Integrative taxonomy in two free-living nematode species complexes

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Integrative taxonomy considers species boundaries from multiple, complementary perspectives, with the main objective being to compare the observed data against the predictions of the methodologies used. In the present study we used three methods for delineating species boundaries within the cosmopolitan nematode species *Rhabditis (Pellioditis) marina* and *Halomonhystera disjuncta*. First, phylogenetic relationships among molecular sequences from the mitochondrial cytochrome oxidase *c* subunit 1 gene (COI), and from two nuclear regions, internal transcribed spacer (ITS) and D2D3, were analysed. Subsequently, multivariate morphometric analysis was used to investigate whether concordant molecular lineages were also morphologically distinct. When morphological differences were found, typological taxonomy was performed to identify fixed or non-overlapping characters between lineages. Interbreeding experiments were conducted between the two closest related lineages of *R. (P.) marina* to investigate potential reproductive isolation. This integrative approach confirmed the presence of several species within each nominal species: molecular lineages were concordant across two independent loci (COI and ITS), and were characterized by significant morphological divergence. Most lineages were also detectable in the D2D3 region, but were less resolved. The two lineages investigated in our study did not produce offspring. Our results highlight that classical taxonomy grossly underestimates species diversity within the phylum Nematoda. © 2008 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2008, **94**, 737–753.

ADDITIONAL KEYWORDS: COI – D2D3 – ITS – marine nematodes – morphology.

INTRODUCTION

The discovery of cryptic species, i.e. genetically divergent but morphologically identical species (Avise & Walker, 1999), has polarized taxonomy between 'classical' and molecular biologists (Blaxter & Floyd, 2003; Sites & Marshall, 2003; Tautz *et al.*, 2003). The communication gap between the different disciplines is an important and neglected problem in the so-called 'taxonomy crisis' (Dayrat, 2005). To solve this crisis, species boundaries should be diagnosed using different methodologies, with clear hypotheses on the criteria used in each methodology (Sites & Marshall, 2004; and references therein). However, different researchers emphasize different criteria, which in turn provide information on different phenomena associated with the separation of lineages. As an alternative to consider each method independently, the 'integrative taxonomy' approach studies species boundaries from multiple, complementary perspectives (Dayart, 2005; Will, Mishler & Wheeler, 2005). Based on such a multidisciplinary perspective, all species concepts are considered variations on the single theme of species as evolutionary lineages, where the main objective is to compare data against the predictions of the various methodologies used (de Queiroz, 1998).

Integrative taxonomy has been efficiently introduced for some vertebrate (e.g. Wiens & Penkrot, 2002; Malhotra & Thorpe, 2004) and invertebrate taxa (e.g. Parmakelis *et al.*, 2003; Wahlberg *et al.*, 2005), but much less so for small-size metazoan groups like nematodes (De Ley *et al.*, 1999, 2005; Gozel *et al.*, 2006). The phylum Nematoda suffers an

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enormous 'taxonomic deficit' (the ratio of expected taxa vs. named taxa) (Lambshead, 1993), which hampers the proper understanding of its evolutionary history and ecological importance. Taxonomical efforts through the integration of various methods for classifying nematode species have hitherto mainly focused on parasitic groups (Blaxter *et al.*, 1998; Nadler, 2002), and on a few terrestrial species (Blouin, 2002; Abebe & Blaxter, 2003). However, the majority of species are free-living and marine based, for which the taxonomy relies entirely on morphological characters.

Morphological species recognition within the phylum Nematoda is problematic, and has been criticized because it uses only a few characters and is mainly based on qualitative methods (Nielsen, 1998). In parasitic groups (Coomans, 1979), and in a few terrestrial species (e.g. Ehlers, 2001; Abebe & Blaxter, 2003), additional information based on breeding experiments and behavioural observations has been introduced in species delineation. However, successful attempts to culture marine nematodes have been limited to a few taxa (Moens & Vincx, 1998). Molecular techniques, on the other hand, deal with many characters, and are practical in virtually all taxa (Blaxter *et al.*, 1998; De Ley *et al.*, 2005). Genome-wide information has proven to be powerful for the separation of nematode sibling species (De Ley, 2000; Derycke *et al.*, 2005), potentially even allowing the development of a DNA barcoding approach in nematology (Blaxter *et al.*, 2005; Bhadury *et al.*, 2006). Nevertheless, the molecular approach is also not free from pitfalls, and inconsistencies have already been observed between conspecific taxa (De Ley *et al.*, 2005). Congruence between independent morphological, molecular, reproductive isolation, and behavioural data is probably the best guide to infer whether species boundaries are accurate (de Queiroz, 1998).

In this study, we investigated the taxonomic status of molecular lineages in two free-living nominal nematode species, *Rhabditis (Pellioditis) marina* (Bastian, 1865) Andrássy, 1983 and *Halomonhystera disjuncta* (Bastian, 1865) Andrássy, 2006, through integrative taxonomy. The delineation of species was based on phylogenetic and evolutionary principles (Adams, 1998, 2001), i.e. species are independent evolutionary lineages, with non-reticulate relationships, harbouring a sufficient number of fixed autapomorphies. However, like other methods of species delimitation, the use of fixed autapomorphies is subject to potential errors. We therefore used phylogenetic concordance criteria between a mitochondrial and a nuclear locus as a first step to delineate species. Several specimens from the resulting lineages were used to screen the variability in the nuclear D2D3 region. The resulting molecular lineages were used as coding factors for morphometric data in a discriminant function analysis. We also included typological taxonomy to investigate the presence of diagnostic characters for morphological identification, and performed breeding experiments with two closely related lineages of *R. (P.) marina*. We argue that such an integrative approach is the best way to overcome potential caveats of any species delimitation method, and is a necessary basis for future studies aiming at pinpointing diversity based on molecular data in the phylum Nematoda.

MATERIAL AND METHODS

TAXONOMICAL BASIS

The nominal species *H. disjuncta* and *R. (P.) marina* have particularly confused histories. *Halomonhystera disjuncta* is the type material of the genus, recently introduced by Andrássy (2006). The new genus comprises only marine species formerly known as *Geomonhystera* (Andrássy, 2006). In the past, *H. disjuncta* has been synonymized with seven other species (Jacobs, 1987). All the *H. disjuncta* populations studied here agreed with the original species description of *H. disjuncta* (Bastian, 1865) Andrássy, 2006, which was later adapted by Chitwood & Murphy (1964). The genus *Halomonhystera* is characterized by the minute labial sensory organs, scarce and minute cephalic setae, a sclerotized buccal cavity, a short pharynx, a thin rectum, a gubernaculum with caudal process, a vulva that is located further back, and a sclerotized spinneret chamber (Andrássy, 2006). In the latest dichotomous key made by Andrássy (2006), *H. disjuncta* is distinguished from *Halomonhystera glaciei* (Blome & Riemann 1999) Andrássy, 2006, and *Halomonhystera chitwoodi* (Steiner, 1959) Andrássy, 2006 by the shorter tail, and by the shorter distance between the vulva and the anus, from *Halomonhystera continentalis* Andrássy, 2006 and *Halomonhystera uniformis* (Cobb, 1914) Andrássy, 2006 by its larger body length, and finally, from its morphologically closest sister species, *Halomonhystera ambiguoides* (Bütschli, 1874) Andrássy, 2006, by the more anterior position of the amphids.

Prior to the review by Inglis & Coles (1961), *R. (P.) marina* was thought to consist of seven varieties (*danica*, *marina*, *kielensis*, *nidrosiensis*, *septentrionalis*, *norwegica*, and *bengalensis*), but the morphological characters and the number of individuals used to describe them were insufficient to attribute them species rank. Only *Rhabditis (Pellioditis) bengalensis* Timm, 1956 was considered to be a separate species. Another subspecies, *Rhabditis (Pellioditis) marina mediterranea* Sudhaus, 1974, has been raised to species level based on its pointed tail tip and smaller

body size compared with *R. (P.) marina* (Andrássy, 1983). At present, *R. (P.) marina* is still considered to be a species complex (Sudhaus & Nimrich, 1989). Species belonging to the subgenus *Pellioditis* Dougherty, 1953 possess a large pharyngeal sleeve, three or five warts on each metarhabdion, a medial vulva, and nine precloacal papillae on the open bursa. *Rhabditis (Pellioditis) marina* is closely related to *Rhabditis (Pellioditis) typica* (Stefanski, 1922) Andrássy, 1983 and *Rhabditis (Pellioditis) littorea* Sudhaus & Nimrich, 1989, as was revealed by the arrangement of the papillae (1+2/3+3) before and after the cloaca (Sudhaus & Fitch, 2001). *Rhabditis (Pellioditis) marina* is distinguishable by its conspicuous cheilorhabdions, by its poorly developed terminal pharyngeal bulb, by having between five and eight lateral longitudinal ridges, and by its bursal papillae arranged in two definite postcloacal groups (Sudhaus & Nimrich, 1989). All the *R. (P.) marina* populations considered in this study agree with the first description made by Bastian (1865), which was recently adapted by Sudhaus & Fitch (2001).

SAMPLING AND SAMPLE PROCESSING

Rhabditis (Pellioditis) marina and *H. disjuncta* were sampled in nine and eight locations, respectively, along the coast of Belgium and the south-western part of the Netherlands. The sampling strategy and sample processing have been described in detail by Derycke *et al*. (2005, 2007). For *R. (P.) marina,* morphological measurements were performed on digital photographs from a subset of the individuals used for molecular analyses. Of these, 18–23 specimens from each molecular lineage were chosen for morphological measurements. In this way, molecular and morphological data were obtained from the same individuals. We also included two populations of *R. (P.) marina* from Boston (MA, USA) and Westroy (Scotland, UK). For each of these two populations, 25–30 specimens were picked randomly for molecular analyses, and the remaining individuals were mounted on slides for morphological measurements. Morphological and molecular data thus stem from different individuals, but the link between morphological and molecular datasets was easily made, because each of these two populations contained a single molecular lineage. For *H. disjuncta*, we applied a different strategy to obtain sufficient resolution for morphological characterization: 100 specimens were isolated from each location. Of these, 50 were randomly picked and preserved on acetone for molecular analysis, whereas the remaining specimens were mounted onto glycerine slides. To access morphological variability within molecular lineages, between ten and 15 specimens (males and females) were selected from populations that contained a single lineage (see Derycke *et al*., 2007: fig. 1 for the distribution of the lineages). This was not possible for lineage Gd5, which always co-occurred with Gd1. To characterize Gd5, we first traced the characters that varied among the other lineages. Then, we used the selected characters to look for the presence of more than one morphological lineage in the respective samples.

INTEGRATIVE TAXONOMY DATA ANALYSIS

Three taxonomical methods were used. First, sequences of the mitochondrial cytochrome oxidase *c* subunit 1 gene (COI) and of the nuclear internal transcribed spacer region (ITS) were analysed. All individuals of each nominal species were expected to form a single exclusive lineage. If several exclusive genetic lineages were concordant between the two loci, they were used as coding factors for the multivariate morphometric analysis (MMA). If the MMA indicated the presence of morphological differences between lineages, typological taxonomy was performed as a next step to establish if molecular and morphological lineages could be separated by fixed or non-overlapping characters. In addition, several specimens from each exclusive genetic lineage were used to investigate the potential of the D2D3 region in species identification.

As a next step, we performed hybridization experiments between the two closest related species of *R. (P.) marina*. Ideally, such hybridization experiments should be performed between all possible species pairs of *R. (P.) marina* and *H. disjuncta*, but this was not feasible because we currently only have two species of *R. (P.) marina* and one of *H. disjuncta* in permanent culture. Nevertheless, if reproductive isolation is found between the pair of genetically closest sister taxa, the same is plausible between genetically more distant lineages.

Molecular phylogenetic analysis
The COI sequences used in this study were obtained after screening 759 *H. disjuncta* and 1604 *R. (P.) marina* specimens from various populations in Belgium and the Netherlands (Derycke *et al*., 2005, 2006, 2007a), and all three genes were amplified from the same set of DNA samples. We refer to these previous studies and to Derycke *et al*. (2008) for a detailed description of the amplification and sequencing protocol of the three gene regions, and for a list of the Genbank accession numbers. We were unable to amplify the complete D2D3 region in *H. disjuncta*, and used an internal forward primer (D2/F1, 5′-TTCGACCCGTCTTGAAACACG-3′), in combination with the D3b primer, yielding a fragment of 307 bp instead of 600–1000 bp (De Ley *et al*., 2005).

The ITS dataset was created as a subset consisting of between three and ten haplotypes of each mitochondrial lineage for each species complex, whereas the D2D3 dataset consisted of between two and eight specimens from each lineage. This resulted in 24 ITS and 27 D2D3 sequences for *R.* (*P.*) *marina*, and in 16 ITS and 12 D2D3 sequences for *H. disjuncta*. Eight ITS sequences of *R.* (*P.*) *marina* (accession numbers AM398811–AM398818), and all D2D3 sequences of *H. disjuncta*, are new (AM900752–AM900754).

The COI, ITS and D2D3 sequences were aligned in ClustalX v1.81 (Thompson *et al.*, 1997) using default alignment parameters (gap opening/gap extension costs of 15/6.66). *Rhabditis* (*Pellioditis*) *marina* trees were rooted with the closely related marine/estuarine species *Rhabditis* (*Rhabditis*) *nidrosiensis* Allgén, 1933, and the congener *Rhabditis* (*Pellioditis*) *mediterranea* was added. The *H. disjuncta* trees were unrooted because of the lack of suitable outgroup sequences. The COI and D2D3 alignments were unambiguous in both nominal species. In contrast, the ITS alignment contained many indels, and we identified the ambiguous sites in SOAP 1.2.a4 (Löytynoja & Milinkovitch, 2001). Gap penalties were allowed to range between 11 and 19, with a two-step increase, and extension penalties ranged between three and 11, also with a two-step increase. This resulted in the exclusion of 55 sites at the 90% confidence level in the ITS alignment of *Rhabditis*. For *H. disjuncta*, we created and used the alignment as described in Derycke *et al.* (2007). Most parsimonious (MP) and maximum likelihood (ML) trees were calculated with PAUP* 4.0b10 (Swofford, 1999) and a Bayesian analysis (BA) with MrBayes v3.1.2 (Huelsenbeck & Roncquist, 2005), as described in Derycke *et al.* (2008). The best substitution model for the COI and ITS datasets was determined with Modeltest 3.7 (Posada & Crandall, 1998) using the Akaike information criterion (Posada & Buckley, 2004: table 1). Mr Modeltest was used to determine the evolutionary parameters for the Bayesian analysis. Phylogenetic relationships among haplotype groups are visualized in a neighbour-joining (NJ) tree, calculated in MEGA v3.1 (Kumar, Tamura & Nei, 2004). Branch lengths are based on pairwise *p*-distances.

Multivariate morphometric analysis (MMA)
All specimens were measured (in μm) by video capture; curved structures were measured along the arch. In total, 27 and 31 morphological characters were considered for *H. disjuncta* and *R.* (*P.*) *marina*, respectively. All the measurements were taken from the anterior to the posterior end.
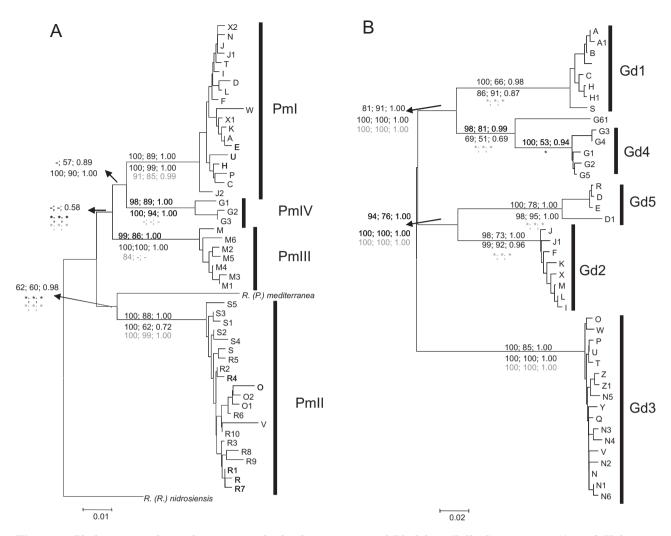
The hypothesis that different lineages within each nominal species did not differ morphologically was tested by a forward stepwise discriminant function analysis (DFA). If significant differences were observed ($P < 0.05$), a posteriori canonical analysis was performed, and the first two roots were plotted in a scatter plot. Finally, in order to detect significant differences between lineages, *P*-values and squared Mahalanobis distances (D^2) for each pairwise comparison were calculated. Lineages were considered different when $P < 0.01$. Characters that were significantly correlated with each other ($P < 0.05$; $r > 0.8$), or characters where the means were significantly correlated with the variance ($P < 0.05$; Cochran test of homogeneity; Sokal & Rohlf (1995), even after log transformation, were not considered for the DFA. Males and females were analysed separately.

Typology
In order to test whether molecular and morphological lineages were separated by fixed or non-overlapping characters, all specimens were observed. Selected characters were coded and presented in a table. One male and one female from each molecular lineage were chosen for representative drawings (see the Appendix).

Breeding experiment
Ulva and *Fucus* fragments collected from Lake Grevelingen and Paulina saltmarsh (Weesterschelde, the Netherlands; Derycke *et al.* 2007) were incubated on marine agar for several days. Adult worms were transferred to fresh agar plates, and were stored at 18 °C for about one month in order to to acclimatize. Monospecific cultures of cryptic lineages from both places were established by transferring one gravid female and between one and three males to a fresh plate. Subsequently, one nematode was handpicked for DNA extraction and PCR-RFLP of the nuclear ITS region. A 5-μL aliquot of each PCR product was digested with 0.5 μL *Alu*I restriction enzyme (10 units μL^{-1}), 1 μL 10x Y+ tango buffer, and 3.5 μL distilled water for 2 h at 37 °C. The digested PCR products were subsequently submitted to electrophoresis, and bands were stained with ethidium bromide. Crosses were performed on agar inoculated with 50 μL of a bacterial suspension, with a density of 2×10^{10} *Escherichia coli* cells per ml as food. In a first experiment, intra- and interspecific crosses were performed with third-stage juvenile females (J3) and adult males from two lineages (PmI and PmIV). All crosses were replicated 20 times. All plates were checked for the first time after 24 (intralineage crosses) or 48 h (interlineage crosses). If dead males were encountered, they were replaced by new ones. Subsequently, all plates were checked every 48 h over a period of 7 days. We then repeated the whole experiment using first-stage juveniles (J1) instead of J3,

Figure 1. Phylogenetic relationships among the haplotype groups of *Rhabditis* (*Pellioditis*) *marina* (A) and *Halomonhystera disjuncta* (B). A neighbour-joining (NJ) tree of 50 and 43 cytochrome oxidase *c* subunit 1 gene (COI) haplotypes with branch lengths calculated on the basis of *p*-distances. Bootstrap values are listed for the following analyses: most parsimonious (MP); maximum likelihood (ML); Bayesian analysis (BA). Values above branches are based on COI; values below branches are based on internal transcribed spacer (ITS) (black) and D2D3 (gray) (–, bootstrap <50; *branch absent).

based on evidence that insemination can sometimes already occur in J3 (TM, pers. obs.). We replicated the intra- and interlineage crosses five and six times, respectively.

RESULTS

MOLECULAR PHYLOGENETIC ANALYSIS

All four methods of phylogenetic inference yielded highly concordant tree topologies for the mitochondrial and nuclear datasets in *R.* (*P.*) *marina* and *H. disjuncta* (Fig. 1). For each method (NJ, MP, ML, and BA), and for each dataset (COI or ITS), sequences of *R.* (*P.*) *marina* were divided into four (Fig. 1A), and sequences of *H. disjuncta* were divided into five, well-supported lineages (Fig. 1B). The same grouping of

R. (*P.*) *marina* specimens was present in the D2D3 region, but very low support was observed for lineages PmIII and PmIV (except in the MP analysis for PmIII, Fig. 1A). All three markers indicated that PmI and PmIV are more closely related to each other than to the other lineages. Other deeper phylogenetic relationships were better resolved in the nuclear regions than in the COI (Fig. 1A). Based on the D2D3 region, lineage PmII was closer related to lineages PmI and PmIV (90, 92, and 0.98 for MP, ML, and BA, respectively) than to PmIII, and *R.* (*P.*) *mediterranea* was pooled with PmI, PmII, and PmIV (although only in ML and BA, and with low bootstrap values of 56 and 0.75, respectively). For the ITS region, *R.* (*P.*) *mediterranea* was closer related to PmIII (with very high bootstrap support: 99, 100, and 1.00 for MP, ML,

Table 1. Phylogenetic parameters, percentage divergence and the substitution model chosen by Modeltest using the Akaike information criterion for cytochrome oxidase *c* subunit 1 gene (COI), internal transcribed spacer (ITS) and D2D3 in *Halomonhystera disjuncta* and *Rhabditis* (*Pellioditis*) *marina* [with inclusion of the congeners *Rhabditis* (*Rhabditis*) *nidrosiensis* and *Rhabditis* (*Pellioditis*) *mediterranea*]

	COI		ITS		D2D3	
	H. disjuncta	*R. (P.) marina*	*H. disjuncta*	*R. (P.) marina*	*H. disjuncta*	*R. (P.) marina*
Alignment length	331	396	892	911*	307	597
Variables sites	117	91	258	387	28	127
Parsimony informative	112	67	245	235	28	35
% divergence	13.8–25.7	5.8–10.6	1.0–24.7	3.3–21.1	0.0–7.2	0.0–19.6
Evolutionary model	HKY + I + G	K81uf + I + G	TrN + G	GTR + G	HKY	TVM + G

*55 excluded by SOAP. HKY: Hasegawa-Kishino-Yano; K81uf: Kimura-1981 with unequal frequencies; TrN: Tamura-Nei; GTR: general time reversible; TVM: transversion model; I: inclusion of the number of invariable sites; G: inclusion of rate variation among sites.

and BA, respectively). For *H. disjuncta*, COI and ITS phylogenies were identical, and showed well-supported interspecific relationships between lineages Gd1-Gd4 and Gd2Gd5, which were more closely related to each other than to any other lineage (Fig. 1A). The 307-bp-long D2D3 sequences were identical between these closely related groups, thereby supporting their phylogenetic relatedness.

Divergence levels of COI within each lineage were low [0.32–2.56% in *H. disjuncta* and 0.25–2.3% in *R.* (*P.*) *marina*], whereas high divergences were observed between lineages. The COI divergences were at least two times higher in *H. disjuncta* than in *R.* (*P.*) *marina* (Table 1). The nuclear ITS variation was also higher in *H. disjuncta* than in *R.* (*P.*) *marina* (Table 1). Each lineage was characterized by a substantial number of fixed differences [between six and 19 in *H. disjuncta*, and between three and seven in *R.* (*P.*) *marina*].Variability of the D2D3 region within each lineage was low [0% in *H. disjuncta* and 0–0.5% in *R.* (*P.*) *marina*]. Interlineage variability ranged between 0–7.2% for *H. disjuncta* and between 0.6–4.7% for *R.* (*P.*) *marina*. No fixed differences were observed between lineages PmI and PmIV, whereas three and four fixed differences were observed in PmIII and PmII, respectively. For *H. disjuncta*, 11 fixed differences were observed in lineage Gd3, and between six and 13 fixed differences were present in the group Gd1-Gd4 and Gd2-Gd5.

section intestine divided by the *abd*, testis length divided by *L*, position of the nerve ring from the anterior end divided by pharynx length, length of the buccal cavity (*BcL*) divided by its width, and *BcL* divided by the head diameter] and nine for females (as for males, except *spic./abd* and testis/*L* were replaced by the position of the vulva divided by *L*, represented as a percentage). There were significant differences between all lineages independent of gender (Table 2). For males and females, the first two roots of the canonical analysis yielded similar results, separating the lineages in four groups (Fig. 2A, B).

Of the 27 characters measured in *H. disjuncta*, only five were selected by the DFA. For females, these characters were tail length, head diameter, *BcL*, ratio *a*, and vulva–anus/tail. The last character was replaced by spicule length in males. In accordance with the molecular results, females from Gd3 were the most divergent, whereas females from Gd1 and Gd4 were morphologically similar (*P* > 0.01; Table 2). All other pairwise comparisons showed significant morphological differences (Table 2). For males, significant differences between lineages were fewer (Table 2). It is important to note, however, that fewer males (*N* = 16) were used for the statistical analysis than females (*N* = 39). For males, the results from the canonical analysis (Fig. 2C) separated all lineages, whereas for the females Gd1 and Gd4 still formed one single cluster (Fig. 2D).

MULTIVARIATE MORPHOMETRICS

Of the 31 characters measured in *R.* (*P.*) *marina*, ten were selected by the DFA for males [body length (*L*), ratio *a*, ratio *b*, ratio *c*, spicules (*spic.*) divided by anal-body diameter (*abd*), length of the posterior-
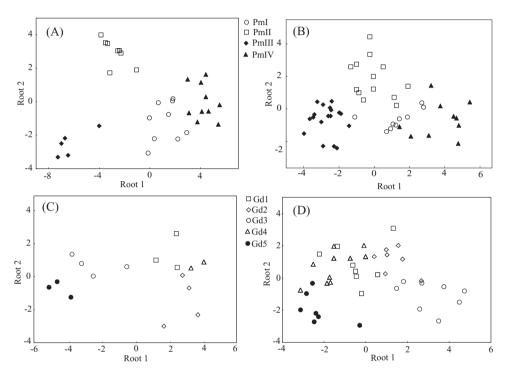
TYPOLOGY

Despite substantial overlap of morphological measurements between all *R.* (*P.*) *marina* lineages, it was possible to discern each lineage based on selected characters (Table 3). For example, the dis-

Table 2. Squared Mahalanobis distances (D^2) between lineages of the *Halomonhystera disjuncta* (Gd) and *Rhabditis* (*Pellioditis*) *marina* (Pm) nominal species

	Gd1	Gd2	Gd3	Gd4	Gd5		PmI	PmII	PmIII	PmIV
Gd1		11.4*	17.8*	3.92	14.5*	**PmI**		8.6*	33.8*	17.3*
Gd2	9.1		13.2*	12.7*	23.4*	**PmII**	37.2*		25.6*	28.7*
Gd3	22.8	33.1*		24.8*	31.4*	**PmIII**	71.3*	45.2*		69.8*
Gd4	6.3	7.6	41.4*		9.4*	**PmIV**	24.1*	61.1*	117.4*	
Gd5	46.3*	54.7*	8.5	68.0*						

Upper right is the distance between females and lower left the distances between males. *$P < 0.01$.

Figure 2. Scatter plot of the canonical measures calculated after the discriminant function analysis (DFA) for the multivariate morphometric data along the first two roots. *Rhabditis* (*Pellioditis*) *marina* lineages: (A) males and (B) females; ○, PMI; □, PmII; ◆, PmIII; ▲, PmIV. *Halomonhystera disjuncta* lineages: (C) males and (D) females; □, Gd1; ◇, Gd2; ○, Gd3; △, Gd4; ●, Gd5.

tribution of characters shows that males from PmIV are distinguished from PmI and PmII by the body length, and from PmIII by the tail length. Considering both genders, PmIV had only one character (ratio *c*) out of the range of the other three lineages, whereas PmII had three (ratio *c*, body length, and *BcL*). All lineages had at least one character that was different from the other species previously described (Table 3).

In total, eight fixed and non-overlapping characters were selected to distinguish all five *H. disjuncta* lineages, and males and females were needed to separate them. Gd3 and Gd5 were the most divergent lineages (Table 4). Gd1 shared more characters with

Gd4, and Gd2 with Gd5. Several measurements from the lineages studied here were within the range described for *H. disjuncta*, and differed from two other closely related species (*Halomonhystera socialis* (Bütschli, 1874) Andrássy, 2006 and *H. ambiguoides*; Table 4).

BREEDING EXPERIMENTS

The PCR-RFLP analyses indicated that all nematodes isolated from Lake Grevelingen belonged to lineage PmIV, and that all nematodes from Paulina were PmI. Interestingly, the monospecific stock cultures of PmIV produced offspring much more quickly than

Table 3. Minimum, maximum, and average (between brackets) values of selected morphological measures from the four *Rhabditis (Pellioditis) marina* specimens studied here (PmI–PmIV), from the *R. (P.) marina* described by Sudhaus (1974), from *Rhabditis (Pellioditis) mediterranea* Sudhaus 1974, from *Rhabditis (Rhabditis) nidrosiensis* Inglis & Coles 1961, from *Rhabditis (Pellioditis) littorea* **Sudhaus & Nimrich 1989**, and from *Rhabditis (Pellioditis) obesa* Gagarin 2001

	Lineages	L	BcL	Tail	a	b	c	%V/spic.	Testis	PI
♀	PmI	1626–1798 (1705) **c**	21–26 (23) **b**	109–123 (117) **c**	19.9–21.9 (20.9) **b, c**	6.2–7.2 (6.7)	13.4–16.4 (14.6) **b**	50–53 (52) **a**	–	S
	PmII	1457–1818 (1638) **b, c**	22–26 (24) **b**	88–103 (95) **b**	19.8–20.4 (20.1) **b**	5.6–6.8 (6.2)	16.6–17.5 (17.0) **b**	52–56 (54) **a, b**	–	F
	PmIII	1095–1514 (1309) **b**	16–20 (18) **a**	77–107 (90) **b**	21.0–28.6 (24.1) **c**	5.8–7.7 (6.7)	13.4–15.9 (14.3) **b**	50–53 (51) **a**	–	F
	PmIV	1160–1548 (1387) **b**	19–24 (21) **a, b**	97–134 (112) **b, c**	18.9–24.5 (21.8) **b**	5.1–6.6 (5.9)	10.5–13.8 (12.3) **b**	47–53 (50) **a**	–	I
	R. (P.) mediterranea	1157–1590 **b**	22–24 **b**	56–91 **a, b**	18.2–25.8 **b, c**	5.2–7.0	15.7–25.0 **b, c, d**	51–55 **a, b**	–	?
	R. (P.) marina	1628–2875 **c**	30–39 **c**	99–139 **b, c**	20–24.5 **b, c**	5.2–8.1	14.7–22.0 **b, c, d**	53–57 **b**	–	S
	R. (P.) ehrenbaumi	1380–1640 **b, c**	? **c**	? –	14.9–20.3 **a, b**	3.6–4.2	18.0–24.8 **d**	52–56 **a, b**	–	F
	R. (P.) littorea	599–900 (731) **a**	16–20 (18) **a**	89–147 (113) **b, c**	14.4–17.8 (15.9) **a**	4.7–6.4 (5.3)	5.1–8.7 (6.4) **b**	47–53 (50) **a**	–	?
	R. (P.) obesa	1422–1619 (1524) **b**	?	52–59 (56) **a**	?	16.0–20.0 (19)	23.2–28.9 (25.6) **c**	57–59 (58) **c**	–	?
♂	PmI	1731–1998 (1864) **IV**	24–28 (26) **III**	65–79 (69) **III**	21.4–27.7 (23.6) **II, III**	5.7–6.4 (6.1)	25.2–28.7 (26.8) **III**	50–54 (53) **III**	1205–1555 (1316) **III**	S
	PmII	1403–1445 (1424) **III**	23–23 (23) **II**	57–60 (58) **II**	22.6–23.16 (22.9) **III**	5.0–5.6 (5.3)	24.0–24.3 (24.2) **II**	57–64 (61) **IV**	1168–1238 (1203) **III**	F
	PmIII	1051–1130 (1090) **II**	14–16 (15) **I**	32–41 (36) **I**	28.4–32.5 (30.5) **IV**	6.3–6.4 (6.4)	25.1–35.2 (30.2) **III**	37–42 (40) **II**	885–948 (917) **II**	F
	PmIV	1043–1210 (1125) **II**	18–20 (19) **II**	56–70 (60) **II**	18.9–21.9 (20.6) **II**	4.9–5.5 (5.3)	17.3–20.0 (18.7) **I**	52–62 (57) **III, IV**	909–1078 (996) **II**	I
	R. (P.) mediterranea	779–1298 (1125) **II**	19–22 **I, II**	22–37 **I**	18.7–33.2 (20.6) **II, III, IV**	4.4–6.6	23.6–41.7 **II, III**	31–45 **II**	?	?
	R. (P.) marina	1337–1978 **II**	21–31 **II, III**	50–59 **II**	20.9–32.4 **II, III, IV**	4.6–7.7	22.4–30.2 **II**	37–57 **II, III**	?	S
	R. (P.) ehrenbaumi	890–1280 **II**	? –	27–40 **I**	17.0–18.3 **I**	3.2–4.1	25.3–32.0 **III**	62–72 **IV**	?	F
	R. (P.) littorea	400–708 (501) **I**	15–20 (16) **I, II**	25–33 (28) **I**	13.6–19.9 (16.3) **I**	3.5–4.8 (3.9)	14.6–17.8 (16.2) **I**	23–30 (26) **I**	201–453 (286) **I**	?
	R. (P.) obesa	1039–1318 (1116) **II**	? –	28–37 (32) **I**	? –	11.0–19.0 (16)	35.2–39.2 (37.1) **IV**	75–84 (79) **V**	?	?

Abbreviations: *L*, total body length; *a*, *L* divided by maximum body width; *b*, *L* divided by Pharynx length; *c*, *L* divided by tail length (*t*); *BcL*, length of the buccal cavity; spic., spicules; %V, position of the vulva from the anterior end divided by *L*; PI, junction between the pharynx and intestine (S, straight; F, folded; I, invaginated). Letters for females (a–d) and roman numbers for males (I–IV) stand for non-overlapping measurements.

Table 4. Maximum, minimum, and average (between brackets) values of selected morphological measures from the five *Halomonhystera disjuncta* specimens studied here (GD1–GD5), from the *H. disjuncta* described by Chitwood & Murphy (1964), and from two other closely related species (Bütschili, 1874)

	Lineages	L	a	b	c	c'	Amph ant./head	v – alt spic./abd	Pre-sup.	Pos-sup.	CPA
♂	GD1	1124.8–844.7 (999.4) a	25.3–21.0 (23.1) a	3.8–2.6 (3.0) a	12.7–9.7 (11.4) a	4.8–3.3 (4.0) a, b	1.68–1.22 (1.40) b	0.48–0.19 (0.3) a, b	–	–	–
	GD2	1090–950.8 (1011.5) a	28.6–25.5 (27.1) b	3.8–3.0 (3.4) b	13.4–9.8 (11.7) a, b	5.3–4.2 (4.5) b	1.46–1.21 (1.29) b	0.49–0.26 (0.37) b	–	–	–
	GD3	1224.0–911.5 (1049.5) a	28.1–21.3 (24.5) a, b	3.3–2.2 (2.7) a, b	11.5–9.3 (10.4) a	5.4–4.3 (5.0) b	1.49–1.08 (1.28) b	0.38–0.29 (0.34) b	–	–	–
	GD4	1150.8–825.7 (960.9) a	26.3–21.1 (24.6) a, b	3.4–2.8 (3.1) a, b	13.0–9.6 (11.5) a	4.7–3.5 (4.2) a, b	1.69–1.04 (1.33) b	0.33–0.17 (0.24) b	–	–	–
	GD5	1005.1–804.7 (897.6) a	23.9–20.7 (22.6) a	3.2–2.1 (2.7) a	15.2–13.1 (13.8) b	3.8–3.1 (3.4) a	1.92–1.32 (1.58) b	0.38–0.28 (0.33) b	–	–	–
	H. disjuncta	1453–635 a	37–13 a, b	10–5.7 b	14.2–8.7 b	4 b	<2 b	0.33 b	–	–	–
	H. socialis	2200–1900 b	50–40 c	9–8 b	11–10 b	5 b	<1 a	0.20 a	–	–	–
	H. ambiguoides	1000 a	33–28 b	7 b	10 b	4–3 a, b	3 c	0.25 a	–	–	–
♀	GD1	1068.7–969.0 (1013.8) I	33.0–27.2 (29.7) II	3.9–3.2 (3.5) I	13.5–11.5 (12.52) II	3.5–2.8 (3.2) I, II	1.06–0.92 (0.97) I, II	1.47–1.17 (1.35) I	P	P	Small
	GD2	1232.4–953.2 (1073.0) I	33.9–29.7 (31.9) II	4.3–3.4 (3.9) I	13.9–11.7 (12.43) II	3.6–3.4 (3.4) II	1.20–0.86 (1.00) I, II	1.57–1.19 (1.35) I	P	P	Large
	GD3	1212.9–775.0 (988.5) I	30.0–26.7 (28.9) II	3.9–3.3 (3.6) II	13.5–11.2 (12.60) I, II	3.4–3.1 (3.2) I	0.96–0.77 (0.86) I	1.67–1.40 (1.53) I, II	A	A	Small
	GD4	1134.8–1037.3 (1086.0) I	32.3–28.9 (30.6) II	3.6–3.4 (3.5) II	11.5–11.1 (11.28) I	3.5–3.4 (3.5) II	0.93–0.86 (0.89) I	1.54–1.34 (1.44) I	P		Small
	GD5	1161.6–873.1 (996.6) I	28.5–25.7 (27.1) I	3.9–3.2 (3.6) I	13.8–11.7 (12.56) II	3.5–3.1 (3.3) I, II	1.13–1.06 (1.10) II	1.78–1.66 (1.71) II	A	A	Large
	H. disjuncta	1520–677 I	35–24 I, II	10.3–6.2 (3.6) I	20–10.9 I, II III	3.1 I	<2 II	1.2 I	P	P	Small–Large
	H. socialis	2200–1900 II	50–40 III	9–8 II	14 III	3 I	<1 I	1.5–1.3 I	A	?	Large
	H. ambiguoides	Male not known									

Abbreviations: *L*, total body length; *a*, *L* divided by maximum body width; *b*, *L* divided by Pharynx length; *c*, *L* divided by tail length (*t*); *c'*, *t* divided by anal body diameter (*abd*); *Amph ant.*, position of the amphids from the anterior end; *cbd*, corresponding body diameter; *spic.*, spicules; *v – a/t*, distance between the vulva and anus divided by the tail length; *head*, head diameter; sup., supplements located before the cloaca (pre) or posterior to the cloaca (pos); CPA, caudal process of the apophysis; P, present; A, absent. Letters for females and roman numbers for males stand for non-overlapping measurements.

Table 5. Number of plates with successful reproduction between lineages PmI and PmIV from two experiments

		PmI ♀	PmIV ♀
1st exp	**PmI ♂**	3(19)	1(18)
	PmIV ♂	0(20)	4(18)
2nd exp	**PmI ♂**	4(6)	0(5)
	PmIV ♂	0(5)	2(5)

Values between brackets stand for the number of replicates.

those of PmI, and we also observed differences in behaviour and motility between both lineages.

The success rate of all the intralineage crosses varied from 16 to 66% (Table 5). Out of 38 interlineage crosses in the first experiment (with J3), only one produced juveniles. Out of ten interlineage crosses in the second experiment (with J1), not a single one produced offspring.

DISCUSSION

The present integrative approach confirmed the presence of multiple species within each nominal species. The concordant patterns in two independently evolving markers (mitochondrial DNA and ITS) indicate that the deeply diverged lineages within *H. disjuncta* and *R.* (*P.*) *marina* are phylogenetic and genealogical species. Each lineage also harbours a substantial number of fixed molecular differences (autapomorphies), and the levels of divergence between the lineages of either nominal species are well within the range of those observed between congeneric nematode species (Radice *et al.*, 1988; Powers *et al.*, 1997; Blouin, 2002).

The ITS sequences are commonly used for species identification in nematology (Powers *et al.*, 1997; Nguyen, Maruniak & Adams, 2001), but divergence between congeneric nematode species may sometimes be too low for reliable species delineation (Ferris, Ferris & Faghihi, 1993; Kaplan, 1994). Alternatively, the application of mitochondrial DNA for species identification in nematodes has been rarely used, even though it quickly reaches reciprocal monophyly between closely related species, with divergences of usually higher than 10% (Blouin *et al.*, 1997; Hoberg *et al.*, 1999; and present study). Mitochondrial genomes are, however, very diverse within the phylum, thereby hampering the use of a phylum-wide primer. Moreover, phylogenetic patterns can be confounded by tokogenetic patterns (Ballard & Rand, 2005), and heteroplasmy, i.e. different copies of mitochondrial DNA in the same individual, may also

confound species delimitation solely based on mitochondrial DNA (Tigano *et al.*, 2005). Therefore, the combination of ITS and mitochondrial DNA provides powerful means of inferring relationships between lineages, and establishing their species identity.

The D2D3 region has successfully been used for investigating phylogenetic relationships between closely related nematode species (De Ley *et al.*, 2005), and even for the detection of cryptic species (De Ley *et al.*, 1999). In our study, the D2D3 fragment was able to distinguish all lineages of *R.* (*P.*) *marina*, albeit sometimes with very low support. For *H. disjuncta*, in contrast, this fragment separated the lineages into three groups (Gd1-Gd4, Gd2-Gd5, and Gd3). Of the three markers used in both species complexes, the D2D3 was the least informative. Within the *R.* (*P.*) *marina* alignment, the first 300 bp was more variable than the 3′ end of the D3 segment, thereby supporting the contention that a combination of the D2 and D3 expansion segments provides more signal (De Ley *et al.*, 2005). This may also explain why not all Gd lineages were recovered in the shorter D2D3 fragment. The lack of a clear gap between intra- and interlineage variability further corroborates the findings of De Ley *et al.* (2005), in that mean differences in the D2D3 fragment do not always correspond reliably with species boundaries.

All molecular lineages detected based on COI and ITS regions in our two nominal species were in agreement with the MMA, except for the lineages Gd1 and Gd4. These lineages were morphologically similar, at least for the measurements in this study, suggesting that they are cryptic species. In the case of *H. disjuncta*, the absence of morphological differences may also be related to the low number of individuals sampled. The set of morphological characters for identifying sibling species may be largely dependent on the number of populations and individuals analysed (Wiens & Servedio, 2000). Consequently, whereas for the molecular data the variation within lineages was lower than between lineages, for the morphological data this pattern was not consistent (Fig. 3). These differences between methodologies can be explained by the particular pattern of morphological variation in which between-species differentiation is small relative to within-species variation (Wiens & Penkrot, 2002). Indeed, the number of diagnostic morphological characters within both nominal species was relatively small, and some of these characters showed extensive variation within populations and lineages. An alternative explanation for the differences between morphology and the molecular data is more theoretical: most of the variation in mitochondrial genes within a species is selectively neutral (Ballard & Rand, 2005), and is independent of morphological

Figure 3. Means and 95% confidence intervals for the similarities of the multivariate morphometric (Euclidean distance) and molecular data (distances), comparing the variability within and between lineages from both species complexes. •, *Halomonhystera disjuncta*; □, *Rhabditis (Pellioditis) marina*.

changes. Therefore, molecular variability within and between lineages is not necessarily a good proxy of morphological variability (Wiens & Penkrot, 2002).

Typological taxonomy supported the molecular results and separated all the groups identified by MMA in both species complexes. However, closely related species were often separated by only one or two non-overlapping characters, which hampers proper species diagnosis (Wiens & Servedio, 2000). Moreover, the relationships between lineages based on typology were not always consistent with the results generated with MMA, highlighting the problem of selecting only a few characters for morphology-based species identification and phylogeny. This is mainly because morphological characters are not necessarily phylogenetically informative, and may also be influenced by ecological factors (Schiemer, 1982; Herman & Vranken, 1988).

Results of the breeding experiment between the two genetically and morphologically most closely related lineages, PmI and PmIV, suggest that they are different biological species, and provides additional evidence for their species status (Dayrat, 2005). Reproduction was only observed in the intralineage crosses, with the exception of a single successful interlineage cross, which was probably caused by insemination of the J3 female just prior to the experiment. In addition, we observed different behaviour and reproduction rates in the stock cultures of PmI

and PmIV, with PmI typically being less motile and reproducing more slowly. In view of the difficulty to isolate and cultivate all the lineages of each species complex, this method is not very feasible for species identification.

For the two species complexes studied here, we may assume that a considerable part of the morphological variability reported in the literature corresponds to a similar variety of sibling species. For instance, we observed body length variation within *H. disjuncta* between 0.77 and 1.2 mm, compared with a range from 0.5 to more than 1.6 mm in the species, *sensu* Andrássy (2006). The length of the male copulatory organs (spicules) varied between 29 and 44 µm in the present study, and from 25 to 42 µm in the literature. In addition, variability in the presence/absence of pre- and postcloacal supplements, and differences in the shape of the apophysis, are commonly observed (Chitwood & Murphy, 1964). Similar examples are also common in *R. (P.) marina*. It was previously assumed that *R. (P.) marina* consisted of two morphological groups occurring in separate regions (Sudhaus, 1974). In our study, the body length ranged from 1.3 to 3.6 mm [from 0.8 to more than 3 mm in the specimens studied by Sudhaus (1974)], whereas *BcL* varied between 14.5 and 28 µm (vs. 16–40 µm). Hence, the many free-living nematode species for which a high morphological variability has been described are likely to represent a substantial, hitherto unrecog-

nized, species diversity, thereby rendering most diversity estimates for the phylum Nematoda hugely inadequate.

We conclude that each nominal species represents a large species complex. In *H. disjuncta*, the original species description (then *Monhystera disjuncta*) by Bastian (1865) is based on a single, poorly described male. Later, Chitwood & Murphy (1964) made a detailed description of the species, covering a very large morphological range. Although their study was recently used to characterize the genus *Halomonhystera* (Andrássy, 2006), two characters differ between our populations and the ones described by Chitwood & Murphy (1964) (Table 4). Thus, without extra taxonomical information from the type material, it is difficult to know whether any of the lineages reported here can be placed within the respective nominal species, and whether the previous descriptions pertain to single species or to multiple cryptic species. The taxonomical status of our *R. (P.) marina* species is also problematic. All the lineages in this study differ morphologically from the original description (Bastian, 1865); however, Bastian described only two individuals (one male and one female). Nevertheless, our lineages are within the ranges described later (Inglis & Coles, 1961; Sudhaus, 1974; Andrássy, 1983). The molecular markers and the morphological observations show that the species *R. (P.) mediterranea* is a sister species of our *R. (P.) marina* species. The ITS data indicates that *R. (P.) mediterranea* is more closely related to lineage PmIII than to the other lineages; this relationship was also observed in the morphological measurements. The ranges of 11 morphological characters of *R. (P.) marina* published by Sudhaus (1974), and his drawings of the male posterior end, are comparable with the specimens from PmII, but they differ in four other morphological characters. Based upon the present molecular and morphological results, and on comparison with the original and subsequent species descriptions, it would be possible to split the current nominal species *H. disjuncta* into five, and *R. (P.) marina* into four different species. However, instead of increasing the taxonomical chaos in these genera, a detailed taxonomical revision of the complete genera is first warranted, and this is beyond the scope of this study.

Confusions in morphological taxonomy are not particular to these two genera, in fact plenty of marine nematode genera are equally or more problematic (e.g. *Thalassomonhystera*, *Daptonema*, *Theristus*, etc.). Taxonomical reviews are urgently required in the phylum Nematoda. Future descriptions should, however, consider an integrative approach to avoid further disorder. The present study largely contributes to the improvement of our taxonomical tools for correct species delineation, and emphasizes the synergy between different methods. We conclude that for taxa where the majority of species remain to be described, and/or where morphological and molecular variability are poorly known, the selection of a few morphological characters, or of a single molecular marker, for delineating species boundaries is inadequate. By contrast, our a priori use of molecular data – based on more than one marker – to code the morphological dataset for multivariate analysis, and, ultimately, for pinpointing morphological identification characters, proved to be very effective. Although such integrative taxonomy requires substantial expertise and time, it is at this point the best way to accurately delimit species in taxa with unknown biodiversity (Dayart, 2005; Will *et al.*, 2005). Hence, it is important that model studies on integrative taxonomy highlight the limitations of various methods in different taxa, allowing us to choose the best strategy for future identifications.

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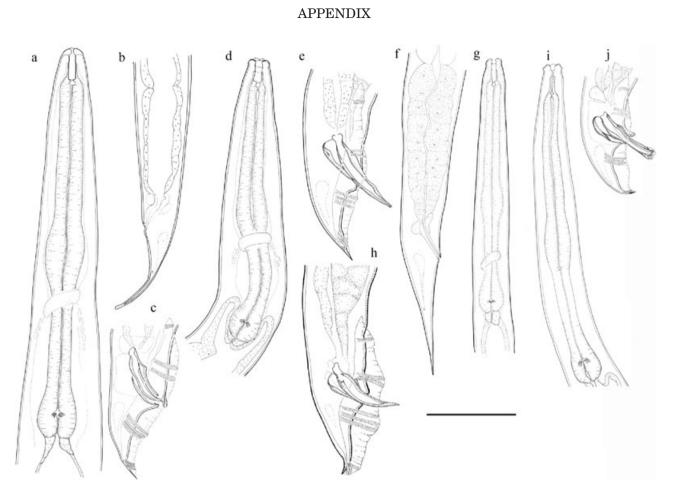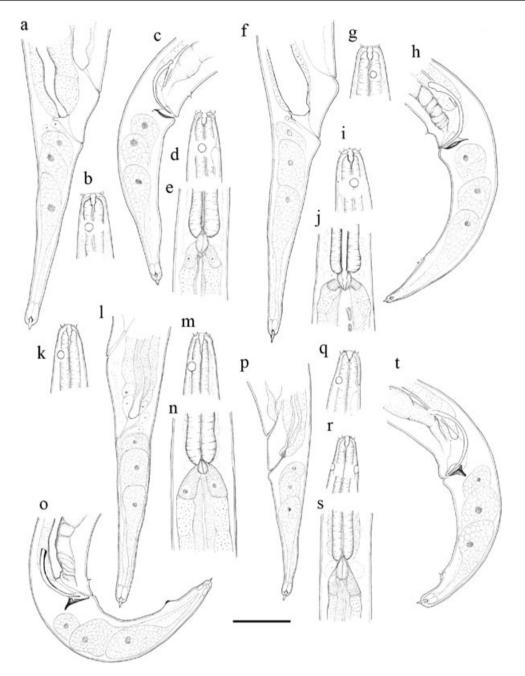
APPENDIX

Figure A1. *Rhabditis (Pellioditis) marina*: (a–c) PmI; (d–e) PmIV; (f–h) PmIII; (i–j) PmII. Scale bar: 60 μm.

Figure A2. *Halomonhystera disjuncta*: (a–b) Gd1♀; (c–e) Gd1♂; (f–g) Gd4♀; (h–j) Gd4♂; (k–l) Gd2♀; (m–o) Gd2♂; (p–q) Gd5♀; (r–t) Gd5♂. Scale bar: 25 μm.
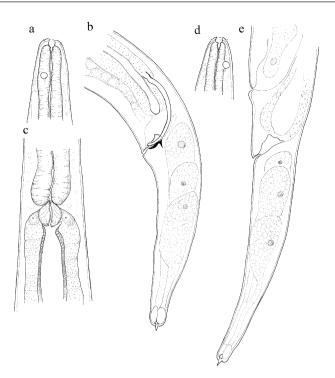
Figure A3. *Halomonhystera disjuncta*: (a–c) Gd3♂; (d–e) Gd3♀.