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Molecular Character of *Mylonchulus hawaiiensis* and Morphometric Differentiation of Six *Mylonchulus* (Nematoda; Order: Mononchida; Family: Mylonchulidae) Species Using Multivariate Analysis

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Abstract: Mononchida members are predatory nematodes with the potential to reduce the number of plant-parasitic nematodes in the soil. During a survey on Mononchida in Iran, several populations of *Mylonchulus* were recovered from various localities. A population of *M. hawaiiensis* was studied using 18S rDNA. The phylogenetic analysis using Bayesian inference placed the sequenced *M. hawiinesis* (OP210758) together with other *M. hawaiiensis* from Japan (AB361438-AB361442) with a 1.00 posterior probability support. In addition, morphological differences between six *Mylonchulus* (Nematoda; order: Mononchida; Family: Mylonchulidae) populations were investigated in Iran using discriminant analyses (DA), PERMANOVA, and principal coordinate analysis (PCoA). The purpose was to evaluate the efficacy of PCoA and DA in separating the *Mylonchulus* species, namely *M. sigmaturus*, *M. paitensis*, *M. lacustris*, *M. brachyuris*, *M. kermaninesis*, and *M. hawaiiensis*. To achieve this, 16 morphometric measurements (body length, *a*, *b*, *c*, c', V, G1, G2, buccal cavity length, buccal cavity width, dorsal tooth apex, dorsal tooth length, neck length, amphid from anterior end, rectum, and tail length) were made on 160 specimens. The analysis of variance showed that all features were significantly different among the species, except a, b, and the amphid position from the anterior end and tail length. The stepwise discriminant analysis revealed that body length, tail length, neck length, and c' value were the four most discriminating variables useful to distinguish clearly the six species of *Mylonchulus*. The variables with strong discriminatory power correctly classified 98.87% of individuals from Iran's sample of known *Mylonchulus* species. The results provide a morphometric basis for effectively distinguishing *Mylonchulus* species.

Keywords: nematode; morphological traits; multivariate analysis; predator

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1. Introduction

The threat group of invertebrates in 1861 by Diesing was considered in Nematoda [1]. In Nematoda, the order Mononchida [2,3] comprises predators found in various habitats, including terrestrial to aquatic [4]. These groups of nematodes play an essential role in the dynamics of the fauna of the soil [5]. Mononchida members play a crucial role in decreasing the plant-parasitic nematodes; however, this predatory behaviour is mainly investigated in the controlled environment [6]. In contrast, *Clarkus papillatus* is being studied for controlling *Meloidogyne* in the sugar beet field [7]. Besides, Mononchids have no food preference and feed on a variety of soil microorganisms, including nematodes [4]. Additionally, the predatory nematodes have a potential for mass rearing on the medium [6]. Conversely, diversity in the prey has been observed for the various genera of Mononchida [4]. Therefore, their precise identification helps to find out a suitable predator to combat the plant-parasitic

nematodes in future research. The genus *Mylonchulus* [8] (Nematoda; order: Mononchida; family: Mylonchulidae), is a broadly distributed taxon with six species being reported from Iran [5,6,9–17]. Morphology, morphometrics, and molecular characters are the primary distinguishing tool for predator nematodes [4,5]. However, in some species of the *Mylonchulus*, the morphology and morphometrics overlap [4], so species identification becomes problematic. Multivariate analysis of morphometric characteristics has been used to differentiate species of the animals, such as fish [18], or different populations belonging to the same species using discriminant analysis for nematodes [19,20]. However, the usefulness of multivariate analysis to distinguish the *Mylonchulus* species has not yet been investigated. Therefore, the present study aimed (1) to study the molecular characteristics of *M. hawaiiensis* using 18S rDNA, and (2) to discriminate the species of *Mylonchulus* using discriminant analysis (DA) and principal coordinate analysis (PCoA).

2. Material and Method

2.1. Nematode Isolation, Processing, and Identification

Soil samples were collected from various localities in Iran (Figure 1 and Table 1). Nematode extraction was achieved using the modified tray method [21]. Extracted nematodes were fixed with a hot 4% formaldehyde solution, preserved in an anhydrous glycerine utilizing the procedure described by De Grisse [22], and mounted on microscopic glass slides. The nematodes were then identified using Ahmad and Jairajpuri [4], up to species level.

Table 1. Geographic locations and coordination of the species studied.

Species	Province	Location	Host	GPS Coordinates
M. brachyuris	Kerman	Lalezar	walnut	N: 29°29′08.5″; E: 56°48′50.0″
	Mazandaran	Qaemshahr	forest soil	N: 36°23′56.75″; E: 52°49′33.97″
M. hawaiiensis	Kerman	Jiroft	soil	N: 28°58′36.77″; E: 57°38′3.80″
M. kermaniensis	Kerman	Jiroft	soil	N: 28°58′36.77″; E: 57°38′3.80″
M. lacustris	Kerman	Jiroft	citrus	N: 28°36′6.17″; E: 57°49′44.1″
M. paitensis	Kerman	Andoohjerd	grassland	N: 30°14′12.10″; E: 57°45′10.9″
	Semnan	Damghan	walnut	N: 36°13′50.29″; E: 54°11′5.34″
	Mazandaran	Qaemshahr	forest soil	N: 36°23′56.75″; E: 52°49′33.97″
M. sigmaturus	Kerman	Kerman	soil	N: 30°15′14.9″; E: 57°6′14.73″
	Fars	Shiraz	ash tree	N: 29°43′45.63″; E: 52°34′56.79″

2.2. DNA Extraction, PCR, and Phylogenetic Analysis

DNA isolation was completed based on the Chelex method [23]. Two individuals of the species were hand-picked with a fine tip needle and transferred to a 1.5 mL Eppendorf tube containing 20 µL nuclease-free water. The nematodes in the tube were crushed with the tip of a fine sterilised needle and vortexed. Thirty microliters of 5% Chelex® 50 and five µL of proteinase K were added to the tube and mixed. The tube with the crushed nematode was set at 56 °C for 2 hours, then 95 °C for 10 min to deactivate the proteinase K, and spun for 2 min at 16000 rpm [24]. The supernatant was extracted from the tube and stored at −20 °C. Afterward, the forward and reverse primers, 988F (5′-CTCAAAGATTAAGCCATGC-3′) and 1912R (5′-TTTACGGTCAGAACTAGGG-3′) [25], were used in the PCR reactions for partial amplification of the 18S rDNA region. PCR was conducted with eight µL of the DNA template, 12.5 µL of 2X PCR Master Mix green (NEB, Hitchin, UK), one µL of each primer (10 pmol μL^{-1}), and ddH_2O for a final volume of 25 µL. The amplification was processed using a bio-rad thermocycler (Hercules, CA, USA), with the following program: initial denaturation for 3 min at 94 °C; 37 cycles of denaturation for 45 s at 94 °C; 54 °C annealing temperature; extension from 45 s to 1 min at 72 °C; and finally an extension step of 6 min at 72 °C followed by a temperature on hold at 4 °C. After DNA amplification, four µL of PCR product was loaded on a 1.5% agarose gel in TBE buffer (40 mM Tris, 40 mM boric acid, and one mM EDTA) for assessment of the DNA bands. The band was dyed with SafeView classic (abm life science, Vancouver,

Pretoria, Canada) and photographed on a UV transilluminator. The PCR product was kept at −20 °C. Finally, Inqaba Biotech company (Pretoria, South Africa) purified and sequenced the PCR product. The ribosomal DNA sequences were analyzed and edited with BioEdit [26] and aligned using CLUSTAL W [27]. A phylogenetic tree was produced using the Bayesian inference method as implemented in the program MrBayes 3.1.2 [28]. Analysis using the GTR+G+I model was started with a random starting tree and ran with the Markov chain Monte Carlo (MCMC) for 10^6 generations for 18S rDNA. The tree was checked with the TreeView software [29]. In addition, as outgroups, based on 18S rDNA, *Bathyodontus mirus* [30,31] (AY284744; FJ969116) was used for the phylogenetic analysis as an outgroup. The original partial 18S rDNA sequence of *M. hawaiiensis* was deposited in GenBank under the accession number OP210758.

Figure 1. Localities of the sampling (red star) for *Mylonchulus* species in Iran.

2.3. Statistical Analysis

The samples were collected from various localities in Iran (Figure 1 and Table 1). A PERMANOVA was performed with the morphometrics obtained from fixed specimens from 16 traits in Primer v7 (Auckland, New Zealand)/PERMANOVA+ [32]. First, a pre-

treatment was conducted to standardize the data using the Log_{10} as used for morphometric data provided by Nattero et al. [33] of the 16 traits analyzed to transform the variables of different measurement units to the same scale. A matrix of Euclidean distances was then constructed, and the PERMANOVA was performed. The statistical significance of the analysis was tested with 999 permutations based on a type III sum of squares. The general patterns of morphological variation of the studied populations in each management category were analyzed using a Principal Coordinates Analysis as implemented in Primer v7/PERMANOVA+ [32]. Therefore, the same pretreatment and Euclidian distance considered in the PERMANOVA design were used. Totally, 160 individuals were analyzed for this study. The morphometric data were extracted from the fixed specimens. Twenty-six specimens of each species, excluding *M. sigmaturus* of which thirty specimens were analyzed. Sixteen morphometric characters, viz. body length (L), "*a*" (body length/greatest body diameter), "*b*" (body length/distance from anterior to pharyngeal-intestinal valve), *c* (body length/tail length), "c'" (tail length/tail diameter at anus), V (% distance of vulva from anterior/body length), G1 (% anterior genital branch length/body length), G2 (% posterior genital branch length/body length), buccal cavity length, buccal cavity width, dorsal tooth length, dorsal tooth apex (% dorsal tooth apex form the anterior end of buccal cavity/buccal cavity length), amphids opening to anterior end, neck length, rectum, and tail length were used for analysis. The morphometrics were obtained from the fixed nematode specimens. Data on the morphometric measurements of the species were analyzed using XLSTAT [34]. Using a stepwise model, the same characters were used for discriminant analysis (DA). Before the examination, the measures were standardized for study with XLSTAT software [34]. The morphometric data were standardized by Log_{10}. Additionally, the hierarchical cluster was studied using the spearman correlation coefficient using XLSTAT.

3. Results

3.1. Molecular Analysis

The phylogenetic tree indicated *Mylonchulus* as a monophyletic group with a 1.00 posterior probability support (Figure 2). The Bayesian tree placed the Iranian population of *M. hawaiiensis* together with the other molecularly identified as *M. hawaiiensis* with 1.00 posterior probability. However, another population of the same species (JQ742964) from Iran was placed differently in the phylogenetic tree under the *Mylonchulus* group (Figure 2).

Pairwise Maximum Composite Likelihood distance for the 18S rDNA region of *M. hawaiiensis* disclosed that the genetic distances ranged from 0.000 to 0.002. Iranian population (OP210758) has the same genetic distance (0.001) with the Japanese (AB361438-AB361442), and the Iranian population (JQ742964) (Table 2). Despite the molecularly identified population as *M. hawaiiensis* placed separately; however, the genetic distance showed no differences among the populations.

Table 2. Genetic pairwise distance of different populations of *Mylonchulus hawaiiensis*.

			1	2	3	4	5	6	7
	Accession Number	**Locality**	**OP210758**	**AB361439**	**AB361441**	**JQ742964**	**AB361438**	**AB361440**	**AB361442**
1	OP210758	Iran		0.002	0.002	0.003	0.002	0.002	0.002
2	AB361439	Japan	0.001		0.001	0.003	0.000	0.000	0.000
3	AB361441	Japan	0.001	0.000		0.003	0.001	0.001	0.001
4	JQ742964	Iran	0.001	0.001	0.002		0.003	0.003	0.003
5	AB361438	Japan	0.001	0.000	0.000	0.001		0.000	0.000
6	AB361440	Japan	0.001	0.000	0.000	0.001	0.000		0.000
7	AB361442	Japan	0.001	0.000	0.000	0.001	0.000	0.000	

Figure 2. Phylogenetic tree using Bayesian inference, including the newly sequenced *M. hawaiiensis* based on 18S rDNA.

3.2. Morphometric Characteristics

The species identified morphologically (Figure 3) resembles the information provided for the species of *Mylonchulus* by Ahmad and Jairajpuri [4]. However, the result indicated that a, b, and amphidial position to anterior end and tail length had no significant effect ($p > 0.05$) on the morphology of the *Mylonchulus* species (Figure 3 and Table 3). Based on F

and Wilks' Lambda, the main discriminant variables in the present study were: c, dorsal tooth apex, G2%, dorsal tooth length, and neck (Table 3).

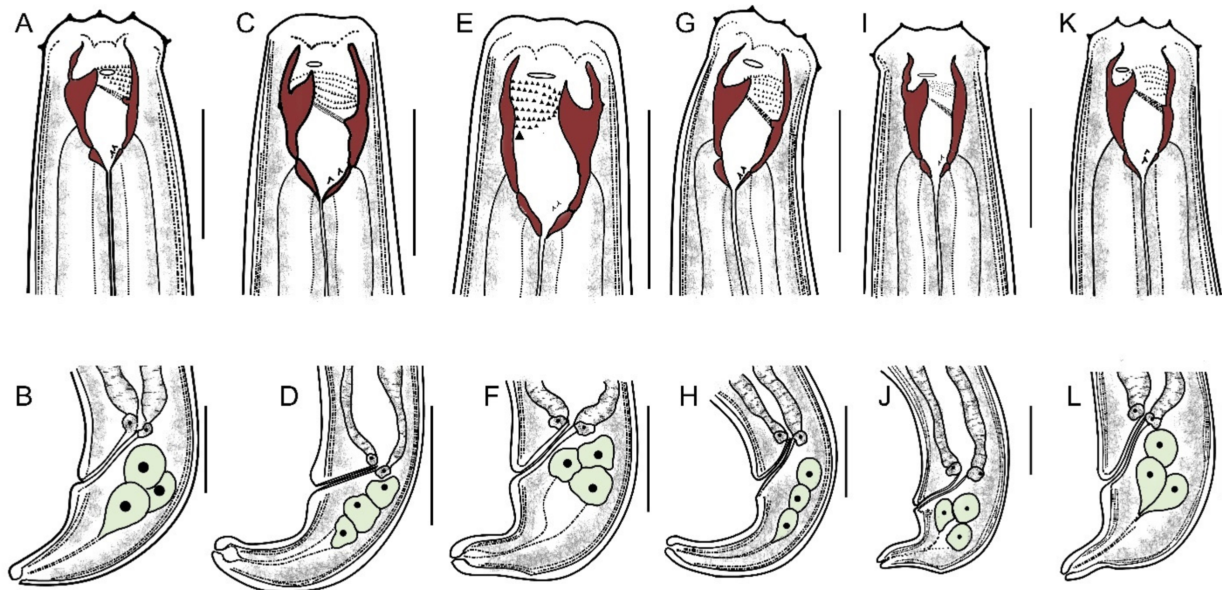

Figure 3. Line illustrations of six *Mylonchulus* species. (**A**,**B**) *Mylonchulus brachyuris* (Bütschli, 1873) Cobb, 1917. (**C**,**D**) *Mylonchulus hawaiiensis* (Cassidy, 1931) Goodey, 1951. (**E**,**F**) *Mylonchulus kermaniensis* Shokoohi, Mehrabi-Nasab, Mirzaei, and Peneva, 2013. (**G**,**H**) *Mylonchulus lacustris* (Cobb in Cobb, 1915) Andrássy, 1958. (**I**,**J**) *Mylonchulus paitensis* Yeates, 1992. (**K**,**L**) *Mylonchulus sigmaturus* (Cobb, 1917) Altherr, 1953. (Up-row: Anterior end. Down-row: posterior end. Scale bar: 25 µm).

Table 3. Discriminant functions for the morphometric variables of six *Mylonchulus* species from Iran.

	Wilks' Lambda	F	Sig.
Body Length	0.434	5.749	0.002
a	0.598	2.961	0.034
b	0.752	1.45	0.246
c	0.208	16.787	0.000
c'	0.441	5.573	0.002
V	0.353	8.08	0.000
G1%	0.561	3.448	0.019
G2%	0.289	10.841	0.000
Neck	0.343	8.434	0.000
Buccal cavity length	0.558	3.479	0.018
Buccal cavity width	0.392	6.818	0.001
Dorsal tooth apex	0.268	12.041	0.000
Amphidial position to ant. end	0.68	2.074	0.107
Dorsal tooth length	0.32	9.36	0.000
Rectum	0.614	2.77	0.044
Tail length	0.776	1.27	0.312

The highest values in the ratio of the *Mylonchulus* species were found in *M. paitensis* (a = 30.0 ± 0.8), *M. hawaiiensis* (b = 24.0 ± 1.5), *M. kermaniensis* (c = 33.1 ± 1.6), *M. lacustris* (c' = 1.9 ± 0.03), and *M. paitensis* (V = 64.4 ± 0.4). Regarding the G1%, *M. paitensis* (18.0 ± 1.0), and G2%, *M. kermaniensis* (20.8 ± 0.2) showed the highest value (Table 4).

Table 4. Descriptive statistics of morphometrics for six *Mylonchulus* species from Iran (Mean ± Standard Error). Characters with similar letters have no significant differences.

Genus	*M. paitensis*	*M. brachyuris*	*M. sigmaturus*	*M. lacustris*	*M. kermaniensis*	*M. hawaiiensis*	*p*-Value
Body length	1164.5 ± 74.1 a	1198.6 ± 50.9 a	1263.1 ± 24.1 ac	979.6 ± 25.8 b	1357.2 ± 31.6 c	1195.8 ± 62.9 a	0.000
a	30.0 ± 0.8 ac	28.2 ± 1.7 abc	31.9 ± 1.9 c	26.0 ± 1.3 ab	29.0 ± 1.6 ac	24.0 ± 1.5 b	0.17
b	3.4 ± 0.1 a	3.5 ± 0.1 ab	3.5 ± 0.2 ab	3.4 ± 0.1 ab	3.6 ± 0.1 ab	3.7 ± 0.1 b	0.222
c	29.7 ± 0.6 a	30.8 ± 0.7 ac	31.8 ± 0.7 ac	21.5 ± 0.3 b	33.1 ± 1.6 c	23.5 ± 1.2 b	0.000
c'	1.4 ± 0.02 a	1.4 ± 0.08 a	1.5 ± 0.08 ac	1.9 ± 0.03 b	1.5 ± 0.07 a	1.7 ± 0.09 c	0.000
V	64.4 ± 0.4 a	61.8 ± 0.6 a	63.4 ± 0.2 a	58.5 ± 1.2 b	63.5 ± 0.7 a	55.3 ± 1.8 c	0.000
G1%	18.0 ± 1.0 a	16.0 ± 0.9 a	16.7 ± 1.0 a	11.4 ± 0.6 b	16.8 ± 0.9 a	16.3 ± 1.4 a	0.002
G2%	16.9 ± 1.2 a	14.3 ± 1.5 a	15.8 ± 1.1 a	9.4 ± 0.2 b	20.8 ± 0.2 c	17.1 ± 1.4 a	0.000
Neck	348.1 ± 24.3 ac	341.8 ± 8.1 c	362.5 ± 3.9 ac	283.9 ± 3.4 b	378.5 ± 7.3 a	289.8 ± 12.1 b	0.000
Buccal cavity length	24.8 ± 0.6 ac	23.4 ± 0.3 c	25.3 ± 0.2 ac	24.9 ± 0.3 ac	25.8 ± 0.4 ab	27.3 ± 1.1 b	0.009
Buccal cavity width	13.9 ± 0.6 ac	12.7 ± 0.5 ce	13.3 ± 0.8 ace	11.5 ± 0.2 e	16.3 ± 0.5 b	14.8 ± 0.9 ab	0.000
Dorsal tooth apex	3.6 ± 0.7 a	4.0 ± 0.1 a	4.5 ± 0.2 ab	5.6 ± 0.2 bd	7.0 ± 0.3 c	6.4 ± 0.3 dc	0.000
Amphidial position to ant. end	10.1 ± 1.3 a	9.5 ± 0.3 a	12.8 ± 0.6 b	9.8 ± 0.2 a	10.4 ± 0.3 a	10.5 ± 0.4 a	0.082
Dorsal tooth length	8.1 ± 0.3 ac	7.7 ± 0.3 a	8.1 ± 0.1 ac	7.6 ± 0.1 a	8.5 ± 0.2 c	6.3 ± 0.3 b	0.000
Rectum	22.5 ± 1.5 ac	23.6 ± 0.7 c	22.0 ± 0.3 abc	20.2 ± 0.7 ab	20.0 ± 0.3 b	20.3 ± 1.1 ab	0.024
Tail	39.7 ± 2.9 a	39.1 ± 2.2 a	40.0 ± 0.7 ab	45.6 ± 0.9 b	42.2 ± 1.8 ab	43.3 ± 1.7 ab	0.131

The morphometrical differences between the six *Mylonchulus* species are shown in Figures 4 and 5. In Figure 4, the hierarchical cluster plot shows the similarity of the different *Mylonchulus* species. The similarity distances obtained from the morphometric measurements are graphically represented, a first cluster grouped *M. lacustris*, a second cluster made up of *M. hawaiiensis*, a third cluster that includes *M. kermaniensis*, and the fourth group made up *M. paitensis*, *M. sigmaturus*, and *M. brachyuris*. The fourth group showed overlapped morphometrics, and morphometrical variation was observed (Figure 4).

The different and distinctive morphometrical models for each species are reflected in Figure 5, which reveals a precise spatial distribution of each *Mylonchulus* species, with an overlap of *M. paitensis*, *M. brachyuris*, and *M. sigmaturus*. The DA plot (Figure 5) clearly separated *M. hawaiiensis*, *M. lacustris*, and *M. kermaniensis*.

Moreover, the PERMANOVA evidenced that 90.5% of the variation was presented among the populations ($p < 0.001$) (Figure 6 and Table 5). Likewise, the PCoA showed a broad variation similar to that identified by the DA and PERMANOVA test among *M. paitensis*, *M. sigmaturus*, and *M. brachyuris*. In PCoA (Figure 6), four groups of species were distinguished. The groups include (1) *M. hawaiiensis*, (2) *M. kermaniensis*, (3) *M. lacustris*, and (4) *M. paitensis*, *M. sigmaturus*, and *M. brachyuris*. Pairwise distance between the populations of *Mylonchulus* (Table 6), showed the same result obtained by the PCoA result displayed in Figure 6. The pairwise distance showed a high similarity of *M. paitensis* with *M. sigmaturus* (r = 0.28), and *M. brachyuris* (r = 0.33). In contrast, *M. hawaiiensis* showed highest distance compared with *M. lacustris* (r = 1.81), and *M. kermaniensis* (r = 0.77) (Table 6).

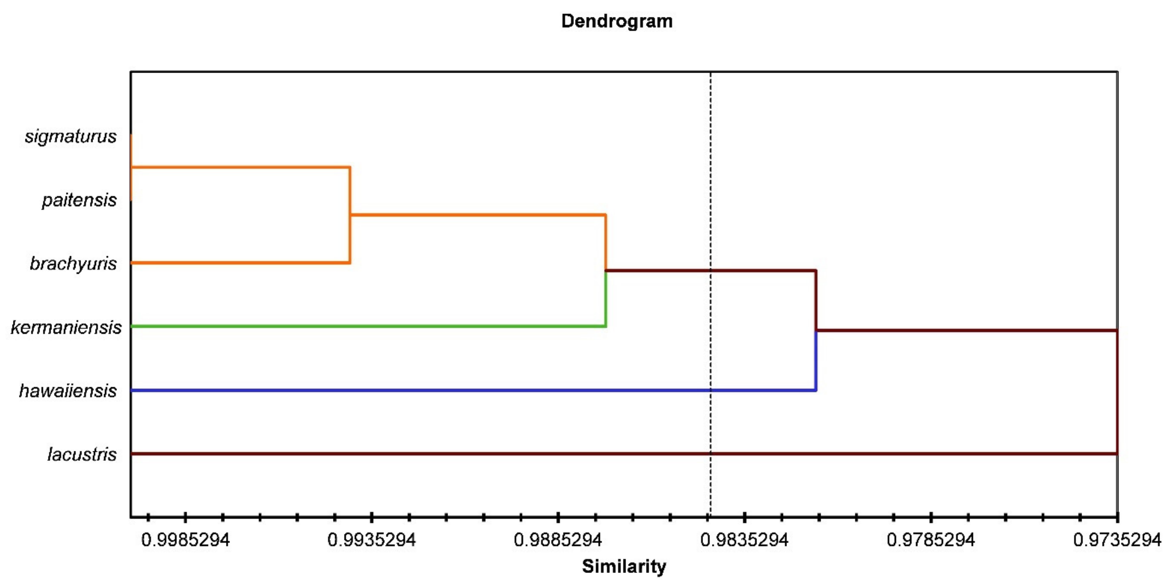

Figure 4. Hierarchical cluster analysis for six species of *Mylonchulus*.

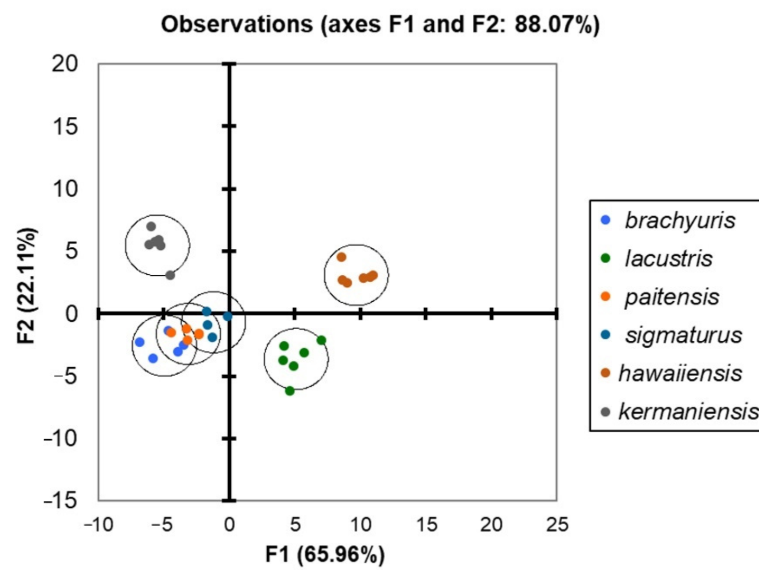

Figure 5. Discriminant analysis plot for six species of *Mylonchulus*.

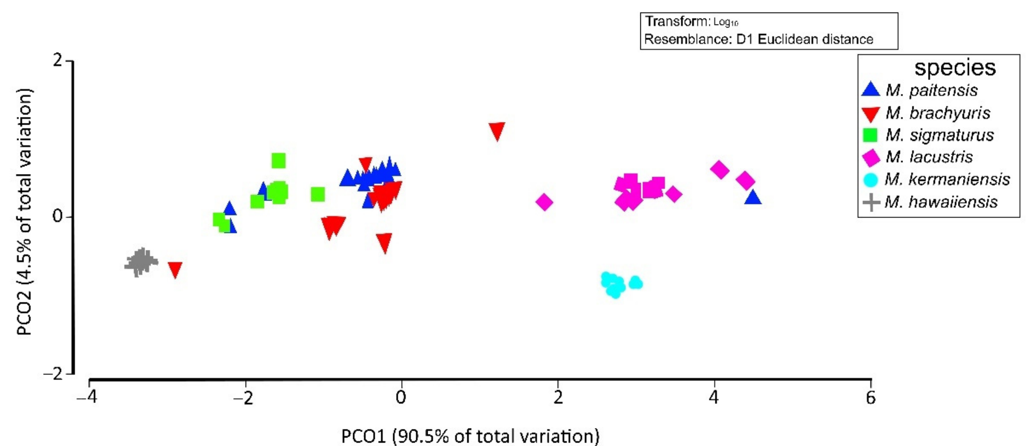

Figure 6. Principal coordinates analysis (PCoA) ordination indicates the relationships among populations for six species of *Mylonchulus*.

Table 5. Permutational analysis of variance (PERMANOVA) for sixteen morphological traits in different populations of *Mylonchulus*.

Source	df	SS	MS	Pseudo-F	p (Perm)	Unique Perms
species	5	895.46	179.09	397.34	0.001	998
Variables	154	69.412	0.45073			
Total	159	964.88				

Table 6. Pairwise distance between the populations of *Mylonchulus* using Primer 7 software.

	M. paitensis	*M. brachyuris*	*M. sigmaturus*	*M. lacustris*	*M. kermaniensis*	*M. hawaiiensis*
M. paitensis						
M. brachyuris	0.33					
M. sigmaturus	0.28	0.38				
M. lacustris	0.93	0.76	0.89			
M. kermaniensis	0.61	0.60	0.74	0.81		
M. hawaiiensis	0.39	0.55	0.51	1.81	0.77	

4. Discussion

The present results indicate that 18S rDNA sequence data are a valuable marker for the phylogenetic analysis within *Mylonchulus* species. This agrees with the previous result obtained [5,11,35]. Tree topology using Bayesian inference shows that *M. hawaiiensis* stand separately, which contrasts with the result obtained by Olia et al. [35]. However, the genetic distance revealed no significant differences among the populations from Iran and Japan. Therefore, the variation that exists may be due to the geographic location. Shokoohi et al. [11] and Koohkan et al. [5] indicated that the populations of *Mylonchulus* make a monophyletic group. The present study obtained the same result.

Overall, the environment and geographic location are key factors in nematodes' morphological variation [36]. They have indicated forest soil to be a more favorable condition for soil nematode dynamics. Besides, the genetic diversity showed to be affected by the geographic location of the nematode species [37]. Therefore, some environmental conditions and expression of genetic differences could be responsible for changes in the morphology of various nematode species. Predator nematodes of the order Mononchida are present in diverse habitats, from cultivated to natural lands [4]. The result indicated that three species, including *M. sigmaturus*, *M. paitensis*, and *M. brachyuris* overlap the morphometrics. They have a similar range for body length (1000–1392 µm for *M. sigmaturus*; 980–1400 µm for *M. paitensis*; and 1100–1580 µm for *M. brachyuris*), and tail length (31–48 µm for *M. sigmaturus*; 31–49 µm for *M. paitensis*; and 39–56 µm for *M. brachyuris*) [9,10]. However, they have different tail morphology which is similar to *M. sigmaturus* and *M. brachyuris* with the terminal spinneret. Tail in *M. sigmaturus* and *M. paitensis* are conoid, bent ventrally, with short and set off digitate portion. Whereas *M. brachyuris* is conical without a set off digitate part and spinneret open sub-terminally.

The results allowed for morphometric differentiation in six *Mylonchulus* species using sixteen features. The species analyzed could be discriminated by the morphometric models generated, demonstrating that discriminant analysis supported differentiating the species. Moreover, these morphological variables could be used to increase the consistency of specimens' classification in each species. Stock and Kaya [38] indicated that PCA and discriminant analysis are helpful tools to differentiate the *Heterorhabditis* species, and they have shown reliable morphometrics to identify the EPN species. Rubtsova et al. [39] showed the efficiency of discriminant analysis and hierarchical analysis in distinguishing the *Longidorus* species. Moreover, Stock and Nadler [40] analyzed the *Panagrellus*, which dif-

ferentiated the species sufficiently using Discriminant analysis. In addition, PCA has been successfully used to separate the *Panagrellus* species [40]. The same results were obtained in the recent work. PERMANOVA and PCoA already been used to study Mexican plants such as *Agave maximiliana* [41] and marine nematodes such as *Paracanthonchus gynodiporata* [42]. A morphological variation has been observed using non-metric Dimensional Scale for *P. gynodiporata* along the coastline of Brazil [42]. The same result was obtained in the present study, in which morphological variation exists among the populations of *Mylonchulus*.

In the present study, canonical plots of females derived from the results obtained by the discriminant analysis, hierarchical clustering, and principal component analysis showed a high degree of clustering among the analyzed species of *Mylonchulus*. The morphometric characters chosen played a vital role in the discrimination process. This suggests that the morphometric features selected should be used to identify *Mylonchulus* species. Therefore, we consider that morphological features, e.g., the body length, buccal cavity length and width, tooth length, G1, and G2%, neck, and tail length, should be considered together with morphometric characters for the identification based on the females for this group of nematodes. Overall, the biology of the animals needs morphometrics [43] which affect the development, evolution, relationship, and adaptation. In some cases, such as *Prionchlus punctatus* and *Mononchus aquaticus*, their life cycle takes 45 and 15 days at 25 °C, respectively [44,45]. Besides, the morphometrics of the above-mentioned species are different, which affects their life cycle. In another study, Cohn and Mordechai [46] observed that a high population of *M. sigmaturus* is significantly correlated with a low population density of *Tylenchulus semipenetrans*. Conversely, the availability of the citrus nematode as prey affects the morphometrics of *M. sigmaturus*. However, due to the lack of information on the biology of mononchids in Iran, discussing the relationship between biology and morphometrics is complicated.

5. Conclusions

This research aimed to analyze morphometric differentiation among six *Mylonchulus* species of *Mylonchulus* from Iran and prove the effectiveness of the multivariate analysis. The six species analyzed could be discriminated by the generated morphometric model, therefore showing that discriminant analysis helped differentiate species. The discriminant analysis approach showed significant differences between species and sameness within each species. However, the discriminant analysis showed overlap between *M. sigmaturus* and *M. paitensis*, which indicates that these two species should be reconsidered for their identification based on morphometrics. Additionally, the result suggests a synonymous potential between those two species. Although, the morphometric and molecular characters used in the present study are still reliable for distinguishing *M. kermaniensis*, *M. lacustris*, and *M. hawaiiensis*. However, more molecular markers in combination with the information derived from morphological features will help study the variability of the species.

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