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ORIGINAL ARTICLE

Species delimitation and phylogeny of *Doto* (Nudibranchia: Dotidae) from the Northeast Atlantic, with a discussion on food specialization

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Abstract

The nudibranch genus *Doto* is taxonomically problematic in particular, and some species are described on the notion of strict monophagy. Here we perform species delimitation on NE Atlantic species, as well as placing them phylogenetically, using two markers: the mitochondrial *COI* and the nuclear *H3*. We also study the morphology of the species including radular ultrastructure and review food specificity. Specimens were first divided into potential species using ABDG on both markers, these groups were used as input species for species delimitation analyses using BPP, and analyses were performed with both markers combined and on H3 only. The analyses delimit 11 and eight species, respectively. With the exception of one species for which only *COI* was available, the differences are found in *D. fragilis*, which is split into three groups when *COI* is included and lumped into one with only H3. *Doto hystrix* is nested within these groups. We also found that specimens from Sweden seemingly close to *D. maculata* in external morphology have identical sequences as *D. coronata*. Analysis of food preferences of the species involved in the study contradicts the notion of strict monophagy within *Doto*.

KEYWORDS
BPP, multi-species coalescent, Northern Ireland, Norway, Sweden, Gastropoda

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1 | INTRODUCTION

The genus *Doto* Oken, 1815 comprises over 90 species of small nudibranchs (Molluscabase, 2021), seldom over a centimeter in length, often only half a centimeter or less. They are generally difficult to identify, with taxonomic uncertainties and species complexes (Lemche, 1976; Morrow et al., 1992; Pola & Gosliner, 2010; Shipman & Gosliner, 2015; Thompson & Brown, 1984), coupled with variation in pattern and a scarcity of defining morphological features. The genus is therefore often viewed as the one of taxonomically most problematic groups among the nudibranchs. The family Dotidae, including four genera, is placed within the Dendronotoidea, combining presence of slender body, smooth oral veil, cone-shaped dorsolateral appenadges and absence of the cuticular lining of stomach (Korshunova, Bakken, et al., 2020; Korshunova & Martynov, 2020; Wägele & Willan, 2000).

The species of *Doto* typically have stout dorsolateral appendages, forming pairs along the body. These are not cerata *sensu stricto* because of radically different structure and function. The appendages have neither cnidosacs nor apical pore, but instead have small tubercles in several circlets, placed one above the other. Some species have small, simple pseudobranchs placed close to the base of the dorsolateral appendages, on the side facing the dorsal midline. They can be distinguished from the tubercles by being transparent and having a different shape. The front of the head has a smooth and often inconspicuous oral veil that usually has two rounded lateral flap-like extensions. The rhinophores are smooth, fingerlike and are placed in distinct, often flared sheaths. The radula is narrow and uniseriate, and the jaws are frail and reduced, or even absent (Lemche, 1976; Lundin et al., 2020; Odhner, 1936; Thompson & Brown, 1984).

Species of *Doto* feed on thecate and athecate hydrozoans and are often claimed as specialized on certain hydrozoan species or genera (Morrow et al., 1992; Picton & Brown, 1981). They may have a notable impact on the marine benthic ecosystem because of the predatory pressure on hydroids, but to our knowledge no studies have been made on this. They do not prey upon the hydroid polyps *per se*, but slice through the perisarc of the hydroid stalks below the polyps and feed on the caenosarc fluid (Thompson & Brown, 1984). In the North Sea area, fully grown specimens are predominantly observed during late winter to early summer, but some species can be observed later in the season. Because of the taxonomic uncertainties of the species complexes, it is difficult to estimate more precise seasonal variation.

The type species of the genus, *Doto coronata* (Gmelin, 1791), was originally described by Johann Gmelin as *Doris coronata* (Gmelin, 1791, p. 3105). Lorenz Oken (1815) introduced the genus name *Doto* Oken, 1815 (Oken, 1815). Nils Odhner made the first attempt at a revision of the species of *Doto* in the world and suggested a division of the genus into three main subgroups, based on the coloration of the tubercles on the dorsolateral appendages (Odhner, 1936). *Doto coronata* had a prominent position in one of these subgroups and *Doto fragilis* (Forbes, 1838) in another. In the 1970, Henning Lemche discovered that *Doto coronata* constitutes a species complex, based on observations from samples of *Doto* collected at numerous localities in the North Atlantic, mainly in the northeast (Lemche, 1976). Based on morphology and species-specific preferences for hydroid prey, Lemche (1976) described five new species from the *coronata* complex; *D. dunnei* Lemche, 1976, *D. eireana* Lemche, 1976, *D. koenneckeri* Lemche, 1976, *D. millbayana* Lemche, 1976, and *D. tuberculata* Lemche, 1976; as well as redescribing older species and establishing a neotype for *D. maculata* (Montagu, 1804), thus doubling the number of known species from the North East Atlantic area. Picton and Brown (1981) acknowledged that *Doto fragilis* constitutes a species complex and described the species *Doto hystrix* Picton & Brown, 1981. Thompson and Brown (1984) indicated that *Doto fragilis* feeds on hydroids of several different genera such as *Halecium* Oken, 1815, *Nemertesia* Lamouroux, 1812, and *Tubularia* Linnaeus, 1758, but did not separate any new species from the *D. fragilis* complex, although "varieties" associated with food were mentioned. They concluded that *D. fragilis* was quote "typically found in association with *Nemertesia* and *Halecium*" (Thompson & Brown, 1984: 32); thus, the taxonomic value for prey preference was partially dismissed. Picton and Morrow (1994, 2016) did not separate any species from within *D. fragilis* either, but mentioned that *D. fragilis* in the British Isles consists of three different forms, feeding on different hydroids. Morrow et al. (1992) introduced genetic methods for studies of *Doto* and used electrophoresis to separate two new species from the *D. coronata* complex; *Doto sarsiae* Morrow et al., 1992 and *Doto hydrallmaniae* Morrow et al., 1992, also indicating that other morphs of *D. coronata* may represent yet other species. It would take another 18 years until genetic studies using Sanger sequencing methods were presented, including North Atlantic and Indo-Pacific *Doto* species (Pola & Gosliner, 2010; Shipman & Gosliner, 2015). Most species separated by Lemche were confirmed to be distinct, but not all, e.g., *D. millbayana* did not reveal significant molecular divergence from *D. dunnei* (Shipman & Gosliner, 2015). The North Atlantic *Doto* still contains unresolved species complexes with minor differences in morphology, but found on different hydroids, such as for *D. fragilis*, and more work needs to be done before the phylogeny of the whole group is elucidated. A promising method to untangle the species complexes is to delimit species using multi-locus analysis based on the multi-species coalescent (MSC) method (Knowles & Carstens, 2007; Yang & Rannala, 2010). This method has for nudibranchs recently been applied to the genus *Amphorina* (see Korshunova, Malmberg, et al., 2020).

2 | MATERIALS AND METHODS

One of the aims of the present study is to test the species limits of the *Doto* species occurring in the North East Atlantic area, by using samples from Sweden, Norway and Northern Ireland (see Table 1, Table S1 and Figure 1 for sampling localities). We particularly wanted to investigate potential species hidden within our current understanding of *D. fragilis* and *D. maculata*, in relation to *D. coronata*. For

TABLE 1 List of material included in this study, with museum voucher numbers, county of origin and GenBank accession numbers, as well as information about which cluster they were placed in in the ABGD analyses based on *COI* and *H3*, and which primary species hypotheses (PSH) they belong to

						GenBank accession no.	
Species	**Museum voucher no.**	***COI* cluster**	***H3* cluster**	**PSH**	**Country**	***COI***	***H3***
Doto coronata	Gastr.9344	–	2	4	SE	–	MZ926919
Doto coronata	Gastr.9345	–	2	4	SE	–	MZ926920
Doto coronata	Gastr.9067	4	2	4	NI	MZ902283	MZ926915
Doto coronata	Gastr.9068	4	2	4	NI	MZ902284	MZ926916
Doto coronata	NTNU-VM-63030	4	–	4	NO	MZ902294	–
Doto coronata	NTNU-VM-62587	4	–	4	NO	MZ902295	–
Doto coronata	NTNU-VM-65472	4	–	4	NO	MZ902285	–
Doto coronata	NTNU-VM-65471	4	–	4	NO	MZ902286	–
Doto coronata	NTNU-VM-67969	4	–	4	NO	MZ902292	–
Doto coronata	NTNU-VM-67970	4	–	4	NO	MZ902293	–
Doto coronata	NTNU-VMc76152	4	–	4	NO	MZ902315	–
Doto coronata	NTNU-VM-76153	4	–	4	NO	MZ902317	–
Doto coronata	NTNU-VM-66933	4	–	4	NO	MZ902300	–
Doto coronata	NTNU-VM-66932	4	–	4	NO	MZ902301	–
Doto coronata	NTNU-VM-66924	4	–	4	NO	MZ902306	–
Doto coronata	NTNU-VM-66931	4	–	4	NO	MZ902307	–
Doto coronata	NTNU-VM-66926	4	–	4	NO	MZ902305	–
Doto coronata	NTNU-VM-76154	4	–	4	NO	MZ902319	–
Doto coronata	NTNU-VM-76180	4	–	4	NO	MZ902321	–
Doto coronata	NTNU-VM-76024	4	–	4	NO	MZ902313	–
Doto fragilis	NTNU-VM–76183	1	–	1	NO	MZ902316	–
Doto fragilis	NTNU-VM-76182	1	–	1	NO	MZ902318	–
Doto cuspidata	Gastr.9057	–	7	10	NI	–	MZ926900
Doto cf. *cuspidata* (*Doto* sp.)	NTNU-VM-66937	6	–	6	NO	MZ902297	–
Doto cf. *cuspidata* (*Doto* sp.)	NTNU-VM-66936	6	–	6	NO	MZ902299	–
Doto cf. *Cuspidata* (*Doto* sp.)	NTNU-VM-66935	6	–	6	NO	MZ902298	–
Doto dunnei	Gastr.9058	5	2	5	NI	MZ902276	MZ926901
Doto dunnei	Gastr.9490	5	2	5	SE	MZ902269	–
Doto dunnei	Gastr.9491	5	2	5	SE	MZ902270	MZ926890
Doto dunnei	Gastr.9492	5	2	5	SE	MZ902271	MZ926891
Doto dunnei	Gastr.9493	5	2	5	SE	MZ902272	MZ926892
Doto dunnei	Gastr.9494	5	2	5	SE	MZ902273	MZ926893
Doto dunnei	Gastr.9495	5	2	5	SE	MZ902274	MZ926894
Doto dunnei	Gastr.9496	–	2	5	SE	–	MZ926895
Doto fragilis neotype	Gastr.9061	1	1	1	NI	MZ902275	MZ926897/MZ926898
Doto fragilis	Gastr.9060	–	1	1	NI	–	MZ926896
Doto fragilis white morph	Gastr.9473	1	1	1	SE	MZ902244	MZ926922
Doto fragilis white morph	Gastr.9474	1	1	1	SE	MZ902245	MZ926923

(Continues)

TABLE 1 (Continued)

Species	Museum voucher no.	*COI* cluster	*H3* cluster	PSH	Country	GenBank accession no.	
						COI	*H3*
Doto fragilis white morph	Gastr.9475	1	1	1	SE	MZ902246	MZ926924
Doto fragilis white morph	Gastr.9476	–	1	1	SE	–	MZ926925
Doto fragilis white morph	Gastr.9477	–	1	1	SE	MZ902247	MZ926926
Doto fragilis white morph	Gastr.9478	1	1	1	SE	MZ902248	MZ926927
Doto fragilis white morph	Gastr.9479	1	1	1	SE	MZ902249	MZ926928
Doto fragilis	NTNU-VM-76138	1	–	1	NO	MZ902314	–
Doto fragilis	NTNU-VM-65470	1	–	1	NO	MZ902287	–
Doto fragilis	NTNU-VM-65538	1	–	1	NO	MZ902289	–
Doto fragilis	NTNU-VM-65537	1	–	1	NO	MZ902290	–
Doto fragilis	NTNU-VM-65539	1	–	1	NO	MZ902291	–
Doto fragilis	NTNU-VM-66940	1	–	1	NO	MZ902302	–
Doto fragilis	NTNU-VM-67129	1	–	1	NO	MZ902308	–
Doto fragilis	NTNU-VM-67128	1	–	1	NO	MZ902309	–
Doto fragilis	NTNU-VM-76223	1	–	1	NO	MZ902320	–
Doto fragilis	NTNU-VM-76222	1	–	1	NO	MZ902322	–
Doto cf. fragilis	Gastr.9499	3	1	3	SE	MZ902280	MZ926906
Doto cf. fragilis	Gastr.9500	3	1	3	SE	MZ902281	MZ926907
Doto cf. fragilis	Gastr.8671	3	1	3	SE	MZ902277	MZ926902
Doto cf. fragilis	Gastr.9497	3	1	3	SE	MZ902278	MZ926903/MZ926904
Doto cf. fragilis	Gastr.9498	3	1	3	SE	MZ902279	MZ926905
Doto cf. fragilis	Gastr.9343	2	1	2	SE	MZ902257	MZ926918
Doto cf. fragilis	NTNU-VM-62667	2	–	2	NO	MZ902296	–
Doto cf. fragilis	NTNU-VM-66941	2	–	2	NO	MZ902304	–
Doto cf. fragilis	NTNU-VM-65469	2	–	2	NO	MZ902288	–
Doto cf. fragilis	NTNU-VM-66939	2	–	2	NO	MZ902303	–
Doto hystrix	NTNU-VM-67896	1	–	1	NO	MZ902310	–
Doto hystrix	NTNU-VM-67895	1	–	1	NO	MZ902311	–
Doto hystrix	NTNU-VM-76126	1	–	1	NO	MZ902312	–
Doto hystrix	Gastr.9480	1	1	1	SE	MZ902250	MZ926929
Doto hystrix	Gastr.9062	1	1	1	NI	MZ902266	MZ926910
Doto hystrix	Gastr.9481	1	1	1	SE	MZ902251	MZ926930
Doto koenneckeri	Gastr.9063	7	4	7	NI	MZ902265	MZ926911
Doto maculata	Gastr.9488	–	3	11	NI	–	MZ926934/MZ926935
Doto cf. *maculata* (*D. coronata*)	Gastr.8990	4	2	4	SE	MZ902260	MZ926908
Doto cf. *maculata* (*D. coronata*)	Gastr.9086	4	2	4	SE	MZ902261	MZ926917
Doto cf. *maculata* (*D. coronata*)	Gastr.9448	4	2	4	SE	MZ902262	MZ926921
Doto cf. *maculata* (*D. coronata*)	Gastr.9486	4	2	4	SE	MZ902259	MZ926931

TABLE 1 (Continued)

Species	Museum voucher no.	*COI* cluster	*H3* cluster	PSH	Country	GenBank accession no.	
						COI	*H3*
Doto cf. *maculata* (*Doto* sp.)	Gastr.9489	5	2	5	NI	MZ902263	MZ926936
Doto cf. *maculata* (*D. coronata*)	Gastr.9487	4	2	4	SE	MZ902258	MZ926932/MZ926933
Doto millbayana	Gastr.8951	—	2	5	SE	—	MZ926899
Doto pinnatifida	Gastr.9064	9	6	9	NI	MZ902267	MZ926912
Doto pinnatifida	Gastr.9065	9	6	9	NI	MZ902268	MZ926913
Doto pinnatifida	Gastr.9066	9	6	9	NI	MZ902282	MZ926914
Doto tuberculata	Gastr.9056	8	5	8	NI	MZ902264	MZ926909
Aeolidia filomenae	Gastr.9482				SE	MZ902252	MZ926937
Aeolidia filomenae	Gastr.9483				SE	MZ902253	MZ926938
Aeolidia filomenae	Gastr.9484				SE	MZ902254	MZ926939
Dendronotus lacteus	Gastr.9446:1				SE	MZ902255	MZ926940
Dendronotus lacteus	Gastr.9446:2				SE	MZ902256	MZ926941

Note: Names given in brackets are identifications and corrections as a result of the analysis. For more information see Table S1.

this analysis, one mitochondrial and one nuclear gene were used. In addition, we are placing the species phylogenetically by analyzing our data together with already published data. Further, we test the results from the molecular analysis against morphology and radular ultrastructure for congruence, using the unified species concept (de Queiroz, 2007). Distance-based single-locus species delimitation was used to generate primary species hypotheses, which then were tested using a MSC-based multi-locus species delimitation method. In this model, genes evolve inside a species phylogeny, the branches are species, and their properties restrict the gene trees. One of these restrictions is that the divergence times between species have to be more recent than the coalescent times for any genes shared between them, assuming no genetic transfer after speciation (Rannala & Yang, 2003). This model can be used for statistical testing of species assignments (Fujita et al., 2012; Rannala, 2015) and has been shown to outperform distance methods (Yu et al., 2017). Thus, we could achieve a more robust model of taxonomy and phylogeny of the genus *Doto* in the Northeast Atlantic than previous studies, as a step on the way to a more conclusive model on a larger scale.

In the molecular study, a total of 82 specimens were included, representing 11 morphospecies of *Doto*, from the Skagerrak, the Irish Sea, the North Sea, the Norwegian Sea and the Barents Sea. Also, five out-group specimens from two species were used; *Aeolidia filomenae* Kienberger et al., 2016 and *Dendronotus lacteus* (Thompson, 1840) (see Table 1 and Table S1 for details). The phylogeny was estimated using our data combined with previously published data.

The Swedish specimens were collected at four different locations on the Swedish west coast, from south to north at the mouth of the Gullmar fjord close to Lysekil, the archipelago outside Smögen, the Väderö archipelago and finally at the Ide fjord at the border to Norway. The specimens from Norway were collected from south to north at the Oslo and Larvik area, at the mouth of the Sognefjord north of Bergen, at the Trondheimsfjord area, at Saltstraumen in Nordland and finally at the Finnmark area in the Arctic. The specimens from Northern Ireland were collected at Strangford Lough close to the Queens University Marine Laboratory at Portaferry. Specimens were deposited at the Gothenburg Natural History Museum (GNM), Gothenburg, Sweden, and at the NTNU University Museum (NTNU-VM) (Bakken et al., 2021), Trondheim, Norway.

2.1 | Morphological analysis

The external and internal morphology of 16 specimens of 14 species was studied under a MBS-10 stereomicroscope, using a Nikon D-810 digital camera.

The fine structure of the radula of 14 species from both Sweden and Northern Ireland was studied to cover regional variations at different parts of the Northeast Atlantic. The coated radulae were examined and photographed using a scanning electron microscope (CamScan Series II and JSM 6380).

2.2 | Molecular analysis

2.2.1 | DNA extraction, amplification and sequencing

DNA was extracted from a small tissue sample taken from the lateral side of the foot or the tail end of the foot on small specimens, using Qiagen's DNeasy Blood & Tissue Kit. Two molecular markers, the mitochondrial gene Cytochrome c oxidase subunit I (*COI*) and nuclear gene Histone H3 (*H3*), were amplified using the primers and PCR programs listed in Table 2. Sequencing was carried out by Eurofins

FIGURE 1 Map of NW Europe showing sampling localities for specimens used in this study, closely situated sampling locations has been combined for clarity. The map is based on Vector Flag of Europe with Countries—Outline available from https://freevectormaps.com/world-maps/europe/WRLD-EU-01-0003?ref=atr

TABLE 2 Primers and programs used for amplification and sequencing of fragments of *COI* and H3 markers

Gene	Amplicon length	Primer	Sequence 5′-3′	Reference	Amplification program
COI	709 (658)	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)	95°C for 5 min, 35 cycles each of 95°C for 40 s, 45°C for 45 s and 72°C for 60 s, finally, 72°C for 8 min
		HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994))	
H3	374 (328)	H3F	ATGGCTCGTACCAAGCAGACVGC	Brown et al. (1999)	95°C for 5 min, 35 cycles each of 95°C for 30 s, 50°C for 30 s and 72°C for 90 s, finally, 72°C for 8 min.
		H3R	ATATCCTTRGGCATKATRGTGAC	Brown et al. (1999)	

Note: The amplicon length is followed, in parentheses, by the length of the fragment with primers removed.

MWG Operon (Ebersberg, Germany), Sequences were assembled into consensus sequences using Geneious v.8.1.9 (Biomatters Ltd). The Norwegian specimens were handled by the Canadian Centre for DNA bar coding (CCDB) (Guelph), following their workflow for DNA bar coding, and only *COI* was sequenced. All new sequences are deposited in GenBank (see Table 1 and Table S1 for accession numbers).

The sequences of each marker were aligned using MAFFT v7.017 (Katoh et al., 2002) as implemented in Geneious. In the *H3* dataset, several individuals showed clear signs of heterozygosity, i.e., distinct double peaks at certain positions in the chromatograms. Due to this, we separated the *H3* alleles using the PHASE algorithm (Stephens & Donnelly, 2003; Stephens et al., 2001) as implemented in DNAsp v.5.10 (Librado & Rozas, 2009), the phasing was run for 100 iterations after 100 initial burn-in iterations, with a thinning interval of 1 using default settings. For homozygous specimens, only one of the two identical alleles was kept. The phased dataset was used in all subsequent analyses.

2.2.2 | Single-locus clustering and distance analyses

Both the *COI* and the *H3* datasets were analyzed with Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012) to divide the specimens into putative species, using the web version of ABGD, using simple distances and default settings. For these analyses, the out-groups were removed. Uncorrected pairwise distances were calculated for both datasets in MEGA 6.06 (Tamura et al., 2013), and missing data and gaps was excluded using pairwise deletions.

2.2.3 | Multi-locus species delimitation

Multi-locus species delimitation analyses were performed using BPP v.3.3 (Yang, 2015). The analyses were performed with *COI* and *H3*, as well as with only H3, with out-groups excluded, the datasets were divided into 11 primary species hypotheses to be tested based on the result of the ABGD analyses. Joint Bayesian species delimitations and species tree estimations were conducted, a method using the MSC model to compare different arrangements of species delimitation and species phylogeny in a Bayesian framework, accounting for incomplete lineage sorting due to ancestral polymorphism and gene tree–species tree conflicts (Rannala & Yang, 2013; Yang & Rannala, 2010, 2014). Two analyses (A and B) with different population size (θs) and divergence time (τ0) priors were performed, using the same settings and priors as in Martinsson and Erséus (2018) (A: θ 2400, τ0 2200; B: θ 21000, τ0 2200). All analyses were performed three times to confirm consistency between runs. We considered species delimited with a PP>0.90 in all analyses to be well supported. For clusters with a PP<0.90, we accepted the best supported more inclusive species.

2.2.4 | Haplotype network

To visualize the relation between different clades within the *Doto fragilis* complex (see results), a *COI* haplotype network was created, using the sequences belonging to this species complex, in PopART v1 (Leigh & Bryant, 2015) using the TCS algorithm (Clement et al., 2002; Templeton et al., 1992). Sites with missing data or gaps were masked and not included in the networks, and due to large amount of missing data, specimens Gastr. 9343 and Gastr. 9474 were excluded altogether.

2.2.5 | Phylogenetic inference

Phylogenies were estimated using Bayesian Inference on both single gene and the concatenated dataset in MrBayes v.3.2.6 (Ronquist et al., 2012). In total, four analyses were performed: one for each marker, one for a concatenated dataset with our data and one for a concatenated dataset with added sequences of several species of *Doto* Oken, 1815 and *Kabeiro* Shipman & Gosliner, 2015 from Pola and Gosliner (2010), Shipman and Gosliner (2015) and Moles et al. (2016). For all analyses, the genes were partitioned according to codon position, and partitions were unlinked. Rate variation across sites was set to gamma distribution with a proportion of invariable sites; model jumping was implemented to integrate over substitution model space. All analyses ran for 10 million generations sampling every 10 000 generations, the first 25% were discarded as burn-in, and a majority rule consensus tree was constructed. Matrices and trees are available on TreeBASE (submission 26369). For the concatenated matrix with our data, a phylogeny was also estimated with maximum likelihood in PhyML 3.0 (Guindon et al., 2010), as implemented at the Montpellier Bioinformatics platform (http://www.atgc-montpellier.fr/), Smart Model Selection (Lefort et al., 2017) with Bayesian information criterion was used for automatic model selection; and nearest neighbor interchange were used for tree improvement. Branch support was calculated with the SH-like (Shimodaira–Hasegawa test-like) approximate likelihood ratio test (aLRT) (Anisimova & Gascuel, 2006) and 1000 bootstrap replicates.

2.3 | Review of *Doto* prey specificity

Thompson and Brown (1984: 28–29) provided at least two different hydroid species as food for four out of 12 *Doto* species from the British Isles. Distantly related species such as *D. fragilis* and *D. pinnatifida* (Montagu, 1804) (Figure 2) were evidently indicated as feeding on the same hydroid species, i.e., *Nemertesia antennina* (Linnaeus, 1758) (Picton & Brown, 1981; Picton & Morrow, 2016; Thompson & Brown, 1984).

To test the for strict prey specificity within the genus *Doto* (e.g., Picton & Brown, 1981; Shipman & Gosliner, 2015), we compiled data on the hydroid associations of the *Doto* species from the available literature sources and own observations (Table 3). For pre-1976 sources (i.e., before Lemche in 1976 showed that "*D. coronata*" is a complex of different species), we generally did not include records of *D. coronata* from the same hydroids, as other species split by Lemche and other authors later on (Morrow et al., 1992; Picton & Brown, 1981) were claimed to be almost monophagous. In cases when a hydroid species was not indicated for some of these narrowly defined species, but for *D. coronata* or other pre-1976 species, we included such hydroids in Table 3. *Clytia hemisphaerica* (Linnaeus, 1767) was added to the list of food sources of *D. coronata*, and *Nemertesia antennina* was added to *D. hystrix* from our own observations (Table 3).

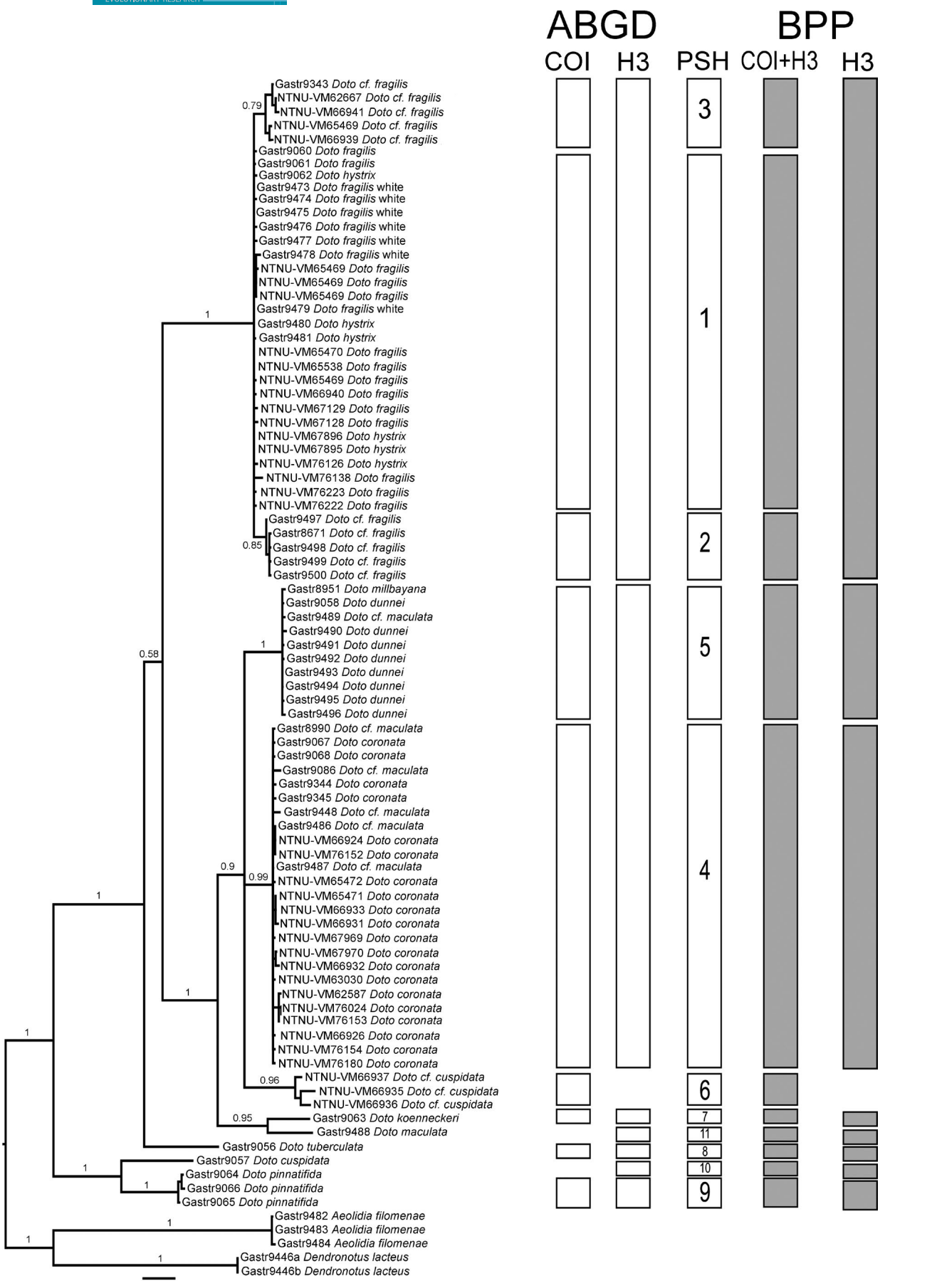
ABGD
BPP
COI
H3
PSH
COI+H3
H3
Gastr9343 Doto cf. fragilis
NTNU-VM62667 Doto cf. fragilis
NTNU-VM66941 Doto cf. fragilis
NTNU-VM65469 Doto cf. fragilis
NTNU-VM66939 Doto cf. fragilis
Gastr9060 Doto fragilis
Gastr9061 Doto fragilis
Gastr9062 Doto hystrix
Gastr9473 Doto fragilis white
Gastr9474 Doto fragilis white
Gastr9475 Doto fragilis white
Gastr9476 Doto fragilis white
Gastr9477 Doto fragilis white
Gastr9478 Doto fragilis white
NTNU-VM65469 Doto fragilis
NTNU-VM65469 Doto fragilis
NTNU-VM65469 Doto fragilis
Gastr9479 Doto fragilis white
Gastr9480 Doto hystrix
Gastr9481 Doto hystrix
NTNU-VM65470 Doto fragilis
NTNU-VM65538 Doto fragilis
NTNU-VM65469 Doto fragilis
NTNU-VM66940 Doto fragilis
NTNU-VM67129 Doto fragilis
NTNU-VM67128 Doto fragilis
NTNU-VM67896 Doto hystrix
NTNU-VM67895 Doto hystrix
NTNU-VM76126 Doto hystrix
NTNU-VM76138 Doto fragilis
NTNU-VM76223 Doto fragilis
NTNU-VM76222 Doto fragilis
Gastr9497 Doto cf. fragilis
Gastr8671 Doto cf. fragilis
Gastr9498 Doto cf. fragilis
Gastr9499 Doto cf. fragilis
Gastr9500 Doto cf. fragilis
Gastr8951 Doto millbayana
Gastr9058 Doto dunnei
Gastr9489 Doto cf. maculata
Gastr9490 Doto dunnei
Gastr9491 Doto dunnei
Gastr9492 Doto dunnei
Gastr9493 Doto dunnei
Gastr9494 Doto dunnei
Gastr9495 Doto dunnei
Gastr9496 Doto dunnei
Gastr8990 Doto cf. maculata
Gastr9067 Doto coronata
Gastr9068 Doto coronata
Gastr9086 Doto cf. maculata
Gastr9344 Doto coronata
Gastr9345 Doto coronata
Gastr9448 Doto cf. maculata
Gastr9486 Doto cf. maculata
NTNU-VM66924 Doto coronata
NTNU-VM76152 Doto coronata
Gastr9487 Doto cf. maculata
NTNU-VM65472 Doto coronata
NTNU-VM65471 Doto coronata
NTNU-VM66933 Doto coronata
NTNU-VM66931 Doto coronata
NTNU-VM67969 Doto coronata
NTNU-VM67970 Doto coronata
NTNU-VM66932 Doto coronata
NTNU-VM63030 Doto coronata
NTNU-VM62587 Doto coronata
NTNU-VM76024 Doto coronata
NTNU-VM76153 Doto coronata
NTNU-VM66926 Doto coronata
NTNU-VM76154 Doto coronata
NTNU-VM76180 Doto coronata
NTNU-VM66937 Doto cf. cuspidata
NTNU-VM66935 Doto cf. cuspidata
NTNU-VM66936 Doto cf. cuspidata
Gastr9063 Doto koenneckeri
Gastr9488 Doto maculata
Gastr9056 Doto tuberculata
Gastr9057 Doto cuspidata
Gastr9064 Doto pinnatifida
Gastr9066 Doto pinnatifida
Gastr9065 Doto pinnatifida
Gastr9482 Aeolidia filomenae
Gastr9483 Aeolidia filomenae
Gastr9484 Aeolidia filomenae
Gastr9446a Dendronotus lacteus
Gastr9446b Dendronotus lacteus
0.79
1
0.85
0.58
1
0.9
0.99
1
1
0.96
0.95
1
1
1
1
1
3
1
2
5
4
6
7
11
8
10
9

FIGURE 2 Combined *COI* and *H3* tree of *Doto* estimated by Bayesian inference. Posterior probabilities shown at branches; Scales show expected number of changes per site. As well as summary of species delimitation results, clusters from ABGD analyses for the *COI* and *H3* datasets, resulting primary species hypotheses (PSH) used as input for the BPP analysis, and results of BPP analysis, where the shaded species are well supported PP > 0.9, whereas the others have less support

3 | RESULTS

From the total 87 specimens, including the out-groups, *COI* sequence data were successfully recovered from 79 specimens, and *H3* from 48 specimens after phasing in the *H3* dataset consisted of 53 sequences. The *COI* alignment was 658 base pairs (bp) long and the *H3* alignment 328 bp long.

3.1 | Single-locus clustering analyses

The *COI* dataset was divided into nine clusters using ABGD, with a prior maximal distance $p = 0.0046$, and with higher P, the number of clusters is lower, with both the initial and recursive partitioning (Figure S3a), whereas the *H3* dataset was divided into seven clusters, with a prior maximal distance $p = 0.0129$, and a single cluster using a higher P (Figure S3b). The highest number of clusters in each marker was used, as downstream analysis only can merge clusters, not divide them further. In *COI*, *Doto fragilis* (Forbes, 1838) formed three clusters, *D. hystrix* Picton & Brown, 1981 grouped with all white morph *D. fragilis* from Sweden, as well as some *D. fragilis* from Norway and Northern Ireland. The other two clusters consist only of *D. cf. fragilis*. Specimens previously identified as "*D. cf. maculata* (Montagu, 1804)" from Sweden, grouped with *D. coronata* (Gmelin, 1791) and one "*D. cf. maculata*" from Northern Ireland clustered with *D. dunnei* Lemche, 1976, whereas *D. koenneckeri* Lemche, 1976, *D. pinnatifida* (Montagu, 1804), *D. tuberculata* Lemche, 1976, and the Norwegian *D. cf. cuspidata* forms separate clusters. In *H3* all *D. fragilis* and *D. hystrix* clustered together, the *D. coronata*/Swedish "cf. *maculata*" group and *D. dunnei* group formed a single cluster also for the latter including *D. millbayana* Lemche, 1976. Further, *D. cuspidata*, *D. koenneckeri* and *D. pinnatifida* form separate clusters. When combining the results from both markers, we find a maximum set of 11 clusters or primary species hypotheses (Table 1, Figure 2). The maximum intracluster genetic distance varies between 0.0% and 2.0% in *COI*, and between 0.0% and 1.8% in *H3*. The minimal intercluster distance within *Doto* varies between 2.3% and 14.4% in *COI* and between 0.0% 12.5% in *H3*, and the distances between *Doto spp*. and the out-groups are generally higher than within *Doto* (Table 4).

3.2 | Phylogenetic inference

In both the *COI* and *H3* gene trees (Figure S1a,b), *Doto* is monophyletic with maximal support, and the groups found in the respective ABGD analyses are recovered. In the *H3* tree, there are no signs of hybridization, and all phased alleles of the same individual are closely related.

In the combined Bayesian tree (Figure 2, Figure S1), *Doto* is monophyletic with maximum support and is divided into four clades: (1) *D. cuspidata* + *D. pinnatifida*, which is the sister to the remaining *Doto*, (2) *D. tuberculata*, (3) *D. hystrix* + *D. fragilis* and (4) *D. coronata*, *Doto* sp. (= "*D. cf. cuspidata*"), *D. dunnei*, *D. maculata*, *D. millbayana* and *D. koenneckeri*. In the last clade, *D. koenneckeri* and one "*D. cf. maculata*" from Northern Ireland are found as sisters, but well separated, and the remaining forms a trichotomy consisting of one clade including *D. dunnei*, *D. millbayana* and one *D. cf. maculata*, another consisting of all *D. coronata* and all of the Swedish *D. cf. maculata* specimens, and the last clade with all Norwegian *D. cf. cuspidata*.

The concatenated Bayesian tree including the extended dataset of *Doto* and *Kabeiro* sequences (Figure 3) is similar to Shipman and Gosliner (2015, fig. 4) and Moles et al. (2016, fig. 7). *Doto cuspidata* is sister to *D. pinnatifida*, and *D. formosa* Verrill, 1875, is placed together with *D. hystrix* and *D. fragilis*. Our *D. coronata* + "*D. cf. maculata*" clade is placed together with additional *D. coronata* sequences (Figure 3, Figure S1), and our *D. dunnei*, *D. millbayana* and the Northern Ireland *D. maculata* are placed together with additional specimens of *D. dunnei*, *D. millbayana*, *D.* sp. *A* and the *D. coronata* specimens from Moles et al. (2016), our *D. maculata* specimen are placed close to one *D. maculata* and one *D. africoronata* Shipman & Gosliner, 2015 specimen, our *Doto* sp. (= "*D.* cf. *cuspidata*") are placed together with *D. paulinae* Trinchese, 1881. The trees with only our data and the tree based on the extended data are mainly congruent; the only difference in the species included in both trees is the position of *D. tuberculata*, which is sister to the *D. fragilis* complex in the extended tree, and found in a tricotomy with the *D. fragilis* complex and *D. coronata* complex.

The ML tree (Figure S2) is congruent with the Bayesian trees.

3.3 | MSC delimitation

All of the 11 input species are well supported and accepted as well-delimited species when both COI and H3 are included (Table 5). However, when only H3 was used the species in the *D. fragilis*/*hystrix* complex were combined (Table 5).

3.4 | Haplotype network

The network (Figure 4) shows that the three groups within *D. fragilis* are separated and that one of the groups also contains *D. hystrix*. In this group, the two mainly have separate haplotypes, only sharing one, but there is no clear structure within the group.

TABLE 3 List of hydroids associated with *Doto* species involved in present study

Hydroid species	*Doto* species	Reference
Abietinaria abietina (Linnaeus, 1758)	*D. coronata* (Gmelin, 1791) *D. tuberculata* Lemche, 1976	Lemche, 1976 Picton & Brown, 1981 Thompson & Brown, 1984
Aglaophenia pluma (Linnaeus, 1758)	*D. coronata* (Gmelin, 1791) *D. koenneckeri* Lemche, 1976 *D. lemchei* Ortea & Urgorri, 1978	Swennen, 1961 Picton & Brown, 1981 Thompson & Brown, 1984 Morrow et al., 1992 Present study
Aglaophenia sp.	*D. koenneckeri* Lemche, 1976 *D. lemchei* Ortea & Urgorri, 1978 *D. millbayana* Lemche, 1976 *D. paulinae* Trinchese, 1881 *D. pinnatifida* (Montagu, 1804)	Ortea & Urgorri, 1978 Urgorri & Besteiro, 1983 Just & Edmunds, 1985 Rudman, 2006 Shipman & Gosliner, 2015 Present study
Aglaophenia tubulifera (Hincks, 1861)	*D. lemchei* Ortea & Urgorri, 1978	Morrow et al., 1992 Picton & Brown, 1981 Thompson & Brown, 1984
Amphisbetia operculata (Linnaeus, 1758)	*D. coronata* (Gmelin, 1791) *Doto eireana* Lemche, 1976 *D. pinnatifida* (Montagu, 1804)	Hecht, 1896 Cornet & Marche-Marchad, 1951 Ortea, 1978 Thompson & Brown, 1984
Bougainvillia muscus (Allman, 1863)	*D. coronata* (Gmelin, 1791)	Miller, 1961 Thompson, 1964
Clava multicornis (Forsskål, 1775)	*D. coronata* (Gmelin, 1791)	Larsen, 1925 Jaeckel, 1952 Miller, 1961
Clytia hemisphaerica (Linnaeus, 1767)	*D. coronata* (Gmelin, 1791)	Present study
Coryne eximia Allman, 1859	*D. coronata* (Gmelin, 1791)	Swennen, 1961
Coryne muscoides (Linnaeus, 1761)	*D. coronata* (Gmelin, 1791)	Miller, 1961
Diphasia fallax (Johnston, 1847)	*D. coronata* (Gmelin, 1791)	Present study
Diphasia rosacea (Linnaeus, 1758)	*D. coronata* (Gmelin, 1791)	Picton & Brown, 1981 Thompson & Brown, 1984
Dynamena pumila (Linnaeus, 1758)	*D. coronata* (Gmelin, 1791)	Alder & Hancock, 1846 Lemche, 1976 Picton & Brown, 1981 Thompson & Brown, 1984
Ectopleura larynx (Ellis & Solander, 1786)	*D. coronata* (Gmelin, 1791) *D. fragilis* (Forbes, 1838)	Walton, 1908 Miller, 1961 Hamond, 1972 Swennen, 1961
Eudendrium ramosum (Linnaeus, 1758)	*D. coronata* (Gmelin, 1791)	Hamond, 1972
Eudendrium spp.	*D. coronata* (Gmelin, 1791)	Picton & Morrow, 1994
Garveia nutans Wright, 1859	*D. coronata* (Gmelin, 1791)	Picton, 1978
Halecium beanii (Johnston, 1838)	*D. coronata* (Gmelin, 1791)	Miller, 1961
Halecium halecinum (Linnaeus, 1758)	*D. coronata* (Gmelin, 1791) *D. fragilis* (Forbes, 1838)	Farran, 1909 Miller, 1961 Thompson, 1964 Hunnam & Brown, 1975 Picton & Brown, 1981 Present study
Halecium muricatum (Ellis et Solander, 1786)	*D. fragilis* (Forbes, 1838)	Picton, 1978 Picton & Brown, 1981 Thompson & Brown, 1984

TABLE 3 (Continued)

Hydroid species	*Doto* species	Reference
Halecium spp.	*D. coronata* (Gmelin, 1791) *D. fragilis* (Forbes, 1838) *D. pinnatifida* (Montagu, 1804)	Walton, 1908 Hamond, 1972 Brown & Picton 1979 Urgorri & Besteiro, 1983 Present study
Halopteris catharina (Johnston, 1833)	*D. maculata* (Montagu, 1804)	Lemche, 1976 Picton & Brown, 1981 Thompson & Brown, 1984
Kirchenpaueria pinnata (Linnaeus, 1758)	*D. dunnei* Lemche, 1876	Lemche, 1976 Picton & Brown, 1981 Thompson & Brown, 1984
Laomedea flexuosa Alder, 1857	*D. coronata* (Gmelin, 1791)	Miller, 1961 Swennen & Dekker, 1987
Lafoea dumosa (Fleming, 1820)	*D. coronata* (Gmelin, 1791)	Miller, 1961
Nemertesia antennina (Linnaeus, 1758)	*D. coronata* (Gmelin, 1791) *D. fragilis* (Forbes, 1838) *D. pinnatifida* (Montagu, 1804) *D. hystrix* Picton & Brown, 1981	Jaeckel, 1952 Miller, 1961 Kress, 1968 Thompson, 1964 Lemche, 1976 Picton & Brown, 1981 Thompson & Brown, 1984 Present study
Nemertesia norvegica (Sars, 1873)	*Doto fragilis*, *Doto cf. fragilis*	Present study
Nemertesia ramosa (Lamarck, 1816)	*D. cuspidata* Alder & Hancock, 1862 *D. fragilis* (Forbes, 1838) *D. millbayana* Lemche, 1976	Miller, 1961 Thompson, 1964 Lemche, 1976 Just & Edmunds, 1985 Thompson & Brown, 1984
Obelia geniculata (Linnaeus, 1758)	*D. coronata* (Gmelin, 1791) *D. paulinae* Trinchese, 1881	Miller, 1961 Lemche, 1976 Schmekel & Kress, 1977 Picton & Brown, 1981 Thompson & Brown, 1984 Lambert, 1991 Martynov et al., 2006 Rudman, 2006 Shipman & Gosliner, 2015 Present study
Obelia dichotoma (Linnaeus, 1758)	*D. coronata* (Gmelin, 1791)	Swennen, 1961 Picton & Brown, 1981 Thompson & Brown, 1984 Lambert, 1991 Shipman & Gosliner, 2015
Obelia longissima (Pallas, 1766)	*D. coronata* (Gmelin, 1791)	Dekker, 1986 Urgorri & Besteiro, 1983 Martynov et al., 2006 Martynov & Korshunova, 2011
Rhizocaulus verticillatus (Linnaeus, 1758)	*D. coronata* (Gmelin, 1791)	Miller, 1961
Plumularia setacea (Linnaeus, 1758)	*D. millbayana* Lemche, 1976	Lemche, 1976 Picton & Brown, 1981 Just & Edmunds, 1985 Thompson & Brown, 1984
Schizotricha frutescens (Ellis & Solander, 1786)	*D. hystrix* Picton & Brown, 1981	Picton & Brown, 1981 Thompson & Brown, 1984 Present study

(Continues)

TABLE 3 (Continued)

Hydroid species	*Doto* species	Reference
Sertularia argentea (Linnaeus, 1758)	*D. coronata* (Gmelin, 1791) *D. dunnei* Lemche, 1876 or *D. millbayana* Lemche, 1876 (recorded as *Doto* sp.)	Miller, 1961 Picton & Brown, 1981 Thompson & Brown, 1984 Shipman & Gosliner, 2015
Sertularia cupressina Linnaeus, 1758	*D. coronata* (Gmelin, 1791)	Swennen, 1961 Thompson, 1964 Thompson & Brown, 1984 Shipman & Gosliner, 2015
Sertularella gayi (Lamouroux, 1821)	*D. tuberculata* Lemche, 1976	Lemche, 1976 Picton & Brown, 1981 Thompson & Brown, 1984
Sertularia sp.	*D. pinnatifida* (Montagu, 1804)	McMillan, 1944 Moore, 1950 Kress, 1968
Symplectoscyphus tricuspidatus (Alder, 1856)	*D. coronata* (Gmelin, 1791)	Martynov et al., 2006
Synthecium sp.	*D. lemchei* Ortea & Urgorri, 1978	Thompson et al., 1990
Tamarisca tamarisca (Linnaeus, 1758)	*Doto cf. fragilis*	Present study
Thuiaria thuja (Linnaeus, 1758)	*D. coronata* (Gmelin, 1791)	Reid, 1846
Tubularia indivisa Linnaeus, 1758	*D. coronata* (Gmelin, 1791)	Jeffreys 1869 Jaeckel, 1952

3.5 | Review of prey specificity

Out of 42 species of hydroids, 11 species were reported to be associated with at least two different *Doto* species (Table 3). This contradicts the notion of monophagy as a major trend of the evolution within the genus *Doto*. Half of the number of hydroid species (21) was recorded solely for *Doto coronata*, *D. dunnei* and *D. millbayana* were recorded from three different hydroids (*Kirchenpaueria pinnata* for *D. dunnei* and *Plumularia setosa* growing upon *Nemertesia antennina* and *N. ramosa* for *D. millbayana*, see Table 3), although these do not show significant genetic divergence (Shipman & Gosliner, 2015; present study).

4 | DISCUSSION

In this study, we use a combination of data sources and methods to test the species limits of northeast Atlantic *Doto* species. For a majority of the species the results are clear-cut. The exceptions are mainly in *D. fragilis*, where we find three groups, one that do not show any genetic separation from *D. hystrix* despite large morphological differences. The three groups are separated in COI, but not H3. It is possible that these groups represent separate species; however, more data are needed to confirm this.

This study is one of few studies on nudibranchs that delimits species using multi-locus analysis based on the MSC method. MSC analyses have been used successfully on a wide variety of taxa (e.g., Delić et al., 2017; Fossen et al., 2016; Leache & Fujita, 2010; Martinsson & Erséus, 2018), and it has been shown to outperform distance methods (Yu et al., 2017), which so far has been the standard for molecular species delimitation in nudibranchs. However, in this study we use the same data for the initial division of specimen into species hypothesis, and for the testing of them using MSC analysis, this is not optimal as it introduces issues of circularity (see e.g., Martinsson & Erséus, 2018; Yang, 2015), and the results should be interpreted with caution.

Specimens of *Doto fragilis* are found in three closely related clades, one that also includes *D. hystrix*. In the *D. fragilis*/*D. hystrix* clade, the *D. fragilis* from Northern Ireland groups with the Swedish *D. fragilis* "white morph" and the *D. hystrix* from Northern Ireland, Norway and Sweden. The spiky tubercles on the dorsolateral appendages of *D. hystrix* are distinctly different from the rounded tubercles of *D. fragilis*. *Doto hystrix* has until now only been reported from the hydroid *Schizotricha frutescens*, but are reported here also observed on *Nemertesia antennina* (Table 3). Species of *Doto fragilis* complex has been found on hydroids from the genera *Halecium*, *Nemertesia* and *Tubularia*, but so far not on *Schizotricha frutescens* (see Table 3). The second *D. fragilis* clade consists of most often red-colored specimens from Sweden. The *D. fragilis* from Shipman and Gosliner (2015) are also found in this clade (Mn33151). That specimen was from Wales and was feeding on the hydroid *Nemertesia ramosa*. The third *D. fragilis* clade consists of five specimens in the analysis: one brownish light white specimen from Sweden and four specimens from Norway.

Our review of the prey species recorded for *Doto* species shows that most species of the genus *Doto* feed on several different hydroids, and hence, the previously mentioned notion of general strict food specialization within *Doto* (e. g. Picton & Brown, 1981; Morrow et al., 1992, 1994) should be abandoned. Not even the species *D. hystrix* show strict monophagy, as previously thought (Picton & Brown,

TABLE 4 Uncorrected pairwise genetic distances (in %) for *COI* (below diagonal) and *H3* (above diagonal), intracluster distances are given as maximum distances, intercluster distances are given as minimal distances

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *D. fragilis/hystrix*	2.0	0.6 0.0	0.0	5.5	5.8	–	6.4	6.1	9.8	10.4	7.3	14.0	11.0
2. *D. fragilis*	2.8	0.5 0.3	0.0	5.8	6.1	–	6.7	6.1	10.4	11.0	7.6	14.3	11.3
3. *D. fragilis*	2.8	2.3 –	1.7	5.8	6.1	–	6.7	6.1	10.4	11.0	7.6	14.3	11.3
4. *D. coronata/maculata*	10.0	11.5	11.0	1.9	0.8	–	3.4	6.7	11.0	11.6	4.9	11.3	11.9
5. *D. dunnei*	9.9	11.3	10.4	4.7	0.8 0.0	–	3.5	6.6	10.8	11.2	4.6	11.2	12.2
6. *D. cf. cuspidata*	10.7	12.4	11.6	6.3	6.5	2.0 –	–	–	–	–	–	–	–
7. *D. koenneckeri*	11.9	12.7	12.1	8.7	8.8	9.4	– –	7.0	11.3	11.6	3.4	10.1	12.2
8. *D. tuberculata*	10.1	12.0	11.9	10.5	.4	10.9	12.0	–	– 10.4	11.6	8.5	15.9	12.8
9. *D. pinnatifida*	12.4	14.4	13.9	11.8	10.5	13.1	14.1	12.0	0.5 0.0	7.3	11.3	15.5	13.7
10. *D. cuspidata*	–	–	–	–	–	–	–	–	–	– –	12.5	15.9	14.3
11. *D. maculata*	–	–	–	–	–	–	–	–	–	–	– 1.8	11.3	11.3
12. *Aeolidia filomenae*	19.8	21.1	21.4	19.0	17.0	19.4	19.9	19.0	16.3	–	–	0.5 0.3	10.7
13. *Dendronotus lacteus*	17.9	19.5	18.1	19.9	18.5	19.0	17.1	18.5	17.6	–	–	21.7	0.0 0.0

1981), but has been documented feeding on both *Schizotricha frutescens* and *Nemertesia antennina* (present study, Table 3). It is possible that different populations within a species of *Doto* have specialized in feeding on separate hydroids. However, the composition of hydroid species is similar along most of the European Atlantic coast, and the fauna is dominated by widely distributed species (Medel & López-González, 1998), which points against differentiation between populations.

There are other examples of nudibranchs with similarly small genetic distances as the ones found between the clusters in *D. fragilis*, such as between some Indo-Pacific *Chromodoris* species, with interspecies COI distances as low as 2.0% (Layton et al., 2018) or between species of *Felimare* along the American Pacific coast with a minimal interspecific COI distance of 2.5% (Hoover et al., 2016), all of these represent cases of recent speciation. It is therefore possible that the clusters we recovered are separate species, but as we have no support from nuclear markers, and the BPP analysis with only H3 did not support a separation, we do not describe them in this paper. More data and analysis are needed to further test the separation of them. "*Doto fragilis*" is a species complex here found in three closely related clades, one that also includes *D. hystrix*, and all, except *D. hystrix*, are externally similar. These potential new species will be described following ongoing revisions of more *Doto* groups. Here, to clearly indicate the clade with true *D. fragilis*, we designate a neotype for it (GNM Gastr. 9061. Strangford lough, Portaferry, United Kingdom, date 20 May 2014, coordinates lat 54°22,00′ long 05°32,00′, depth 20–30 m, collector: Klas Malmberg, Figure 5). The specimen is deposited at the Gothenburg Natural History Museum in Sweden. The original types of *D. fragilis* are lost (Mollusca Type in Great Britain, 2020; Natural History Museum, 2020). Therefore, to define true *D. fragilis* we here designate a neotype for this species. According to ICZN 1999 article 75.3.6. "…evidence that the neotype came as nearly as practicable from the original type locality…" The neotype of *D. fragilis* originates from the Northern Ireland near Portaferry, just 60 km from the original type locality of *D. fragilis* on the western coast of Isle of Man (Forbes, 1838), thus fulfilling the ICZN requirements. According to the original description (Forbes, 1838), *D. fragilis* does not contain red-colored morphs.

The genetic markers used in our study show no separation between *D. hystrix* and *D. fragilis*, despite them being distinct morphologically and ecologically. *Doto hystrix* has long, pointed tubercles on the rim of the rhinophore sheaths (Figure 6) and, mostly feeds on the hydroid *Schizotricha frutescens* (Picton & Brown, 1981, Table 3), whereas the food preferences are wider for *D. fragilis*. There are also differences in the spawning between the two species. This can be seen as a parallel to what is reported from, e.g., some blue butterflies (Lepidoptera: Lycaenidae) where distinct species are mixed in *COI* (Wiemers & Fiedler, 2007) and also from recently described species from the eubranchid nudibranchs of the genus *Amphorina* (Korshunova, Malmberg, et al., 2020). However, the differences in ecology and morphology are large and points toward *D. fragilis* and *D. hystrix* being separate entities, in conflict with the genetic data.

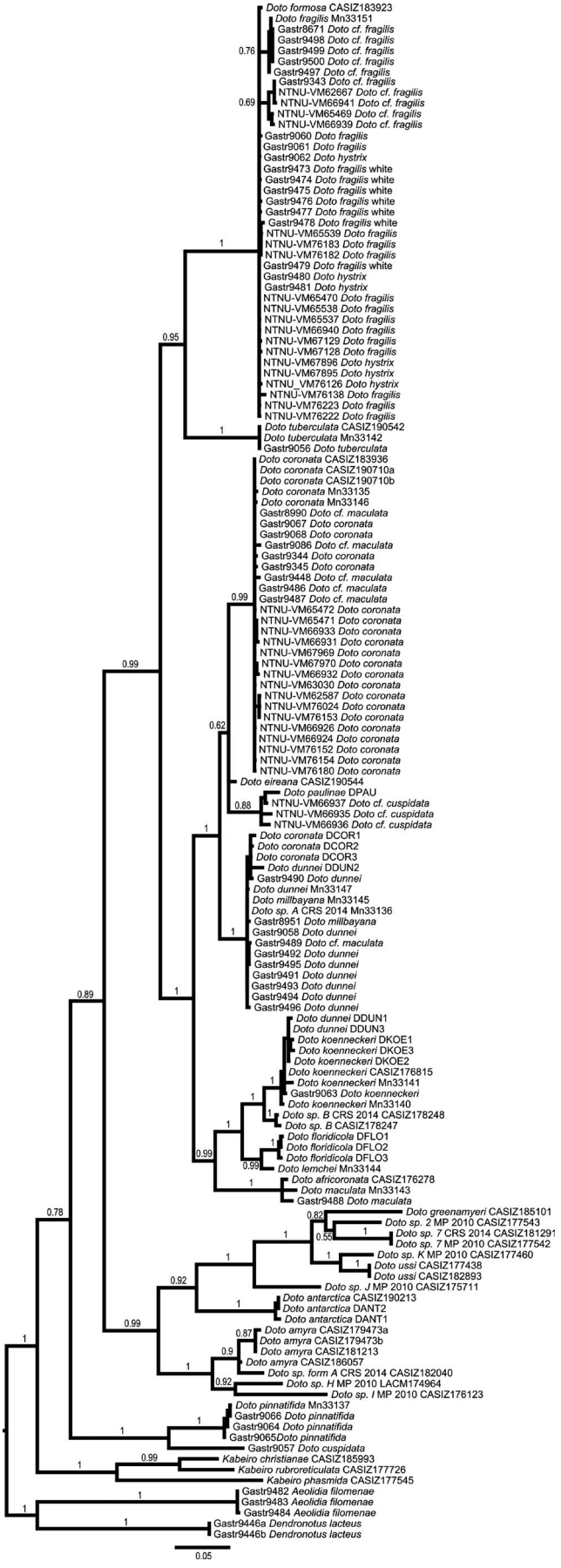

FIGURE 3 Phylogeny of *Doto*, estimated by Bayesian inference, based on *COI* and *H3* with a combined dataset of our studied specimens (with a Gastr. or an NTNU-VM prefix) and data from GenBank. Posterior probabilities shown at branches. Scales show expected number of changes per site

TABLE 5 List of delimited species and their mean posterior probabilities, from BPP analyses

	COI+H3		H3 only	
Species	**A (PP)**	**B (PP)**	**A (PP)**	**B (PP)**
1. *D. fragilis/hystrix*	**1.000**	**1.000**	0.023	0.016
2. *D. fragilis*	**0.999**	**1.000**	0.020	0.013
3. *D. fragilis*	**0.999**	**1.000**	0.010	0.006
4. *D. coronata/maculata*	**0.999**	**0.955**	**1.000**	**1.000**
5. *D. dunnei/millbayana*	**0.999**	**0.955**	**1.000**	**1.000**
6. *D. cf. cuspidata*	**0.998**	**0.955**	—	—
7. ***D. koenneckeri***	**0.990**	**0.955**	**0.972**	**0.996**
8. *D. tuberculata*	**1.000**	**0.994**	**1.000**	**1.000**
9. *D. pinnatifida*	**1.000**	**1.000**	**0.999**	**1.000**
10. *D. cuspidata*	**0.999**	**1.000**	**0.999**	**1.000**
11. *D. maculata*	**0.990**	**0.955**	**0.972**	**0.996**
1+2+3	0.000	0.000	**0.955**	**0.968**
1+2	0.000	0.000	0.007	0.004
1+3	0.000	0.000	0.016	0.012
2+3	0.001	0.000	0.019	0.015
4+5+6+7+11	0.000	0.039	0.000	0.000

Note: Posterior probabilities are means of three runs. Posterior probabilities in bold are considered significant and species in bold are accepted.

Therefore, we do not synonymize them in this study. Further studies are needed to better test whether the lack of genetic separation in combination with ecological and morphological differences are due to recent speciation, with a rapid evolution of morphological characters, or interspecific differences, possibly due to adaptation to different prey species.

The northwest Atlantic species *Doto formosa* groups with *D. fragilis* and *D. hystrix*, but unfortunately only *H3* was available for *D. formosa* on GenBank. Closer studies of *D. formosa* are needed not only in sequence data, but in morphology and ecology. The original description of *D. formosa* by Verrill in 1875 is very meager and could as well cover *D. fragilis* as well. The results from our study show that these species form a clade, but more studies are needed to resolve the taxonomical status of the taxa involved.

Prey species is an indirect method for indicating morphologically similar species of nudibranch molluscs, as the species are partially specific in their choice of prey, through coevolution with the prey (see Goodheart et al., 2017). In some cases, this is justified, as, for

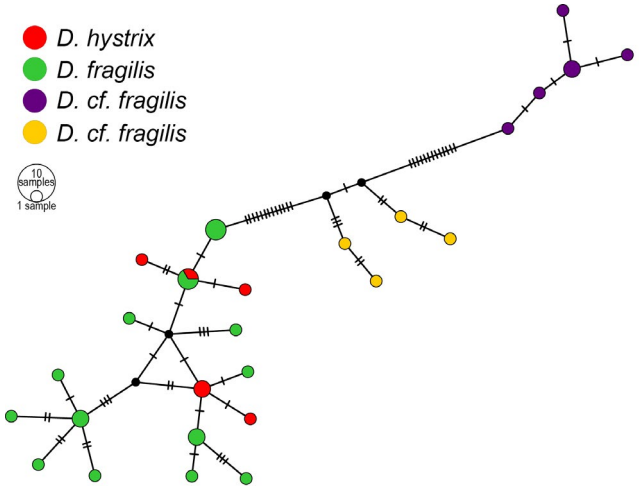

FIGURE 4 *COI* haplotype network of specimen in the *D. fragilis*/*D. hystrix* species complex. The size of the circles is relative to the number of sequences sharing that haplotype, the hatch marks correspond to the number of substitutions between haplotypes, and the haplotypes are colored based on the results pf the BPP analysis of the combined COI and H3 data, but with *D. hystrix* separated

FIGURE 5 *Doto fragilis* (GNM Gastr. 9061), dorsal view of the neotype. Blackbox studio photograph of live specimen by Klas Malmberg

example, the prey of *D. maculata* is the hydroid *Halopteris catharina*, but the concept of one *Doto* species/one hydroid species (with the exception of *D. coronata*) needs to be reconsidered (see below and Table 3). We also illuminate some problems in the *D. coronata* complex (see Shipman & Gosliner, 2015 for a discussion about this complex).

The analysis of the hydroid associations suggests that the evolution of morphological traits and that of food preference within the genus *Doto* were not associated processes. For example, the morphologically and genetically well-supported species *D. pinnatifida*, *D. fragilis* and *D. coronata* were found associated with the same hydroid species *Nemertesia antennina*, whereas *D. koenneckeri* and *D. lemchei* were found on the same hydroid species *Aglaophenia pluma* (Table 3). The present analysis also implies that possibly other species from *D. coronata* complex than solely *D. coronata* s.str. already were recorded from several various hydroids, but these records can still be hidden under the "*D. coronata*" name, as a precise species identification within this complex is a considerable challenge, even for experts.

The morphology of the radula is linked to the prey specialization in any molluscs, including nudibranchs. All the studied species of *Doto* have the radula formula 0.1.0 (Figures 7 and 8). Though the radula in the genus *Doto* does not show significant differences, our data on various species reveal several promising patterns. For every species, there is some minor differences in the details of the general shape of the teeth as well as the number and arrangement of the lateral denticles. The radula of *Doto millbayana* (Figure 8d2–d4) has a very distinct, sharp outline of the denticles and readily distinguishes from the majority of studied here species (Figures 7 and 8), supporting its status as a separate species. In addition, there is a slight similarity between *D. coronata* and the *D.* cf. *maculata* from Sweden in the small central cusp.

The specimens collected in Sweden and identified as *Doto* cf. *maculata* based on coloration, were never found on *Halopteris catharina* even though this hydroid species occurs in both the Skagerrak and the Kattegat areas, but instead on hydroids that could be identified during the scuba dives as belonging to the family Plumulariidae. There were also morphological characters that did not fully match *D. maculata*. This led us to suspect that the Swedish specimens could be a separate species, or at least not *D. maculata*, and this was confirmed by the genetic analysis. Contrary to the *D. fragilis*/*D. hystrix* case, between putative "*D.* cf. *maculata*" and real *D. coronata* there are no reliable morphological and ecological differences. The dorsolateral appendages of "*D.* cf. *maculata*" from Sweden is slightly different from that of *D. coronata* in the absence of a pigment dot on the apical tubercle. Further, the "*D.* cf. *maculata*" specimens lack the red markings at the inner side (toward the midline of the dorsal side of the animal) of the base of the dorsolateral appendages (Figure 6) that is commonly mentioned to be "typical" for *D. coronata*. However, *D. coronata* is a highly variable species (Martynov & Korshunova, 2011; Martynov et al., 2006) (Figure 6) and for *D. maculata* the apparent "key features" were ambiguously indicated in the redescription by Lemche (1976: 697 "*Round reddish or dark brownish spots are placed on the tips of the tubercles on the cerata, except in many cases the end one*"). Hence, coloration patterns should be used with care and always be checked against other morphological characters. *Doto coronata* evidently feeds on a variety of hydroids (Lemche, 1976; Picton & Brown, 1981; Table 3). This was supported by the molecular analysis (Shipman and Gosliner (2015), which showed that the true *D. coronata* has a wide prey specificity, as it was collected from the hydroids *Sertularia cupressina*, *Obelia geniculata*

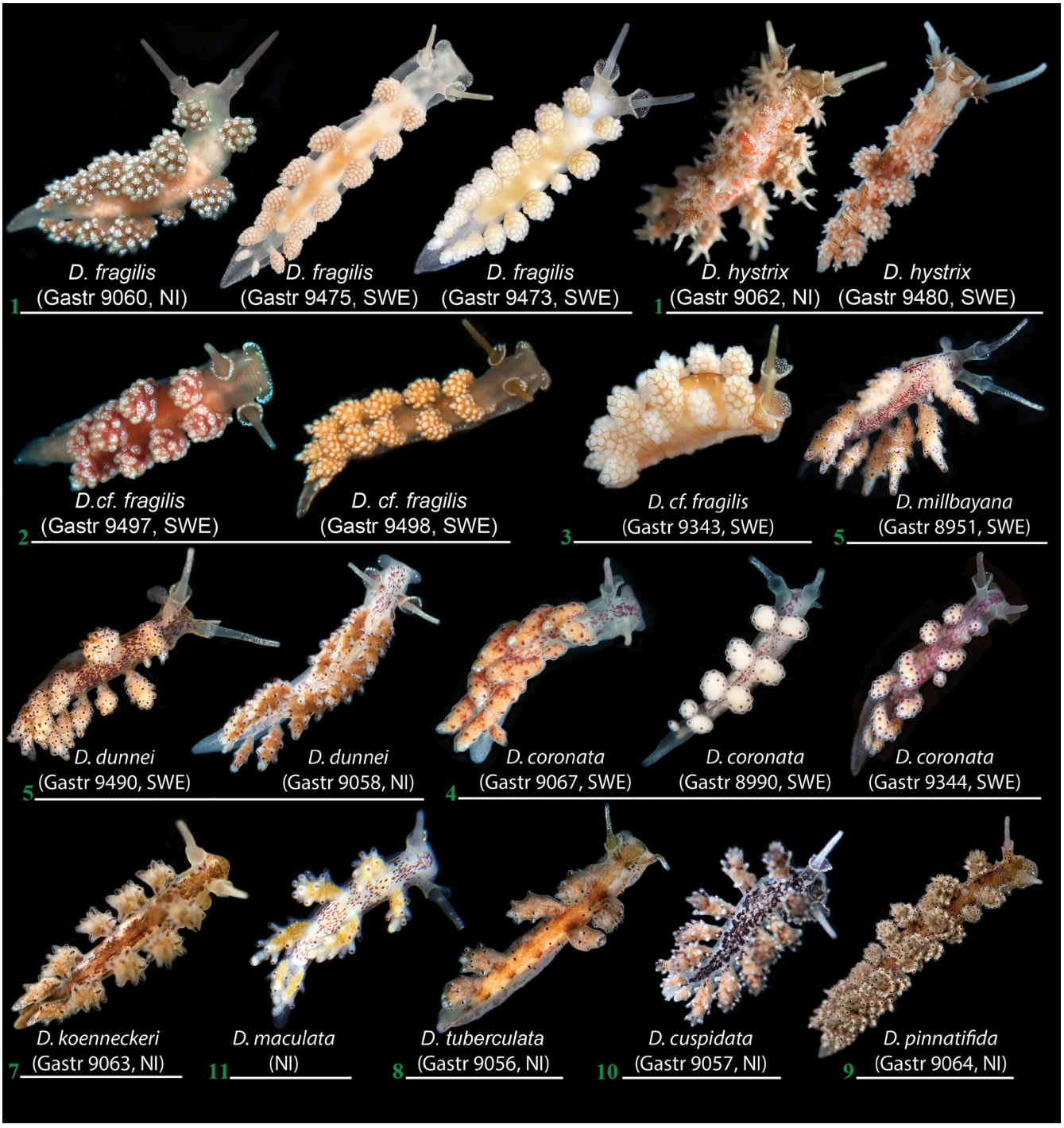

FIGURE 6 Photographs of *Doto* species, showing morphological variation on the studied taxa. All blackbox studio photographs of live specimens by Klas Malmberg

and *O. dichotoma*. The two species *D. hydrallmaniae* Morrow et al., 1992 and *D. sarsiae* Morrow et al., 1992 were not included in the study. According to an earlier work of Morrow et al. (1992), electrophoretic methods show that they represent distinct species, but are closely related to the *D. coronata* species complex; however, this is not yet confirmed by other molecular phylogenetic studies.

The study by Shipman and Gosliner (2015) could not resolve any genetic difference between *Doto dunnei* and *Doto millbayana* from sequences of *COI*, *H3* and 16S, which led them to suggest that the two could be the same species. In our analysis, we got similar result from *H3*, but that does not provide any further support to synonymize the two species. However, we found minor radular characters supporting the separation. In the review of hydroid prey they have only been

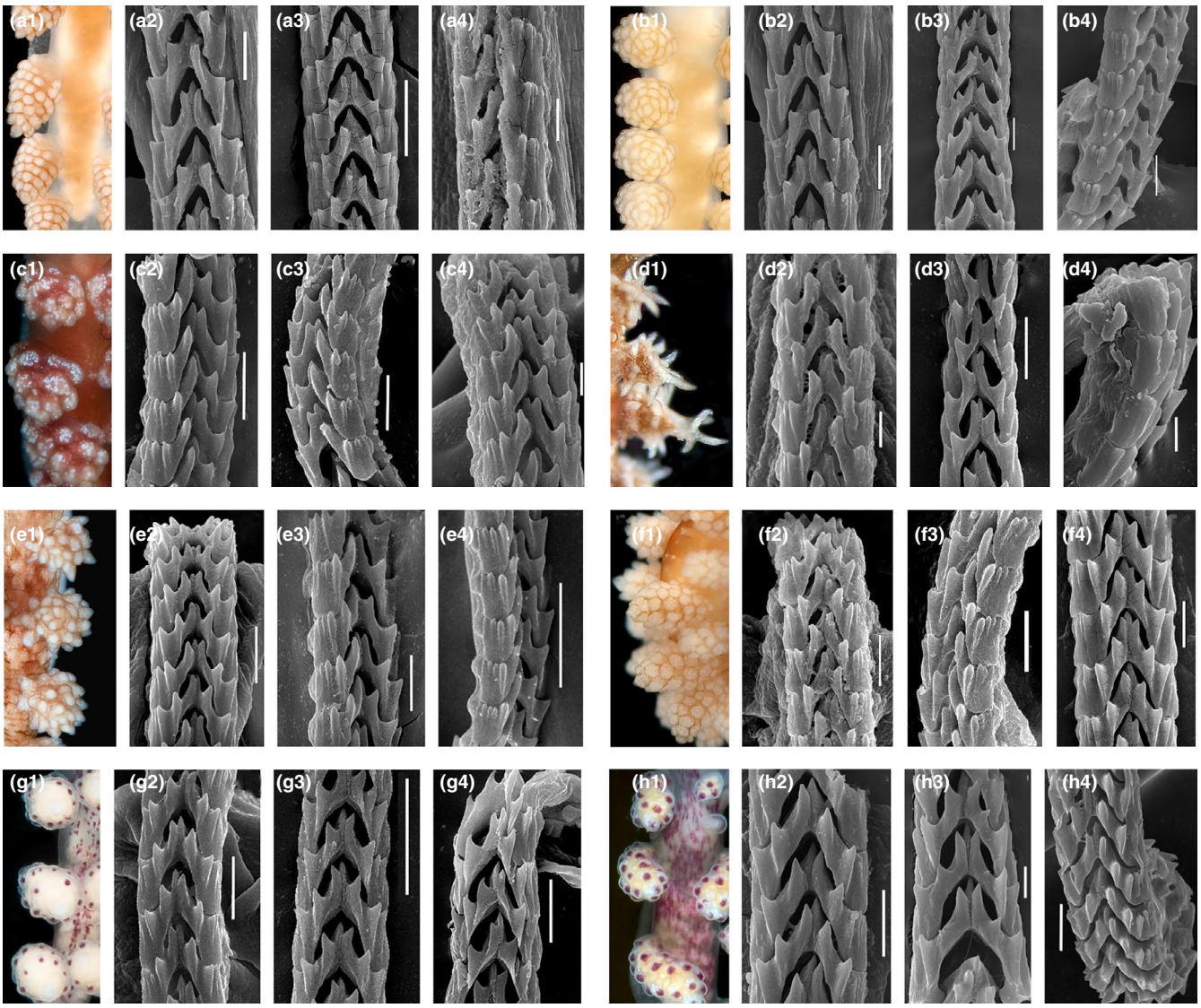

FIGURE 7 SEM micrographs of the studied *Doto* species, combined with the diagnostic characters of the dorsal appendages. (a) *Doto fragilis* (Gastr. 9475); a1—appendages; a2(a) −10 μm; a3 (p) −10 μm; a4(pl)—20 μm. (b) *Doto fragilis* "white morph" (Gastr. 9474); b1—appendages; b2(a) – 10 μm; b3(p) – 10μm; b4(pl)—10 μm. (c) *Doto* cf. *fragilis* (Gastr. 9497); c1—appendages; c2(a) −10 μm; c3(a1)-l0 μm; c4(p) −5 μm. (d) *Doto hystrix* (Gastr. 9062); d1—appendages; d2(a) −5 μm; d2(p) −10 μm; d3(pl)—5 μm. (e) *Doto hystrix* (Gastr. 9480); e1—appendages; e2(a) −10 μm; e3 (p) −10 μm; e4(pl)—20 μm. (f) *Doto* cf. *fragilis* (Gastr. 9343); f1—appendages; f2(a)—10μm; f3 (al) −10μm; f4(p)—10μm. (g) *Doto coronata* (Gastr. 8990); g1—appendages; g2(a) −10 μm; g3 (p) −20 μm; g4(pl)—10 μm. h. *Doto coronata* (Gastr 9344); h1—appendages; h2(a) −10 μm; h3 (p) −5 μm; h4(pl)—10 μm. Photographs of dorsal appendages from live specimens by Klas Malmberg, SEM micrographs by Alexander Martynov

reported from their supposed prey species, so they appear monophagous. There is a distinct difference in the shape of the pseudobranchs (rounded in *D. dunnei*, pointed in *D. millbayana*). The dark red pigment spots on the dorsum are often numerous and partly fusing in *D. dunnei* compared to *D. millbayana* in which the pigment spots are fever and generally more separated. We therefore suggest that the two should remain separate species until further study.

One *D. maculata* specimen from Northern Ireland groups with *D. africoronata* Shipman & Gosliner, 2015 and the *D. maculata* from Shipman and Gosliner (2015) (Figure 6), unfortunately both specimens of *D. maculata* lack one of the genes, which could explain why they are not forming a monophyletic group, rather than being nested with *D. africoronata*.

As a conclusion, the multi-locus species delimitation is shown as a valuable tool in nudibranch systematics, and that *D. fragilis* is a species complex, including *D. hystrix* and *D. formosa*. *Doto fragilis and D. hystrix* have mixed *COI* haplotypes, possibly due to recent speciation. The present results are concordant with the concept of multi-level organismal diversity (Korshunova, Bakken, et al., 2020; Korshunova et al., 2019; Martynov et al., 2020).

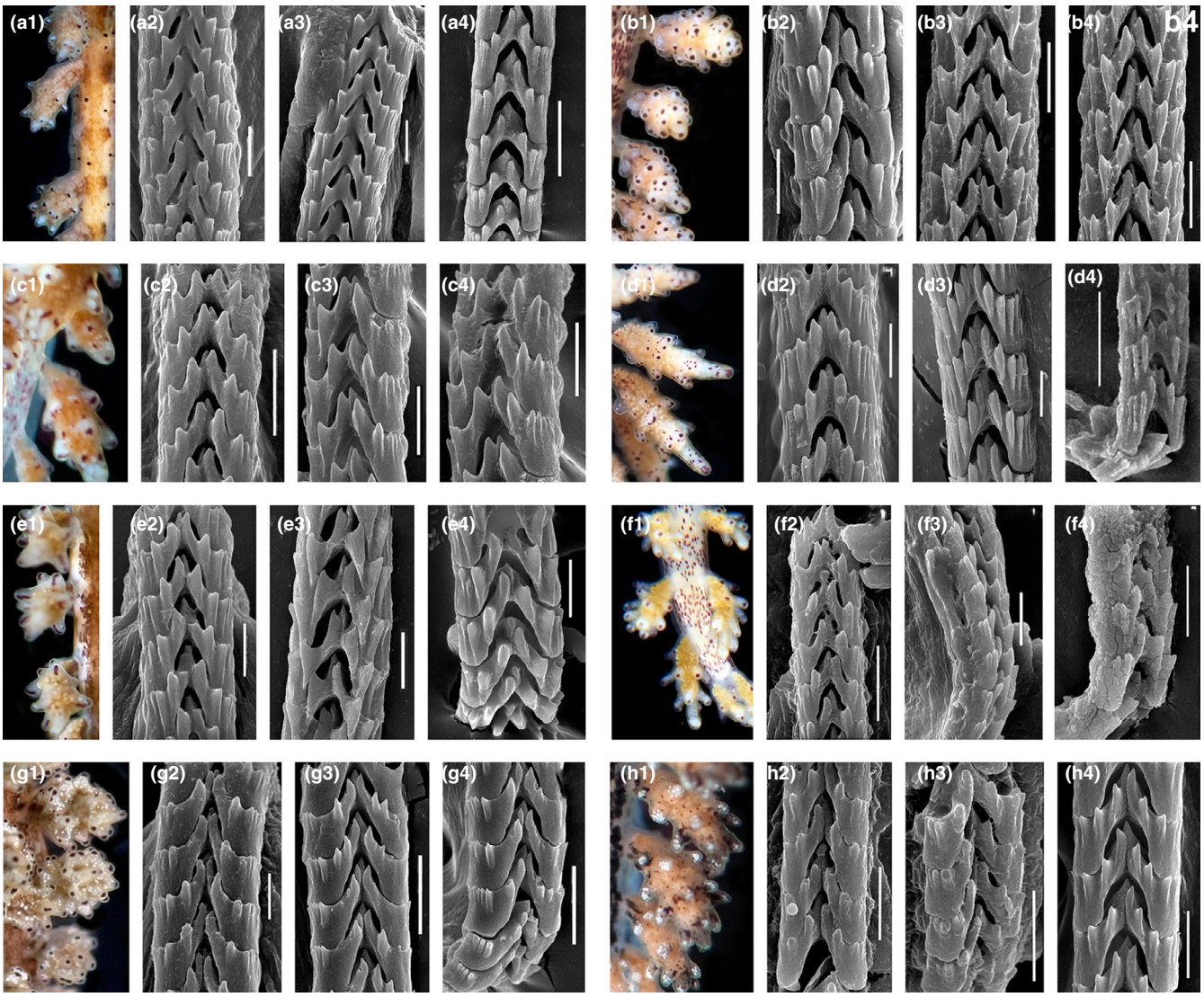

FIGURE 8 SEM micrographs of the studied *Doto* species, combined with the diagnostic characters of the dorsal appendages. (a) *Doto tuberculata* (Gastr. 9056); a1—appendages; a2(a)—10μm; a3 (al) −10 μm; a4(p)—20 μm. (b) *Doto dunnei* (Gastr. 9490); b1—appendages; b2(a) −10 μm; b3 (p) −10 μm; b4(pl)—10 μm. (c) *Doto dunnei* (Gastr. 9058); c1—appendages; c2(a) −10 μm; c3 (p) −10 μm; c4(pl)—10 μm. (d) *Doto millbayana* (Gastr. 8951); d1—appendages; d2(a) −10 μm; d3 (p) −10 μm; d4(pl)—25 μm. (e) *Doto koenneckeri* (Gastr. 9063); e1—appendages; e2(a) −10 μm; e3 (p) −10 μm; e4(pl)—10 μm. (f) *Doto maculata* (Gastr. 9488); f1 – appendages; f2(a)—10 μm; f3 (p)—10μm; f4(pl)—10μm. (g) *Doto pinnatifida* (Gastr 9064); g1—appendages; g2(a)-10 μm; g3 (p)-20 μm; g4(pl)-20 μm. (h) *Doto cuspidata* (Gastr. 9057); h1—appendages; h2(a) −10 μm; h3 (al) −10 μm; h4(p)—10 μm. Photographs of dorsal appendages from live specimens by Klas Malmberg, SEM micrographs by Alexander Martynov

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DATA AVAILABILITY STATEMENT

Specimens were deposited at the Gothenburg Natural History Museum (GNM), Gothenburg, Sweden, and at the NTNU University Museum (NTNU-VM) (Bakken et al., 2021), Trondheim, Norway. All new sequences are deposited in GenBank (see Table 1 for accession numbers). Matrices and trees are available on TreeBASE (submission 26369), matrices are also available as Additional Data S1–S5.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Figure S1. Gene trees of *Doto* estimated by Bayesian inference (a) *COI* gene tree. (b) *H3* gene tree (specimens marked with "-1" or "-2" after the species name are heterozygous and the numbers indicate different alleles from the same specimen).

Figure S2. Phylogeny of *Doto*, estimated by maximum likelihood, based on a concatenated matrix of *COI* and *H3* of our studied specimens.

Figure S3. Output from ABGD analyses, showing no. groups the data was divided into with varying prior intraspecific divergence.

Table S1. List of material included in this study, with museum voucher numbers, collection data with GPS coordinates, and GenBank accession numbers, as well as information about which cluster they were placed in in the ABGD analyses based in *COI* and *H3*, and which primary species hypotheses (PSH) they belong to.

Data S1. Alignment of COI and H3 sequences produced in this study.

Data S2. Alignment of COI and H3 sequences produced in this study and from GenBank.

Data S3. Alignment of COI sequences produced in this study.

Data S4. Alignment of H3 sequences produced in this study.

Data S5. Alignment of COI sequences of specimens from the *Doto fragilis* complex.

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