

**MORPHOLOGICAL AND MOLECULAR CHARACTERISATIONS
OF *Scutellonema brachyurum* (Steiner, 1938) Andrassy, 1958
FROM THE WESTERN HIGHLANDS, VIETNAM**

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ABSTRACT

The genus *Scutellonema* consists of about 50 species that are phytophagous (ecto- and partially endo-parasites). Among species of the genus *Scutellonema*, *Scutellonema brachyurum* was reported to be one of the three most damaging ones, causing serious symptoms to numerous host plants worldwide. In the past, *Scutellonema* species have been described using morphological and morphometric characterizations, however, cryptic species have been proven to exist in the genus. Therefore, molecular data is clearly needed in identifying *Scutellonema* species to ensure correct outcomes. In this study, we provide morphological and molecular characterizations of *S. brachyurum* recovered from the rhizosphere of Vietnamese ginseng for the first time. Morphological comparison and PCA analysis indicated that our nematode population is closer to *S. brachyurum* type B, however, molecular data of D2-D3 of 28S rRNA region indicates that the Vietnamese population of *S. brachyurum* belongs to type A. Our study also provides the 18S rRNA sequence of *S. brachyurum* for the first time in GenBank (only 18S sequences of *Scutellonema bradys* are available in the genus *Scutellonema* before this study). Phylogenetic trees based on 18S and D2-D3 sequences are also provided for a better understanding of the relationships between *Scutellonema* species. Besides, this species was found in association with tuber rot symptom of the host plant, implying potential pathogenicity, and thus, more attention needs to be paid to better understand this pest.

Keywords: first report, Western Highlands, taxonomy, D2-D3, 28S, molecular, Vietnam.

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INTRODUCTION

Andrássy (1958) established the genus *Scutellonema* from the genus *Rotylenchus* Filipjev, 1936 to accommodate the species having large phasmids (scutella) located at the level of the anus or cloacal aperture. Currently, about 50 species of the genus *Scutellonema* have been described over the world (Kolombia et al., 2017; Nguyen, 2017; Sen, 2019). However, only three species, i.e. *Scutellonema bradys* (Steiner & LeHew, 1933) Andrássy, 1958, *Scutellonema cavenessi* Sher, 1964, and *Scutellonema brachyurum* (Steiner, 1938) Andrássy, 1958, are reported frequently as agricultural pests (Sikora et al., 2018). For *S. brachyurum*, this species has been recorded in many countries, such as the US, Zaire, South Africa, India, Vietnam, Greece, Belgium (Nguyen et al., 2019d; Nguyen & Nguyen, 2000; Sikora et al., 2018; Van den Berg et al., 2017). Strikingly, Van den Berg et al. (2013) and Van den Berg et al. (2017) clearly defined the presence of cryptic species in the *S. brachyurum* group using an integrative approach. Additionally, the authors also revealed two types of *S. brachyurum* that are clearly separated into two distant clades on phylogenetic trees based on *COI* mtDNA, ITS, and D2-D3 of 28S rRNA regions. In terms of morphology, *S. brachyurum* type A can be differentiated from type B by the main following traits: lip region with 4 to 6 annuli vs mainly three, rarely 4 to 5 annuli; 4 to 12 blocks on basal annulus vs 8 to 20 blocks; secretory–excretory pore located opposite anterior part to mid-region of overlapping pharyngeal lobe vs from rarely opposite mid-isthmus to mostly opposite the posterior part of pharyngeal gland lobe up to its posterior border; and other morphometric differences (Van den Berg et al., 2013). Therefore, any report of *S. brachyurum* in the world, thereafter, should apply both morphological and molecular approaches to clarify its species type and ensure a correct identification.

In Vietnam, ten species, including *Scutellonema amabile*, *S. brachyurum*, *Scutellonema brevistyletum*, *Scutellonema*

dentivaginum, *Scutellonema hoabinhensis*, *Scutellonema paramovovi*, *Scutellonema sheri*, *Scutellonema siamense*, *Scutellonema tanlamense*, *Scutellonema vietnamense*, have been reported (Nguyen, 2017; Nguyen & Nguyen, 2000). Although *S. brachyurum* has been recorded in association with a number of host plants in Vietnam, including cabbage (*Brassica oleracea* var. *capitata* L.), potato (*Solanum tuberosum* L.), carrot (*Daucus carota* subsp. *sativus* (Hoffm.)), sweet potato (*Ipomoea batatas* L.), radish (*Raphanus raphanistrum* subsp. *sativus* L.), onion (*Allium fistulosum* L.), black turtle bean (*Phaseolus vulgaris* L.), mung bean (*Vigna radiata* (L.) Wilczek), and coffee (*Coffea arabica* L.), banana (*Musa paradisiaca* L.), orange (*Citrus sinensis* L.) (Nguyen, 2017; Nguyen & Nguyen, 2000). However, the reports of *S. brachyurum* from Vietnam have never been incorporated with molecular data. Furthermore, the population of *S. brachyurum* from a previous study in Vietnam was also reported with very limited morphological and morphometric data, hampering its species declamation (Nguyen & Nguyen, 2000). Therefore, detailed morphological studies and molecular analyses need to be exploited in studying *S. brachyurum* from Vietnam to clarify its species status. In addition, Vietnamese ginseng (*Panax vietnamensis* Ha & Grushv) is known as one of the most precious and rare ginsengs in the world with a very high content of saponins (23 known and 14 new saponins compared to other *Panax* spp.) (Yamasaki, 2000), thus, study on the pathogen of this precious ginseng could contribute to its sustainable development.

MATERIALS AND METHODS

Sampling and extraction

Soil and root samples were collected from the Western Highlands in Vietnam, during a study on plant-parasitic nematodes associated with forest and agricultural crops. Approximate 1kg of soil and 20g of roots were taken at each sampling site after the removal of the detritus layer (Nguyen et al., 2019b). The soil and root samples were

reserved in nylon bags and brought to the Department of Nematology in the Institute of Ecology and Biological Resources for further analysis.

Nematodes were extracted from soil and root samples using the modified tray method (Whitehead & Hemming, 1965). A *Scutellonema* species was observed in a number of collected samples and a nematode population associated with Vietnamese ginseng was selected for detailed morphological and molecular study.

Morphological characterizations

The nematode specimens were killed using hot water (60 °C) and fixed in TAF to make permanent slides following Nguyen et al. (2019a). The microphotographs and measurements were taken using a Carl Zeiss Axio Lab.A1 light microscope equipped with a ZEISS AxioCam ERc5s digital camera. Permanent slides of *Scutellonema* species in this study are kept in the Department of

Nematology in the Institute of Ecology and Biological Resources.

PCA analysis

Raw data were processed initially using Excel 2013. Principal Component Analysis (PCA) in Primer version 6.1.12 was used to provide an overview of linear relationships between different *S. brachyurum* populations and measurement variables (Anderson et al., 2008). Eighteen morphometric measurements of 13 *S. brachyurum* populations from different localities (Tables 1, 2) were included in the PCA analysis. All measurements were normalized before their analysis following Shokoochi (2021). The scores values were calculated for each nematode population based on each of the principal components, and the scores for the first two components were used to form a two-dimensional plot (SCORE1 & SCORE2).

Table 1. Eigenvectors (Coefficients in the linear combinations of variables making up PC's)

Variable	PC1	PC2	PC3	PC4
L	-0,258	0,252	-0,117	0,144
a	-0,348	-0,009	0,222	0,148
b	-0,214	0,177	0,347	0,269
b'	-0,258	0,004	-0,357	0,244
c	0,141	-0,165	0,432	0,367
c'	-0,294	0,066	-0,135	-0,178
V%	0,325	0,045	0,264	-0,108
Lip height	0,133	-0,385	-0,002	-0,099
Lip width	0,303	-0,206	-0,211	-0,092
Stylet lenth	0,142	0,371	0,055	0,132
o	-0,004	-0,302	-0,288	0,411
DGO	0,133	-0,125	-0,256	0,594
EP	0,087	0,390	0,035	0,083
Pharynx length	0,272	0,293	-0,050	0,114
MBD	0,338	0,095	-0,250	-0,076
ABD	0,229	0,246	-0,227	-0,003
Tail length	-0,267	0,160	-0,316	-0,179
Scutellum length	0,143	0,321	-0,039	0,176

Table 2. Principal Component Scores

Sample	SCORE1	SCORE2	SCORE3	SCORE4
Vietnam; this study	8,87E-2	4,07	3,73	0,289
Belgium; Nguyen et al. (2019)	0,808	-2,66	2,86	0,341
Type A 1; US; Van den Berg et al. (2013)	4,95	-0,647	-1,21	1,44
Type A 2; US; Van den Berg et al. (2013)	4,18	0,991	-0,486	-1,02
Type A 3; US; Van den Berg et al. (2013)	2,94	-1,71	0,145	-1,27
Type B 1; SA; Van den Berg et al. (2013)	-0,932	1,53	-0,685	-4,01E-2
Type B 2; SA; Van den Berg et al. (2013)	-0,404	2,23	-1,04	-0,107
Type B 3; SA; Van den Berg et al. (2013)	-1,55	0,364	-1,04	-0,45
Type B 4; SA; Van den Berg et al. (2013)	-1,24	-0,488	-0,679	2,9
Type B 5; SA; Van den Berg et al. (2013)	-2,13	2,48	-1,77	-0,396
SA, Shokoohi (2021)	-0,981	-2,28	-1	-1,12
SA, Shokoohi (2021)	-2,8	-1,5	0,846	-1,74
SA, Shokoohi (2021)	-2,93	-2,38	0,33	1,18

Molecular characterizations

A single individual of living nematodes was used for DNA extraction. The primers MN18F/Nem_18S_R (5'-CGCGAATRGCTCATTACAACAGC-3'/5'-GGGCGGTATCTGATCGCC-3') and D2A/D3B (5'-ACAAGTACCGTGGGAAAGTTG-3'/5'-TCGGAA GGAACCAGCTACTA-3') were used to amplify the 18S and D2-D3 of 28S rRNA, respectively (De Ley et al., 1999; Nunn, 1992). The thermal profile to amplify 18S and D2-D3 regions was one cycle of 94 °C for 4 min, followed by five cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 2 min, and 45 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min and finished at 10 °C for 10 min. The newly obtained sequence was viewed and edited using Geneious R11 (www.geneious.com) and submitted to

GenBank under the accession numbers ON164666 (D2-D3) and ON171477 (18S). For phylogenetic analysis, the closely related sequences of other species were obtained using BLAST search (Altschul et al., 1997). Multiple alignments were made using MUSCLE and a Bayesian phylogenetic tree was constructed using MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001) under the GTR+G+I model (Abadi et al., 2019) in Geneious R11 (https://www.geneious.com) with 10⁶ generations of Markov chains (4 runs, 20% burn-in) (Nguyen et al., 2019c).

RESULTS

Measurements

All measurements of *Scutellonema brachyurum* from Vietnam were given in Table 3.

Table 3. Measurements of *Scutellonema brachyurum* (Steiner, 1938) Andrassy, 1958 from Vietnam and the world. All measurements are in µm (except for ratio) and in the form: mean ± sd (range)

Source	<i>Scutellonema brachyurum</i>				
	Vietnam (Present study)	Belgium (Nguyen et al., 2019d)	US (type A of Van den Berg et al. (2013))	South Africa (type B of Van den Berg et al. (2013))	US Type population (Steiner, 1938)
n	11	11	11	15	-
Body length (L)	798 ± 42 (709–844)	699 ± 50 (625–774)	734 ± 45.2 (656–805)	783 ± 47.3 (692–876)	(720–890)

Source	<i>Scutellonema brachyurum</i>				
	Vietnam (Present study)	Belgium (Nguyen et al., 2019d)	US (type A of Van den Berg et al. (2013))	South Africa (type B of Van den Berg et al. (2013))	US Type population (Steiner, 1938)
a = L/MBD	29 ± 1.9 (25–32)	28 ± 7 (24–44)	21 ± 0.9 (19.5–23)	28 ± 1.6 (25–32)	(22.4–29)
b = L/ anterior to pharyngo-intestinal valve	7.2 ± 0.4 (6.6–7.7)	6.6 ± 0.44 (5.9–7.2)	5.0 ± 0.3 (4.6–5.6)	5.8 ± 0.3 (5.3–6.5)	(5.6–6.4)
b' = L/ anterior to base of pharyngeal gland	5.8 ± 0.3 (5.3–6.2)	5.5 ± 0.42 (4.9–6.1)	6.1 ± 0.5 (5.5–6.8)	7 ± 0.5 (6.3–7.9)	-
c = L/Tail length	71 ± 6 (61–81)	79 ± 44 (49– 177)	69 ± 11.3 (59.4–95.3)	55.5 ± 9.6 (42.9–74.8)	(47–80)
c' = Tail length/ABD	0.6 ± 0.04 (0.5–0.6)	0.7 ± 0.06 (0.6–0.8)	0.5 ± 0.06 (0.4–0.6)	0.7 ± 0.01 (0.5–0.9)	-
V%	59 ± 1 (58–60)	59 ± 1.5 (57–62)	60 ± 1 (58–61)	57.5 ± 1.5 (54–60)	(59–61)
Lip height	4.6 ± 0.2 (4.5–4.9)	5.7 ± 0.4 (5.1–6.2)	5.5 ± 0.3 (5–6)	5.0 ± 0.3 (4.5–6.0)	-
Lip width	7.4 ± 0.2 (7.2–7.5)	9.1 ± 0.37 (8.7–9.6)	10 ± 0.5 (9–11)	8.0 ± 0.6 (7.5–9.0)	-
Stylet length	31 ± 1 (29–32)	27 ± 1.1 (25–29)	29 ± 0.8 (28.5–30.5)	28.5 ± 1.0 (26.5–30)	(26–29)
o=DGO*100/Stylet	14.3 ± 1.5 (12.8–16.9)	19.4 ± 2.0 (17.1–23)	21.6 ± 2.6 (17.9–26)	17.8 ± 2.4 (13–21.5)	-
Distance from dorsal gland orifice to stylet base (DGO)	4.4 ± 0.4 (4.1–4.9)	5.2 ± 0.53 (4.6–6.0)	6.0 ± 0.7 (5–7)	5.1 ± 0.8 (3.5–6.0)	-
Anterior end to secretory- excretory pore (EP)	131 ± 3 (127–137)	116 ± 5.2 (110–124)	125 ± 6.5 (111–136)	122 ± 9.7 (105–134)	-
Anterior end to nerve ring	93 ± 2.6 (89–97)	84 ± 1.5 (82–86)	-	-	-
Pharynx length	139 ± 7 (129–149)	127 ± 4 (120–134)	147 ± 6.4 (139–156)	135 ± 10.3 (114–153)	-
Diam. at mid-body (MBD)	28 ± 1.8 (26–32)	27.1 ± 2.4 (23.8–31)	35 ± 2.6 (31.5–38)	28 ± 3 (19.5–31)	-
Diam. at anus (ABD)	20 ± 1.4 (18.1–21.9)	17 ± 0.9 (16.2–18.3)	22 ± 2.1 (18.5–24.5)	21 ± 1.6 (18.5–22.5)	-
Tail length	11.3 ± 0.8 (10.4–12.6)	11.2 ± 1.2 (9.8–13.3)	11 ± 1.6 (7.5–12.5)	14.5 ± 2.7 (10.5–19.5)	-
Scutellum length	4.6 ± 0.4 (4.3–5.5)	3.4 ± 0.28 (3.1–3.8)	4.0 ± 0.5 (3.5–5.0)	4.0 ± 0.5 (3.0–4.5)	-

Morphological characterization

Females of *Scutellonema brachyurum* recovered from ginseng in Vietnam are

characterized by having spiral-shaped body after heat-killed (Fig. 1A); hemispherical lip region with 3–4 annuli, offset from body contour (Fig. 1B); lateral field with four

lines at mid-body, areolated at pharyngeal region; robust stylet with rounded basal knobs (Fig. 1B); oval median bulb (Fig. 1B); slender isthmus encircled by nerve ring (Fig. 1B); sacciform pharyngeal glands, overlapping intestine dorsally (Fig. 1B); secretory-excretory pore located at level of pharyngeal gland end (Fig. 1B); distinct hemizonid ca 1.5 to 2 body annuli long, 1–2

annuli anterior to secretory-excretory pore; didelphic-amphidelphic reproductive system with equally developed branches; outstretched ovaries with a single row of oocytes; vulva at mid-body level, epiptygma folded into vagina (Fig. 1C); small spermatheca without sperm; rounded tail tip with rounded scutellum located at anus level (Fig. 1D).

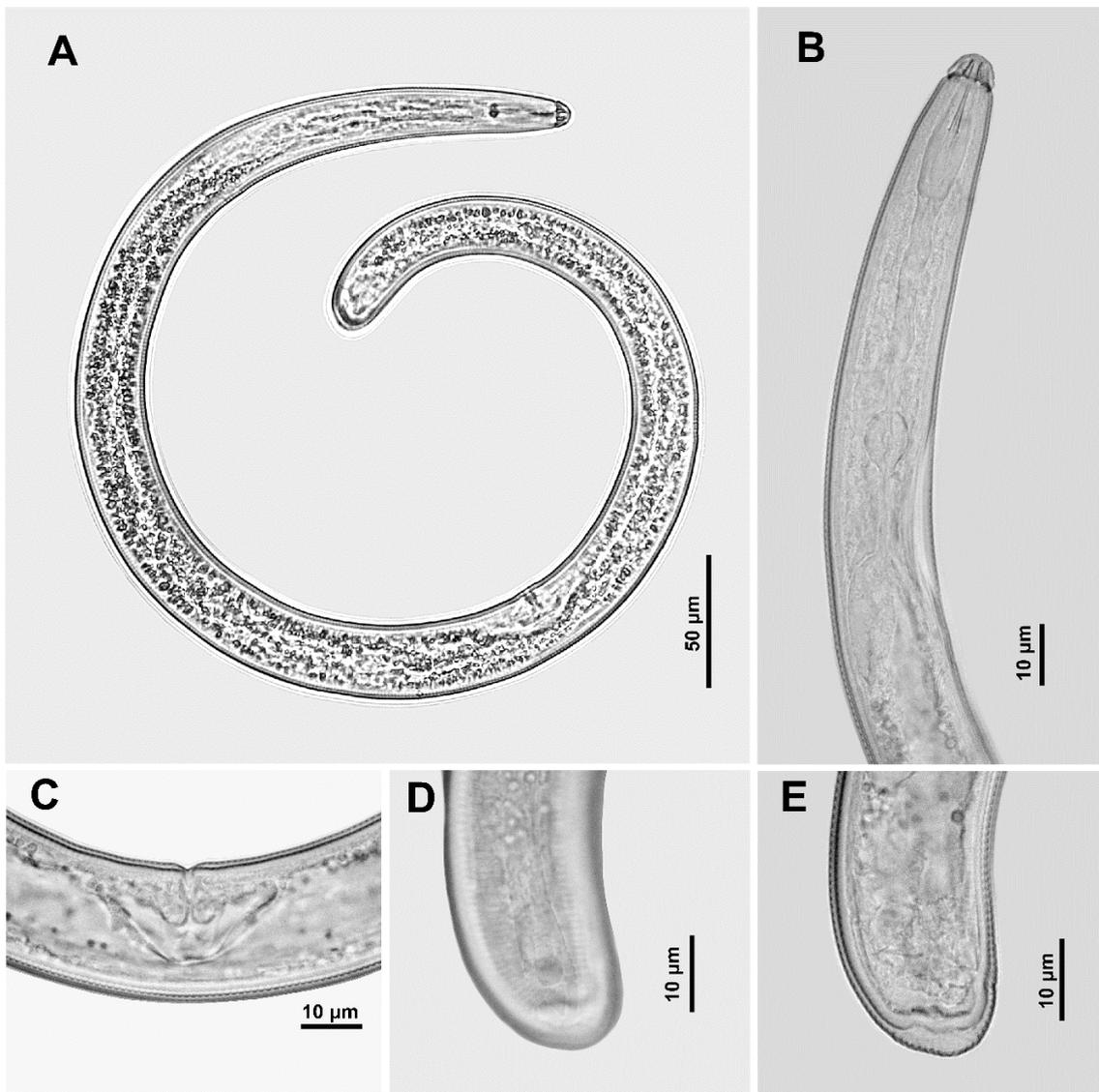


Figure 1. Females of *Scutellonema brachyurum* (Steiner, 1938) Andrassy, 1958 from Vietnam. A: Entire body; B: Anterior region; C: Vulval region; D: Tail region showing scutella; E: Tail region (Scale: A: 50 µm; B-E: 10 µm)

Males were not found.

PCA analysis

In the Principal Component Analysis based on 18 morphometric indices in this study, the eigenvalues reflect how much variation in the data is explained by the different axes. The first axis (PC1) explains 36.2%, and the second axis (PC2) explains 25.4% of the variation in the data (PC1 and PC2 together explain 61.6%). Although the observed correlations were small, the first axis (PC1) is mainly related to V%, lip width, MBD, and a value. The second axis (PC2) has the main association with lip

height, o value, stylet length, distance from anterior end to secretory-excretory pore, and scutellum length. The ordination of *Scutellonema brachyurum* populations on a two-dimensional scale was shown in Figure 2, in which, a relatively clear separation between type A and type B of *Scutellonema brachyurum* from Van den Berg et al. (2013) can be seen. Three South African populations from Shokoohi (2021) are closer to type B based on PC1, but they seem to be closer to type A based on PC2. The Vietnamese population of *Scutellonema brachyurum* was placed closer to the type B according to both PC1 and PC2 axes.

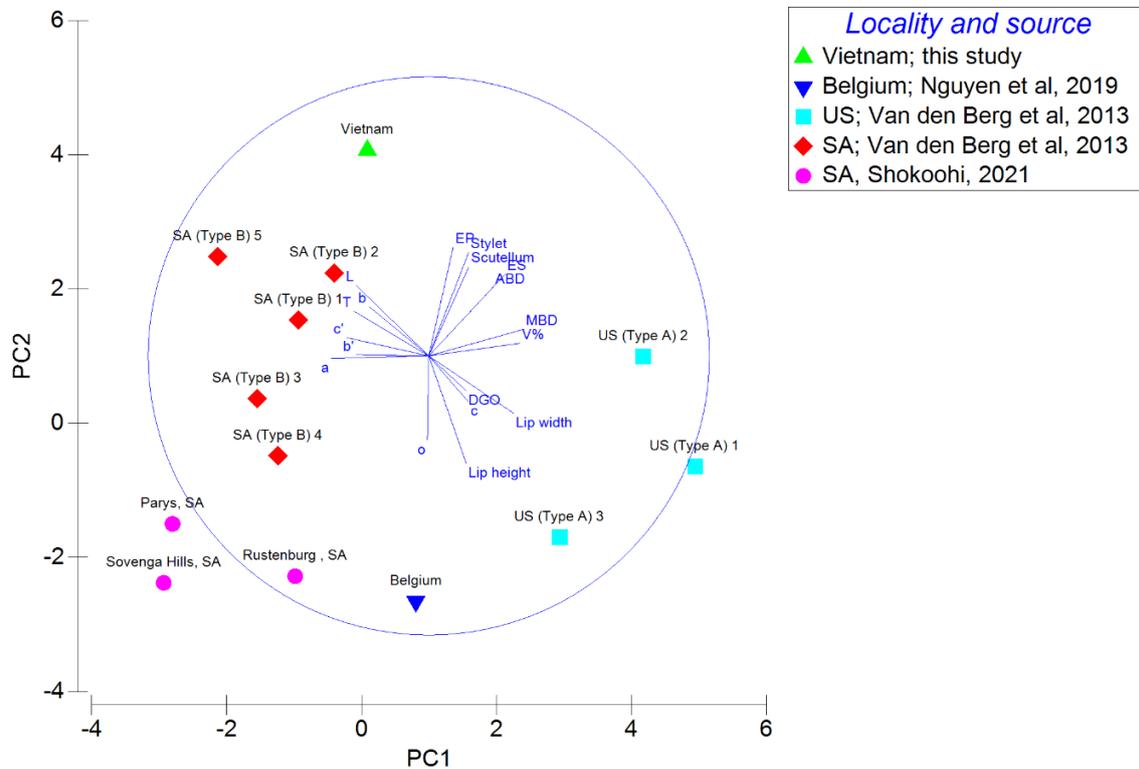


Figure 2. PCA analysis of *Scutellonema brachyurum* from 13 populations

Molecular characterization

The D2-D3 of 28S rRNA sequence of Vietnamese *Scutellonema brachyurum* was 738 bp long. It was found to be closest to the sequences of *Scutellonema brachyurum* (accession number: KX959263, KU059494, KX959259, KX959261) with 99.1-99.7%

similarity (2-6 bp difference). These sequences were referred to as *Scutellonema brachyurum* type A according to Van den Berg et al. (2013). On the contrary, the D2-D3 sequence of *Scutellonema brachyurum* from Vietnam was only 93.3-93.9% similar (39-44 bp difference) to those of *Scutellonema*

brachyurum type B (MW504476, MW504474, JX472051, JX472050, JX472048, JX472049). The inferred phylogenetic tree showed that the sequence of *Scutellonema brachyurum* from Vietnam was embedded in a clade together with the sequences of *Scutellonema brachyurum* type

A with maximal supported posterior probability. The clade of *Scutellonema brachyurum* type A is clearly separated from its sister clades, including *Scutellonema brachyurum* type B and *Scutellonema clavicaudatum* (Fig. 3).

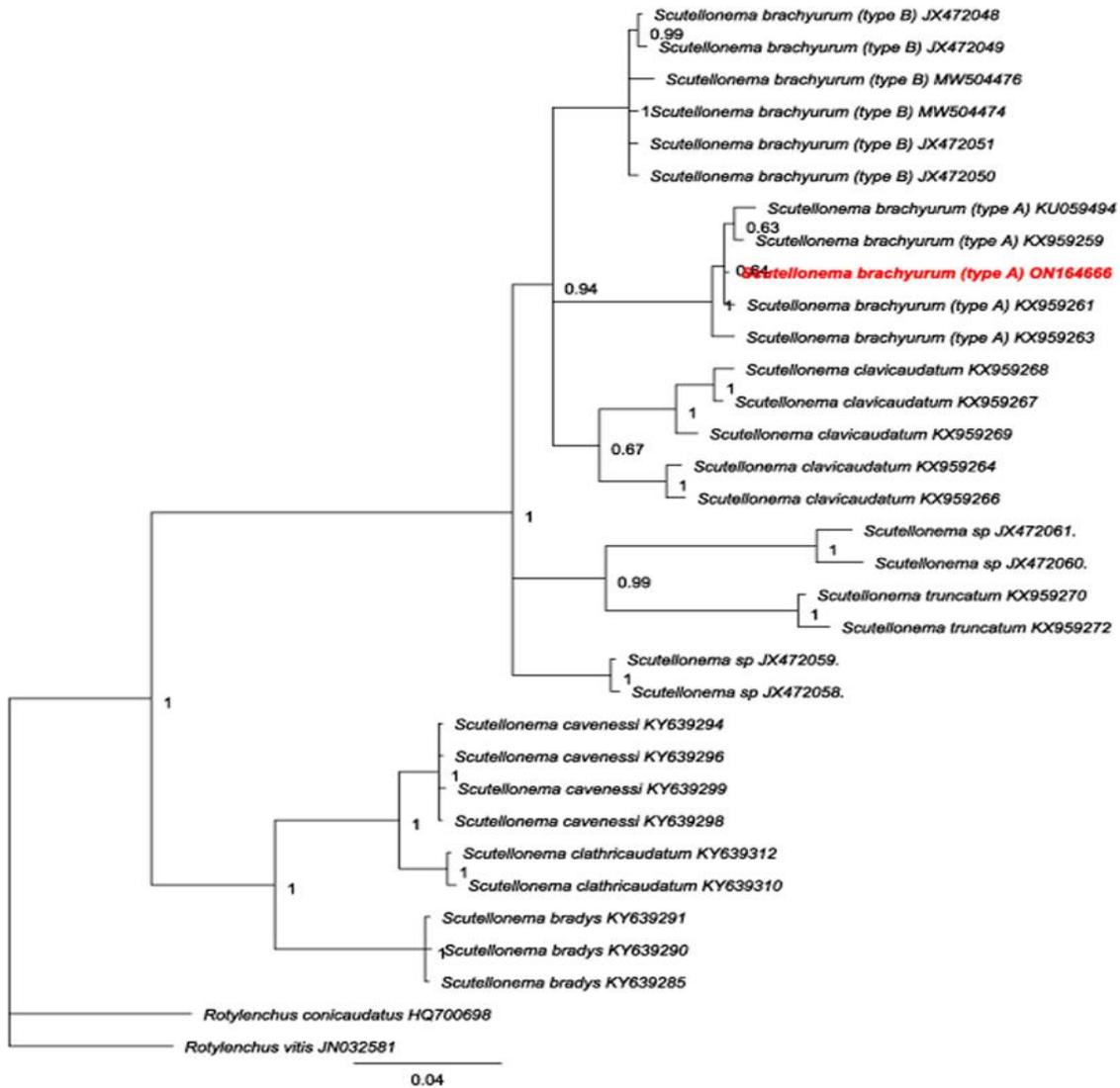


Figure 3. Bayesian phylogenetic tree generated using D2-D3 sequences of *Scutellonema* species under the GTR+G model. Sequence of *Scutellonema brachyurum* from Vietnam was indicated by bold font and red color

One 18S rRNA sequence of *S. brachyurum* from Vietnam was obtained, 925 bp long. This sequence is most similar to the

sequences of *Rotylenchus vitis* (MK348060), *Rotylenchus unisexu* (MK809259), *Rotylenchus robustus* (MW672529) and

identical diagnosis characterization compared to the original description of *S. Brachyurum* (Steiner, 1938). Interestingly, Van den Berg et al. (2013) differentiated two types of *Scutellonema brachyurum* based on both morphological and molecular evidence, i.e. type A and B. Based on morphological features, it is noted that *S. brachyurum* type A differs from type B by the following traits: lip region with 4–6 annuli vs mainly 3 annuli (rarely 4–5 annuli); 4–12 blocks on basal annulus vs 8–20 blocks; secretory–excretory pore located opposite anterior part to mid-region of overlapping pharyngeal lobe vs from rarely opposite mid-isthmus to mostly opposite the posterior part of pharyngeal gland lobe up to its posterior border; shorter tail length (7.5–14 μm vs 7.5–23 μm) and other morphometric differences (as in Table 1) (Shokoohi, 2021; Van den Berg et al., 2013). The Vietnamese population of *Scutellonema brachyurum* in this study was found to be more similar to *Scutellonema brachyurum* type B based on the number of lip annuli (mainly 3), the position of the secretory-excretory pore (at the level of pharyngeal gland end), and having slightly larger body length, larger a value, larger b value, smaller o value, shorter pharynx length, smaller MBD compared to type A (Table 1). Interestingly, the similarity between *Scutellonema brachyurum* from Vietnam and *Scutellonema brachyurum* type B was also relatively well-supported by PCA analysis. Nonetheless, the D2-D3 sequence of *Scutellonema brachyurum* from Vietnam was embedded in a clade together with all other sequences of *Scutellonema brachyurum* type A. Moreover, three South African populations of Shokoohi (2021) did not show a consistent separation into two types of *Scutellonema brachyurum*. Therefore, regardless of the high molecular difference between *Scutellonema brachyurum* type A and B, our result agrees with Shokoohi (2021) that the compared morphological characters are insufficient to differentiate *Scutellonema brachyurum* type A from type B.

Our study provides the first 18S sequence of *Scutellonema brachyurum* that is unequivocally linked to detailed morphological and other molecular data. It is remarkable that, within the genus *Scutellonema*, only 18S sequences of *Scutellonema bradys* are available in GenBank before our study. Albeit our 18S sequence of *S. brachyurum* is highly similar to *Rotylenchus vitis* (MK348060), *Rotylenchus unisexus* (MK809259), and *Rotylenchus robustus* (MW672529 and MK348059), none of the aforementioned *Rotylenchus* sequences is available in a published paper, suspecting the status of these sequences. Similarly, our blast search for closely related 18S sequences also forwarded several other 18S sequences of *Rotylenchus robustus* (KJ636415, KJ636429, KJ636365, AJ966503) that were placed on other distant clades compared to the clade consists of 18S sequence of *S. brachyurum* from Vietnam. However, to the best of our knowledge, all 18S sequences of *R. robustus* are not linked to morphological data in any published paper. Therefore, other studies on the 18S rRNA region of *Scutellonema* and *Rotylenchus* species are recommended to better understand the evolution of these nematode groups.

Scutellonema brachyurum is widely distributed from temperate to tropical areas, causing damage to a large number of host plants including fruits, vegetables, ornamental plants, and other plants (Sikora et al., 2018; Van den Berg et al., 2013). Notably, *Scutellonema brachyurum* has been found to cause serious damage to tuber crops such as yam, cassava, and sweet potato (Coyne et al., 2003). In this study, we recorded the presence of *S. brachyurum* in association with tuber rot symptom of Vietnamese ginseng (one of the most precious and rare ginseng varieties in the world), indicating its potential pathogenicity. The symptoms change to brown, dark brown and black while tubers remain firm which can be referred to dry rot symptom.

Therefore, more detailed studies on the relationship between *S. brachyurum* and its host, Vietnamese ginseng, are needed to better understand the damaging potential of this pest.

CONCLUSION

Our study provides the first report of *Scutellonema brachyurum* associated with Vietnamese ginseng in Vietnam. The integrative approach used in our study indicated that *Scutellonema brachyurum* type A can be differentiated from type B based on molecular data, however, compared morphological data are insufficient to delimitate these two types. In addition, this study provided the phylogenetic trees of *Scutellonema* species and the first 18S sequence of *Scutellonema brachyurum* to better understand the relationship among *Scutellonema* species. Due to its association with tuber rot symptom of the host plant, *Scutellonema brachyurum* can be a potential pest on Vietnamese ginseng that needs to be monitored carefully.

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