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Abstract: Coral reefs constitute the most diverse ecosystem of the marine realm and an increasing number of studies are focusing on coral species boundaries, distribution and on processes that control species ranges. However, less attention has been paid to coral associated species. Deep-sea sponges dominate cold-water coral ecosystems, but virtually nothing is known about their molecular diversity. Moreover, species boundaries based on morphology may sometimes be inadequate, since sponges have few diagnostic characters. Within the present study, we investigated the molecular diversity within the genus Hexadella (Porifera, Demospongiae, Verongida, Ianthellidae) from the European shallow-water environment to the deep-sea coral ecosystems. Three molecular markers were used: one mitochondrial (COI) and two nuclear gene fragments (28S rDNA and the ATPS intron). Phylogenetic analyses revealed deeply divergent deep-sea clades congruent across the mitochondrial and nuclear markers. One clade contained specimens from the Irish, the Scottish and the Norwegian margins and the Greenland Sea (Hexadella dedritifera) while another clade contained specimens from the Ionian Sea, the Bay of Biscay and the Irish margin (Hexadella cf. dedritifera). Moreover, these deeply divergent deep-sea clades showed a wide distribution suggesting a connection between the reefs. The results also point to the existence of a new deep-sea species (Hexadella sp.) in the Mediterranean Sea and of a cryptic shallow-water species (Hexadella cf. pruvoti) in the Gorringe Bank. In contrast, low genetic differentiation between H. cf. dedritifera and Hexadella pruvoti from the Mediterranean Sea was observed. All Hexadella racovitzai specimens from the Mediterranean Sea (shallow and deep) to the Atlantic were monophyletic.

Suggested Reviewers: Gert Wörheide Prof. Dr.
Chair, Department for Geo- and Environmental Sciences, Section Palaeontology, Ludwig-Maximilians-Universitaet Muenchen
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Prof. Dr. Gert Wörheide and his student Bastian Bentlage were the first to use the second intron ofthe nuclear ATP synthetase beta subunitgene (ATPSbeta-iII) on sponges, and to highlight the highresolution of this new nuclear marker system in sponge evolutionary studies.

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Dr.Andrea Blanquer and her department used mitochondrial and nuclear genes to report crypticspeciation in marine sponges, and recently emphasized on the work needed to be 'a posteriori'done by taxonomists in order to make these cryptic species available to the scientific community.

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Dr. Marina Cunha is specialist in the deep-sea fauna of the Gulf of Cadiz, the Portuguese marginand the Azores. She combines morphologic and molecular identification (barcoding) to assess thedistribution of marine invertebrates, to reveal cryptic speciation and to report new species from thedeep-sea.

Opposed Reviewers:

Ghent, Belgium, the 14.09.2009

Dear Editor

A former version of the manuscript 'Species boundaries and phylogenetic relationships between Atlanto-Mediterranean shallow-water and deep-sea coral associated Hexadella species (Porifera, Ianthellidae)' has been submitted for publication in Molecular Phylogenetics and Evolution in February 2007 (referenced Ms. No.: MPE-09-104; edited by Pr.Dr. Bernd Schierwater). Although one reviewer was almost completely positive, the relevant criticism raised by the second reviewer impeded you to accept this manuscript as a research paper in MPE. In your final decision mail from 30/06/2009 you were however willing to receive a revised manuscript if an effort could be done to address the sampling size problem.

As MPE seems the most relevant journal according to its aim and scope to publish these data, a significant effort was made by me and the other authors in order to improve the sampling scheme, the laboratory protocols and the manuscript content, as suggested by reviewer II.

A total of 14 additional shallow and deep-sea samples, including a better Atlanto-Mediterranean coverage for the shallow-water species *H. racovitzai* clearly improved the dataset, with a current sampling size of 46 (instead of 32), and a doubling of the number of populations (14 instead of 7).

The design of a new 28S primer and of robust amplification protocols allowed us to fill the gaps in the 28S and the ATPS database (Table 1). As suggested by reviewer II, all the phylogenetic analyses were done using only different sequences.

The use of ATPS to check the value of this gene for taxonomic and phylogenetic purposes and to validate the mitochondrial phylogeny is now explicitly stated in the Ms in the introduction and the ATPS "section" in M & M which has been extended and clarified. It is there explained that putative problems with different nuclear intron copies within individuals are treated cautiously. Length variation and the number of double peaks in the chromatogram of both forward and reverse sequences were checked for each sample. At most 3 positions (out of 235 bp) were observed to show double peaks, which were subsequently encoded using the IUPAC ambiguity code. No length variation of individual sequences was observed.

All the other remarks raised by reviewer II were carefully checked and revised, such as providing the combined tree figure, providing detailed explanations about the BLAST searches, the use of the term haplotype if alleles are not analyzed, the removal of misspelled species names, the use of unique name along the MS for commercial brand and markers, a thorough revision of the whole reference section, etc…

We hope that with these major revisions of the manuscript, it will now be positively received by the reviewers and that you will be able to accept this work as a research paper in MPE.

Yours sincerely,

Julie Reveillaud

Species boundaries and phylogenetic relationships between Atlanto-Mediterranean shallow-water and deep-sea coral associated *Hexadella* species (Porifera, Ianthellidae)

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Abstract

Coral reefs constitute the most diverse ecosystem of the marine realm and an increasing number of studies are focusing on coral species boundaries, distribution and on processes that control species ranges. However, less attention has been paid to coral associated species. Deep-sea sponges dominate cold-water coral ecosystems, but virtually nothing is known about their molecular diversity. Moreover, species boundaries based on morphology may sometimes be inadequate, since sponges have few diagnostic characters. Within the present study, we investigated the molecular diversity within the genus *Hexadella* (Porifera, Demospongiae, Verongida, Ianthellidae) from the European shallow-water environment to the deep-sea coral ecosystems. Three molecular markers were used: one mitochondrial (COI) and two nuclear gene fragments (28S rDNA and the ATPS intron). Phylogenetic analyses revealed deeply divergent deep-sea clades congruent across the mitochondrial and nuclear markers. One clade contained specimens from the Irish, the Scottish and the Norwegian margins and the Greenland Sea (*Hexadella dedritifera*) while another clade contained specimens from the Ionian Sea, the Bay of Biscay and the Irish margin (*Hexadella* cf. *dedritifera*). Moreover, these deeply divergent deep-sea clades showed a wide distribution suggesting a connection between the reefs. The results also point to the existence of a new deep-sea species (*Hexadella* sp.) in the Mediterranean Sea and of a cryptic shallow-water species (*Hexadella* cf. *pruvoti*) in the Gorringe Bank. In contrast, low genetic differentiation between *H.* cf. *dedritifera* and *Hexadella pruvoti* from the Mediterranean Sea was observed. All *Hexadella racovitzai* specimens from the Mediterranean Sea (shallow and deep) to the Atlantic were monophyletic.

Keywords: Porifera; cold-water coral; COI; Nuclear intron; Partial 28S rDNA; phylogenetic resolution; Atlanto-Mediterranean.

Introduction

Coral reefs constitute one of the most diverse but also one of the most vulnerable ecosystems of the marine realm (Hughes et al., 2003; Pandolfi et al., 2003). Recent evidence suggests that deep-sea coral ecosystems may compare in species richness and abundance to their shallow-water counterparts (Freiwald et al., 2004; Roberts et al., 2006; Cairns, 2007), and act as speciation centers (Roberts et al., 2006; Lindner et al., 2008). Understanding the origin and evolution of this marine biodiversity is essential for its conservation and sustainable management (Palumbi, 2004). Species boundaries and processes that control the distribution of coral species are hence receiving increasing interest, from the shallow-water

environment to the deep-sea (France and Hoover, 2002; Le Goff-Vitry et al., 2004; Reveillaud et al., 2008; Lindner et al., 2008). However, less attention has been paid to the associated species, despite the fact that they represent the highest biodiversity in deep-sea reefs (Roberts et al., 2006). Sponges constitute an important and dominant invertebrate group in hard-bottom benthic communities and play key roles on ecosystem functioning (Bell, 2008). Although ca 200 Porifera species dominate the deep-water coral reef ecosystems, deep-water sponge species remain until today a reservoir of diversity barely explored (e.g. Vacelet, 1969; Jensen and Frederiksen, 1992; Rogers, 1999; Longo et al., 2005; Van Soest et al., 2007).

Sponges are a group with numerous but considerably plastic morphological features. Characters such as texture, form and coloration are not reliable as they are frequently influenced by environmental factors (Palumbi, 1984; Jones, 1984; Barthel, 1991; Schönberg and Barthel, 1997; Carballo et al., 2006). Spicule morphology and skeletal arrangement traditionally are the diagnostic characters to identify sponge species (Bergquist, 1978). Unfortunately, spicule size and micromorphology can also be influenced by the environment (Palumbi, 1986; Maldonado et al., 1999; Bell et al., 2002). Consequently, taxonomic and systematic uncertainty may prevail, especially in species with a low number of informative characters (Klautau et al., 1999; Knowlton, 2000). Molecular analyses repeatedly revealed cryptic species and proved valuable in delineating species boundaries in Porifera (Solé-Cava et al., 1992; Boury-Esnault et al., 1992, 1999; Klautau et al., 1994, 1999; Lazoski et al., 2001; Wulff, 2006; Blanquer and Uriz, 2007; Cárdenas et al., 2007). However, the number of sponge genetic studies from remote environments, such as the deep-sea, remains scarce. Knowledge on speciation within sponges from cold-water coral ecosystems has potentially great consequences for the efficient conservation and economic use of this group (Van Soest and Lavaleye, 2005). Moreover, peculiar life-history traits of sponges, such as a restricted larval dispersal (Maldonado, 2006) which induces high genetic structure (Duran et al., 2004;

Calderón et al., 2007; Blanquer et al., 2009) make sponges a particularly interesting model group to determine the degree of connectivity vs. isolation between deep-sea reef populations and may prove very useful for establishing baselines of deep-water coral reefs biodiversity along the European margins.

In the present study we examine the species boundaries and phylogenetic relationships between members of the genus *Hexadella* Tospent, 1896 (Order Verongida, Family Ianthellidae) collected along the European margins in both shallow-water and deep-sea habitats. Verongid sponges are know so far, as single taxonomic group to produce dibromotyrosine secondary metabolites (Wu et al., 1986; Bergquist and Cook, 2002; Erwin and Thacker, 2007), potential antithyroidic and antibiotic agents, and are therefore of particular interest for biotechnological applications (see also Erpenbeck and Van Soest, 2007). Chemical analyses of *Hexadella* species sampled in shallow-water and deep-sea suggested the production of different secondary metabolites at different depths (Morris and Andersen, 1989). However, due to their lack of mineral skeleton, verongid identifications are particularly challenging at the intra-ordinal and especially species level. Taxa are distinguished almost exclusively by the structure and arrangement of their spongin fibers (Bergquist and Cook, 2002), and in the case of fiberless species such as *Hexadella* spp. by the type and size of the choanocyte chambers. Taxonomists are left with very few morphological diagnostic characters difficult to observe (Topsent, 1913). Consequently, these and similar species have been widely reported simply as 'crustose sponges' (Mortensen et al., 1995). Especially, *H. dedritifera* Topsent, 1913 is a common species with the thin/soft habitus in deep-water coral ecosystems along the European margins. Fine crusts of *H. dedritifera* are found in the deep-sea on top of rocks, large sponges (e.g. astrophorids) or coral rubble in the Mediterranean Sea (Longo et al., 2005), the Gulf of Cadiz, the Bay of Biscay, the Porcupine Seabight (Van Soest et al., 2007), the Rockall Bank (Van Soest and Lavaleye, 2005), along the Norwegian margin

144 and in the Greenland Sea. However, such wide geographical distribution, over a distance of
145 more than 8,700 km (from the Mediterranean Sea to the Greenland Sea) raises the question
146 whether *H. dedritifera* actually represents a distinct taxonomic unit or a complex of (cryptic)
147 species. The two other Atlanto-Mediterranean species of the genus, *Hexadella pruvoti*
148 Topsent, 1896 and *Hexadella racovitzai* Topsent, 1896 occur in shallow-water or in caves,
149 although *H. pruvoti* has also been observed in deeper water (J. Vacelet, pers. communication).
150 A subtle pink color distinguishes *H. racovitzai* from *H. dedritifera* and *H. pruvoti*, both bright
151 yellow when alive and turning deep purple when taken out of the water (aerophobic reaction).
152 Furthermore, the deep-sea *H. dedritifera* has larger choanocyte chambers than both *H.*
153 *racovitzai* and *H. pruvoti* (Topsent, 1913). With such subtle differences, it remains unclear
154 whether *H. dedritifera* and *H. pruvoti*, are two separated species or not.
155 We used in the present study phylogenetic concordance criteria between the Folmer
156 partition of the mitochondrial cytochrome c oxidase subunit I (COI) gene, the D3-D5 region
157 of the nuclear large ribosomal subunit (28S rDNA) and the second intron of the nuclear ATP-
158 synthetase beta subunit gene (ATPS) to delineate the three European *Hexadella* species and to
159 investigate the presence of cryptic species within this genus. The ATPS marker was tested for
160 the first time for its applicability in sponge species delimitation.
161
162 **Material and methods**
163 ***Sampling***
164 Species of the genus *Hexadella* were collected at various locations throughout their
165 distribution range (Table 1 and Fig. 1). Samples of *H. dedritifera* were collected with
166 boxcores or Remote Operated Vehicle (ROV) during 7 deep-sea cruises (see Table 1). The
167 deep-sea Mediterranean sample (180 m) collected in a canyon close to la Gabinière, Port-
168 Cros, France (CRO) could not be unambiguously identified because it formed some

degenerating crusts alive. Subsequent phylogenetic analyses (see below) revealed relationships to *H. racovitzai*. Three deep-sea specimens collected at (ION) location could not be unequivocally assigned to *H. dedritifera* due to a slight color variation when fixed in ethanol (dark green instead of dark violet). Hereafter, these three specimens will be referred to as *Hexadella* sp.. Samples were preserved in 100% ethanol and stored at room temperature until further processing.

DNA extraction

Total genomic DNA was extracted from sponge tissue using the DNeasy Blood and Tissue Kit (Qiagen) following the instructions of the manufacturer. The standard protocol was optimized by starting with a 25-minute centrifugation step in the Savant Speed Vacuum System to eliminate ethanol prior to lysis and increase final DNA yield. Amplifications by polymerase chain reaction (PCR) were performed in a total volume of 45 µl, with 5 µl 10 x PCR buffer (Qiagen), 1 µl MgCl2 (25mM), 1 µl dNTP (10mM), 0.5 µl of BSA (10µg/µl), 1 µl of forward and reverse primer (25µM), 0.25 µl TopTaq DNA polymerase (Qiagen) and 1 µl of template DNA and 34.25 µl of distilled water.

Amplification of COI fragment

PCR amplification of the 5' partition (Folmer et al., 1994) of the cytochrome c oxidase subunit I (COI) mtDNA was performed using the degenerated primers from Meyer et al. (2005) dgLCO 5'-GGT CAA CAA ATC ATA AAGAYA TYG G -3', and dgHCO 5'-TAA ACT TCA GGGTGA CCA AAR AAY CA-3' with PCR cycling parameters: 94°C for 2 min, followed by 35 cycles of (94°C for 40s, 42°C for 40s, 72°C for 60s) and a final extension at 72°C for 10 min. The nuclear markers were investigated for phylogenetic congruence with the mitochondrial tree topology. Therefore, representatives of each COI haplotype were sequenced for both the nuclear ATPS and 28S rDNA. A variable number of individuals

193 (between 33% and 75%) were sequenced for each haplotype, depending on the haplotype
194 frequency.

195 ***Amplification of 28S rDNA fragment***

196 PCR primers RD3A 5'-GAC CCG TCT TGA AAC ACG A-3' and RD5B2 5'- ACA
197 CAC TCC TTA GCG GA-3' (McCormack and Kelly, 2002) were used for amplification of
198 the D3-D5 fragment of the 28S rDNA gene under a temperature regime of 3 min at 94 °C,
199 followed by 35 cycles of 30 s at 94 °C, 20 s at 45 °C, 1 min at 72 °C and a final elongation of
200 10 min at 72 °C. Because PCR-amplification of some individuals was problematic, we
201 designed more specific forward and reverse primers Hex28F (5'-CCG AAC AGG GTG AAG
202 CCA GG-3') and Hex28R (5'-TTACA CAC TCC TTA GCG G-3') and used the same PCR
203 cycle conditions as described above.

204 ***Amplification of ATPS fragment***

205 A fragment of 235 bp of the nuclear ATPS was amplified using the sponge specific
206 primers ATPSb_Ph_Fwd 5'-TGT CTT GGA AAA GGA AGG ATC AAA GG-3' and
207 ATPSb_Ph_Rev: 5'-CGT TCA TTT GAC CAT ACA CCA GCG-3' (Bentlage and Wörheide,
208 2007) and the following cycling parameters: 3 min at 94°C, then 35 cycles of (94°C for 45s,
209 50°C for 45s, and 72°C for 45s) and a final elongation of 10 min at 72°C. To obtain the
210 largest possible fragment of the flanking exons of the ATPS gene (Bentlage and Wörheide,
211 2007) and to facilitate verification by BLAST search (Altschul et al., 1990), the degenerated
212 exon primed intron crossing (EPIC) primers ATPSBF1/ ATPSBR1 (Jarman et al., 2002) were
213 used on eight random specimens. They yielded a bigger fragment of 295 bp containing 123 bp
214 (73 bp at the 5' extremity and 50 bp at the 3' extremity) of the exon sequence, flanking the
215 phase 0-intron that follows the GT-AT rule. PCR cycling conditions included 2 min at 94°C,
216 35 cycles of 94°C for 20 s, 55°C for 60 s, and 72°C for 50 s, and a final extension step at

72°C for 10 min. These eight EPIC PCR products were checked for identity with the shorter fragments amplified with the sponge specific primers and their homology with sponge sequences was investigated using a BLAST similarity search (Altschul et al., 1990) with the sequences published by Wörheide and colleagues (2008).

Putative different nuclear intron copies within individuals require the ATPS marker to be treated cautiously, and to resolve the two alleles for analyses at the population-level. In the present study, where the nuclear gene ATPS is used for taxonomic and phylogenetic purposes, chromatograms of both forward and reverse sequences were checked for length and sequence variants. No length variation was observed within individuals and at most three positions out of 235 bp (representing a maximum ambiguity of 1.3%) showed double peaks in the chromatogram. These ambiguous positions were encoded using the IUPAC ambiguity code (Cornish-Bowden, 1985) and were insignificant for the outcome, as there were too many other variable sites, which were unambiguous.

PCR product processing and sequencing

PCR products were loaded onto a 1% agarose gel to check the size of the amplified product. The PCR products were then sequenced directly in both directions through a Perkin-Elmer ABI 3130 capillary DNA sequencer. PCR products were purified using exonuclease I, *E. Coli* (20 U μl^{-1}; Fermentas) and Calf Intestine Akaline Phosphate (CIAP) (1 U μl^{-1}; Fermentas). Sequence files were read and assembled using the SeqMan software (Lasergene). Because of the enhanced chance of amplifying symbionts or ingested DNA templates in encrusting sponges, sequences were verified for their poriferan origin by BLAST searches against the GenBank database (http://blast.ncbi.nlm.nih.gov/BLAST/) and with a cladistic tree-reconstruction as described in Erpenbeck et al. (2002).

All sequences are deposited in the GenBank nucleotide sequence database under accession numbers XXXX to XXXX. (Accession numbers will be provided upon manuscript acceptance).

Sequence alignment and phylogenetic analyses

Nucleotide data of COI, 28S and ATPS fragments were used for phylogenetic analyses. Alignments were performed using the multiple alignment software MAFFT (Katoh et al., 2002) at http://www.ebi.ac.uk/Tools/mafft/index.html. As outgroups, we included sequences from *Aplysina fistularis* (AY561987 and AY561864) and *Porphyria flintae* (Erpenbeck et al, unpublished), from the closely related family Aplysinidae and Aplysinellidae respectively. Due to high evolutionary rates, ATPS intron sequences obtained from closely related Verongida species used as outgroup were unalignable. Therefore midpoint rooting was used in the phylogenetic reconstruction of the ATPS fragment.

Phylogenetic reconstructions were performed under Maximum Likelihood (ML) and Bayesian inference (BI) criteria on each of the 28S, COI and ATPS nucleotide datasets. BI analyses were performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) under the best-fit evolutionary model estimated for each independent gene under the Akaike Information Criterion (AIC) with MrModeltest 1.1 (Nylander, 2002). The models selected by AIC were TVM+I+G for the COI partition, HKY+G for the ATPS partition and K80+I for the 28S rDNA partition. Four Markov chains were run for 1 million generations and sampled every 1,000 generations. The remaining trees after a burnin of 25% of sampled trees were used to generate a 50% majority rule consensus tree. Only posterior probabilities >95% were considered to significantly support clades. Phylogenetic relationships using the ML criterion were estimated with PAUP* 4.0b10 (Swofford, 2002) under the best-fitting evolutionary model estimated for each independent gene using the Akaike Information Criterion (AIC) with Modeltest 3.06 (Posada and Crandall, 1998). ML trees were computed using heuristic

searches with 100 replicates of random taxon addition sequence and tree bisection and reconnection (TBR) branch swapping. Nodal support was estimated with a bootstrap procedure computed after 10,000 replicates of heuristic search. Bootstrap values >80% were considered high enough to support clades in ML reconstructions.

A partition homogeneity test was conducted using PAUP (Swofford, 2002) to statistically compare the congruence between all gene trees. When data were found to be congruent, gene partitions were combined for specimens from which the three sequences were available. The combined dataset was analyzed using both Bayesian (BI) and Maximum likelihood (ML) inference criteria. Separate substitution models corresponding to data partitions were used for the concatenated data set in BI whereas a new evolutionary model was estimated in ML.

Uncorrected p-distances between clades for the COI, ATPS and 28S gene fragments were calculated using MEGA 4.0 (Tamura et al., 2007).

Results

mtDNA COI dataset

The resulting dataset comprised 46 sequences and 11 different haplotypes, with 657 nucleotides (81 variable, 45 of which parsimony informative). We observed 7 non-silent mutations in the COI amino-acid dataset which all resulted from a change in the first codon-position. *Hexadella* species formed a well supported monophyletic group, with high Bayesian posterior probabilities and ML bootstrap support (100/100) (Fig. 2). *Hexadella* sp. was clearly divergent from the other *Hexadella* specimens (Seq1). Three highly supported clades indicated that only *H. racovitzai* was monophyletic (clade H2). Clade H2 contained all *H. racovitzai* specimens, with representatives from the Mediterranean Sea (mam, ban), and the

289 Atlantic (gor, ire, eng) sharing a common haplotype. Two specimens from the Mediterranean
290 Sea (mam and the deep-sea sample CRO) differed in 16 bp from the previous haplotype.
291 Hereafter, the CRO specimen will be referred to as *H. racovitzai*. Clade H1 comprised
292 specimens identified as *H. pruvoti* from Monaco and Marseille (mam) and *H. dedritifera* from
293 the Ionian Sea (ION), the Bay of Biscay (BIS), and from the Irish margin (D23ROC). The two
294 haplotypes differed by only a single base mutation. The third well supported clade (H3)
295 corresponded to the deep-sea species *H. dedritifera* from the North East Atlantic (NEA),
296 including samples from the Irish (ROC), the Scottish (MIN), and the Norwegian margin
297 (BER, ROS) as well as from the Greenland Sea (GRE). Within clade H3 some substructuring
298 was observed, with a highly supported Irish-Scottish-Norwegian subclade. Clearly, *H.*
299 *dedritifera* from clade H3 is highly divergent from *H. dedritifera* from clade H1. Similarly, *H.*
300 *pruvoti* specimens from the Gorringe Bank (P67gor, P73gor) located off the southwest coast
301 of Portugal (hereafter Seq2) were clearly divergent from the *H. pruvoti* specimens occurring
302 in the Mediterranean Sea (H1).

303 Genetic divergence values between the three supported COI clades (H1-3) and the two
304 divergent sequences (Seq1, 2) ranged from 3.5 % to 6.6 % (uncorrected *p*-distance).
305 Surprisingly, genetic variation within H1 containing specimens from the shallow-water *H.*
306 *pruvoti* and the deep-sea *H. dedritifera* was very low (0.5 %) while genetic divergence
307 between specimens morphologically identified as *H. dedritifera* (H1 and H3) were much
308 higher (4.3 %). In addition, genetic divergence between specimens identified as *H. pruvoti*
309 (H1 vs. Seq2) was as high as 3.5 % (Table 2).

310 ***ATPS dataset***

311 The resulting dataset comprised 33 sequences with 235 nucleotides (82 variable, 42 of
312 which parsimony informative), collapsed into 11 different haplotypes unambiguously

alignable. The three clades (H1-3), previously detected with COI, were also recovered with high Bayesian posterior probabilities and ML bootstrap support in the analysis of the ATPS intron (Fig. 3). The deep-sea *H. dedritifera* specimens from Clade H1 now formed a well supported subclade (99/96), while the *H. pruvoti* specimens from the Marseille and Monaco regions (mam) differed by 15 to 19 bp from this subclade. H2 was shown as a subclade of a larger clade now including the Ionian *Hexadella* sp. sequences (Seq 1). This subclade (H2) contained again all *H. racovitzai* specimens, with the deep-sea *H. racovitzai* specimen from Port-Cros (CRO) differing from the remaining specimens by 9 bp. Clade H3 contained the deep-sea *H. dedritifera* specimens from the Irish (ROC), the Scottish (MIN) and the Norwegian margins (BER, ROS), represented by a single haplotype and the samples from Norway (ROS), and the Greenland Sea (GRE), represented by another haplotype differing 3 bp from the previous. The *H. pruvoti* specimens from the Gorringe Bank (Seq2) were again highly divergent from the *H. pruvoti* samples from the Mediterranean Sea (H1).

The genetic divergence between the three supported ATPS clades (H1-3) and the two divergent sequences (Seq1, 2) were about twofold the values found with COI, and ranged from 9.6 % to 21.3 %. Intra-clade variation within H1 was low (3.3 %) supporting the lack of genetic divergence between the shallow-water *H. pruvoti* specimens (P1mam, P2mam, P3mam) and the deep-sea *H. dedritifera* specimens in the Ionian Sea, the Bay of Biscay (BIS) and the Rockall Bank (ROC). Similarly, the low variation observed within H3 (1.3%) suggests a lack of genetic divergence between the Irish, the Scottish and the Norwegian margins. The high genetic divergence values between H1 and H3 (9.6 %) and between H1 and Seq 2 (16.2%) confirmed the occurrence of sharp genetic breaks within *H. dedritifera* and within *H. pruvoti*, respectively.

28S rDNA dataset

The resulting dataset comprised 33 sequences with 411 characters (9 variable, 3 of which parsimony informative), collapsing into 10 different haplotypes. Phylogenetic resolution in the nuclear ribosomal 28S gene was clearly lower than in the two other markers, but species of the genus *Hexadella* were nevertheless confirmed as a monophyletic group (Fig. 4), with high Bayesian posterior probabilities and high ML bootstrap support (100/99). This marker was, however, unable to discriminate the different *Hexadella* species. For instance, *H. pruvoti* (mam) and *H. dedritifera* specimens from the Bay of Biscay (BIS), Scotland (MIN) and Norway (ROS, BER) were shown as a single haplotype. Values of sequence divergence (uncorrected *p*-distance) were about 10-fold smaller for 28S rDNA than for the COI and 25-fold smaller than for ATPS (data not shown).

Concatenated COI-ATPS-28S dataset

A partition homogeneity test conducted on COI and ATPS sequences *(p=0.11),* on COI and 28S sequences (*p=0.25)* and on ATPS and 28S sequences (*p=0.68*) indicated that data partitions were significantly congruent and therefore sequences of the three markers were concatenated for further analyses.

The resulting dataset comprised 25 sequences, with 1303 nucleotides. *Hexadella* spp. were confirmed as a monophyletic group, with high Bayesian posterior probabilities and high ML bootstrap support (100/100). The three clades (H1-3) and the two divergent sequences (Seq1, 2) were recovered in both analytical methods with higher support from the combined dataset than from the individual marker's datasets. *H. dedritifera* from clade H3 is confirmed in the concatenated dataset as highly divergent from *H. dedritifera* from clade H1. Similarly, Seq 2 is confirmed in the combined dataset as clearly divergent from the *H. pruvoti* specimens occurring in the Mediterranean Sea (H1) (Fig. 5).

Discussion

Our results demonstrate for the first time the presence of deeply divergent lineages in a major group of benthic marine invertebrates associated with deep-water coral ecosystems along the European margin. The deep-sea species *H. dedritifera* is polyphyletic, as shown by phylogenetic reconstructions of COI, ATPS, and concatenated data which revealed two highly divergent clades H1 vs H3 (uncorrected p-distances of 4.3% in COI and 9.6% in ATPS). No COI threshold was defined to separate Evolutionary Significant Units because great differences in rates of evolution between sponges groups were reported (Solé-Cava and Boury-Esnault, 1999; Cárdenas et al., 2007). Nevertheless, the genetic divergence values observed between H1 and H3 for ATPS are in the range of the maximum p-distance values reported in previous molecular analyzes between sponge species (Wörheide et al., 2008). The material from the Irish, the Scottish, the Scandinavian margins and the Greenland Sea in the NEA (Clade H3) probably represents *H. dedritifera sensu* Topsent, 1913 because of its origin close to Bear Island (Norway), the type locality. Following this, *H. dedritifera* specimens from clade H1 should be called *Hexadella* cf. *dedritifera* until proper redescription.

At the same time, *H. pruvoti* and *H.* cf. *dedritifera*, both bright-yellow colored, form a strongly supported monophyletic clade (H1). The low intra-clade variation within H1 in CO1 and in ATPS (maximum uncorrected p-distance 0.5% and 3.3% respectively) stresses the lack of genetic divergence between the shallow-water *H. pruvoti* specimens and the deep-sea *H.* cf. *dedritifera* specimens in the Mediterranean Sea. We may consider that one single species, *H.pruvoti*, occupies a wide bathymetric range in the Mediterranean Sea. However, morphological discriminating criteria such as choanocyte chamber size or incorporation of foreign material need to be reassessed, in our opinion, before synonymizing both species.

Besides, the results highlight the occurrence of significantly divergent lineages of *H. dedritifera*: H3 vs. D23ROC (H1) within the Irish cold-water coral reefs (ROC), indicating

independent evolutionary history of deep-sea sponges in the same cold-water coral ecosystem. Similarly, a putatively new *Hexadella* species (Seq 1) associated with the deep-sea reefs in the Ionian Sea was found to diverge from the *Hexadella* cf. *dedritifera* with genetic variation values of about 3.6 % in the COI fragment and 13.1 % in the ATPS fragment. This suggests that the deep-sea coral ecosystems are not only a source of diversity, but that these habitats have accumulated species and lineages over time.

Despite their morphological similarity, the shallow-water individuals from the Atlantic Gorringe Bank (Seq 2) were well differentiated in both the COI and the ATPS (maximum uncorrected p-distance around 3.5 % and 16.2% respectively) from the Mediterranean Sea *H. pruvoti* (Clade H1). The type locality of this species is Banyuls (France, Mediterranean Sea, Topsent, 1896), and therefore specimens from the Gorringe Bank most likely represent a new species, hereafter called *Hexadella* cf. *pruvoti,* that will require proper taxonomic description. In contrast to *H. dedritifera* and *H. pruvoti*, the specimens of *H. racovitzai* from the Mediterranean Sea (mam, ban, CRO), the Atlantic Gorringe Bank (gor) and the United Kingdom area (ire, eng) were found to be genetically closely related (monophyletic). The deep-sea specimen (CRO) was the first *H. racovitzai* reported from the deep-sea and was shown as a divergent sequence in both the ATPS and the combined dataset. Nevertheless, more individuals per populations would be necessary to unravel the intra-specific diversity of this species, and to elucidate possible cryptic speciation patterns within the species.

The mitochondrial cytochrome c oxidase subunit I (COI) gene and the D3-D5 region of the nuclear large ribosomal subunit (28S rDNA) have been repeatedly used in sponge systematics (Erpenbeck et al., 2002; Duran and Rützler, 2006; Erpenbeck et al., 2006; Wulff, 2006; Blanquer and Uriz, 2007). The second intron of the nuclear ATP-synthetase beta subunit gene (ATPS) has only recently been shown to provide a high resolution at the intra-specific level in sponge evolutionary studies (Bentlage and Wörheide, 2007; Wörheide et al.,

2008). Although mitochondrial genes are maternally inherited and follow different evolutionary pathways than their nuclear counterparts (Ballard and Whitlock, 2004) we found a topological congruency between the trees obtained with COI and the ATPS. Besides, the ATPS marker showed more than two-fold higher substitution rates than the mitochondrial one. This analysis of ATPS sequences proved the intron marker very useful for taxonomic purposes within the genus *Hexadella* and suggested the use of ATPS of great interest to complement mitochondrial markers in sponge taxonomic studies. Nevertheless, two ATPS haplotypes instead of five in the COI dataset were shown in clade H3, possibly because a highly variable position of the ATPS partition showed some double peaks in the chromatogram and could not be resolved. It suggests that the ATPS marker is of great potential for future phylogeographic studies within *Hexadella* species and for deeper insights into gene flow patterns, provided that the two alleles of the intron are retrieved. As expected, the 28S (D3-D5) gene fragment showed the lowest resolution (max. 1.8% pairwise genetic distance). Our results show that this gene partition alone is inappropriate to address the phylogenetic relationships among the studied material. Our 28S partition being only 411 bp long, the use of a longer fragment might increase the resolution of the marker. Or one could use the 28S (D1-D2) partition instead, shown to evolve at a slightly higher rate than COI in Geodiidae sponges (Cárdenas et al., 2009). Despite high distances, COI exhibited a low resolution at the deeper phylogenetic nodes among the different *Hexadella* spp.. The observed polytomy might be the result of either insufficient sampling of taxa and/or data (i.e. soft polytomy). Given the vastness of ocean margins and the wide distribution of sponge habitats, the existence of other undiscovered species remains very likely.

Interestingly, no single polymorphic site was observed in the COI sequences between the H1 specimens from the Ionian Sea (ION), the Bay of Biscay (BIS) and the D23ROC specimen (ROC), and only 3% genetic divergence was observed in the ATPS dataset for those

H. cf. *dedritifera.* This very low genetic divergence values across more than 5200 km (Ionian Sea-Rockall Bank) may represent the first genetic evidence suggesting the connection of deep-water coral associates from the Mediterranean Sea up to the Irish margin. This connection could be the result from a restricted gene flow between intermediate (unsampled) localities. Reveillaud et al. (2008) suggested the Biscay canyons and slope to presumably act as a semi-continuous habitat for larval dispersal of stony corals, while low gene flow has already been reported in sponges (e.g., Duran et al. 2004). Furthermore, it has been recently shown in laboratory conditions (Maldonado, 2009) that larvae in the order Verongida (*Aplysina aerophoba*) can swim for seven days before settling, indicating potential great dispersal phase in at least some aspiculate demosponges. This is also in accordance with the sporadic gene flow described for the coral species *Lophelia pertusa* between reefs along the similar NEA continental margin (Le Goff-Vitry et al., 2004; Le Goff-Vitry and Rogers, 2005). A presumably post-glacial deep-sea colonization event from the Mediterranean to the high-latitude reefs may possibly be explained by the process of species emigration in association with the warm saline Mediterranean Outflow Water (MOW) mass (Huvenne et al., 2005; De Mol et al., 2005), which can be identified throughout a large part of the North Atlantic as far as the Porcupine Seabight, south-west of Ireland (Millot et al., 2006), at depths of 800 to 1200 m. Paleoecological analyses also suggest that recent glaciations have forced some fauna towards more southern refugia such as the Mediterranean Sea, the region off NW Africa, and the mid-Atlantic ridge beyond the southern limit of the ice sheets, where the oldest coral U/th datings are observed (Schröder-Ritzrau et al., 2005). From these putative speciation centers, post-glacial currents may have driven cold-water corals and associated fauna towards northern latitudes (Roberts et al., 2006). Given these two oceanographical and paleoecological hypothesis, it is likely that the colonization pattern for the deep-sea *H.* cf. *dedritifera* results from a post-glacial expansion from the Mediterranean Sea into the North East Atlantic. The

co-occurrence in the Mediterranean Sea of two highly divergent deep-sea species *H.* cf. *dedritifera* (H1) and *Hexadella* sp. (Seq 1) further reinforces the putative role of this region as an important glacial refugium and speciation center.

Similarly, the well-supported clade H3 including specimens from the Irish, the Scottish, the Norwegian margins and the Greenland Sea suggests the connection of the northern latitudes specimens. A single COI haplotype shared between the Irish, the Scottish and the Norwegian margin can raise some discussion about its significance. However a single ATPS haplotype shared between these geographically distant regions, taking into account that one critical mutational step could not be resolved due to the absence of allele resolution in this study, is suggesting a putative gene-flow between these reefs. Further sampling of *H. dedritifera* from northern populations and exploration of each ATPS allele will be needed to fully understand the putative ongoing gene-flow within this species.

Conclusion

This phylogenetic study illustrates the evolutionary distinctiveness of different lineages within the genus *Hexadella* in both shallow-water environments and deep-water coral systems. Before this study, three species of *Hexadella* were described from the Northeast Atlantic and Mediterranean Sea: *H. dedritifera*, *H. pruvoti* and *H. racovitzai*. Our phylogenetic analyses, based on the congruence of three independent nuclear and mitochondrial markers have revealed three new cryptic species: *Hexadella* cf. *dedritifera* (maybe a junior synonym of *H. pruvoti*), *H.* cf. *pruvoti* (from the Gorringe Bank) and *Hexadella* sp. (at the moment only known from a deep-sea coral bank in the Ionian Sea). Now that they were revealed by molecular markers, an ‘a posteriori’ search of diagnostic phenotypic characters and description of these new cryptic species is the next step for

taxonomists (Blanquer and Uriz, 2008). Sharp genetic breaks as well as connections in the deep-sea species *H. dedritifera* and *Hexadella* cf. *dedritifera* confirm that speciation centers provide opportunities for the evolution and diversification of taxa that subsequently colonize other regions. These results stress the need to protect multiple lineages of cold-water coral reefs. This important aspect in the conservation of deep-sea resources will ensure the maintenance of various sources of biodiversity.

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Figures and tables

Table 1. *Hexadella* specimens analyzed in the present study. Information regarding the sampling (region, localities, sampling method, coordinates, depth), number of individuals studied for each marker, and number of different haplotypes (No. h) is provided. Sampling location abbreviations are given in uppercase letters for deep-sea samples (>100m) and in lowercase letters for shallow-water samples.

Table 2. Genetic divergence (uncorrected p-distance) between terminal clades (below diagonal) and between individuals within terminal clades (on diagonal) for mtDNA (COI) and nuclear (ATPS intron) markers. The different clades are presented in Figs. 2-5.

Fig 1. Map showing sampling localitions of *H. dedritifera* (star shape), *H. pruvoti* (round shape) and *H. racovitzai* (square shape). For abbreviations of sampling localities see Table1. Map was provided by the project Hotspot Ecosystem Research and Man's Impact on European Seas (HERMIONE).

Fig 2. Strict consensus tree of the mtDNA COI fragment. Bayesian posterior probabilities (when > 95%) and the ML bootstrap values (when >80%) are indicated above and below branches, respectively. For information on the specimens see Table 1.

Fig 3. Strict consensus tree of the nuclear ATPS intron. Bayesian posterior probabilities (when > 95%) and the ML bootstrap values (when >80%) are indicated above and below branches, respectively. The tree is midpoint rooted. For information on the specimens see Table 1.

Fig 4. Strict consensus tree of the rDNA 28S fragment. Bayesian posterior probabilities (when > 95%) and the ML bootstrap values (when >80%) are indicated above and below branches, respectively. For information on the specimens see Table 1.

Fig 5. Strict consensus tree of the concatenated dataset (COI-ATPS-28S). Bayesian posterior probabilities (when > 95%) and the ML bootstrap values (when >80%) are indicated above and below branches, respectively. For information on the specimens see Table 1.

Figure1


GREENLAND SEA
RØST REEF
BERGEN
MINGULAY
Rathlin Island
ROCKALL
Plymouth
BISCAY
Marseille
Monaco
Banyuls
PORT-CROS
IONIAN SEA
Gorringe

Figure2

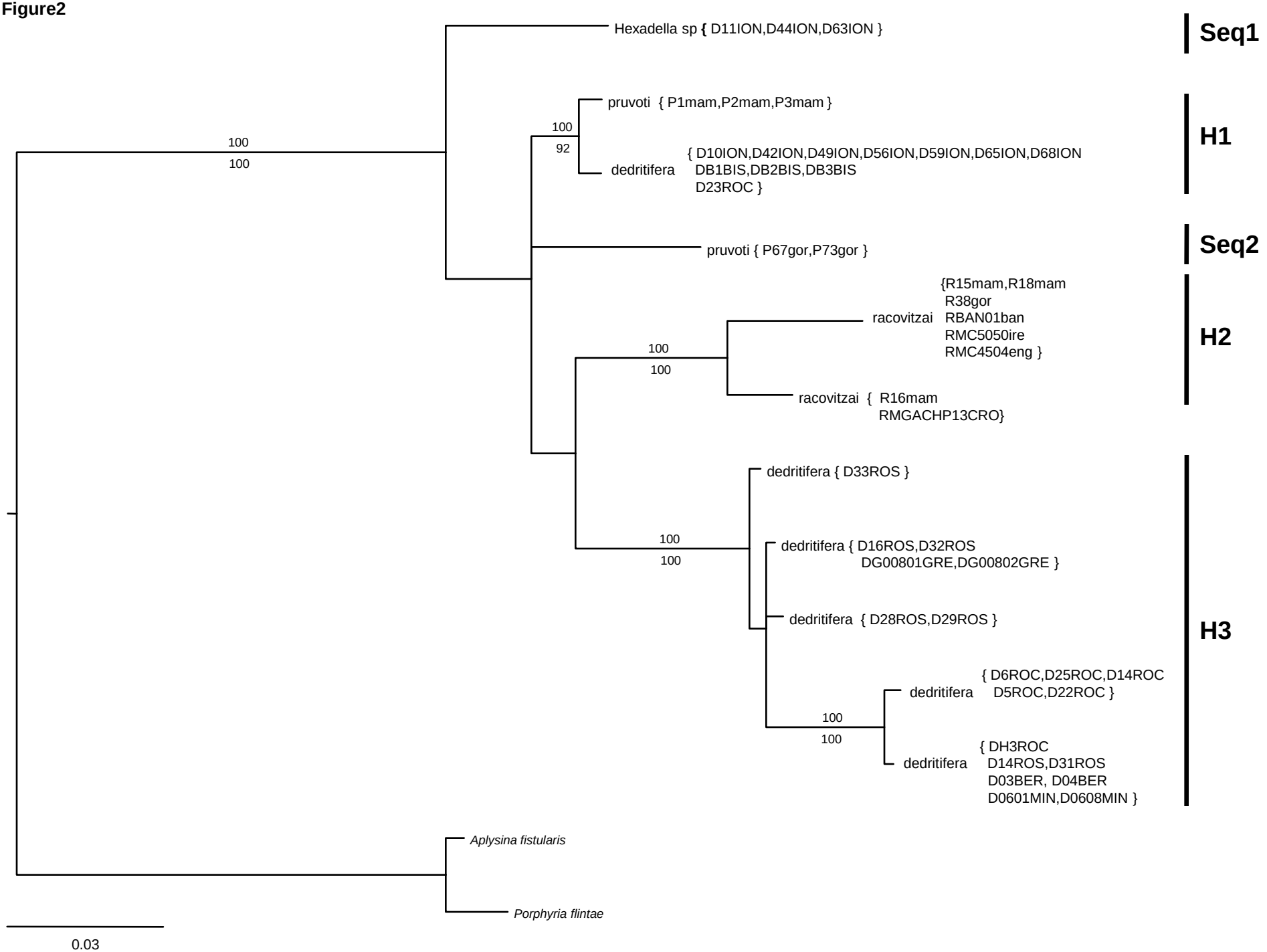
Hexadella sp { D11ION,D44ION,D63ION }
Seq1
pruvoti { P1mam,P2mam,P3mam }
100
92
dedritifera { D10ION,D42ION,D49ION,D56ION,D59ION,D65ION,D68ION
DB1BIS,DB2BIS,DB3BIS
D23ROC }
H1
100
100
pruvoti { P67gor,P73gor }
Seq2
{R15mam,R18mam
R38gor
racovitzai RBAN01ban
RMC5050ire
RMC4504eng }
100
100
racovitzai { R16mam
RMGACHP13CRO}
H2
dedritifera { D33ROS }
100
100
dedritifera { D16ROS,D32ROS
DG00801GRE,DG00802GRE }
dedritifera { D28ROS,D29ROS }
{ D6ROC,D25ROC,D14ROC
dedritifera D5ROC,D22ROC }
100
100
{ DH3ROC
D14ROS,D31ROS
dedritifera D03BER, D04BER
D0601MIN,D0608MIN }
H3
Aplysina fistularis
Porphyria flintae
0.03

Figure3

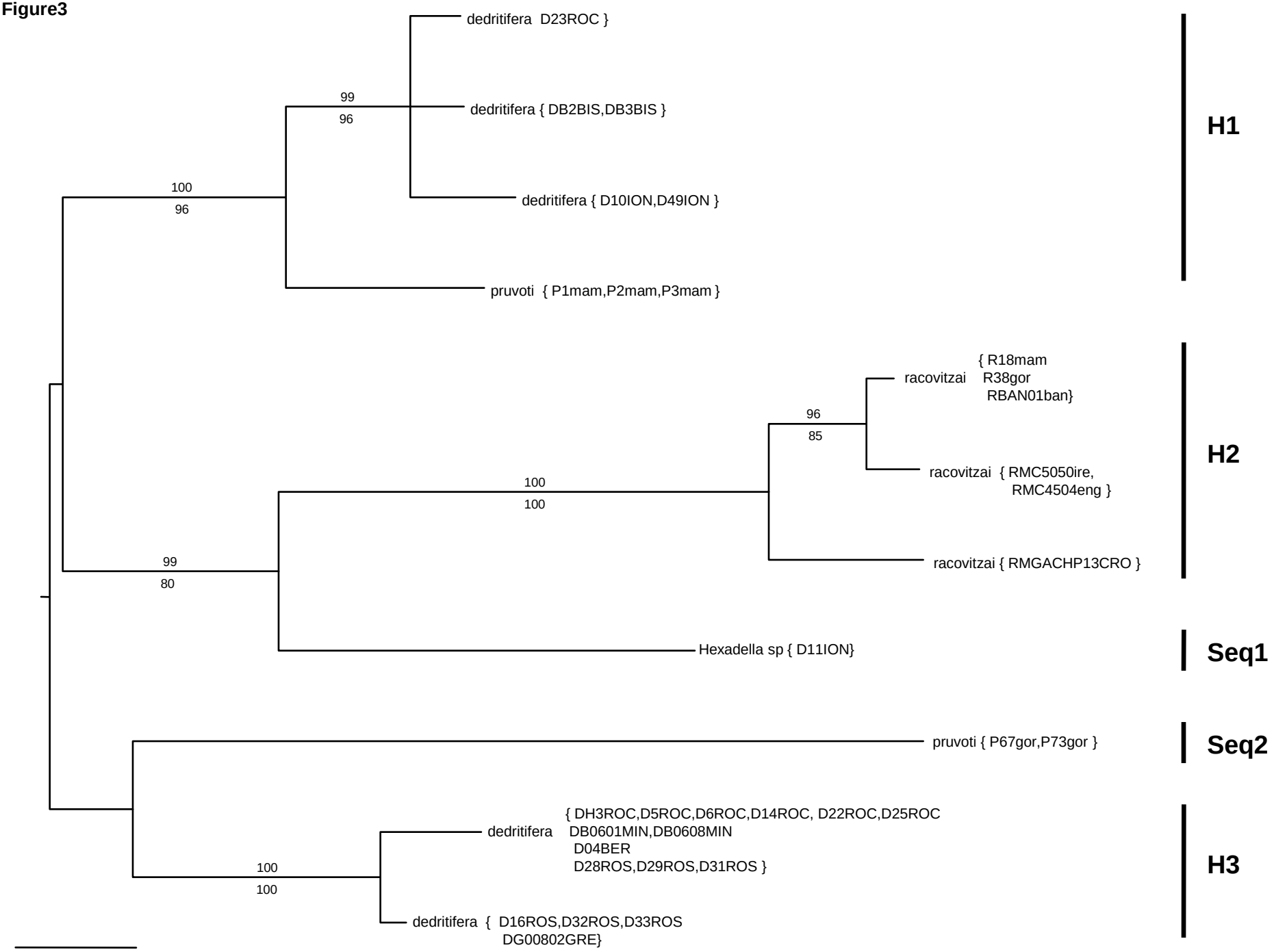
dedritifera D23ROC }
99
96
dedritifera { DB2BIS,DB3BIS }
100
96
dedritifera { D10ION,D49ION }
pruvoti { P1mam,P2mam,P3mam }
H1
{ R18mam
racovitzai R38gor
RBAN01ban}
96
85
racovitzai { RMC5050ire,
RMC4504eng }
100
100
racovitzai { RMGACHP13CRO }
99
80
H2
Hexadella sp { D11ION}
Seq1
pruvoti { P67gor,P73gor }
Seq2
{ DH3ROC,D5ROC,D6ROC,D14ROC, D22ROC,D25ROC
dedritifera DB0601MIN,DB0608MIN
D04BER
D28ROS,D29ROS,D31ROS }
100
100
dedritifera { D16ROS,D32ROS,D33ROS
DG00802GRE}
H3
0.02

Figure4

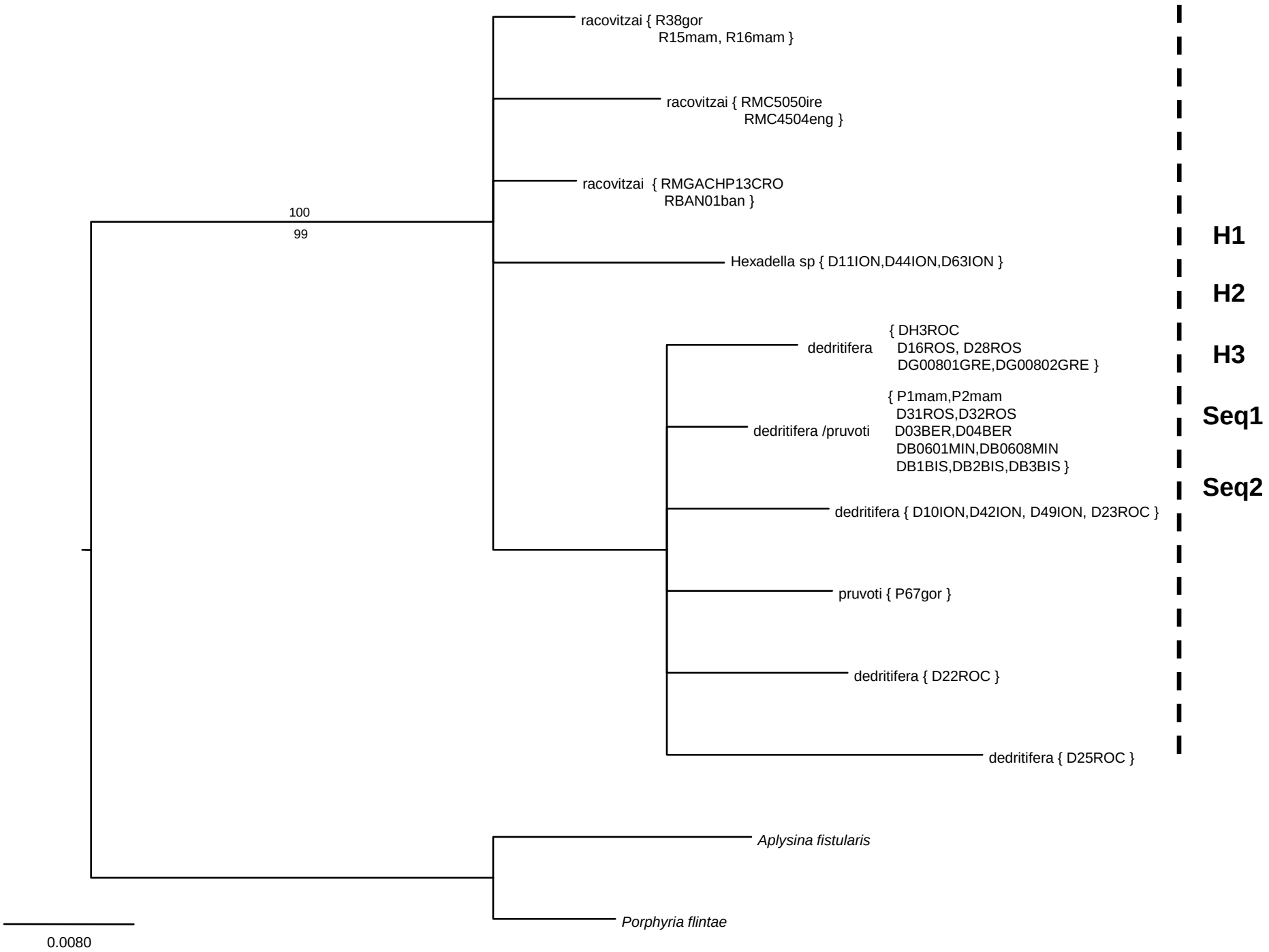
racovitzai { R38gor
R15mam, R16mam }
racovitzai { RMC5050ire
RMC4504eng }
racovitzai { RMGACHP13CRO
RBAN01ban }
100
99
Hexadella sp { D11ION,D44ION,D63ION }
dedritifera { DH3ROC
D16ROS, D28ROS
DG00801GRE,DG00802GRE }
dedritifera /pruvoti { P1mam,P2mam
D31ROS,D32ROS
D03BER,D04BER
DB0601MIN,DB0608MIN
DB1BIS,DB2BIS,DB3BIS }
dedritifera { D10ION,D42ION, D49ION, D23ROC }
pruvoti { P67gor }
dedritifera { D22ROC }
dedritifera { D25ROC }
Aplysina fistularis
Porphyria flintae
0.0080
H1
H2
H3
Seq1
Seq2

Figure5

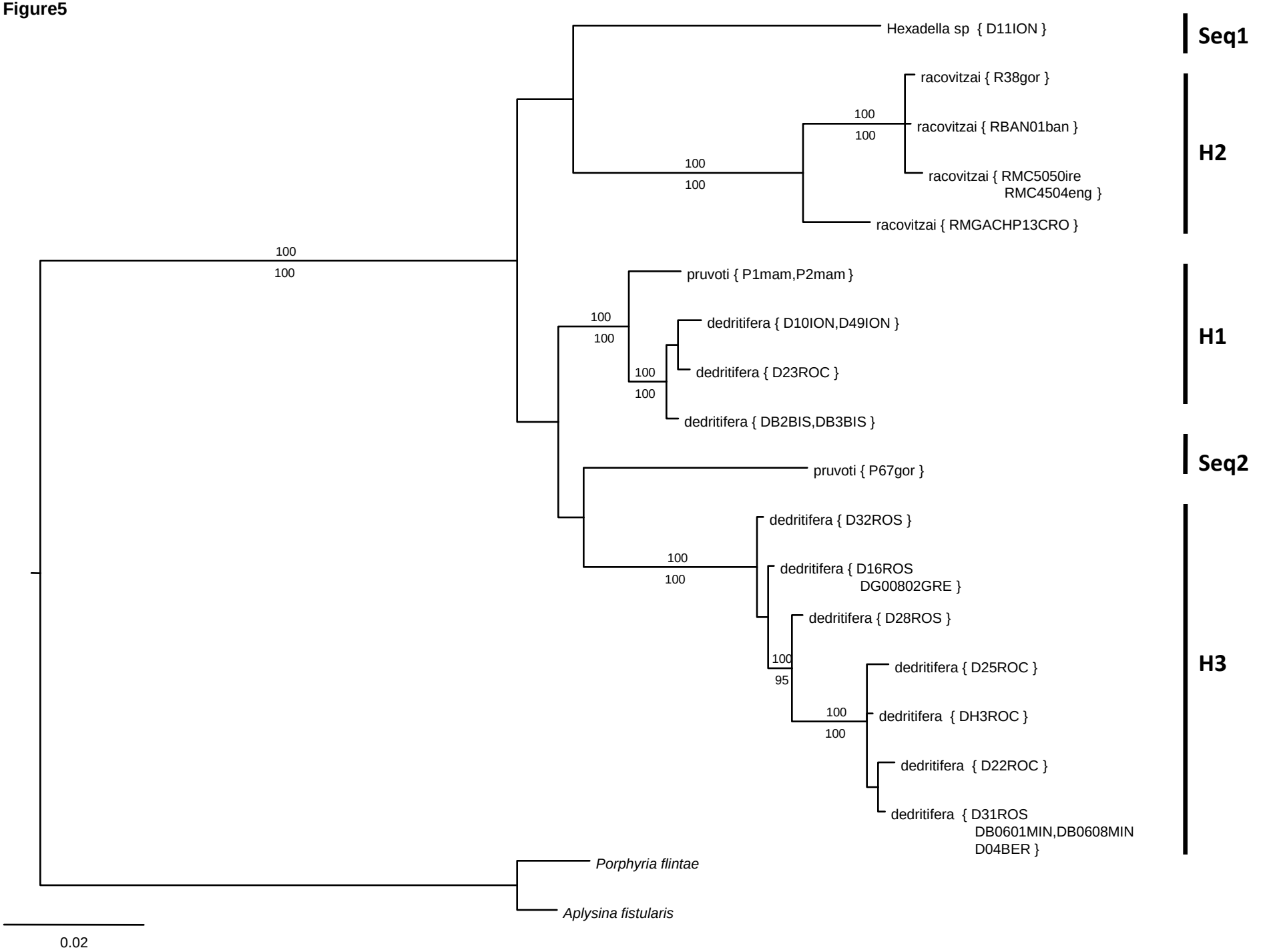
Hexadella sp { D11ION }
racovitzai { R38gor }
racovitzai { RBAN01ban }
racovitzai { RMC5050ire
RMC4504eng }
racovitzai { RMGACHP13CRO }
pruvoti { P1mam,P2mam }
dedritifera { D10ION,D49ION }
dedritifera { D23ROC }
dedritifera { DB2BIS,DB3BIS }
pruvoti { P67gor }
dedritifera { D32ROS }
dedritifera { D16ROS
DG00802GRE }
dedritifera { D28ROS }
dedritifera { D25ROC }
dedritifera { DH3ROC }
dedritifera { D22ROC }
dedritifera { D31ROS
DB0601MIN,DB0608MIN
D04BER }
Porphyria flintae
Aplysina fistularis
100
100
100
100
100
100
100
100
100
100
100
100
100
95
100
100
Seq1
H2
H1
Seq2
H3
0.02

Table1

Species	Region	Localities	Deep-sea cruise, shallow water sampling (collector) or Museum	Lat.[N]	Lon.	Depth[m]	COI	No.h	ATPS	No.h	28S	No.h
H. dedritifera (D)	North East Atlantic	Ireland, Rockall Bank (ROC)	Moundforce 2004 (R/V *Pelagia*), BIOSYS/HERMES 2005 (R/V *Pelagia*)	55,45/55,50	-15,78/-16,11	575-773	7	3	7	2	4	4
H. dedritifera (D)	North East Atlantic	Norway, Røst reef (ROS)	ARK-XXII/1a 2007 (R/V *Polarstern*)	66,96/67,50	9,42/11,13	319-345	7	4	6	3	4	2
H. dedritifera (D)	Mediterranean Sea	Italy, Ionian Sea (ION)	Medeco 2007 (R/V *Pourquoi Pas?)*	39,56/39,61	18,43/18,50	561-649	7	1	2	1	3	1
H. dedritifera (D)	North East Atlantic	France, Bay of Biscay (BIS)	Biscosystem 2008 (R/V *Belgica*)	48,90/48,91	-5,32/-5,33	676	3	1	2	1	3	1
H. dedritifera (D)	North East Atlantic	Norway, Bergen (BER)	ZMA (Zoological Museum of Amsterdam)	60,30	5,10	100	2	1	1	1	2	1
H. dedritifera (D)	North East Atlantic	Scotland, Mingulay (MIN)	BIOSYS/HERMES 2006 (R/V *Pelagia*)	56,82	-7,37/7,39	128-137	2	1	2	1	2	1
H. dedritifera (D)	North East Atlantic	Greenland Sea, Schultz Seamount (GRE)	H2DEEP 2008 (R/V *G.O.Sars*)	73,95	7,71	688	2	1	1	1	2	1
H. pruvoti (P)	North East Atlantic	South Portugal, Gorringe Bank (gor)	Scuba diving (Joana Xavier)	36,51/36,71	-11,16/-11,56	39-42	2	1	2	1	1	1
H. pruvoti (P)	Mediterranean Sea	France, 3PP coral caveMarseille-Monaco (mam)	Scuba diving (Thierry Perez)	43,21/43,73	5,33/7,42	15-20	3	1	3	1	2	1
H. racovitzai (R)	Mediterranean Sea	France, 3PP coral cave Marseille-Monaco (mam)	Scuba diving (Thierry Perez)	43,21/43,73	5,33/7,42	15-20	3	2	1	1	2	1
H. racovitzai (R)	Mediterranean Sea	France, Banyuls (ban)	ZMA (Zoological Museum of Amsterdam)	42,50	3,13	35	1	1	1	1	1	1
H. racovitzai (R)	Mediterranean Sea	France, Port Cros (CRO)	MedSeaCan (R/V *Minibex*)	43,00	6,39	180	1	1	1	1	1	1
H. racovitzai (R)	North East Atlantic	South Portugal, Gorringe Bank (gor)	Scuba diving (Joana Xavier)	36,51	-11,56	32	1	1	1	1	1	1
H. racovitzai (R)	North East Atlantic	England, Plymouth, Outer Heybrook Bay (eng)	Scuba diving (Bernard Picton and Claire Goodwin)	50,31	-4,12	30	1	1	1	1	1	1
H. racovitzai (R)	North East Atlantic	Northern Ireland , Rathlin Island (ire)	Scuba diving (Bernard Picton and Claire Goodwin)	55,29	-6,25	30	1	1	1	1	1	1
Hexadella sp.	Mediterranean Sea	Italy, Ionian Sea (ION)	Medeco 2007 (R/V *Pourquoi Pas?)*	39,61	18,50	648	3	1	1	1	3	1

Table2

COI	H3	H1	Seq2	Seq1	H2
H3	0.012				
H1	0.043	0.005			
Seq2	0.057	0.035	0.000		
Seq1	0.060	0.036	0.054	0.000	
H2	0.061	0.049	0.052	0.066	0.029

ATPS	H3	H1	Seq2	Seq1	H2
H3	0.013				
H1	0.096	0.033			
Seq2	0.154	0.162	0.000		
Seq1	0.13	0.131	0.192	X	
H2	0.147	0.151	0.213	0.137	0.025