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Three Biological Species Closely Related to *Rhabditis (Oscheius) pseudodolichura* Körner in Osche, 1952

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Abstract: The *Oscheius* subgenus (Nematoda: Rhabditidae) comprises several common free-living hermaphroditic species. Morphological identification is difficult due to a lack of reliable characters to discriminate species. We studied 32 strains that are closely related to *Rhabditis (Oscheius) pseudodolichura* and *R. (O.) tipulae*. We present results from mating experiments between the strains and sequence data from the internal transcribed spacer region of ribosomal RNA, allowing discrimination of three closely related biological species.

Key words: ITS, nematode, *Oscheius,* PCR-RFLP, systematics.

Within genus *Rhabditis* sensu Sudhaus (1976) (family Rhabditidae), the *Oscheius* subgenus includes some common hermaphroditic species that are easily isolated from soil samples. A number of strains are available in culture. Their morphological identification is difficult due to a lack of good characters to discriminate species.

The *Oscheius* subgenus has been divided in two groups: *Dolichura* and *Insectivora* (Sudhaus and Hooper, 1994). From new molecular sequencing data of 18S RNA genes, it is unclear whether these two groups form a monophyletic clade (Sudhaus and Fitch, 2001). They may be split into two (sub)genera and the *Dolichura* group called genus *Dolichorhabditis* (Carta, pers. comm., Blaxter et al., 1998; Evans et al., 1997; Félix et al., 2000). The *Dolichura* group further comprises three subgroups—the first represented by the monodelphic species *Rhabditis (Oscheius) guentheri* (Sudhaus and Hooper, 1994); a second including the species *R. (O.) dolichura* and others; and a third containing the species *R. (O.) pseudodolichura, R. (O.) sechellensis,* and *R. (O.) tipulae* (Sudhaus, 1976; Sudhaus and Fitch, 2001; Carta, pers. comm.). We address here only the latter subgroup. Members of this subgroup are extremely common in the soil (unpubl. obs.; Carta, pers. comm.), but species identification is difficult (Sudhaus and Hooper, 1994).

One strain of this subgroup, called CEW1, is used by us and other investigators for molecular and genetic studies pertaining to vitellogenin sequence evolution (Winter, 1992; Winter et al., 1996), transsplicing (Evans et al., 1997), and vulva development in comparison with that of *Caenorhabditis elegans* (Dichtel et al., 2001; Félix et al., 2000); another strain, PS1131, also was used for vulva studies (Félix and Sternberg, 1997; Sommer and Sternberg, 1995). Moreover, we recently found large variations in vulval lineages between strains of this subgroup (Delattre and Félix, 2001). It is therefore important to define within this subgroup which strains belong to the same species. Here we present mating and DNA sequence data that may define three biological species that are closely related to each other in this subgroup.

Materials and Methods

Cultures: The nematodes were cultured as described in Wood (1988), using the *Escherichia coli* strain OP50 as a food source.

Origin of the strains: All strains were isolated from soil, except SB128, which was isolated from larvae of the leatherjackets *Tipula paludosa* (Diptera: Tipulidae) as described in Sudhaus (1993). The strains come from a wide geographic area (Table 1). Details on the origin of each strain are available from M.-A. Félix.

Mating tests: Rare spontaneous males were isolated, and a male-containing culture of a given strain was maintained continuously by mating. For matings, approximately 5 males and 5 hermaphrodites (L4 larvae or young adults) were placed on a 55-mm agar dish seeded with a small drop of *E. coli* OP50. The plates were incubated at 23 °C. The success of the cross was assessed by the presence of young males one generation later. If hermaphrodites were from a strain producing males at a relatively high frequency (1–5%), the cross was counted as positive if a much larger number of males was observed relative to this spontaneous frequency.

ITS sequencing: The internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) was amplified by the polymerase chain reaction (PCR). Five nematodes were transferred to 30 µl of Lysis Buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP40, 0.45% Tween 20, 60 µg/ml proteinase K), frozen at −80 °C for 15 minutes, and incubated at 65 °C for 1 hour and at 95 °C for 10 minutes. Five µl were used per PCR reaction.

The PCR primers are Vrain 2F: 5′-CTT TGT ACA CAC CGC CCG TCG CT-3′ and Vrain 2R: 5′-TTT CAC TCG CCG TTA CTA AGG GAA TC-3′ (Fig. 3A). PCR conditions were 30 seconds at 94 °C, 1 minute at 54 °C, and 1 minute 45 seconds at 72 °C for 40 cycles, followed by a 7-minute extension at 72 °C. Sequencing was performed using a Perkin Elmer ABI Prism 377 automated DNA sequencer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). PCR product was treated with shrimp alkaline phosphatase (1 U/µl, Amersham) and

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TABLE 1. Mating experiments define three biological species among the 32 strains in this subgroup of *Oscheius*.

Strain	Geographic origin	CEW1 males	PS959 males	PS966 males	PS2068 males	JU75 males
CEW1	Brazil	4/4	4/4	4/4	0/4	0/4
JU134	Hawaii	2/2				
JU136	Hawaii	2/2				
JU142	Hawaii	2/2				
JU149	Madagascar	2/2				
JU170	Spain	4/4				
JU177	New York	2/2				
JU178	New York	2/2				
PS320	California	2/4[a]			0/4	
PS959	New York	4/4[a]	4/4		0/4	
PS966	Ohio	2/2		4/4		
PS986	Kansas	4/4				
PS1005	Washington D.C.	2/2				
PS1131	Japan	2/2				
PS1170	Alabama	2/2				
PS1181	Michigan	2/6[a]			0/5	
PS1305	Pennsylvania	4/4[a]				
PS2069	Washington State	4/4				
PS2070	Paris area, France	4/4[a]	4/4		0/4	
PS2280	Indre, France	2/2				
BA1009	Arizona	4/4				
BC4783	British Columbia	2/2				
SB128	Germany	3/3				
PS2068	Oregon	0/4	0/4	0/4	4/4	0/4
JU76	Paris area, France	0/4			4/4[a]	
JU77	Paris area, France	0/4			3/3[a]	0/4
JU171	Brittany, France	0/4			4/4[a]	
JU179	Paris area, France	0/4			0/8	0/4
JU181	Paris area, France	0/2			4/4	
PS335	California	0/4			3/3[a]	
VT683	New York	0/4			4/4[a]	
JU75	Paris area, France	0/4			0/4	4/4

For each cross, the hermaphrodites are derived from strains listed in the first column and the males from strains listed in the first line. The numbers indicate the number of plates scored positive for the cross over the total number of plates. The different mating groups are separated by dotted lines (JU179 is placed in sp. 2 because of molecular data).

[a] Also successful backcrosses to both parents.

exonuclease I (20 U/μl, Epicentre Technologies, Madison, WI Pharmecïa, Piscataway, NJ) for 15 minutes at 37 °C, followed by 15 minutes at 80 °C to inactivate the enzymes. This material was then used for cycle sequencing without any further purification, using the ABI Prism BigDye Terminator Cycle Sequencing kit. The sequencing conditions were 30 seconds at 96 °C, 15 seconds at 50 °C, and 4 minutes at 60 °C for 27 cycles. Primers used for sequencing are Ferris2F: 5′-RGY AAA AGT CGT AAC AAG GT-3′ and 28R1: 5′-TGA TAT GCT TAA NTT CAG CGG GT-3′. Cycle sequence products were precipitated by adding 25 μl of 95% ethanol and 1 μl 3M sodium acetate pH 4.6 to each cycle-sequencing reaction (10 μl). The samples were placed at 20 °C for 15 minutes and centrifuged at 14,000 rpm for 15 minutes. After precipitation, an additional pellet wash was performed with 125 μl of 70% ethanol and centrifuged at 14,000 rpm for 5 minutes. The pellet was dried in a Speedvac concentrator (Savant Instruments, Hicksville, NY), redissolved in loading buffer, and run on a 48-cm 4.25% acrylamide:bisacrylamide (29:1) gel. Sequences are deposited with EMBL under Accession Nos. AJ297889-2978999 and AJ291722 for the isolate JU179.

Sequence alignment and tree building: The sequences (approximately 820 bp) were aligned with Treealign (Hein, 1990), followed by limited manual editing to improve inferences of positional homology. The entire alignment is available from A. Vierstraete and J. Vanfleteren on request. The alignment was analyzed with PAUP* 4.0b8 (Swofford, 1998) to construct neighbor joining, maximum parsimony (MP), and maximum likelihood (ML) trees. Neighbor joining and maximum likelihood were performed with the Hasegawa-Kishino-Yano (HKY) 85 model of DNA evolution. Heuristic maximum parsimony searches were performed using tree-bisection-reconnection as branch-swapping algorithm for MP and ML. Bootstrap analyses comprised 3,000, 1,000, and 100 replicates for NJ, MP, and ML, respectively. Although hierarchic likelihood ratio tests, run under ModelTest 3.05 (Posada and Crandall, 1998), suggested that HKY85, adapted to account for invariable sites (I) and unequal (gamma distributed) rates among sites (HKY+I+gamma), provided best fits to the data set, we found that the less sophisticated HKY85 model provided superior phylogenetic inference. Maximum likelihood and NJ trees constructed using the HKY+I+gamma model of DNA evolution gave identical overall results to the tree shown in Figure 2B, except that there is no longer bootstrap support (>50%) linking JU75 to the clade defining sp. 1 (data not shown).

PCR-RFLP test: The ITS region was amplified by PCR as described above. One microliter of the appropriate restriction enzyme buffer (10×) and 0.5 μl of restriction enzyme were added to 20 μl of PCR reaction, and incubated for 1 hour at 55 °C (Bcl I) or 37 °C (Bgl II and Hinc II).

RESULTS

Mating groups: All strains in this species group are self-fertilizing hermaphrodites with facultative males. Spontaneous males are very rare in most strains, whereas mating with males produces a high proportion of males (close to half of the animals). We therefore assessed successful crosses between two strains by scoring for the presence of male progeny. In some cases, we also crossed the male progeny with hermaphrodites of either parental strain (backcrosses) and scored for male progeny at the next generation, thereby checking that the hybrid males were fertile.

The results of crosses define three mating groups (Table 1). A first mating group, which we provisionally call species 1, contains 23 strains, including CEW1. A second group, which we call sp. 2, contains 8 strains, including PS2068. A third group, which we call sp. 3, includes only the isolate JU75. CEW1 is taken as the

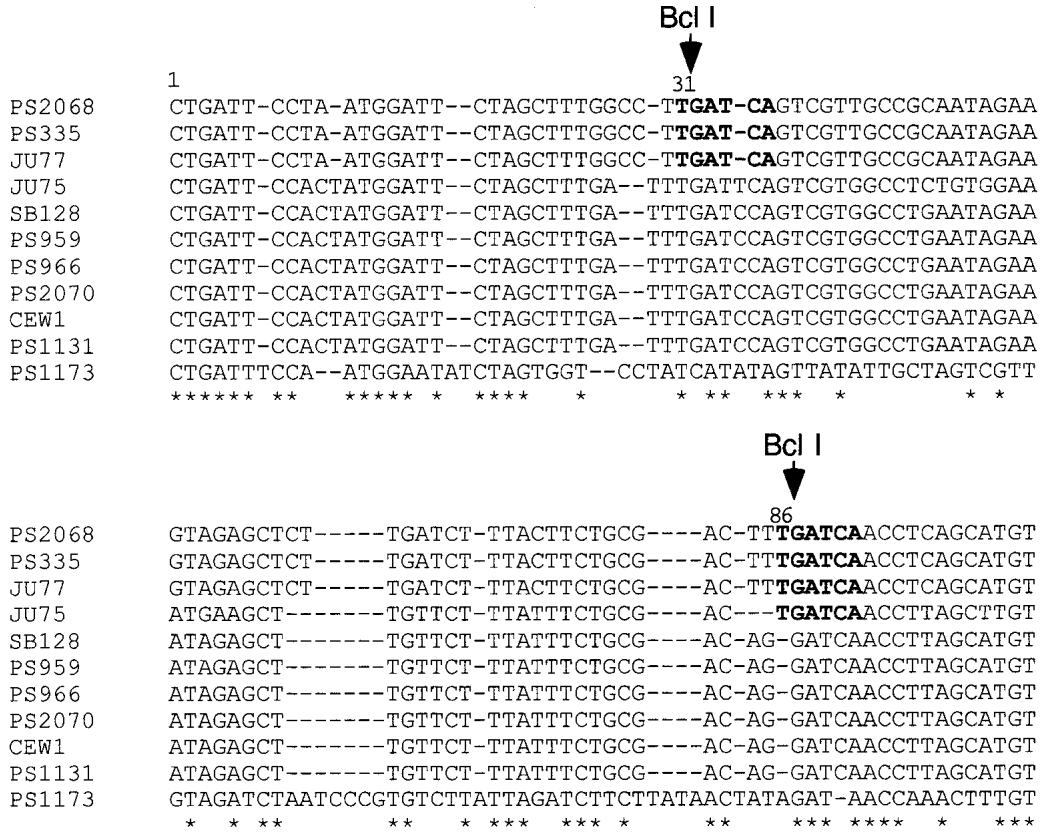

FIG. 1. Alignment of the internal transcribed spacer (ITS) 5′ end region. The first 100 nucleotides are aligned (out of approximately 820 nucleotides for the entire ITS sequence). Dashes indicate gaps introduced for alignment purposes. Nucleotides that are conserved between all strains (including outgroup PS1173) are indicated by stars. The Bcl I cleavage sites are shown in bold. Nucleotide numbers are indicated for PS2068.

reference strain for sp. 1 because it has been used for several molecular and genetic studies (Dichtel et al., 2001; Evans et al., 1997; Félix et al., 2000; Winter, 1992; Winter et al., 1996). PS2068 is taken as the reference strain for sp. 2 because it grows best (its fecundity is high).

We also checked that PS2068 and JU75 were cross-sterile with CEW1 by using recessive marker mutations isolated in the CEW1 strain: an Uncoordinated (Unc) mutant and a Dumpy (Dpy) mutant (Félix et al., 2000). Neither PS2068 nor JU75 males give non-Unc cross-progeny with *unc-6(sy474)* hermaphrodites (0/4 plates for each). JU75 males gave only one sterile non-Dpy animal on 4 plates when incubated with *dpy-6(sy518)* hermaphrodites.

JU179 hermaphrodites did not produce male cross-progeny with males of any of the three species (Table 1). The few spontaneous males that we obtained in this strain did not produce progeny with JU179 hermaphrodites. Because we could not obtain male mating from this strain, we cannot assign a fourth mating group to it and place it provisionally in sp. 2 (see below).

Sequences of the ITS region of ribosomal RNA: We amplified by PCR the internal transcribed spacer (ITS) of ribosomal RNA in a subset of these strains, namely, CEW1, PS1131, PS959, PS966, and PS2070 for sp. 1; PS2068, PS335, and JU77 for sp. 2; and JU75 for sp. 3. This subset represents a geographic variety within each mating group. PS1173 is a hermaphroditic strain of the *dolichura* subgroup, which is used as an outgroup representative. Nucleotide sequence of the ITS region from PS1173 is very divergent and cannot always be aligned easily with the others. We tried to find a closer outgroup representative by sequencing the ITS locus from PS1017, another strain of this *dolichura* subgroup (Carta, pers. comm.), as well as *Rhabditis (Oscheius) guentheri* SB133, but they did not align more closely.

A partial (5′ end) alignment is shown in Figure 1, and the relationships deduced between the full ITS sequences of these strains are displayed in Figure 2. It is clear that the three mating groups are also defined by a number of sequence characters. Furthermore, it appears that the ITS sequence of sp. 3 is closer to that of sp. 1 than to that of sp. 2. Within sp. 1, SB128, PS959, and PS966 have identical sequences along the entire ITS length. Strain PS2070 differs from SB128 by one nucleotide substitution. Including indels, CEW1 and PS1131 reveal seven and six changes, respectively, relative to SB128 (three of which are shared between CEW1 and PS1131).

A rapid molecular identification of the three species by PCR-RFLP: Polymorphisms among the ITS sequences al-

A.

	PS1173	JU75	PS2068	PS335	JU77	SB128	PS959	PS966	PS2070	CEW1	PS1131
PS1173		0.346	0.365	0.363	0.363	0.338	0.338	0.338	0.339	0.341	0.338
JU75	0.472		0.147	0.146	0.148	0.081	0.081	0.081	0.082	0.083	0.078
PS2068	0.506	0.165		0.001	0.001	0.143	0.143	0.143	0.144	0.144	0.141
PS335	0.501	0.163	0.001		0.002	0.141	0.141	0.141	0.142	0.143	0.140
JU77	0.502	0.167	0.001	0.002		0.144	0.144	0.144	0.145	0.145	0.143
SB128	0.456	0.086	0.159	0.157	0.161		0.000	0.000	0.001	0.006	0.005
PS959	0.456	0.086	0.159	0.157	0.161	0.000		0.000	0.001	0.006	0.005
PS966	0.456	0.086	0.159	0.157	0.161	0.000	0.000		0.001	0.006	0.005
PS2070	0.458	0.087	0.161	0.159	0.162	0.001	0.001	0.001		0.007	0.006
CEW1	0.462	0.089	0.161	0.159	0.162	0.006	0.006	0.006	0.007		0.006
PS1131	0.456	0.083	0.157	0.155	0.159	0.005	0.005	0.005	0.006	0.006	

B.

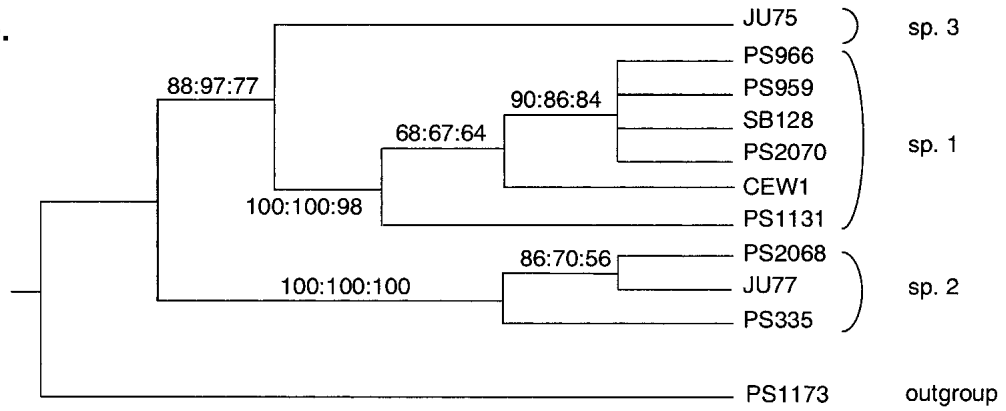

FIG. 2. Analysis of ITS alignments. A) Matrix of mean pairwise differences between the ITS sequences. Gaps were pairwise excluded from the calculation. The observed differences are indicated above the diagonal, and the distances corrected according to the HKY85 model of DNA evolution are indicated below. B) Dendogram summary from different ITS sequence analyses. Numbers above branches (a:b:c) indicate bootstrap support percentages for neighbor-joining using the HKY85 model of evolution (a), maximum parsimony with heuristic search (b), and maximum likelihood (c) analyses of the ITS sequences (3,000, 1,000, and 100 replicates, respectively). Branch lengths have no meaning.

lowed us to identify restriction sites within the ITS region that are present in one species and absent in another. For example, the Bcl I enzyme recognizes a TGATCA sequence, which is found in the ITS fragment at positions 31 and 86 in the sequenced sp. 2 strains but is present only once (at the second position) in sp. 3 JU75, and is not found at all in the sp. 1 strains (Figs. 1; 3A,B). Species-specific restriction patterns are also found with two other common restriction enzymes: a unique Bgl II site is found in the sp. 1 and 3 strains but not in sp. 2, and a unique HinC II site is found in sp. 1 and 2 strains but not in sp. 3. The predicted digestion pattern is obtained after digestion of the ITS fragment of CEW1, PS2068, and JU75 with appropriate enzymes (Fig. 3B).

We amplified the ITS region by PCR in all strains of the three mating groups that we had not sequenced and digested the PCR product with each of the three restriction enzymes. We found a perfect congruence between the mating group and the restriction pattern of the ITS region (Fig. 3C; data not shown). In the case of JU179, the restriction pattern corresponds to sp. 2. Because we could not assign it to a mating group, we sequenced the full ITS region and found it to be identical to that of PS2068 except for one indel. These molecular data strongly suggest that JU179 can be placed in sp. 2.

DISCUSSION

Definition of three species and nomenclature: We have defined three biological species in the subgroup *sechellensis/pseudodolichura/tipulae* of the *Dolichura* group in the *Oscheius* subgenus. We provisionally call these species sp. 1 (reference strain CEW1), sp. 2 (reference strain PS2068), and sp. 3 (JU75), from the most to the least common. The assignment of species names to these three species is beyond our scope. The strain SB128 has been used to redescribe the species *R. (O.) tipulae* in Sudhaus (1993). This species was found in leatherjackets, i.e., larvae of *Tipula paludosa* (Diptera: Tipulidae), as in its original description (Lam and Webster, 1971). However, we find this species to be common in soil samples. Although we cannot rule out association with leatherjackets at some stage in the life cycle, it makes it improbable that it is an obligate associate of leatherjackets. Previous species name assignments in this subgroup are discussed in Sudhaus and Hooper (1994)

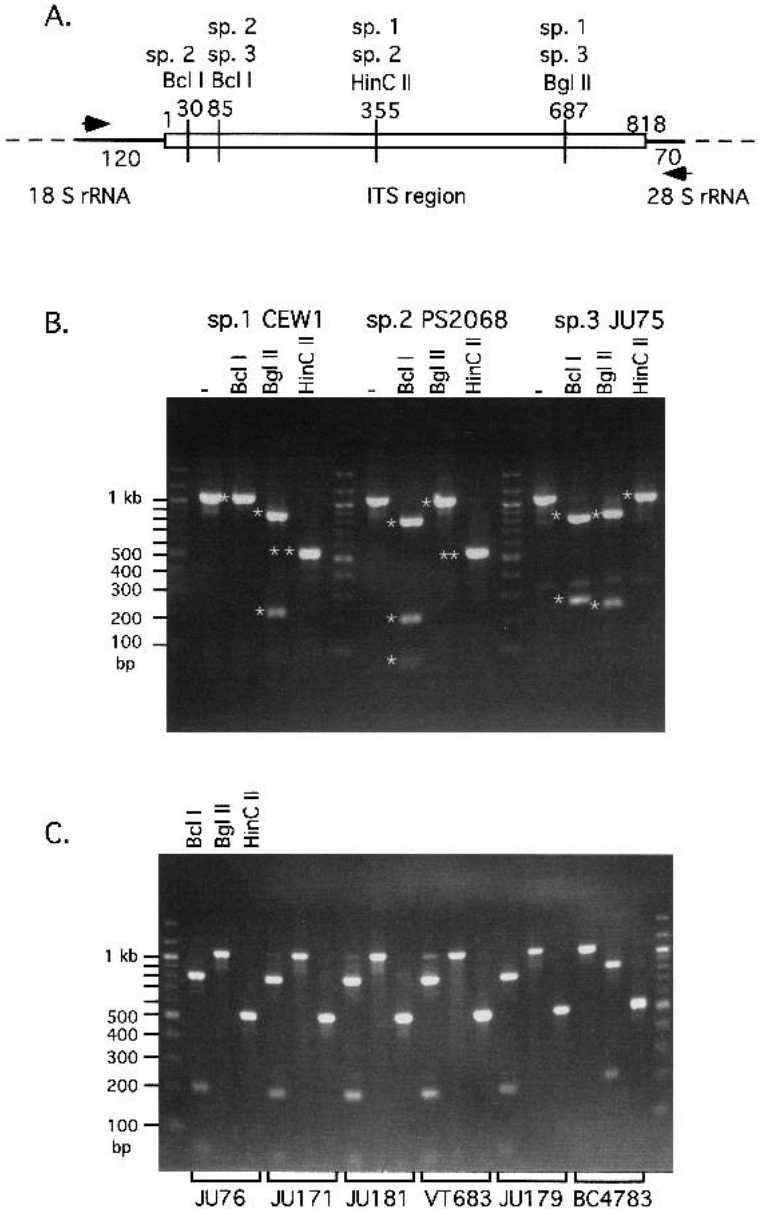

FIG. 3. Species identification by PCR-RFLP. A) Schematic arrangement of the ITS region (white rectangle), PCR primer targets (arrows), and diagnostic restriction sites used in this study. B) Agarose gel electrophoresis of restriction enzyme-treated PCR products. PCR products from reference strains of each species are shown from left to right: the undigested PCR product (''-'') and the PCR product after digestion with Bcl I, Bgl II, or HinC II. Each band after digestion is indicated by a star. Digestion of CEW1 and PS2068 by HinC II produces two bands of almost equal sizes. Molecular weight markers are in the lefthand lane. C) Restriction enzyme treatment of PCR products from strains in Table 1 that were not subject to nucleotide sequence analysis. For each strain, the three lanes correspond to the Bcl I, Bgl II, and HinC II digestions (from left to right).

and include *R. (O.) sechellensis* Potts, 1910 and *R. (O.) pseudodolichura* Körner in Osche, 1952. From the key in Sudhaus and Hooper (1994), discriminating characters between these species are tail length, body color, relative position of the excretory pore compared to the basal bulb, and number of lines in the lateral field (2 or 4). Although there may be small differences in some of these characters between the strains, they do not seem to consistently distinguish these three species. We have not undertaken a systematic study of the number of lateral lines, but it varies along the length of one individual, between individuals in some strains, and between strains of the same species. We therefore do not assign species names to the three biological species we have defined.

We noticed that the length of the life cycle may be a character discriminating these species. At 23 °C in our culture conditions (see Materials and Methods), sp. 1 has a life cycle of approximately 3 to 3.5 days (from egg to egg), sp. 2 has one of 4 to 5 days, and sp. 3 has one of 3.5 to 4 days.

A molecular test to discriminate the three species: Because

these species are very common and exhibit similar morphologies, we have devised a fast, easy, and inexpensive test to discriminate the three species in this subgroup.

In practice, we first identify a newly isolated member of the family Rhabditidae to be in this subgroup by scoring its vulva lineage in L4-stage larvae (Félix et al., 2000; Sommer and Sternberg, 1995). The only other species in the family Rhabditidae (and other families) so far with the same vulval lineage are in the *Insectivora* group of *Oscheius* (Sommer and Sternberg, 1995; Delattre and Félix, 2001), but these nematodes, and especially old adults, are larger (Flemming et al., 2000). In the other subgroups in the *Dolichura* group the P4.p and P8.p cells undergo fewer divisions (Delattre and Félix, 2001).

Once a nematode has been assigned to this subgroup by morphological and (or) developmental criteria, variations in the ITS region that discriminate the three species can be revealed by PCR followed by digestion by a specific restriction enzyme (PCR-RFLP; Fig. 3). These variations appear to be species-specific. We obviously cannot rule out a within-species polymorphism or the existence of additional species in this subgroup, which may be revealed by these tests in the future. In practice, the digestion with Bcl I is sufficient to discriminate between the three species because it cuts the ITS region twice in sp. 2, once in sp. 3, and never in sp. 1. The test can be performed on a single nematode. This method thus provides a quick test to assign a newly isolated nematode of this subgroup to a species (although it does not fully replace a mating test).

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