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Comparison of Biological, Molecular, and Morphological Methods of Species Identification in a Set of Cultured *Panagrolaimus* Isolates

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Abstract: We have developed a molecular barcode system that uses the small subunit ribosomal RNA (SSU) sequence to define molecular operational taxonomic units (MOTU) of soil nematodes. Here we attempt to differentiate five cultured isolates of a taxonomically difficult genus, *Panagrolaimus,* using morphological, molecular, and biological (breeding) criteria. The results indicated that the five culture populations belonged to two reproductively isolated species. The available morphological criteria, including scanning electron microscopy (SEM), were insufficient to differentiate among them, and all five could be classified as one morphospecies. Within-culture variation of the morphometrical data did not discern between the two biological species. Sequence data clearly separated the populations into two groups that supported the breeding results. Given this study represented only five populations of one genus, we suggest a congruence of MOTU analysis with the biological species concept. This multifaceted approach is promising for future identification of nematodes as it is simple, comparable, and transferable.

Key words: biological species concept, breeding, culture isolate, method, molecular barcode, morphology, morphospecies, nematode, *Panagrolaimus,* rRNA, scanning electron microscopy, taxonomy.

The search for a singularly comprehensive and theoretically sound species concept that can be applied for all living things and at same time be useful for practical species recognition purposes has now given way to the distinction between theoretical species concepts and the more operational species recognition methods (Adams, 2002) or, as Mayden (1997) categorizes them, the primary and secondary species concepts. To recognize the limitations of the various theoretical concepts and attempt to use a combination of species recognition methods in conjunction with a sound theoretical concept undoubtedly is a step forward in better refining nematode systematics (De Ley et al., 1999; Hunt, 1997). The polarity between practicality and theoretical appeal in the "species problem" is well recognized (Adams, 2002; Hull, 1997; Hunt, 1997; Mayden, 1997). Hunt (1997) and Mayden (1997) clearly indicated the role of secondary concepts or recognition methods as the bridge between the primary concept and the subjects of the concept, species. Choice of recognition methods depends on various factors, including our level of technology in extracting information with regard to characters and character-states.

Despite the well-recognized limitations of the biological species recognition with regard to apomictic species, its inability to distinguish between potential and actual isolations, and its technical impracticality, reproductive isolation remains to be one of the most widely advocated species recognition methods in amphimictic groups. The main problem in defining a species when mainly morphological criteria are employed to measure similarity/difference is the difficulty in defining the point at which the similarity/difference is taken to indicate distinct taxa. The biological species concept defines the needed similarity/difference between populations in a less subjective way than morphological recognition. Nevertheless, the majority of nematode species identification is not based on confirmed reproductive isolation. As an alternative, morphological difference is sought, but the staggering list of synonymized species in the literature is a monumental witness to the extent of subjectivity of this method. Consequently, despite the abundance and diversity of nematodes in terrestrial ecosystems, their use in ecological and diversity assessment studies has until now been neglected, mainly because of the need for expert knowledge and the large amount of time required for identification. Moreover, in some groups morphological characters that would help experts discern reproductively isolated populations are still wanting. Some species may be morphologically indiscernible but reproductively isolated (De Ley et al., 1999; Ferris, 1983; Kaplan et al., 2000; Nguyen et al., 2001) or could be genetically similar but phenotypically different, as is known in some plant-parasitic forms. As a result, data on supplementary criteria or recognition methods, such as genomic makeup and development, have come to be recognized more than ever as vital tools in the discovery and diagnosis of nematode species (De Ley et al., 1999; Sommer et al., 1996).

The cross-consideration between the different species recognition methods is of interest in examining the interplay between different modes of biodiversity measurement (Heywood, 1995). Given this, it would be useful to know whether the nematode molecular barcode-derived molecular operational taxonomic units (MOTU) (Floyd et al., 2002) invariably indicate biological species sensu Mayr (1963). Here we present the results of our attempt to identify five culture isolates of a taxonomically difficult genus, *Panagrolaimus,* using a combination of recognition methods—molecular barcode, relating this information to morphological species discrimination methods, and relating it biologically through breeding.

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Role of morphology and morphometry in species identification in Panagrolaimus: The genus *Panagrolaimus* was erected by Fuchs (1930). In his most recently compiled comprehensive list of species, Andrássy (1984) provided a key for the 35 species he considered valid. Andrássy's (1984) review showed that differentiation of the nominal species within the genus was primarily on morphometrical criteria. Few species within the genus have distinctive morphological characteristics. Intraspecific variability of morphometrical characteristics, inconsistencies in original descriptions of morphological characters, the small size of the structures being observed under the light microscope (LM), and the effect of other external factors on morphometrical characters have made the use of these characters at best subjective. Identification of species in the genus remains unreliable (Kozlowska and Mianowska, 1971; Mianowska, 1977; Wharton, 1998; Williams, 1987).

Recognizing these drawbacks, Williams (1987) studied 32 *Panagrolaimus* populations from the United Kingdom and United States using scanning electron microscope (SEM). Based on the morphology of the lip region, as an informative characteristic for each group, four taxa were catalogued. These four groups corresponded in morphology and in the light microscope (LM) original description of their lip region to four species, three of which were considered valid by Andrássy (1984). Breeding experiments also were conducted using the various populations. Despite their reproductive isolation, Williams grouped them within the same species, mainly because they had similar lip region morphology and for "practicality." Lip region morphology was apparently weighted more heavily than reproductive isolation. Therefore, objective morphological criteria for the identification of populations within the genus remain wanting.

Materials and Methods

Culturing: Five cultured isolates of *Panagrolaimus,* each originating from a single female, were derived from soil samples collected in July 1999 at control plot number five, Sourhope Farm of Natural Environmental Research Council for "Soil Biodiversity and Ecosystem Function" experimental site, United Kingdom. Each isolate was designated with a unique code (ED2013, ED2021, ED2041, ED2042, and ED2043) following the guidelines of the *Caenorhabditis* Genetics Center. Isolates ED2013 and ED2041 were found in subplot S, isolates ED2042 and ED2043 in subplot T, and isolate ED2021 in subplot U (information on the Sourhope field experimental site is available at http://mwnta.nmw.ac.uk/soilbio/Sourhope.htm).

Cultures were maintained at 15 °C on 20% modified Youngren's only bactopeptone (MYOB) agar (per 10 liters: 1.1 g Tris-HC1; 0.48 g Tris base; 6.2 g peptone; 4 g NaCl; 16 mg cholesterol; 210 g agar) in 50-mm-diam. media dishes seeded with *Escherichia coli* OP50, and were sub-cultured to fresh cultures once every 4 to 6 weeks.

Molecular identification: We used the 5′ segment of the small subunit ribosomal RNA (SSU) gene. Single nematodes were picked into 20 µl of 0.25 M NaOH in 0.2 ml tubes and kept at 25 °C for 3 hours (Stanton et al., 1998). The lysate was then heated at 95 °C for 3 minutes, and neutralized by 4 µl of HCl and 10 µl of 0.5M Tris-HCl (pH 8.0). Five microliters of 2% Triton X-100 was then added, and the lysate was heated for 3 minutes at 95 °C. We used primers SSU 18A (AAAGATTAAGCCATGCATG) and SSU 26R (CATTCTTGGCAAATGCTTTCG) (Blaxter et al., 1998) to amplify the segment using the polymerase chain reaction (PCR). The PCR conditions were: 94 °C for 5 minutes, 35 cycles of 94 °C for 1 minute, 52 °C for 1 minute, 30 seconds at 68 °C for 2 minutes, and then a 68 °C extension for 10 minutes. The PCR product was cleaned with exonuclease I and shrimp alkaline phosphatase, and DNA sequence was determined using Applied Biosystems BigDye sequencing reaction employing the sequencing primers SSU18A and SSU9R (AGCTGGAATTACCGCGGCTG) (Blaxter et al., 1998). An Applied Biosystems 377 sequencer was used to collect sequence chromatograms.

We used phred (Ewing and Green, 1998; Ewing et al., 1998) to trim poor-quality data, CLUSTAL_X (Jeanmougin et al., 1998; Thompson et al., 1997) to align the quality sequences, and PAUP* 4.0b10 (Swofford, 1999; Swofford et al., 1996) to define MOTU using the neighbor joining algorithm. Each MOTU defines a cluster of sequences that differ from each other by less than three bases over the aligned region, which was ~500 bases of the 5′ end of SSU (Floyd et al., 2002).

Morphological identification: Nematodes were fixed in hot (± 60 °C) 4% formaldehyde and transferred to anhydrous glycerine according to Seinhorst's (1959) method as modified by De Grisse (1969). Permanent slides were prepared according to Cobb (1918). We used Zeiss Axiovert (Carl Zeiss, UK) and Olympus BX 50 (Olympus, UK) light microscopes to study all specimens. All measurements are in micrometers (µm) unless otherwise specified.

For SEM, nematodes were transferred into pure distilled water and treated with ultrasonic vibration to remove attached foreign particles. However, this method did not remove all attached particles. Washed nematodes were dehydrated in a concentration gradient of ethanol, then dried using the critical-point drying procedure, attached to self-adhesive tape, coated with gold, and studied under a JEOL JSM-840 (Japan 1985) SEM.

Breeding experiment: We made sure that females used for the experiment were virgins by isolating 60 second-stage juveniles (J2) of each culture isolate and transfer-

ring them into new culture dishes to grow and become adults. Their sex was determined using a dissecting microscope. From this exercise, we also calculated the sex ratio of each culture isolate. We also picked additional males from the cultures to supplement the ones isolated, as we needed more males than females for every set of experiments.

To determine breeding between two isolates (e.g., ED2013 and ED2021), three males of ED2013 were transferred onto a dish that already contained a virgin female of ED2021 and vice versa (three males of ED2021 with a virgin female of ED2013). Each set of breeding experiments was performed in duplicate, and the entire experiment was carried out twice. Thus, breeding between each combination of isolates was tested a total of eight times except for the combination ED2013 vs. ED2041, which was tested 12 times.

Breeding experiments using one male and one female also produced similar results to those using the three males and one female, except in cases where migrating males climbed the side of the plates and died before mating. Using three males ensured reproduction in each dish by increasing probability of survival.

Ten virgin females of each isolate were kept individually and left to mature to determine if eggs were laid and if eggs would hatch. Since unmated females of all isolates did not lay eggs, we considered breeding "positive" when females laid eggs and the F_1 eggs hatched.

For the successfully interbred culture isolates we tested the fertility of the F_1 generation. We isolated 10 F_1 J2 of each successfully cross, allowed them to mature into adults, and re-crossed them with each other as described above. The sex ratio of the F_1 generation was calculated using these data.

Fecundity: Isolates were typically slow growing and their fecundity low. Ideally, a count of total number of eggs and juveniles of mating pairs would have been most appropriate, but our attempt to count eggs daily by moving more than 30 mature, fertilized females separately from one dish to new dishes was unsuccessful; females did not lay eggs, despite their age. It may be that the nematodes needed a certain lag phase to recover from the trauma before resuming normal behavior. As a result, we counted number of eggs and juveniles after 7 days to assess fecundity.

Results

Breeding: Isolates ED2013 and ED2041 interbred and produced a viable and fertile F_1 generation with a sex ratio (male:female) of 1:1.5 (i.e., the F_1 generation had slightly more females compared to the parent populations) (Table 1). Subsequent crossing of the F_1 generation with each other produced similarly viable and fertile progeny with a similar sex ratio. Isolates ED2021, ED2042, and ED2043 interbred among themselves and produced a viable and fertile F_1 generation. However, the first two isolates, ED2013 and ED2041, did not interbreed with the remaining three isolates ED2021, ED2042, and ED2043. Sex ratio of these three later isolates ranged between 1:1 and 1:1.4 (Table 1). The sex ratio of the F_1 generation of these later isolates was within the range of the parent populations.

Mating behavior: All isolates exhibited a similar pattern of mating. Males generally moved actively around the dish, and the time required to encounter females varied from 5 to 35 minutes. Mating commenced as soon as nematodes of the opposite sexes found each other. In a type of pre-mating courtship, the posterior part of the male coiled around the female into a hook-shape and slid repeatedly from anterior side to posterior side and vice versa in search of the vulva. The male remained in copulation with the female for about 5 minutes after successful insertion of spicules into the vulva. Rarely, a male abandoned a female after repeated trials without mating. Generally, males tended to stay close to females, though it was not uncommon to see the different sexes moving separately. Nevertheless, in cases where we found absence of breeding, males and females did not stay together and males seemed not to attempt to mate.

Fecundity: Isolates ED2013, ED2021, ED2041, ED2042, and ED2043 had a mean fecundity of 16, 23, 19, 21, and 13 eggs and juveniles per week per female, respectively.

Morphological and morphometrical identification: LM as well as SEM studies (Fig. 1) of the five culture isolates did not reveal any substantial difference. Characters such as body size, position of the excretory pore in relation to base of pharynx, shape of tail, position of phasmids, appearance of lateral line, distance of male

Table 1. Breeding between the five *Panagrolaimus* isolates.[a]

	ED2013	ED2041	ED2021	ED2042	ED2043
ED2013	+4/4 (50:50)	+5/7 (40:60)	–	–	–
ED2041	+6/8 (40:60)	+4/4 (44:56)	–	–	–
ED2021	–	–	+4/4 (50:50)	+3/3 (50:50)	+4/4 (50:50)
ED2042	–	–	+4/4 (50:50)	+4/4 (43:57)	+4/4 (44:56)
ED2043	–	–	+4/4 (50:50)	+4/4 (44:56)	+4/4 (42:58)

[a] Positive breeding is represented by a plus sign and absence of breeding by a minus sign. The ratios represent numbers of times breeding was positive in relation to the total number of breeding experiment sets for the pair. Sex ratio (male:female) of progeny are given in brackets.

copulatory papillae from cloaca, and shape of vulva in females showed variation even within the same isolate population. The shape of lip region also showed variation depending on whether the mouth was open or closed (Fig. 1A-F).

Morphometrical data showed variation within each isolate and overlap between the different isolates in most of the parameters measured (Tables 2, 3). There was no difference in morphometrics among females of ED2013, ED2041, or ED2043. The only difference between ED2042 and ED2043 was the longer isthmus in the former, and thus a lower value for the corpus/isthmus ratio.

The isolate that showed differences in measurements of a number of parameters compared to the remaining four isolates in both sexes was ED2021. Females of this isolate differed from the other isolates in size—the longer and wider with a more posteriorly situated vulva. Furthermore, comparison of this isolate with each of the other four also showed that this isolate differed from all but ED2041 in a more posteriorly situated excretory pore; from ED2013 in length of pharynx, vulva-anus distance, and the corpus/isthmus ratio; from ED2041 in vulva-anus distance and the ratio 'b'; from ED2042 in corpus and isthmus length, ratios 'b', and corpus/isthmus; and from ED2043 in corpus length, vulva-anus distance, and ratios 'b' and 'c'.

Males of isolate ED2021 differed from those of ED2042 in length of body, stoma, corpus, and gubernaculum, and in the ratios 'b' and corpus/isthmus; from those of ED2043 in length of body, pharynx, and corpus, and in lip region and cloacal body width and corpus/isthmus ratio; and from those of ED2013 in lip region width (Table 3). Males of isolate ED2041 differed from those of ED2043 in their longer pharynx and wider body at cloaca, and males of ED2013 differed

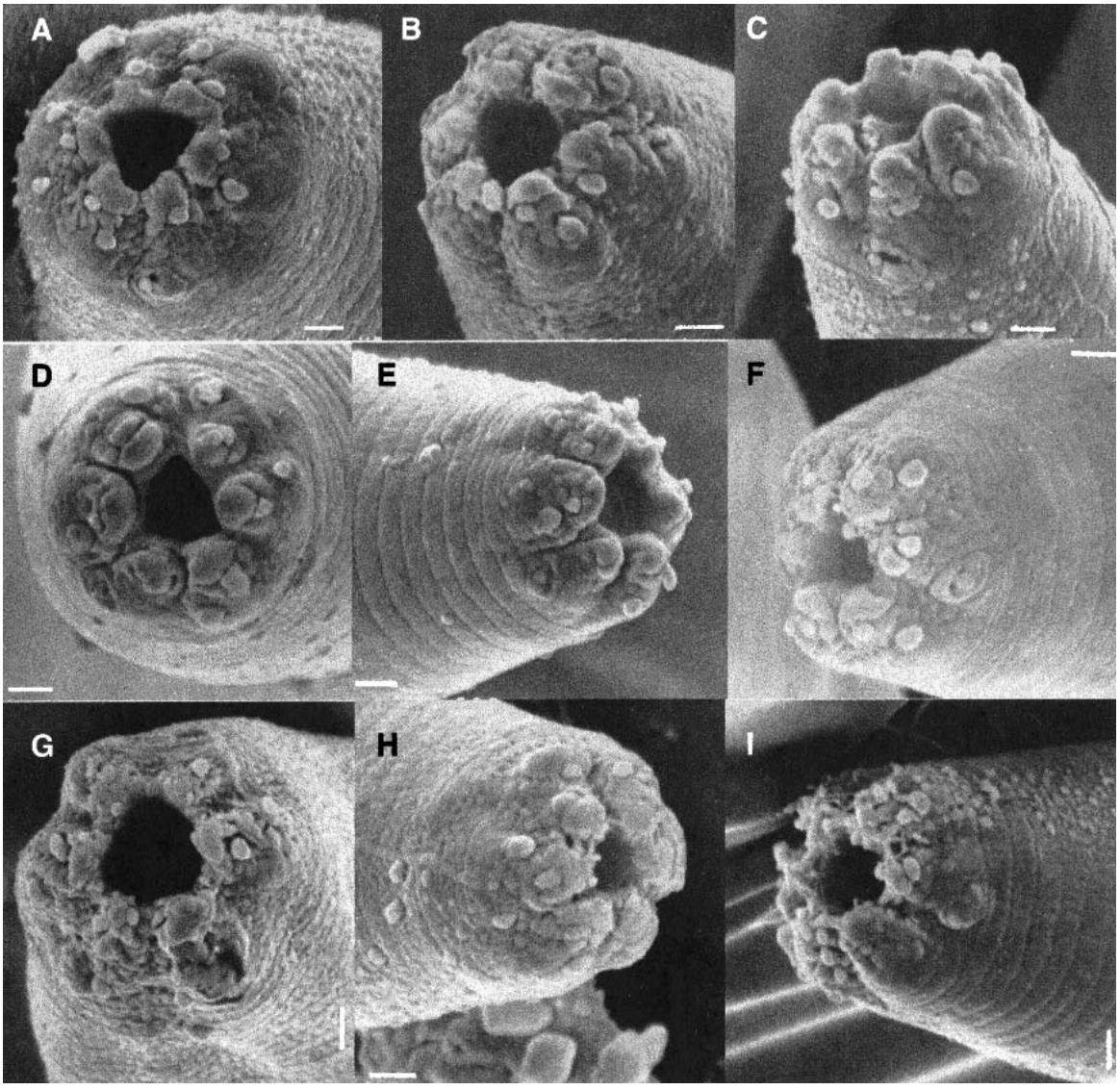

FIG. 1. Scanning electron micrographs of the lip region of the five *Panagrolaimus* isolates. A-C) Isolate ED2013. D-F) Isolate ED2021. G) Isolate ED2041. H) Isolate ED2042. I) Isolate ED2043. Scale bar = 1μm.

TABLE 2. Morphometric data of females of the five *Panagrolaimus* isolates.[a]

	MOTU1						MOTU2								
	ED2013			ED2041			ED2021			ED2042			ED2043		
	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
Lengths															
Body	1,048.0	286.5	674–1,323	1,048.0	180.5	833–1,245	1,404.8	62.5	1,380–1,495	1,065.7	227.3	850–1,303	1,035.9	133.8	825–1,170
Pharynx	164.4	5.0	159–168	178.3	8.4	166–185	181.5	6.6	173–188	174.0	12.1	161–185	167.0	7.6	156–176
Corpus	90.2	7.1	83–100	97.2	5.6	88–105	103.0	2.4	100–105	88.7	6.0	83–95	92.9	3.4	88–93
Isthmus	38.8	3.1	36–44	43.3	2.8	39–46	38.8	5.4	32–42	45.3	1.2	44–46	37.4	1.8	34–39
Tail	54.6	5.2	47–60	52.5	5.4	48–61	59.8	7.9	49–68	52.7	7.2	48–61	49.7	4.2	44–56
Excretory Pore	152.0	9.2	144–163	148.0		148–148	182.0	1.4	181–183	153.8	21.0	122–181	154.2	8.8	141–163
Vulva	611.6	181.1	363–784	633.8	114.0	490–744	841.5	36.6	813–895	614.0	122.8	500–744	607.6	77.1	485–685
Vulva-Anus	382.0	103.7	242–479	362.3	68.5	275–451	497.8	26.4	480–535	404.3	104.0	300–508	378.6	56.5	296–440
Post-uterine branch	32.0	7.0	20–37	30.5	4.1	27–38	28.5	3.7	24–32	24.7	2.5	22–27	29.1	4.3	24–34
Stoma	9.2	1.6	7–11	9.3	2.3	6–11	10.5	0.6	10–11	6.8	0.6	6–8	9.1	1.6	7–11
Widths															
Maximum body	41.2	9.1	28–49	35.7	10.4	26–52	57.8	2.4	56–61	39.3	16.2	22–54	38.1	5.9	29–44
Lip region	7.8	1.5	6–10	8.3	1.4	7–10	8.5	0.6	8–9	7.0	1.0	6–8	7.6	0.8	7–9
Anal body	22.4	4.2	16–27	25.0	5.3	19–31	27.3	2.1	25–30	20.7	3.1	18–24	20.1	2.3	17–24
Ratios															
a	25.1	2.2	21.6–27.0	23.3	1.9	21.5–25.2	24.0	0.9	23.1–24.6	30.0	7.6	24.1–38.6	27.3	1.9	23.5–29.4
b	6.3	1.9	3.5–7.7	5.8	0.8	4.9–6.7	7.6	0.3	7.3–8.0	6.1	0.9	5.3–7.1	6.2	0.6	5.3–6.6
c	19.2	3.9	13.5–23.1	20.1	3.8	15.1–24.1	23.8	3.0	22.0–28.5	20.1	3.5	17.7–24.1	20.8	1.4	18.8–21.7
c′	2.5	0.5	2.2–3.3	2.2	0.4	1.7–2.5	2.3	0.2	2.0–2.4	2.5	0.2	2.3–2.7	2.5	0.2	2.3–2.8
Corpus/Isthmus	2.4	0.3	1.9–2.6	2.2	0.3	1.9–2.7	2.7	0.4	2.5–3.3	1.9	0.2	1.8–2.1	2.5	0.1	2.3–2.6
V%	58.4	1.3	56.1–59.3	60.4	1.5	58.8–62.8	59.9	0.2	59.7–60.1	58.5	1.3	57.1–59.7	58.7	1.4	56.5–60.4
V-An/Tail L	6.9	1.5	4.6–8.4	7.0	1.4	5.2–8.4	8.1	0.9	7.0–9.3	7.4	1.7	6.1–9.4	7.6	0.8	6.7–8.2

[a] $n = 10$ in each case.

TABLE 3. Morphometric data of males of the five *Panagrolaimus* isolates.[a]

	MOTU1						MOTU2								
	ED2013			ED2041			ED2021			ED2042			ED2043		
	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
Lengths															
Body	866.5	143.4	706–1,078	1,010.8	126.2	870–1,134	1,066.3	47.4	1,010–1,117	906.5	73.3	843–970	800.7	126.6	662–910
Pharynx	151.8	6.7	154–161	169.2	8.8	161–183	167.2	6.4	160–176	159.5	7.5	153–166	141.3	31.5	105–158
Corpus	87.0	7.4	85–100	90.3	4.8	83–98	94.7	6.2	90–107	83	2.3	81–85	85.8	0.0	85–87
Isthmus	38.0	4.0	37–41	41.8	3.1	37–46	39.5	2.7	37–44	43	3.5	40–46	43.1	0.7	42–44
Tail	38.8	2.6	35–42	42.0	4.3	34–46	46.5	3.6	41–52	37.5	4.0	34–41	35.0	8.7	25–41
Excretory pore	127.2	4.1	117–134	147.5	12.5	139–166	161.0			133.4	7.7	120–139	120.7	26.9	90–140
Spicule	26.3	2.3	24–30	26.4	3.0	23–30	27.3	1.7	25–29	27.5	4.0	24–31	29.5	3.5	27–32
Gubernaculum	9.6	1.1	8–11	10.5	1.6	9–13	9.0	0.8	8–10	12	1.2	11–13	9.5	0.7	9–10
Stoma	8.7	2.0	6–11	9.2	1.7	7–11	10.2	0.4	10–11	6.5	0.6	6–7	8.0	1.0	7–9
Widths															
Lip region	6.8	0.4	6–7	7.7	0.5	7–8	8.0	0.0	8–8	6.5	0.6	6–7	6.0	1.0	5–7
Cloacal body	23.7	3.2	20–30	26.0	3.0	23–30	24.0	1.7	22–27	21.5	1.7	20–23	19.3	2.1	17–21
Maximum body	28.5	6.7	24–31	35.8	5.7	29–44	37.0	1.4	35–39	31.5	2.9	29–34	29.3	4.9	26–35
Papillae															
Pre-cloacal	1 pair			1 pair			1 pair			1 pair			1 pair		
Adanal	1 pair			1 pair			1 pair			1 pair			1 pair		
Caudal															
Lateral	1 pair			1 pair			1 pair			1 pair			1 pair		
Ventro-lateral	2 pairs			2 pairs			2 pairs			2 pairs			2 pairs		
Dorso-lateral	10 pair			1 pair			1 pair			1 pair			1 pair		
Ratios															
a	31.0	2.8	29.4–34.3	28.4	1.7	25.7–30.4	28.8	1.5	27.9–31.6	29.1	5.0	24.8–33.4	28.2	8.3	18.9–35.0
b	5.7	1.1	4.5–7.1	6.0	0.6	5.2–6.9	6.4	0.4	6.1–6.8	5.45	0.2	5.3–5.6	5.7	0.6	5.2–6.3
c	22.2	4.0	16.9–28.4	24.1	1.9	21.2–26.0	23.8	2.0	21.2–26.6	24.55	4.6	20.6–28.5	23.3	2.8	22.2–26.5
c′	1.7	0.3	1.3–2.1	1.6	0.1	1.5–1.8	2.0	0.3	1.7–2.4	1.8	0.1	1.7–1.9	1.8	0.3	1.5–2.0

[a] $n = 10$ in each case.

from those of ED2041 in having a more anteriorly situated excretory pore.

On the basis of morphological and morphometrical criteria, these isolates are close to *P. superbus* Fuchs, 1930. The only difference compared with the original description of the species is in the possession of a small protrusion (possibly a denticle) on the dorsal wall of the stoma. This morphological characteristic has until now been reported in only two species within the genus. *Panagrolaimus davidi* Timm, 1971 and *P. superbus* (De Ley et al. 1999). A detailed description of our populations has been deliberately excluded here because the primary aim of this paper is not to give a descriptive account of the isolates.

Molecular identification: Sequence data of the isolates revealed the presence of two MOTU based on the criterion that "populations belong to the same MOTU if they have differences of less than three bases" (Floyd et al., 2002). One group included isolates ED2013 and ED2041, and the other included the remaining three isolates, ED2021, ED2024, and ED2043. Alignment showed no sequence difference between isolates ED2013 and ED2041, nor between isolates ED2042 and ED2043 (Table 4). However, sequence of isolate ED2021 had a difference of one base (0.2% difference) with ED2042 or ED2043. These five sequences have been deposited in GenBank (accession numbers AF430483-AF430487).

Discussion

Morphological species recognition within the genus *Panagrolaimus* has long been considered difficult (Boström, 1995; Williams, 1987). Though the lip region was considered by Williams (1987) to be a useful character, its use may be limited to representing four species-groups rather than four separate species (Williams, 1982). The degree of variation we observed in lip region morphology within each of the five isolates was not to an extent that would violate its usefulness in the proposed classification system for the genus (Williams, 1987). A more recent addition to the characters already in use was the protruding vulva (Boström, 1995). The possession of a protruding vulva was used to erect *P. magnivulvatus* Boström, 1995 as a new species from Antarctica. This character varied among individuals of the same isolate in the present work; in some, the vulval lips protruded prominently while in others they were less protruding. In *Caenorhabditis elegans,* the vulval lips protrude more with age of hermaphrodites.

The use of morphometry alone for species recognition in the genus *Panagrolaimus* has been strongly criticized (Williams, 1987) due to intraspecific variability (Mianowska, 1977). Furthermore, species that include both large and small individuals have been described (Boström, 1995), implying that size may not be an important identifying character within the genus. Therefore, based on established morphological and morphometrical criteria, all five of our *Panagrolaimus* isolates belong to the same morphospecies. Isolate ED2021, which had a noticeable morphometric difference when typed by morphometrics, and originated from a separate sub-plot, perhaps could be considered as a distinct type. This designation does not correspond to the results of our breeding experiment, which showed the presence of two reproductively isolated population-groups (Table 1). Neither morphological nor morphometric data grouped the five isolates similarly to reproductive isolation.

Though "the species problem" continues to be discussed actively in the literature (Adams, 1998, 2002; Ferris, 1983; Hull, 1997; Mayden, 1997; Mayr and Ashlock, 1991), as yet no simple and unifying method of recognizing a species has been developed beyond the recognition of the theoretical difficulties and technical limitations (Adams, 2002). The difficulty of finding a species concept that has a universal theoretical appeal and can at the same time be of practical value has now given way to the understanding that the use of a combination of species recognition methods framed on a sound theoretical species concept is the way forward. The use of a combination of the evolutionary and phylogenetic species concepts has been advocated by Adams (1998). Despite its theoretical and operational shortcomings, such as the potential of underestimating the number of phylogenetic species (Adams, 1998), reproductive isolation among populations in truly amphimictic nematode species remains a relatively testable method of defining species (Coomans, 1979). Typically, nematode species are still diagnosed mainly on morphological characteristics. The use of morphological and morphometric characters in some groups has become difficult due to their within-population variability. Therefore, easier and faster methods of diagnosing species are being sought. Currently, a shift from the

Table 4. Uncorrected ("p") distance matrix of the pair-wise comparison between the 5′ SSU DNA segment sequence of the five culture isolates. Actual number of base differences are given in parentheses.

	ED2013	ED2041	ED2021	ED2043	ED2042
ED2013	—				
ED2041	0.00000 (0)	—			
ED2021	0.05538 (28)	0.05527 (28)	—		
ED2043	0.05679 (29)	0.05328 (27)	0.00205 (1)	—	
ED2042	0.06005 (31)	0.05330 (27)	0.00205 (1)	0.00000 (0)	—

traditional Linnean to a more molecular-oriented taxonomy or a combination of both methods is being adopted in nematology, especially for plant-parasitic nematodes (Powers et al., 1997).

Some recent nematode species descriptions have included molecular characterization (Sommer et al., 1996). Powers et al. (1997) recommended that studies attempt to link morphological variation with molecular patterns for parthenogenetic species. De Ley et al. (1999) and Felix et al. (2001) used a combination of molecular characterization and reproductive isolation to diagnose species with indistinguishable anatomy.

In other cases, molecular data have been used for various purposes—to identify species and assess variability within species (Blok et al., 1997; Blok et al., 1998; Fallas et al., 1996), and within genera (Beckenbach et al., 1999; Vrain, 1993); and to construct phylogenetic relationships of variants within species (Kaplan et al., 2000), species within a genus (Nguyen et al., 2001; Powers et al., 2001; Sabo et al., 2001), among a group of genera (Ferris et al., 1999), or of higher groups (Blaxter et al., 1998; Litvaitis et al., 2000; Nadler, 1995).

Previously described molecular methods of defining species or analyzing phylogeny have employed the intergenic spacer (IGS) region of the ribosomal repeats (Blok et al., 1997), the D2/D3 region of the large subunit rRNA gene (De Ley et al., 1999; Litvaitis et al., 2000), PCR-RFLP patterns for the internal transcribed spacer (ITS) (Powers et al., 1997; Powers et al., 2001; Wendt et al., 1993), or sequencing the ITS region rDNA (Felix et al., 2001; Nguyen et al., 2001; Sabo et al., 2001).

The variability of the IGS and ITS regions has been regarded as an advantage over using other parts of the rDNA for the study of a group of closely related species (Nguyen et al., 2001) but not for phylogenetic analysis of distantly related taxa. The site also was considered by Nguyen et al. (2001) to be "too variable to reliably infer relationships among all species in the genus" they studied. In addition, Ibrahim et al. (1994), Felix et al. (2001), and Zijlstra et al. (1995) indicated the possibility of within-species polymorphism in the corresponding groups they studied, while Powers et al. (1997) identified ITS heterogeneity in individuals and populations in several nematode taxa and recommended careful use of the marker even for taxonomic purposes.

Unlike the ITS region, within the SSU gene there are deeply conserved stem regions and rapidly evolving loops that allow discrimination at order, family, genus, and species level from the same molecule (Blaxter et al., 1998, Fitch et al., 1995). The general expectation in the nematological literature has been that SSU rRNA sequences should be informative for defining relationships among major lineages, but uninformative for closely related groups such as species within a genus (Nadler, 1995; Wendt et al., 1993). Our results, however, clearly show that this marker can unequivocally distinguish between reproductively isolated populations within a genus when morphology cannot. Given the advantage of its utility for phylogenetic analysis at various levels, coupled with its potential use for species delimitation in taxonomically difficult genera such as *Panagrolaimus,* the 5′ end of SSU may have wider applicability than previously used markers. We are aware that we have explored its applicability in only five populations of one genus, and suggest a wider testing in other genera. A clear advantage of this molecular marker, however, is that it is easy, fast, transferable, and therefore applicable in extensive nematological studies that address questions of biodiversity and biogeography. Moreover, once the sequence is generated, it can be used further for inferring phylogeny at various levels.

While the SSU MOTU method concurred with biological species recognition, morphological characteristics and morphometrics in the genus *Panagrolaimus* did not correspond to reproductive isolation. This implies that characters discovered within this genus are currently insufficient to identify reproductively isolated populations. We do not propose that morphology is a less useful tool in taxonomy and systematics relative to molecular techniques. It should be noted that "when competent taxonomic work based on morphological evidence is re-examined in light of findings in behaviour or biochemistry, it is confirmed in its entirety" (Mayr and Ashlock, 1991). Whether this statement holds true for nematode systematics, however, needs to be verified. De Ley et al. (1999) recommended a combination of molecular and morphological approaches for species diagnosis and description in Cephalobidae. Though it may be difficult to make wider generalizations at this early stage, this molecular method now has a verified potential to contribute to solving some of the taxonomic problems within morphologically indistinguishable species and genera complexes within the family Panagrolaimidae. As such, the "barcode" approach could be a crucial tool in our understanding of the diversity and phylogeny of this group. Therefore, we advocate its test as a tool for delimiting evolutionary species in other genera of this family as well as in other groups within the Nematoda.

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