen No	MOTU	Country	Canton/ Department	Locality	Habitat	Long.	Lat.	Station ID	Sample ID	Collection date	Collector	COI
RBINS	IG 34562	23.003.23	20	7	Switzerland	Vaud	Ollon, near L'Aiguille	groundwater: aquifer	7.0661	46.3187	20	290_K_4_olig	8/27/2021	KAA	OQ351764
RBINS	IG 34562	23.003.24	21	7	Switzerland	Vaud	Ollon, near Roc à l'Ours	groundwater: aquifer	7.0745	46.3214	21	290_F_3_olig	8/20/2021	KAA	OQ351765
RBINS	IG 34562	22.238.01	22	7	Switzerland	Grisons	Laax, near Val Fraissen	groundwater: aquifer	9.2474	46.8122	22	578_F_26_olig	9/10/2021	KAA	OQ351766
RBINS	IG 34562	23.003.25	23	7	Switzerland	Grisons	Laax, near Val Fraissen	groundwater: aquifer	9.2472	46.8109	23	578_F_27_olig	9/10/2021	KAA	OQ351767
RBINS	IG 34562	23.003.26	24	7	Switzerland	Grisons	Domleschg, near Scheid	groundwater: aquifer	9.4525	46.7868	24	583_F_4_olig	8/18/2021	KAA	OQ351768
RBINS	IG 34562	23.003.27	25	6	Switzerland	Valais	Val d'Illiez, near Les Essertis	groundwater: aquifer	6.9175	46.1993	25	494_F_1_olig	8/5/2021	KAA	OQ351769
RBINS	IG 34562	23.003.28	26	7	Switzerland	Valais	Val d'Illiez, near Champoussin	groundwater: aquifer	6.8563	46.2085	26	494_F_4_olig	8/4/2021	KAA	OQ351770
RBINS	IG 34562	23.003.29	27	5	Switzerland	Valais	St. Niklaus, near Alpja	groundwater: aquifer	7.8339	46.1621	27	473_F_3_olig	9/16/2021	KAA	OQ351771
RBINS	IG 34562	23.003.30	28	5	Switzerland	Valais	St. Niklaus, near Alpja	groundwater: aquifer	7.8339	46.1621	28	473_F_7_olig	9/30/2021	KAA	OQ351772
RBINS	IG 34562	23.003.31	29a	7	Switzerland	Grisons	Albula/Alvra, near Vazerol	groundwater: aquifer	9.5807	46.6719	29	560_K_4_olig	9/14/2021	KAA	OQ351773
RBINS	IG 34562	23.003.32	29b	7	Switzerland	Grisons	Albula/Alvra, near Vazerol	groundwater: aquifer	9.5807	46.6719	29	560_K_4_olig	9/14/2021	KAA	OQ351774
RBINS	IG 34562	23.003.33	30	7	Switzerland	Grisons	Domat/Ems, near Samun	groundwater: aquifer	9.4503	46.8175	30	575_K_1_olig	10/26/2021	KAA	OQ351775

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TABLE 1. (Continued)

POD	IG No	Voucher	Specimen No	MOTU	Country	Canton/ Department	Locality	Habitat	Long.	Lat.	Station ID	Sample ID	Collection date	Collector	COI
RBINS	IG 34562	22.241.03	31	5	Switzerland	Grisons	Domat/Ems, near Val Aulta	groundwater: aquifer	9.4814	46.8150	31	575_F_5_olig	11/18/2021	KAA	OQ351776
RBINS	IG 34562	23.003.34	34a	3	Switzerland	Bern	Cortébert, in the Graben River, near the source of the river	fine/sandy sediments; possible groundwater exfiltration	7.1052	47.1776	34		3/31/2021	RV	OQ351777
RBINS	IG 34562	23.003.35	34b	3	Switzerland	Bern	Cortébert, in the Graben River, near the source of the river	fine/sandy sediments; possible groundwater exfiltration	7.1052	47.1776	34		3/31/2021	RV	OQ351778
RBINS	IG 34562	23.003.36	34c	3	Switzerland	Bern	Cortébert, in the Graben River, near the source of the river	fine/sandy sediments; possible groundwater exfiltration	7.1052	47.1776	34		3/31/2021	RV	OQ351779
RBINS	IG 34562	23.003.37	35	3	Switzerland	Neuchâtel	Fleurier at the source of the Fleurier River	fine/sandy sediments; possible groundwater exfiltration	6.5777	46.8914	35		3/31/2021	RV	OQ351780
RBINS	IG 34562	23.003.38	36	7	Switzerland	Obwald	Klein Melchtal	cave	8.2154	46.7864	36		2021	MT	OQ351781
RBINS	IG 34562	23.003.39	37	7	Switzerland	Obwald	Klein Melchtal	cave	8.2154	46.7864	37		2021	MT	OQ351782

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TABLE 1. (Continued)

POD	IG No	Voucher	Specimen No	MOTU	Country	Canton/ Department	Locality	Habitat	Long.	Lat.	Station ID	Sample ID	Collection date	Collector	COI
RBINS	IG 34562	23.003.40	44	3	Switzerland	Orbe	Vallorbe, in the Orbe River	coarse sediments, groundwater exfiltrations	6.3823	46.7116	44		2021	RV	OQ351783
RBINS	IG 34562	23.003.41	45	4	Switzerland	Zurich	near Dickbuch	groundwater: aquifer	8.8227	47.4899	45	35_F_ 19_olig	1/13/2022	KAA	OQ351784
RBINS	IG 34562	23.003.42	46	3	Switzerland	Geneva	in the Hermance River, near Hermance	fine/sandy sediments; possible groundwater exfiltration	6.2487	46.2976	46		2016	RV	LT598615
RBINS	IG 33953	15.294.18	LM18		Benin	Zou	Zogbodomey, Massi Dokpa	traditional hand-dug well	2.2427	6.9690	BEN008	F0015	26/07/15	ML	OQ249703
RBINS	IG 33953	15.294.48	LM48		Benin	Atlantique	Abomey Calavi, Akassato Pharmacie	traditional hand-dug well	2.3619	6.5111	BEN003	F0005	23/07/15	ML	OQ249705
RBINS	IG 33953	15.299.56	LM57		Benin	Plateau	Pobè, Isheko	traditional hand-dug well	2.6657	6.9871	ISH		13/06/14	ML	OQ249704
RBINS	IG 34181	16.288.01	LM60		Benin	Plateau	Adja-Ouèrè, Oke-Ola	traditional hand-dug well	2.5039	7.2222	BEN031	F0212	2/08/16	ML	OQ249702
RBINS	IG 34181	16.288.09	LM68		Benin	Collines	Savalou, Tchètti Lema	traditional hand-dug well	1.6719	7.8161	BEN072	F0219	10/08/16	ML	OQ249701

DNA analyses

Specimens. We analysed a dataset consisting of 50 *Haplotaxis* specimens, of which 5 were collected in Benin (Martin *et al.*, 2017) and included as outgroup (Table 1).

DNA extraction and sequencing. The 658 bp fragment of the 5'end of the cytochrome oxidase *c* subunit I gene (COI), which is recommended as a standard barcode fragment in animals (Hebert *et al.*, 2003), was amplified and sequenced for both the Swiss and Beninese material. The DNA of Beninese specimens was extracted and the COI fragment amplified according to Martin and Ohtaka (2008) and subsequently sequenced bidirectionally at Macrogen Europe BV (Amsterdam, The Netherlands).

For the Swiss *Haplotaxis*, a slightly different protocol was applied, as follows. Total genomic DNA was extracted using guanidine thiocyanate as described by Tkach and Pawłowski (1999). PCR amplifications were performed in a total volume of 20 µl containing 0.2 µl of Taq polymerase 5U/µl (Roche, Basel, Switzerland), 2 µl of the PCR buffer (10x concentrated) with MgCl2 (Roche), 0.5 µl of each primer (10 µM each), 0.4 µl of a mix containing 10 mM of each dNTP (Roche) and 1 µl of DNA template. The PCR comprised an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 40 s, annealing at 44°C for 45 s and elongation at 72°C for 1 min and a final elongation step at 72°C for 8 min.

COI products were then bi-directionally Sanger sequenced on an Applied Biosystems 96-capillary 3730xl instrument by the company Fasteris (Switzerland) using the same primers (mentioned above) and following the manufacturer's protocol. Raw sequence editing and contiguous sequence generation were performed using CodonCode Aligner (CodonCode Corporation).

We used the COI sequence of one specimen (Site No 46) found in the Geneva area (Hermance River) in 2016 from Vivien *et al.* (2017).

Molecular phylogeny. A phylogenetic tree was inferred by maximum likelihood using IQ-TREE v. 2.2.0 for macOS (Nguyen *et al.*, 2015), with the best-fit model, GTR+F+I+R2, automatically selected by the software via ModelFinder (Kalyaanamoorthy *et al.*, 2017), as well as optimisation of its parameters, and data partitioned according to codon position. Branch support was obtained with the ultrafast bootstrap with 1000 replicates (Hoang *et al.*, 2018).

Distance analysis. Uncorrected pairwise genetic distances were calculated using MEGA 11 (Tamura *et al.*, 2021). Genetic distances were calculated between sequences, and between and within MOTUs (Molecular Operational Taxonomic Units) as identified by the single-locus approaches.

Single-locus species delimitation. Species were delineated using single-locus methods following two complementary approaches, ASAP, a distance-based method (Puillandre *et al.*, 2021) and GMYC, a tree-based method (Pons *et al.*, 2006). Assembling species by automatic partitioning was done in selecting p-distances and both the Jukes-Cantor (JC69) and the Kimura (K80) substitution models to compute the distances, in order to investigate the possible impact of different distance estimates on the partitioning.

For the GMYC analysis, transition points between inter- and intra-species branching rates were estimated on a time-calibrated ultrametric tree reconstructed using BEAST v. 2.7.2 (Bouckaert *et al.*, 2019). The Bayesian inference of phylogeny was performed using the General time-reversible (GTR) nucleotide substitution model, a gamma category count of 4 with a shape parameter estimated by the software, the strict molecular clock model and the Yule prior with default parameters. The analysis was run with a Markov chain Monte Carlo (MCMC) length of 10 million. The first 10% of the trees were discarded as "burn-in" and marginal posterior estimates were checked with Tracer v1.7.2. (Rambaut *et al.*, 2021). The maximum credibility tree obtained from the BEAST analysis was imported in R v4.2.2 and submitted to the gmyc function available in the R package splits v1.0-20 (Ezard *et al.*, 2021).

Results

Species delimitation

Whatever the method (ASAP or GMYC), the single-locus approach identifies MOTUs that all correspond to supported clades (bootstrap values above 70) (Fig. 2). ASAP analyses consistently suggested the same partitioning into 7 different candidate species, regardless of how the distances were estimated (p-distances, JC69, K80). In contrast, the species

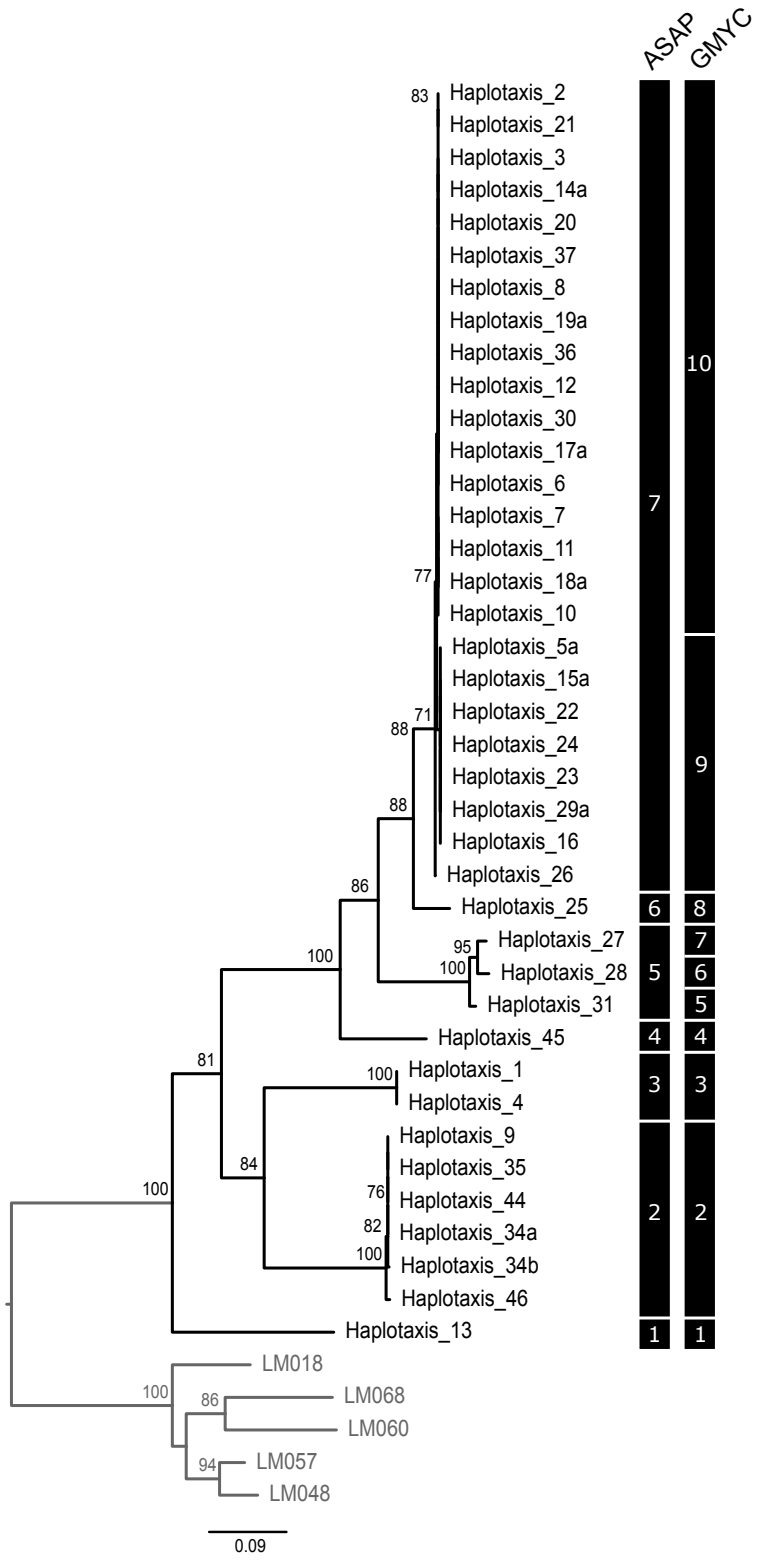

FIGURE 2. Molecular phylogeny constructed using the maximum likelihood method and COI gene fragment. Numbers at nodes are ultrafast bootstrap values (BV). Nodes were considered as supported if BVs were higher or equal to 70. The numbers following "*Haplotaxis*" refer to the identifiers of the specimens listed in Tab. 1. Partitions at the right side of the figure represent the results of the species delimitation analyses with single-locus methods (ASAP, GMYC).

delimitation analysis performed based on the generalized mixed Yule-coalescent (GMYC) model suggested 10 candidate species. By comparison with the ASAP method, the clade identified as MOTU 5 in the ASAP analysis was split into three distinct species hypotheses. Similarly, the large clade identified as MOTU 7 in the ASAP analysis was considered as two distinct MOTUs in the GMYC analysis, separating specimens (5a, 15a, 16, 22, 23, 24, 26 and 29a) from the rest of the group.

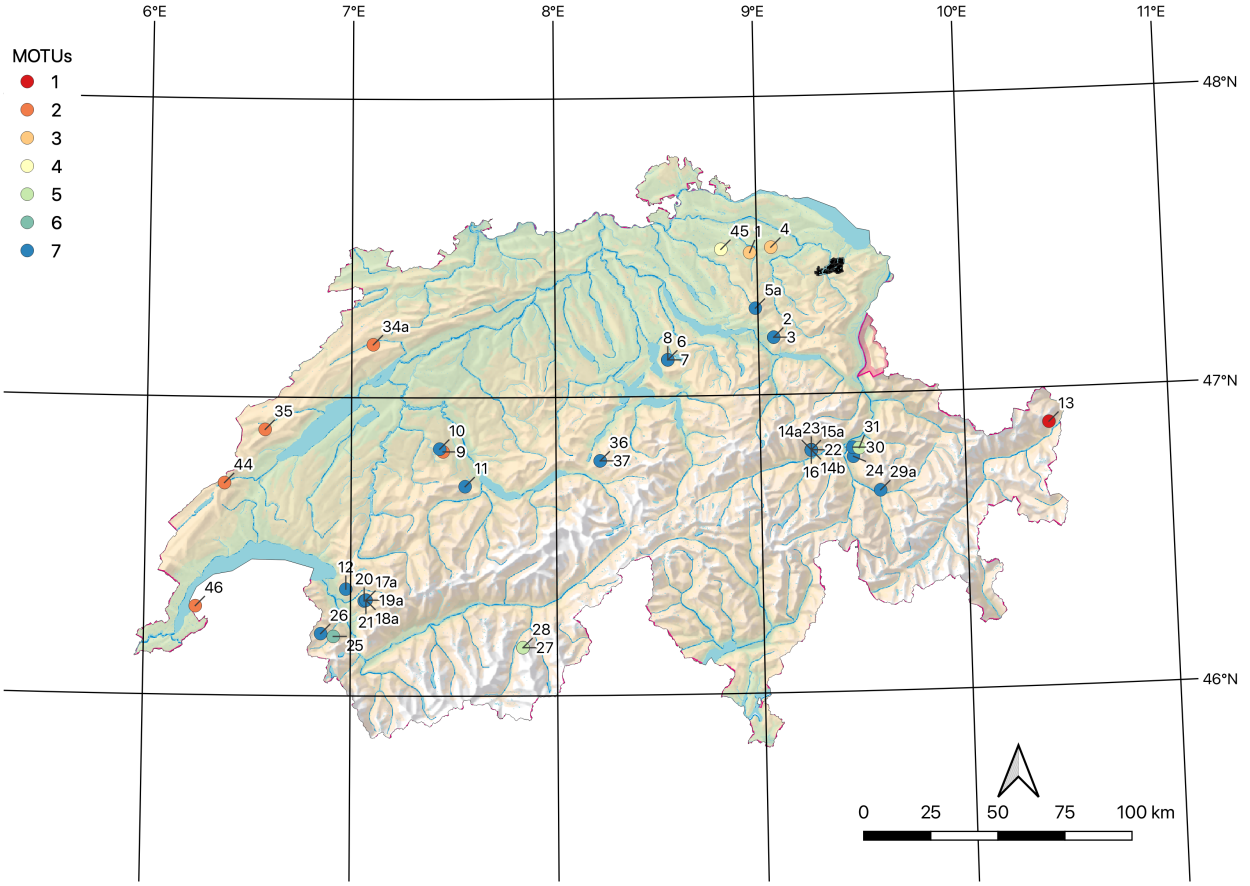

FIGURE 3. Location of the stations (in Switzerland) where the different Molecular Operational Taxonomic Units (MOTUs) were found (see colour codes). The MOTUs shown correspond to the partitioning obtained with ASAP (see Fig. 2). The numbers on the map refer to the identifiers of the specimens listed in Tab. 1. The agglomeration of St. Gallen is coloured in black (in the northwest of Switzerland).

Distance analysis

Several sequences were found to be 100% identical. When they corresponded to specimens from the same station, they were discarded from all subsequent analyses (5b, 14b, 14c, 15b, 15c, 29b, 34c). Uncorrected pairwise distances between specimens vary between 0.0 and 20.0 per cent. Considering the 7 MOTUs defined according to the outcome of the ASAP analysis (Fig. 2), the maximal intraspecific distances varied between 0.0 (MOTU 3) and 2.3% (MOTU 5) while the minimal interspecific distances were between 5.3 (MOTU 6 and MOTU 7) and 17.2% (MOTU 1 and MOTU 2) (Tab. 2). In contrast, if the maximal intraspecific distances were 0.3% for all MOTUs when GMYC results were considered, the minimal interspecific distances varied between 0.6% (MOTU 9 and MOTU 10) and 17.3% (MOTU 1 and MOTU3).

TABLE 2. Estimates of evolutionary divergence over sequence pairs between and within MOTUs identified with the ASAP analysis (average uncorrected pairwise distances in per cent).

	MOTU 1	MOTU 2	MOTU 3	MOTU 4	MOTU 5	MOTU 6	MOTU 7
MOTU 1	n/c						
MOTU 2	17.2	0.3					
MOTU 3	17.3	14.8	0.0				
MOTU 4	18.1	18.1	17.6	n/c			
MOTU 5	19.2	18.4	19.4	12.3	2.3		
MOTU 6	18.3	17.7	16.3	12.5	11.9	n/c	
MOTU 7	17.8	16.3	16.9	11.9	11.0	5.3	0.3

n/c = not calculated

Discussion

Species delimitation

It is generally accepted that, as a rule of thumb in clitellates, if two clusters differ from each other with more than 10% uncorrected distances, they are likely to belong to different species, and if they differ with less than 5%, they are likely to belong to one species (Schmelz *et al.*, 2017) (but see Liu *et al.* (2017) for exceptions within the *Limnodrilus hoffmeisteri* species complex). In this respect, all MOTUs identified with the ASAP approach are consistent with this observation as all of them, but one (MOTU 6 vs. MOTU 7) are separated by mean p-distances higher than 12% or even much more (Tab. 2). In contrast, the GMYC approach provided some species hypotheses that are highly unlikely when the method suggests MOTUs separated by interspecific distances as small as 0.6% (MOTU 9 and MOTU 10) or less than 3% (MOTUs 5, 6, 7).

The performance of each method is variable and subject to its own errors, resulting in either oversplitting or overlumping (Dellicour & Flot, 2018), and the GMYC approach is well known to belong to the first category (Luo *et al.*, 2018; Puillandre *et al.*, 2021). All methods of species delimitation perform poorly when the number of sampled individuals per species is too low (Ahrens *et al.*, 2016). This could explain an oversplitting bias of GMYC on our data, and the unlikely delimitation of specimen No 25 as a species hypothesis by both methods. In a recent study, Goulpeau *et al.* (2022) showed that distance-based methods (notably ASAP) are better suited to delimit earthworm species with DNA barcodes than phylogenetic methods, the latter, and in particular GMYC, having a strong tendency to oversplit species.

Provided that our results are further confirmed by testing them against other evidence in an integrative taxonomic framework, we can reasonably conclude that the so-called sub-cosmopolitan species *Haplotaxis gordioides* consists, in Switzerland, of a species complex of at least 6 species.

Haplotaxis gordioides as a species complex

The main objective of this preliminary study was to test the hypothesis of *H. gordioides* as a single species with a sub-cosmopolitan distribution versus a complex of cryptic species with a narrow distribution. For this, an accurate delimitation of species is not yet required, although it is highly desirable in the future. In this respect, the use of a single locus approach as a first step in a species delimitation is justified, despite its well-known weaknesses. Even if a single locus may not follow the history of the species, due to introgression and incomplete lineage sorting (Puillandre *et al.*, 2021), it nevertheless provides an overview on the diversity within a group. The present study should be seen as a first step of the integrative taxonomy process dedicated to the *Haplotaxis* genus.

The first results obtained on the genus *Haplotaxis* in Switzerland are particularly revealing: instead of a single supposed species, our results based on the COI barcode region suggest that this genus harbours at least six or seven highly divergent lineages, that could be potential distinct species. This does not come as a surprise: it has appeared, in recent decades, that the aquatic groundwater environment is a significant ecosystem in terms of macrobiological

diversity, endemism and relict species (Borko *et al.*, 2021; Dole-Olivier *et al.*, 2005; Gibert *et al.*, 2009; Gibert, Danielopol, *et al.*, 1994). For example, in Switzerland alone, the stygobiotic crustacean genus *Niphargus* contains at least 22 species, many of which were described or only even discovered within the last decade (Altermatt *et al.*, 2014; Altermatt *et al.*, 2019; Alther *et al.*, 2021; Fišer *et al.*, 2018). This has two important implications, one concerning the identity of *Haplotaxis gordioides* sensu stricto and the other in questioning the validity of all *Haplotaxis* species described before they were placed in synonymy by Michaelsen (1899).

Despite a large sampling countrywide, our results do not allow us to elucidate the exact identity of *Haplotaxis gordioides* (Hartmann in Oken, 1819) in the absence of specimens from the type locality at St. Gallen. As the two stations nearest to the agglomeration, stations 1 and 4, harbour the single MOTU No 3, it is tantalizing to consider this lineage as the same described by Hartmann two centuries ago. However, three distinct MOTUs (3, 4 and 7) are observed in a slightly wider geographical area, so that it would be hazardous to decide with the present data. Additional samples, ideally from St. Gallen, are required to check the species diversity at the level of the city itself. If it turns out that a few distinct species are present there, this will mean that we would never know the exact identity of the species described by Hartmann, justifying an arbitrary neotypification of the species.

By synonymising all *Haplotaxis* species described before him as *Phreoryctes* (=*Haplotaxis*) *gordioides*, Michaelsen (1899) made a taxonomic decision that affected all species recorded in Germany, whose type localities were sometimes separated by several hundred kilometres, but also species described from different countries (England, France, Switzerland, Czech Republic) and North America (as *Phreoryctes (= Haplotaxis) emissarius* Forbes, 1890). The present results clearly question this decision and, if the observation that *H. gordioides* s. str. is a cryptic species complex is confirmed by future studies in these different countries, a thorough taxonomic revision of the genus will be required.

Further efforts are currently being made to complete our dataset, by obtaining additional material in Switzerland, and in particular in the St. Gallen area, studying the morphology of specimens, and obtaining additional genetic data, including nuclear markers, to refine species hypotheses within the genus *Haplotaxis*. It is hoped that these will enable the problem of the identity of *Haplotaxis gordioides* s. str. to be solved in the near future and a taxonomy of the genus to be rebuilt on a sound basis. In the meantime, given the uncertainties about the exact morphological nature of *H. gordioides*, it is advisable to designate as "*H.* cf. *gordioides*" specimens that meet the following list of diagnostic characters: all chaetae simple-pointed, often hooked, dissimilar, with large single ventral chaetae, small dorsal and frequently reduced in number or absent, Timm's glands, gizzard in segments IV–V, and 2 segments with developing ovaries.

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