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Integrative taxonomy of the freshwater worm *Rhyacodrilus falciformis* s.l. (Clitellata: Naididae), with the description of a new species

Svante Martinsson, Ainara Achurra, Marcus Svensson & Christer Erséus

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The genetic and morphological variation within *Rhyacodrilus falciformis* Bretscher, 1901 (Clitellata: Naididae) in Europe was explored using an integrative approach, with three unlinked genetic markers [the mitochondrial cytochrome *c* oxidase subunit I (COI), the nuclear histone 3 (H3) and internal transcribed spacer region (ITS)] combined with morphology, to investigate whether this taxon constitutes a single or several species. Using Automatic Barcode Gap Discovery on the COI data set, the specimens were divided into seven clusters, used as hypothetical species that were further tested with the other data sources. Single-gene trees were estimated for all three markers, using coalescence analysis and they were in many parts incongruent with each other. Only one of the clusters was supported by all trees; it was also morphologically differentiated from the other clusters by the shape of its modified penial chaetae. This group consists of two specimens from the Crotot Cave in south-eastern France, and morphologically they fit a previously described but invalid variety, '*pigueti*', which is here described as a new species, *Rhyacodrilus pigueti* Achurra & Martinsson sp. n. The study highlights the fact that a single data source (e.g. COI barcodes) seldom provides a sufficient basis for taxonomic decisions such as species delimitation.

Corresponding author: *Svante Martinsson, Department of Biological and Environmental Sciences, University of Gothenburg, Box 463, SE-405 30 Göteborg, Sweden. E-mail: svante.martinsson@bioenv.gu.se*

Ainara Achurra, Station d'Ecologie Expérimentale du CNRS à Moulis, USR 2936, 09200, Moulis, France and Department of Zoology and Animal Cell Biology, Faculty of Science and Technology, University of the Basque Country, Box 644, 48080, Bilbao, Spain. E-mails: ainara.achurra@ehu.es

Marcus Svensson, Christer Erséus, and Svante Martinsson, Department of Biological and Environmental Sciences, University of Gothenburg, Box 463, SE-405 30, Göteborg, Sweden. E-mails: boo_marcus@hotmail.com, christer.erseus@bioenv.gu.se, svante.martinsson@bioenv.gu.se

Introduction

Cryptic species are species that are morphologically indistinguishable or so similar that they have been classified under the same species name (Bickford *et al.* 2007). This seems to be a common phenomenon among several organismal groups, not the least among clitellate worms (see review by Erséus & Gustafsson 2009). Due to the lack of externally discernible characters, especially in immature specimens, many clitellate species have proved hard to distinguish without the aid of molecular markers, and the diversity has in many cases been shown to be underestimated when based on morphology alone (e.g. Gustafsson *et al.* 2009; Envall *et al.* 2012; Matamoros *et al.* 2012; Novo *et al.* 2012).

The mitochondrial (mt) genome is haploid and almost exclusively transmitted maternally, which reduces the effective population size (N_e) to generally one-fourth of that of nuclear markers (Birky *et al.* 1989), increasing the genetic drift fourfold, resulting in faster lineage sorting and shorter time to monophyly (Neigel & Avise 1986). This together with the fact that mt genes normally evolve several times faster than nuclear genes (Brown *et al.* 1979, 1982; Lin & Danforth 2004) has made such markers widely used in studies of recent divergence and species delimitation in

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several animal groups including clitellates (e.g. Heethoff *et al.* 2004; James *et al.* 2010; Dózsa-Farkas *et al.* 2012). The cytochrome *c* oxidase subunit I (COI) gene is the most often used marker in such studies, and a fragment of COI has been proposed as the standard 'DNA barcode' for identifying animal taxa (Hebert *et al.* 2003). The identification is often based on the assumption that intraspecific divergence is distinctly lower than interspecific divergence (the so-called barcoding gap) and is performed by comparing the sequence of an unidentified individual with known sequences in a database (see review by Taylor & Harris 2012). However, it has been shown that the use of single mt markers for species delimitation can overestimate the numbers of species if used alone, that is, without nuclear markers (e.g. King *et al.* 2008; Dasmahapatra *et al.* 2010; Torres-Leguizamon *et al.* 2012; Achurra & Erséus 2013).

Species delimitation and species conceptualisation have long been debated among biologists, mostly regarding the species criteria. Different schools within the field have put forward their own different species concepts, based on, for example, reproductive isolation (Mayr 1942), specific mate recognition (Paterson 1985), different ecological niches (Van Valen 1976), monophyly (Rosen 1979) and diagnosability (Nelson & Platnick 1981). De Queiroz (2007) proposed a *unified species concept*, where being a separately evolving metapopulation lineage is the sole requirement of a species and where all other criteria (secondary species criteria) are used for assessing lineage separation (species delimitation). The greater the number of secondary species criteria supporting a divergence, the stronger the case is for speciation. However, one piece of evidence, if properly examined, may be enough to establish lineage separation. De Queiroz's view, which will be shared throughout this paper, is useful when dealing with cryptic species.

Rhyacodrilus falciformis Bretscher, 1901 (Clitellata: Naididae *sensu* Erséus *et al.* 2008) is widespread in the Holarctic region. It is found in freshwater bodies, but also in wet soil and seems to be associated with ground water (Timm *et al.* 1996; Erséus *et al.* 2005; Dumnicka 2006; Achurra & Rodriguez 2008), and it may therefore be regarded as a stygophilic species (Giani *et al.* 2011). The worm is about 5 mm long and can be identified by the sickle-shaped penial chaetae, which have given the species its name. These chaetae are located in close proximity to the male pores in sexually mature specimens. A variety, '*pigueti*', with straight penial chaetae was described by Juget (1967) from Lake Léman on the border between France and Switzerland. However, as the description of this variety was published after 1960, the name is not available and has no nomenclatorial status (ICZN 1999: §15.2). In the course of the study of the diversity of European freshwater clitellates using DNA barcoding, large genetic variation was observed in *R. falciformis*.

This study's aim is to explore the genetic and morphological variation within *Rhyacodrilus falciformis* in order to investigate whether this taxon comprises a complex of cryptic species or not.

Material and methods

Taxa and specimens

Thirty-six specimens of *Rhyacodrilus falciformis* were collected from various locations in France, Great Britain, Spain and Sweden, between 2003 and 2012 (Table 1). Wet soil samples and sandy sediments were taken in aquatic habitats (e.g. groundwater springs, caves, streams, ponds and lakes) for sieving and sorting under a dissecting microscope in the laboratory. After preliminary identification, specimens were divided into two parts; the anterior parts were stained with paracarmine and mounted in Canada balsam as outlined by Erséus (1994), as hologenophore vouchers (*sensu* Pleijel *et al.* 2008), and for morphological examinations, whereas the rear ends were transferred into 95% ethanol for subsequent DNA extraction and sequencing. For specimen CE14049, only a fragment was available and used for DNA extraction.

DNA sequencing and assembly

The posterior parts of all 36 individuals were selected for DNA extraction, using Qiagen's DNeasy Blood & Tissue Kit or Epicentre QuickExtract DNA Extraction Solution 1.0, following the manufacturer's instructions. One specimen was handled by Canadian Centre for DNA Barcoding (CCDB; Guelph, ON, Canada), with data stored at the Barcode of Life Data systems (BOLD). Parts of the mitochondrial cytochrome *c* oxidase subunit I (COI), the nuclear histone 3 (H3) and complete internal transcribed spacer region (ITS; ITS1, 5.8S rDNA and ITS2) were amplified using primers and PCR programs listed in Table S1. After amplification by PCR, the existence of the target genes was tested using 1% agarose gel electrophoresis. PCR products were purified using exonuclease I (Fermentas, Burlington, Canada) and FastAP thermosensitive alkaline phosphatase (Fermentas). Sequencing was carried out by Macrogen Inc. (Seoul, Korea) and Eurofins MWG Operon (Ebersberg, Germany). Sequences were assembled in GENEIOUS PRO v. 5.6.3 (Biomatters Ltd., Auckland, New Zealand) and aligned using MAFFT v6.814b (Katoh *et al.* 2002) as implemented in Geneious using the auto algorithm.

Distance analyses

Pairwise genetic distances were calculated for the COI data set in MEGA 5.1 using both uncorrected p-distances and the Tamura–Nei, 1993 (TN93) model, using pairwise deletion

Table 1 List of specimens included in the study, collection localities and dates, GenBank accession nos. and voucher nos

Species	ID no.	COI cluster	Collection locality	Collection date	GenBank accession no.			Voucher no.
					COI	ITS	H3	
Rhyacodrilus falciformis	CE631-1	E	Vitärtkällan Spring, Gotland, SE	September 2003	KF267920	KF267973	KF267939	SMNH130504
Rhyacodrilus falciformis	CE637	E	Vitärtkällan Spring, Gotland, SE	September 2003	KF267935	KF267974	KF267940	SMNH130505
Rhyacodrilus falciformis	CE795-1	F	Wet soil, Hällekis, Götene, SE	May 2004	KF267926	KF267990	KF267941	SMNH130506
Rhyacodrilus falciformis	CE795-2	F	Wet soil, Hällekis, Götene, SE	May 2004	KF267927	KF267991	KF267942	SMNH130507
Rhyacodrilus falciformis	CE795-3	F	Wet soil, Hällekis, Götene, SE	May 2004	KF267928	–	KF267943	SMNH130508
Rhyacodrilus falciformis	CE836	F	Säveån River, Lerum, SE	June 2004	KF267929	KF267988	KF267944	SMNH130509
Rhyacodrilus falciformis	CE1203	D	Swamp, Rya skog, Hisingen, SE	November 2005	KF267910	–	KF267945	SMNH130510
Rhyacodrilus falciformis	CE3868	F	Aspen Lake, Lerum, SE	April 2008	KF267913	KF267975	KF267956	SMNH130511
Rhyacodrilus falciformis	CE3869	F	Aspen Lake, Lerum, SE	April 2008	KF267914	–	KF267946	SMNH130512
Rhyacodrilus falciformis	CE3870	F	Aspen Lake, Lerum, SE	April 2008	KF267915	KF267976	KF267947	SMNH130513
Rhyacodrilus falciformis	CE4387	G	Stream, Omberg, Stocklycke, Ödeshög, SE	July 2008	KF267916	KF267977	KF267948	SMNH130514
Rhyacodrilus falciformis	CE4388	G	Stream, Omberg, Stocklycke, Ödeshög, SE	July 2008	KF267917	KF267978	KF267949	SMNH130515
Rhyacodrilus falciformis	CE4563	F	Stream, Frösön, Östersund, SE	July 2008	KF267918	KF267989	KF267950	SMNH130516
Rhyacodrilus falciformis	CE4564	G	Stream, Frösön, Östersund, SE	July 2008	KF267919	KF267980	KF267951	SMNH130517
Rhyacodrilus falciformis	CE6611	F	Wet soil, Uppsala Botanical Garden, SE	June 2009	IN260080[a]	–	–	SMNH130518
Rhyacodrilus falciformis	CE6612	F	Wet soil, Uppsala Botanical Garden, SE	June 2009	KF267921	KF267979	KF267957	SMNH130519
Rhyacodrilus falciformis	CE6615	D	Wet soil, Uppsala Botanical Garden, SE	June 2009	KF267922	–	KF267958	SMNH130520
Rhyacodrilus falciformis	CE6631	E	Prästfjärden Lake, Åkersberga, SE	June 2009	KF267923	KF267987	KF267952	SMNH130521
Rhyacodrilus falciformis	CE6632	E	Prästfjärden Lake, Åkersberga, SE	June 2009	KF267924	–	KF267953	SMNH130522
Rhyacodrilus falciformis	CE7737	F	Stream, Guldheden, Göteborg, SE	May 2010	KF267925	–	KF267954	SMNH130523
Rhyacodrilus falciformis	CE8714	C	Borehole, Abbotshood Farm, Devon, GB	January 2010	KF267930	KF267983	KF267955	SMNH130524
Rhyacodrilus falciformis	CE10955	C	Spring, Greatcombe Head Farm, Devon, GB	January 2010	KF267901	–	KF267959	SMNH130525
Rhyacodrilus falciformis	CE11311	G	Ögonakällan Spring, Östansjö, Hallsberg, SE	April 2011	KF267902	KF267981	KF267960	SMNH130526
Rhyacodrilus falciformis	CE11339	G	Spring, Öglunda, Skara, SE	April 2011	KF267903	KF267982	KF267961	SMNH130527
Rhyacodrilus falciformis	CE11351	F	Stream, Guldheden, Göteborg, SE	April 2011	KF267904	–	KF267962	SMNH130528
Rhyacodrilus falciformis	CE11366	F	Ditch, Guldheden, Göteborg, SE	April 2011	KF267900	–	KF267963	SMNH130529
Rhyacodrilus falciformis	CE11405	E	Stream,Vitsippsdalen, Göteborg, SE	March 2011	KF267905	–	KF267970	SMNH130530
Rhyacodrilus falciformis	CE11406	E	Stream,Vitsippsdalen, Göteborg, SE	March 2011	KF267906	KF267984	KF267964	SMNH130531
Rhyacodrilus falciformis	CE11451	F	Ditch, Guldheden, Göteborg, SE	March 2011	KF267907	KF267992	KF267965	SMNH130532
Rhyacodrilus falciformis	CE11452	D	Ditch, Guldheden, Göteborg, SE	March 2011	KF267908	KF267985	KF267966	SMNH130533
Rhyacodrilus falciformis	CE11489	E	Ground water seepage, Brännås, Lerum, SE	April 2011	KF267909	KF267986	KF267967	SMNH130534
Rhyacodrilus pigueti sp. n.	CE14048	A	Crotot Cave, Besançon, Dep. Doubs FR	January 2011	KF267911	KF267993	KF267968	SMNH Type-8475[b]
Rhyacodrilus pigueti sp. n.	CE14049	A	Crotot Cave, Besançon, Dep. Doubs FR	January 2011	KF267912	KF267994	KF267969	No voucher
Rhyacodrilus falciformis	CE16690	B	Jivero Cave, Matienzo, Cantabria, ES	October 2012	KF267932	–	KF267938	SMNH130535
Rhyacodrilus falciformis	CE16691	B	Jivero Cave, Matienzo, Cantabria, ES	October 2012	KF267931	–	KF267937	SMNH130536
Rhyacodrilus falciformis	CE16692	B	Jivero Cave, Matienzo, Cantabria, ES	October 2012	KF267933	–	KF267936	SMNH130537
Rhyacodrilus coccineus	CE623	–	Stream, Alingsås, SE	July 2003	GU902110[c]	KF267996	KF267971	No voucher
Rhyacodrilus subterraneus	CE10572	–	Spring, Willesleigh Farm, Devon, GB	January 2010	KF267934	KF267995	KF267972	SMNH130538

SE, Sweden; GB, Great Britain; FR, France, ES, Spain.
[a]BOLD accession no: ENSWD081-11, [b]Holotype, [c]From Erséus *et al.* (2010).

for missing data. Models were chosen after model testing conducted in MEGA 5.1 (Tamura *et al.* 2011) using the Bayesian information criterion (BIC). Histograms of distances were drawn in Microsoft Excel.

The TN93 distances were analysed with the online version of ABGD (Automatic Barcode Gap Discovery; Puillandre *et al.* 2012; available at http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) with default settings, to delimit

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mitochondrial clusters. The ABGD method detects the first significant gap (the barcoding gap) between the two modes of pairwise distance distributions, where the lower distance values presumably reflect the intraspecific differences and the higher the interspecific differences. The delimited COI clusters were used as hypothetical species that were further tested.

Coalescent analyses

Genealogies were estimated for the COI, ITS and H3 data sets under the null hypothesis that all the included specimens of *R. falciformis* constitute a single species. Estimations were performed using Bayesian coalescence analysis as implemented in the BEAST package (Drummond & Rambaut 2007; Drummond *et al.* 2012). All .xml input files were created using BEAUTI v1.7.4 (Drummond & Rambaut 2007), with the TN93 + I model for COI, Jukes-Cantor, 1969 (JC69) + Γ model for ITS and Hasegawa–Kishino–Yano (HKY) + Γ model for H3; the model testing was conducted in MEGA 5.1 using the Bayesian information criterion (BIC). For all analyses, the following settings were used: base frequencies 'estimated'; clock model 'lognormal relaxed clock (uncorrelated)'; tree prior 'coalescent/constant size'; constant.popSize 'lognormal: Log(Mean) = 0, Log(Stdev) = 1, offset = 0'. For other priors, default settings were used. The analyses were run in BEASTMC3 v. 1.7.4 (Drummond & Rambaut 2007; Drummond *et al.* 2012), using three chains, a delta value of 1.00 and swapping chains every 100 generations. The COI and H3 analyses were run for 10 million generations and the ITS analysis for 20 million generations, sampling every 1000th generation. The number of generations was chosen to obtain sufficient effective sample size (ESS). TRACER v1.5 (Rambaut & Drummond 2007) was used for examining ESS for parameters and determining the burn-in. Trees were summarised using TREEANNOTATOR v1.7.4. (Drummond & Rambaut 2007), discarding the first 10% as burn-in.

Molecular species delimitation

The gene trees were imported into Geneious, and in the cases where the previously delimited clusters were found monophyletic, the species delimitation plug-in (Masters *et al.* 2011) was used to calculate (i) *P* (*Randomly Distinct*), that is, the probability for clades to have the observed degree of distinctiveness due to random coalescent processes (Rodrigo *et al.* 2008) and (ii) *Rosenberg's* P_{AB} that expresses the probability of reciprocal monophyly of the clade of interest and its nearest defined group, under random branching (Rosenberg 2007). Both statistics were used to test whether or not the null hypothesis of random coalescence can be rejected as the explanation for phylogenetic structure.

Morphological study

The microscopically studied material included 24 mature specimens and seven immatures or fragments. For the detailed morphological analysis of the reproductive organs, only completely mature specimens (i.e. with eggs or with sperm in the spermathecal ampulla) were selected, while for external characters, we included all available specimens. This selection guarantees that differences in measurements are not due to incompletely developed organs. Only anterior parts (approximately segments I–XV) were examined because posterior parts were used for the molecular study. External characters are related to the number and size of somatic chaetae, and internal characters are related to the reproductive organs: spermathecae, male ducts and penial chaetae. The width of segment XI was included to represent the size of each specimen.

Concatenated phylogenetic analysis

To test whether the two species found (see Results) were reciprocally monophyletic, a phylogenetic analysis was performed on a concatenated data set.

The three single-gene matrices were concatenated into one data matrix using GENEIOUS PRO v. 5.6.3, with one specimen each of *Rhyacodrilus coccineus* and *R. subterraneus* added as out-groups (see Table 1). A phylogenetic tree was estimated using maximum likelihood; the analysis was performed with PHYML 3.0 (Guindon & Gascuel 2003; Guindon *et al.* 2010) as implemented at the ATGC Montpellier bioinformatics platform (http://www.atgc-montpellier.fr/). The TN93 model with Γ parameter estimated from the data was used after model testing in MEGA 5.2, and SPR+NNI was used for tree improvement. Branch support was calculated with the chi-square-based approximative likelihood ratio test (aLRT; Anisimova & Gascuel 2006) in PHYML.

Data deposition

All new sequences generated in this study are deposited in GenBank, and submission numbers are listed in Table 1; all xml files used in the BEAST analyses, as well as log files from the analyses, are deposited in the Dryad Data Repository (http://www.datadryad.org/) at DOI:10.5061/dryad.1fb3d; trees and matrices are deposited in TREEBASE (http://treebase.org/), submission TB2:S14391. Vouchers are deposited in the Swedish Museum of Natural History, Stockholm; accession numbers are given in Table 1.

Results

DNA sequencing

DNA from 36 samples of *Rhyacodrilus falciformis* s.l. was successfully amplified and sequenced with regard to COI, whereas in the cases of ITS, 22 specimens, and H3, 35

specimens were successfully sequenced (Tables 1 and S2). More details on the alignments are given in Table S2.

Distance analysis

The maximum pairwise distance values within *R. falciformis* s.l. for COI were 12.5% using uncorrected p-distances and 14.0% using TN93 distances. A barcoding gap was observed between 2.9 and 4.6% pairwise differences using uncorrected p-distances and between 3.0 and 4.9% using TN93 distances (Fig. 1A). The ABGD analysis of the COI data set yielded seven mitochondrial clusters, further used as hypothetical species, assuming maximal intraspecific variation between 1.3% and 6.0%. One cluster contains the specimens from France (cluster A), one the Spanish specimens (cluster B), one the English specimens (cluster C) and four groups includes specimens from Sweden (clusters D–G).

Coalescent analyses

In the COI tree (Fig. 1B), each of the seven groups recognised by the ABGD analysis was found monophyletic with maximum support. The French specimens (cluster A) are the sister group to all other *R. falciformis* [posterior probability (pp) 0.94], whereas the clade with the British

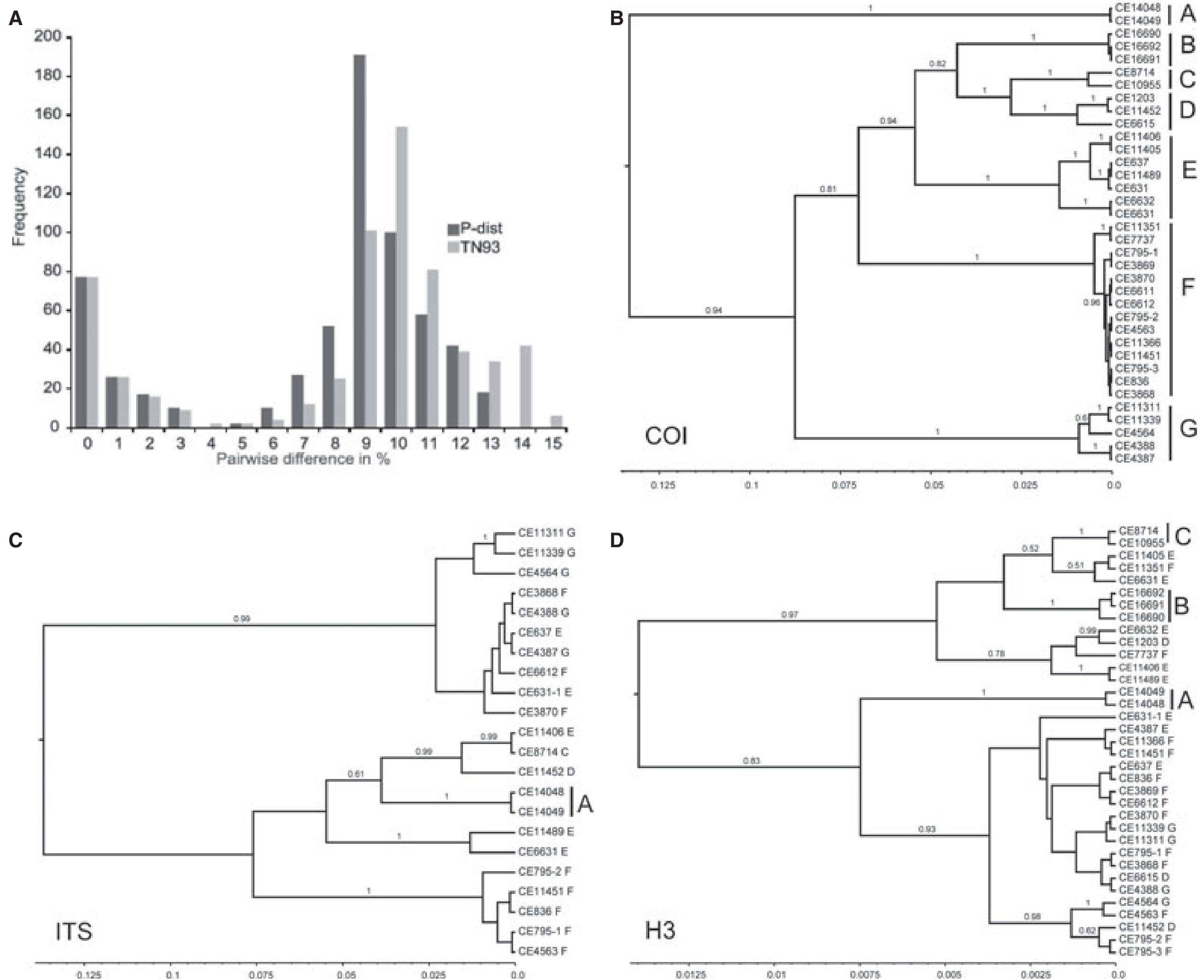

Fig. 1 Distribution of the genetic variation within *Rhyacodrilus falciformis* s.l. —A Histogram of pairwise COI distances given in both uncorrected p-distances and corrected TN93 distances. —B, C Gene trees estimated with Bayesian coalescent analysis in BEAST. Letters right of specimen codes represent COI cluster, if clusters are found monophyletic is a line followed by the letter shown right of the specimens. Letter A represents *Rhyacodrilus pigueti* sp.n. and B–G *R. falciformis s.s.* Numbers above branches are posterior probability only values above 0.50 are given. Scale shows expected numbers of substation per site. —B COI gene tree. —C ITS gene tree. —D H3 gene tree.

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specimens (cluster C) is found as sister group to one of the clades from Sweden (cluster D; pp 1), and the Spanish cluster B is sister group to them with low support (pp 0.82).

In the ITS tree (Fig. 1C), there are five well-supported clades (pp > 0.99). One of them is identical to the French cluster (A), while the other clades either consist of specimens from several clusters or contain only some of the specimens from one cluster. Unfortunately, none of the specimens from Spain and only one specimen from England was successfully sequenced; the latter specimen (CE8714) having the same ITS haplotype as one Swedish specimen (CE11406). The French cluster A is nested among the other clades, but the relationships between most of the clades are unsupported.

In the H3 tree (Fig. 1D), clusters A, B and C are monophyletic with maximum support. None of the other mt clusters are recovered in the analysis, and the relationships between groups are generally without support.

Molecular species delimitation

In the Geneious species delimitation analyses on the COI genealogy, the null hypothesis of observing reciprocal monophyly by chance could be rejected for all groups [*Rosenberg's* $P_{AB} \leq 0.05$], and the null hypothesis that the observed degree of distinctiveness is due to random coalescence processes could be rejected [*P* (*Randomly Distinct*) <0.05] for all groups except cluster G [*P*(*Randomly Distinct*) = 0.10]. In the ITS genealogy, all five well-supported clades (including cluster A) had a *Rosenberg's* $P_{AB} \leq 0.05$, whereas cluster A had a *P* (*Randomly Distinct*) of 0.12 and *P* (*Randomly Distinct*) varied between 0.11 and 0.45 for the other clades. In the H3 genealogy, only clades A–C were monophyletic and could be tested. For these groups, *Rosenberg's* $P_{AB} \leq 0.05$, whereas *P*(*Randomly Distinct*) was between 0.47 and 0.73.

Morphological study

All examined mature specimens fit with previous descriptions of *Rhyacodrilus falciformis* Bretscher, 1901 (Piguet 1906; Hrabě 1935; Juget 1967; Kasprzak 1979; Chekanovskaya 1981; Timm *et al.* 1996) except for individual CE14048, which shows different genital chaetae (Fig. 2).

We compared the ranges of the values of morphological characters across the COI clusters to search for congruence with molecular results (Table S3). There is no evidence for morphological differentiation in reproductive characters among the clusters, except the different genital chaetae in specimen CE14048 (cluster A). Slight differences in number and size of somatic chaetae (external characters) are frequent among populations and are thus not considered species specific. At specimen level, such differences may be due to the use and replacement of chaetae. The number of genital chaetae as well as their shape and size is considered to be a diagnostic character in species of the genus *Rhyacodrilus* Bretscher, 1901 (Timm 2009; Rodriguez & Fend 2013).

Concatenated phylogenetic analysis

The maximum likelihood tree based on combined COI, H3 and ITS (Fig. S1) shows a coherent, maximally supported group of all specimens of *Rhyacodrilus falciformis* s.l. All clusters except cluster E are recovered with maximal support, and cluster E is paraphyletic with respect to a clade consisting of clusters B–D. Cluster A, that is, *Rhyacodrilus pigueti* sp.n., is found as sister group to all other clusters, *Rhyacodrilus falciformis* s.s. with a support of 0.99.

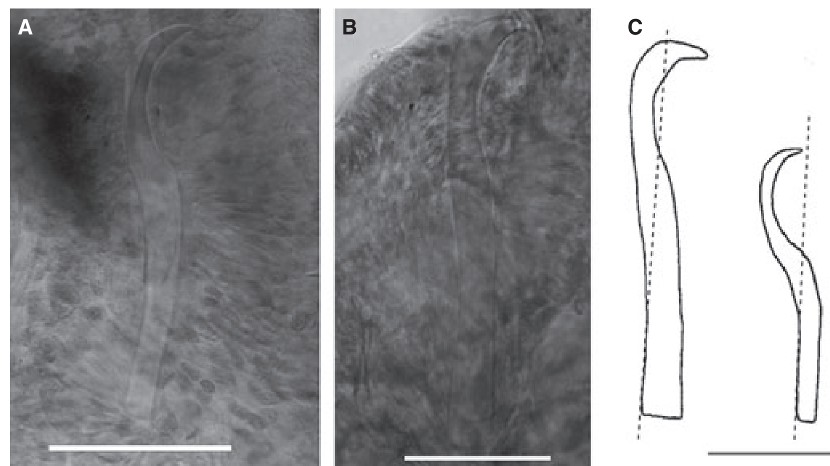

Fig. 2 Differences in the penial chaetae between *Rhyacodrilus pigueti* sp.n. and *R. falciformis*. —A. *Rhyacodrilus falciformis*. —B. *Rhyacodrilus pigueti*, Holotype. —C Drawings of the penial chaetae in *R. pigueti* (straight chaeta) and *R. falciformis* (sickle-shaped chaeta). Scale bars = 50 μm.

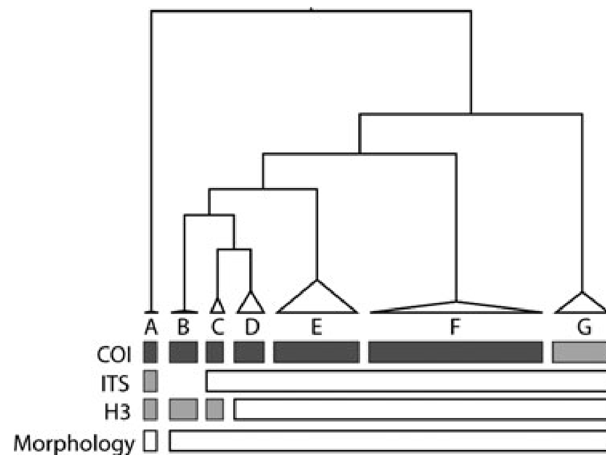

Fig. 3 Summary of the results, indicating the amount of support for each COI cluster. For COI, ITS and H3 clusters that were monophyletic are indicated with a grey bar, pale grey indicates a *Rosenberg's* $P_{AB} \leq 0.05$ and dark grey both a *Rosenberg's* $P_{AB} \leq 0.05$ and a *P*(*Randomly Distinct*) ≤0.05. Cluster A represents *Rhyacodrilus pigueti* sp.n. and clusters B–G *R. falciformis s.s.*

Therefore, in the light of molecular and morphological data (results summarised in Fig. 3) and further discussed in Discussion, we consider cluster A as a separate species from *R. falciformis s.s.* The shape of the penial chaetae of CE14048 is in accordance with the description of *R. falciformis* var. *pigueti* Juget, 1967. However, a new name published after 1960 expressly as a 'variety' is considered to be infrasubspecific and as such does not formally exist (ICZN 1999: §15.2). Therefore, a new species is proposed below for this form, including the two specimens in cluster A.

Taxonomy

Genus *Rhyacodrilus* Bretscher, 1901

Rhyacodrilus pigueti **Achurra & Martinsson, sp. n.** (Figure 2A, C).

Rhyacodrilus falciformis, var. *pigueti* Juget, 1967: Fig. 2B, Table 1.

Holotype. CE14048, whole-mounted worm, anterior part (segments I–XVI only), stained in paracarmine and mounted in Canada balsam. Collected by Michel C. des Chatelliers, 26 Jan 2011. Deposited in Swedish Museum of Natural History (SMNH), Stockholm, accession no. SMNH Type-8475. COI barcode sequence, GenBank acc. no. KF267911; ITS sequence, GenBank acc. no. KF267993; H3 sequence, GenBank acc. no. KF267968.

Type locality. Crotot Cave, north-east of Besançon, Départment du Doubs, France (47°26′44.52″N,6°24′31.64″ E, DMS datum WGS84; elevation: 375 m).

Other material, genetically examined. CE14049, only molecular data, no voucher available. COI barcode sequence, GenBank acc. no. KF267912; ITS sequence, GenBank acc. no. KF267994; H3 sequence, GenBank acc. no. KF267969.

Etymology. Named after Émile Piguet, a specialist on aquatic oligochaetes of the early 20th century. The new material fits the description of *R. falciformis* var. *pigueti* Juget, 1967, but as varieties published after 1961 are invalid, Juget's name *pigueti* is not available (§15.2 and §45.5 ICZN 1999). Therefore, the name *pigueti* is here proposed as new for this taxon, now regarded as a species. According to Juget 1967, Émile Piguet had once found specimens with the characters of Juget's variety, but Piguet only identified them as *Rhyacodrilus* sp. and never formally described them taxonomically.

Diagnosis. This species can be distinguished from other species in *Rhyacodrilus* by the shape of the penial chaetae (Fig. 2) and by genetic data (Fig. 3). See also Remarks and Discussion below.

Description. Incomplete specimen, with 16 anteriormost segments 1.93 mm long, body width 265 μm in segment V, 380 μm in segment XI. Prostomium 120 μm long, 132 μm wide. Coelomocytes abundant within coelomic cavity of all segments, round, 27 μm diameter, granulated, when nuclei stained with paracarmine. Clitellum indistinguishable (poorly developed?). Spermathecal pores midlateral, located anteriorly in segment X. One pair of male pores open ventrally in segment XI.

Dorsal and ventral chaetae of identical shape, bifid, with distal tooth somewhat thinner and longer than proximal. Anteriorly 3–5 chaetae per bundle, 1–2 in segments XI–XV and 2–3 in segment XVI; maximum length 92 μm, 2–3 μm thick. Modified penial chaetae in segment XI, a single chaeta per 'bundle', straight, somewhat spoon-shaped (Fig. 2A, C); chaeta 150 μm long, 15 μm thick in proximal and middle parts, narrowing to 10 μm before widening to 15 μm in the distal portion. Proximal end of penial chaeta associated with conspicuous chaetal gland (diameter 75 μm) and strong muscular strands. Distal end located within male pore. Penial chaetae orientated with their distal ends towards segment X.

Atria paired in segment XI, close to septum XI/XII, elongate, pear-shaped, ampullae 150 μm long, maximum diameter 50 μm, with about 10 μm-thick muscular layer. Proximal end of atrium barely observed due to the presence of the assembly of penial chaeta + chaetal gland + muscular strands. Abundant prostatic cells covering proximal and middle parts of atrium. Spermathecae paired,

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ampullae 96 μm diameter, 60 μm long, with a single-layer epithelium 5–10 μm thick; lumen filled with random mass of sperm; duct 80 μm long, 60 μm diameter.

Distribution and habitat. Crotot Cave is a large cavity (5800 m long), in Jurassic limestone, on a plateau near the valley of the Doubs River (Departement du Doubs), eastern France. Following Juget (1967), the new species has also been found in the deep zone of Lake Léman (Lake Geneva), in Switzerland and France, approximately 100 km away from the Crotot Cave.

Remarks. *Rhyacodrilus pigueti* sp. n. is well distinguished from *R. falciformis s.s.* by the shape of the penial chaetae. The chaetae are sickle-shaped in *R. falciformis*, while they are straight and somewhat spoon-shaped in the new species (Fig. 2). In *R. falciformis* s.s., the ratio of the distance from the focal point of the sickle to the dorsal axis of the shaft (indicated in Fig. 2C) and the chaetal length (mean ratio is 0.06 in *R. falciformis*, 0.02–0.10, $n = 7$; ratio is 0.03 in *R. piguet*, $n = 1$). However, this measurement should be interpreted with caution as it seems to vary with the orientation of the chaeta. The distal end of the penial chaeta is more pointed and slender in *R. falciformis* than in the new species, in which the distal part is widening proximal to the apex. In addition, the penial chaetae are 150 μm long in the new species, while they are between 100 and 140 μm long in our specimens of *R. falciformis*. Other measurements in the literature also show slightly shorter penial chaetae for *R. falciformis*: 100–138 μm (Piguet 1906; Hrabě 1935; Juget 1967; Kasprzak 1979; Chekanovskaya 1981; Timm *et al.* 1996), but no measurements of the penial chaetae are given in the original description (Bretscher 1901).

Discussion

Based on the results from the ABGD and species delimitation tests on the COI data set, it seems that *Rhyacodrilus falciformis* consists of 6–7 different species, one of which morphologically conforms to the '*pigueti*' form reported by Juget (1967). However, the barcoding gap is small, 1.7–1.9% depending on model, and there is no barcode gap between *Rhyacodrilus pigueti* and *R. falciformis s.s.* Several other studies have found a much larger barcoding gap between clitellate species (e.g. Gustafsson *et al.* 2009; Kvist *et al.* 2010; Richard *et al.* 2010; Envall *et al.* 2012; Matamoros *et al.* 2012). The difference in barcoding gap between our study and others may be explained by the age of the lineages. For instance, the split between *Rhyacodrilus pigueti* and *R. falciformis s.s.* could be distinctly younger than the splits between the taxa in the other studies. In the nuclear gene trees, members of the hypothetical species suggested by the mtDNA analyses are in many cases found scattered and mixed in different clades. In the case of ITS, five well-supported clades were found, with a *Rosenberg's* $P_{AB} \leq 0.05$, whereof one constitutes cluster A, that is, *Rhyacodrilus pigueti*.

The molecular operational taxonomic units (MOTUs; Floyd *et al.* 2002) that will be defined thus differ considerably between the genes, both in numbers and constitution. This highlights the problems with using a single genetic marker for delimiting taxonomic units. Instead using an integrative approach (see review by Schlick-Steiner *et al.* 2010), combining several genetic markers as well as other data sources can result in more robust taxonomic hypotheses.

The support for the hypothesis that the delimited COI-based groups represent separately evolving lineages varies. Some of the groups are supported by COI only, others by two loci, but only cluster A, here regarded as *Rhyacodrilus pigueti* sp. n., is supported by all genes as well as morphology (Fig. 3). The differences in topology between the gene trees suggest that gene flow is present between some of the different COI-based groups and that speciation – if at all in progress – is not yet complete between them. However, recent speciation events, where lineage sorting of nuclear genes still is incomplete and morphological differences not yet have accumulated to any discernible degree, cannot be ruled out, especially not as some of the COI clusters show a clear geographical pattern.

The shape of the penial chaetae is the only morphological diagnostic character that we found to separate the new species from *R. falciformis s.s.*, but together with the molecular data, it gives us strong support for *R. pigueti* being a separately evolving lineage. The integration of independent data sources is here crucial for a proper determination of the species boundaries in *R. falciformis* s.l.; neither individual gene trees nor the morphological data alone provide enough support to clearly delimit the two species. New techniques may reveal other putative diagnostic anatomical structures that are currently not observable. For instance, Cuadrado & Martinez-Ansemil (2001), using scanning electron microscopy (SEM), found several bundles of cilia near the male pores in specimens of *R. falciformis* that had not been observed with other techniques. Such ultrastructural details may deserve attention in future taxonomic work on cryptic species of aquatic clitellates. However, SEM is a destructive and rather expensive technique and may therefore be of little help in specimen identification.

It is possible that there are physiological and/or ecological differences between the two species as has been shown for the different lineages within *Tubifex tubifex* (Sturmbauer *et al.*, 1999; Beauchamp *et al.* 2002).

The separation of *R. pigueti* seems to render *R. falciformis* paraphyletic in the ITS and H3 trees, but the support for

the topologies is weak or lacking, and in the concatenated analysis, *R. falciformis* is found monophyletic with high support. However, even if the topologies should prove to be correct despite the low or lacking support, non-monophyly of species in single-gene trees is not uncommon (Crisp & Chandler 1996; Funk & Omland 2003), and the reason in this case is likely to be recent separation and incomplete lineage sorting. The N_e of the nuclear genes is greater than that of mitochondrial genes and it takes a longer time for such genes to reach complete lineage sorting and reciprocal monophyly (see Introduction). Evidence of reciprocal monophyly in several gene trees is a strong indication that groups are separately evolving lineages, that is, different species. However, reciprocal monophyly is not a necessity for species delimitation (see e.g. Doyle 1995; Helbig *et al.* 2002) except under a strict monophyletic species concept.

The divergence in COI sequences observed in the Swedish specimens of *R. falciformis* could be due to allopatric divergence with secondary contact. This could be the result of recolonisation from different refugia after deglaciation, as has been discussed for other animal groups (e.g. Verovnik *et al.* 2005; De Wit & Erséus 2010; Achurra & Erséus 2013). The combination of higher mutation rate and a faster genetic drift in mitochondrial markers compared with nuclear ones could explain why divergent haplotype clusters are formed to a higher extent in the COI data set compared with the nuclear data sets. These haplotype clusters will then be retained after a secondary contact, but if gene flow between them resumes, the unlinked nuclear and mitochondrial haplotype clusters will be mixed in the populations. The nuclear genome will also further be affected by recombination increasing the blending between haplotype clusters. Specimens from different Swedish COI clusters are found at the same locality in several cases (see Table 1).

The western Alps and adjacent areas seem to have a rich *Rhyacodrilus* fauna both in surface and in ground water (e.g. Juget 1984, 1987; Lafont & Juget 1993) including the here described *R. pigueti*, which together with *R. falciformis* is also found in Lake Léman (Juget 1967). From Lake Léman, also a seemingly undescribed species was reported and illustrated by Piguet and Bretscher (1913: Fig. 13). It has straight penial chaetae of approximately the same length as the somatic chaetae and lack hair chaetae, like both *R. falciformis* and *R. pigueti*. Future studies of the clitellate fauna in this area may reveal an even larger diversity of *Rhyacodrilus*.

Finding unexpected genetic divergence within a morphospecies can be a starting point for further investigation using an integrative approach with various data sources. However, COI alone should not be used to claim that cryptic speciation has occurred, as several studies have found deep mitochondrial divergence within populations with retained gene flow (e.g. Webb *et al.* 2011; Hogner *et al.* 2012; Torres-Leguizamon *et al.* 2012). Nevertheless, we certainly recognise the usefulness of DNA barcoding in specimen identification, once species boundaries have been properly established.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Maximum Likelihood tree based on combined COI, H3 and ITS data from *Rhyacodrilus falciformis* s.l. obtained with PhyML, numbers at branches are aLRT branch support.

Table S1. Primers, sequences and programs used for amplification of the mitochondrial COI and nuclear ITS and H3.

Table S2. Details of alignments of the mitochondrial COI and the nuclear H3 and ITS genes.

Table S3. Ranges (μm) for morphological characters of the COI-clusters (A–G).

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