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A novel use of a geometric morphometric technique to distinguish human parasite eggs of twelve different species

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ABSTRACT

Copro-microscopic diagnostic methods are the most common approach for screening patients with parasitic infections. However, expertise is required to identify helminthic eggs from fecal specimens. Consequently, new methods are required to support accurate species identification. Novel technologies have recently been developed for the classification of organisms, including geometric morphometric (GM) approaches. In this study, the outline-based GM approach was used to distinguish the eggs of 12 common human parasite species, including *Ascaris lumbricoides, Trichuris trichiura, Enterobius vermicularis*, hookworm, *Capillaria philippinensis, Opisthorchis* spp., *Fasciola* spp., *Paragonimus* spp., *Schistosoma mekongi, Taenia* spp., *Hymenolepis diminuta* and *Hymenolepis nana.* The GM analysis revealed that the size cannot be used as the main variable in the identification of parasite species at the egg stage, producing only 30.18% overall accuracy. However, comparisons of shape based on the Mahalanobis distances between pairs of parasite species showed significant differences in all pairs ($p < 0.05$). The shape analysis produced 84.29% overall accuracy. This is the first time that outline-based GM has been preliminarily confirmed as a valuable approach to support copro-microscopic analysis, in order to effectively screen helminth eggs. However, further studies with a larger set of helminth eggs and artefacts should be carried out to increase confidence in the identification of parasite species in the absence of local experts.

1. Introduction

Parasitic infections are a major public health problem worldwide, especially in less industrialized countries in tropical and subtropical regions (Suntaravitun and Dokmaikaw, 2018). The World Health Organization (WHO) reported that more than 1.5 billion people, representing approximately 24% of the world's population, are infected with parasitic helminths (WHO, 2020).

Parasitic helminths are multicellular metazoan organisms that must survive by taking advantage of their hosts, such as by taking nutrients from the host body, leading them to illness (Ásbjörnsdóttir et al., 2017). Parasitic worms can be divided into three main groups: Nemathelminthes (nematodes), Acanthocephala (acanthocephalans) and Platyhelminthes (flatworms). Nematodes (commonly called roundworms) are a class of Nemathelminthes that include human and animal parasitic worms (Tantaleán and Chavez, 2004). Acanthocephalan parasites have a broad range of vertebrate and invertebrate hosts but human parasitic infections are rare (Mathison et al., 2021). While, flatworms are further divided into four subgroups, two of which, trematodes (flatworms or flukes) and cestodes (tapeworms) are extremely important in public health (Hahn et al., 2014). Each helminth species has different virulence and different effects on human health. Most infections of humans with soil-transmitted helminths such as *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworms (*Necator americanus* and *Ancylostoma duodenale*) are asymptomatic or produce mild symptoms (Bethony et al., 2006). However, some human parasites, such as the liver fluke (*Opisthorchis viverrini*) can cause death by inducing cholangiocarcinoma. This parasite has its highest incidence in the population of northeastern Thailand (Sripa and Pairojkul, 2008; Kamsa-Ard et al., 2018). However, the severity of parasitic diseases in patients can be reduced if they are accurately diagnosed as quickly as possible (Ndao, 2009; Momčilović et al., 2019).

The copro-microscopic method is currently the gold standard for the diagnosis of soil-transmitted and other intestinal helminths from their

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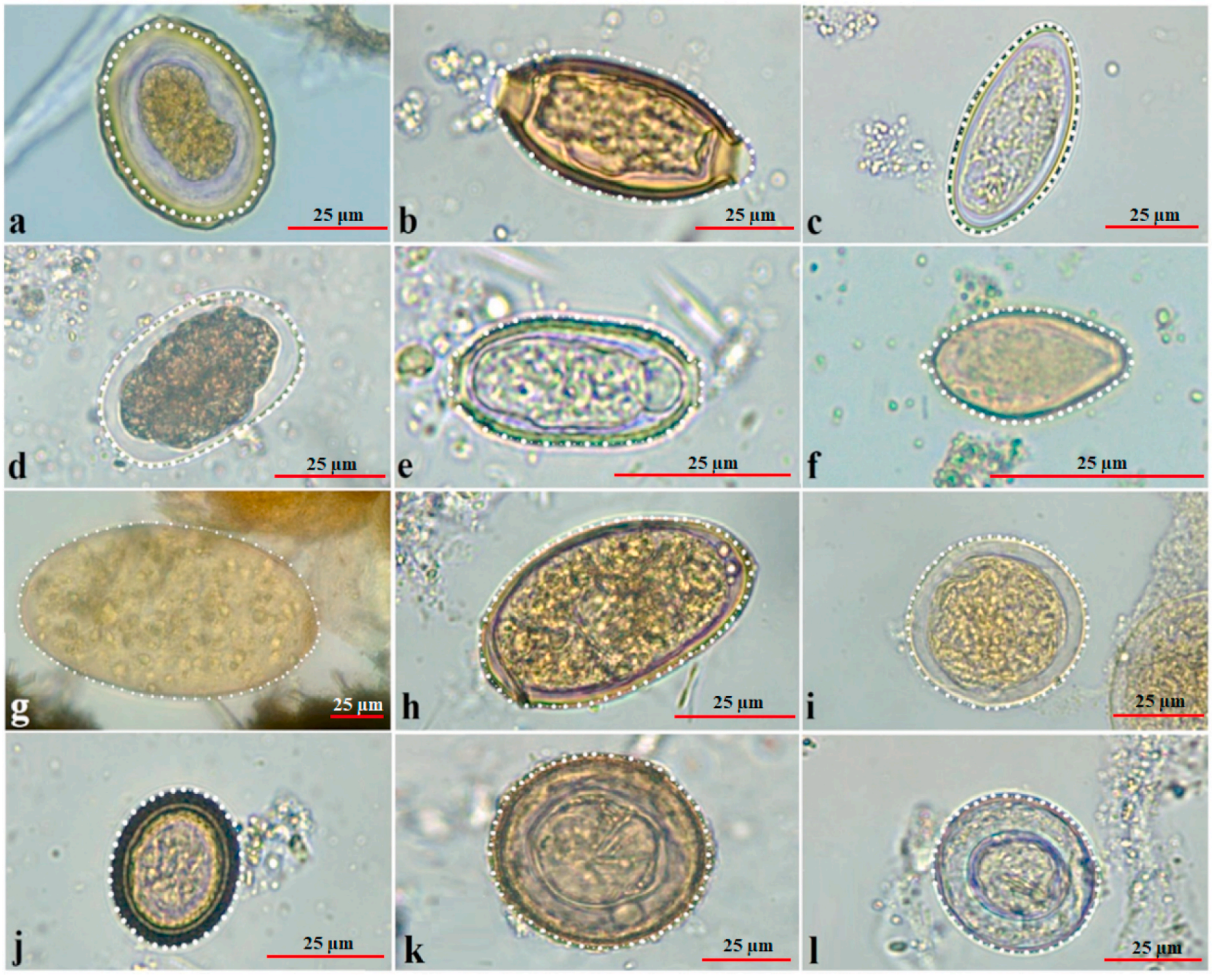

Fig. 1. Digitized external contours of 12 parasite eggs. a: *A. lumbricoides*, b: *T. trichiura*, c: *E. vermicularis*, d: hookworm, e: *C. philippinensis*, f: *Opisthorchis* spp., g: *Fasciola* spp., h: *Paragonimus* spp., i: *S. mekongi*, j: *Taenia* spp., k: *H. diminuta*, and l: *H. nana*. All scale bars represent 25 µm, adjusted from a 0.1 mm scale (100 µm).

eggs or larvae in the stools of patients. Copro-microscopic approaches are mainly performed after processing using concentration techniques such as the formalin-ether concentration technique (FECT), McMaster (McM) method, FLOTAC, and Mini-FLOTAC bedside direct microscopy (Nikolay et al., 2014). However, these techniques have a significant limitation, they require highly skilled technicians because the diagnosis must be assessed using the microscopy. This approach can lead to misidentification of results, and subsequent failure of treatment (Ngwese et al., 2020). In the last 20 years, molecular techniques based on conventional PCR, quantitative PCR (qPCR), nested PCR, and multiplex PCR have been used to confirm the presence of parasitic infections with high sensitivity and specificity. Unfortunately, they are expensive and advanced laboratory equipment is needed (Ngwese et al., 2020; Khurana and Sethi, 2017). In addition, pre-specified diagnostic suspicion is needed in order to know what to test for. Often the molecular diagnosis is limited to a selection of parasites. Therefore, the molecular techniques are not appropriate for the identification of helminth eggs in rural regions. Alternative methods are required to rapidly and accurately diagnose the presence of parasites and identity them, especially in rural areas without advanced technology and expertise.

In studies carried out by Georgi and McCulloch (1989), traditional morphometric methods were used to identify the eggs of 12 strongylid species, and achieved a relatively high percentage of correct classification rate in seven species (72%–100%) including *Nematodirus filicollis* (100%), *N. battus* (98%), *N. spathiqer* (96%), *Oesophagostomum. venulosum* (100%), *Oe. columbianum* (72%), *Trichostrongylus axei* (76%), and *T. colubriformis* (73%). However, this technique has limitations for egg shape analysis, as these methods involve calculating multiple inter-landmark distances without taking into account the geometric configuration of the landmarks. Geometric morphometrics (GM) is a newly developed morphometric technique that uses analysis based on size and shapes variables separately. Quantitative geometric data are compared and calculated using mathematical and statistical approaches (Dujardin, 2011; Lorenz et al., 2017). This technique is becoming increasingly popular and widely accepted in studies of animals that are hard to identify, such as mosquito species (Wilke et al., 2016; Chaiphongpachara, 2018; Chaiphongpachara et al., 2019), fireflies (Sumruayphol and Chaiphongpachara, 2019), fish (Ponton, 2006; Santos et al., 2019), bats (Schmieder et al., 2015), *Odontomachus* ants (Samung et al., 2022), and mollusks as intermediate hosts of some parasites (Vaux et al., 2017, 2018). The GM approach also has important advantages: it is easy to use, does not require expertise, and can be performed rapidly. The outline-based GM approach is a modern morphometric technique which involves assessing and analyzing shapes, contours, concavities, and curves, and is suitable for outlines without landmarks or without enough landmarks (Dujardin et al., 2014). This approach can be used to distinguish different animal species. The outline-based GM approach has been reported to be able to classify species of animals in life stages that have species-specific shapes, such as eggs of different kinds of birds (Attard et al., 2018), eggs of triatomine bugs (Santillán-Guayasamín et al., 2017), and the pupal stage of flies (Chaiphongpachara and Tubsamut, 2019). The outline or shape of helminth eggs is an important contributor to the identification of nearly all helminth eggs. Recently, GM techniques have been used to identify *Trichuris* parasite eggs from

non-human primates, and have been found to be highly effective (García-Sánchez et al., 2020).

In this study, the outline-based GM approach was used to distinguish the eggs of 12 common human parasites, including *Ascaris lumbricoides, Trichuris trichiura, Enterobius vermicularis,* hookworm*, Capillaria philippinensis, Opisthorchis* spp.*, Fasciola* spp.*, Paragonimus* spp., *Schistosoma mekongi, Taenia* spp., *Hymenolepis diminuta*, and *Hymenolepis nana.* This study is the first GM experiment with human parasite eggs, and the results could serve as a guideline for the detection of helminth eggs of patients.

2. Materials and methods

2.1. Ethics statement

The present study was approved by the Human Ethics Committee of Suan Sunandha Rajabhat University Ethics Committee. The ethical certificate number of this research is COE. 1–052/2020.

2.2. Parasite eggs obtaining

The helminth eggs in this study were obtained from two sources; 1) fresh specimens obtained from 50 people who were screened for parasitic disease in Ban Don Yang, Na Khamin sub-district, Phon Sawan district, Nakhon Phanom Province, Thailand, between January and April 2021. Stool samples were examined within 2 h by simple direct smear microscopy. Feces were mixed with a drop of 0.85% sodium chloride solution on a slide, covered with a coverslip, and observed directly with a light microscope (Nikon, Eclipse - E200) for parasitic diagnosis which have been identified by three parasitologists in our team. In the case of parasite eggs found in the stool, 30–50 complete parasite eggs of each species were photographed using a digital camera (DS-Fi3; Nikon, Tokyo, Japan) connected to a light microscope (Nikon, Eclipse - E200) and a scale bar of 0.1 mm was added to all images. 2) Egg specimens from permanent slides for the medical parasitology study were taken from the Department of Parasitology, Phramongkutklao College of Medicine and Department of Helminthology, the Faculty of Tropical Medicine, Mahidol University, Thailand. The permount (Fisher Scientific GmbH, Schwerte, Germany) was used as a permanent mounting medium for eggs of parasites. In addition, parasite samples in the permanent slides were collected from patients in Thailand over the past five years. Thirty to fifty complete parasite eggs from the permanent slides were photographed using a digital camera (DS-Fi3; Nikon, Tokyo, Japan) connected to a light microscope (Nikon, Eclipse - E200), and a scale bar added.

2.3. Egg contour digitization

The external contours of the eggs of each helminth species were digitized (Fig. 1). Only complete shapes with correct contours according to the species of helminth were used for contour digitization. Deformed or uncompleted contours that had lost species-specific characteristic were immediately discarded, to prevent discrepancies in the converted digital data.

2.4. Repeatability

Before analyzing the helminth eggs using the GM technique, it was necessary to explore the measurement error, to assess the accuracy of contour digitization in the test image set. In the present study, repeatability index was used for detection of measurement error (Arnqvist and Mårtensson, 1998). Ten eggs per species were randomly selected, digitized twice by the same person and computed both image sets based on Procrustes analysis.

2.5. Contribution of size to species variation

The contribution of size to shape, also called allometry, is the relationship between the size and shape variables which causes shape changes in response to size differences (Lorenz et al., 2017). Allometry was evaluated using the linear determination coefficient after regressing the principal components of the normalized elliptic Fourier coefficients on the global size (Santillán-Guayasamín et al., 2017).

2.6. Size-based discrimination

Before GM analysis, all parasite egg images were separated and grouped based on parasites/genus/species. After that metric properties including size and shape were extracted in order to examine each variable separately. For species discrimination based on egg size, the global size was defined based on half the major axis of the first ellipse (first harmonic) which contained information about the perimeter and the square-root area of the contour (Dujardin and Dujardin, 2019). To make it easier to understand, global size variations of parasite eggs were illustrated by color quantile boxes. Subsequently, one-way ANOVA was used to examine the differences between the mean global sizes of the egg of parasite species. In order to evaluate the statistical significance of the one-way ANOVA, a non-parametric procedure (1000 permutations) with Bonferroni *post hoc* correction was performed at p-values < 0.05. After that, each individual was then classified based on global size, to verify the correct assignment, which was evaluated using maximum likelihood based on a validated reclassification procedure (Dujardin and Dujardin, 2019) for checking the potential of species identification based on size GM analysis.

2.7. Shape-based discrimination

For species discrimination based on egg shape, elliptical Fourier analysis was used to construct the egg shape variables. Shape variables derived from the normalized elliptic Fourier coefficients were used for principal component analysis. After that, the results of principal components were used as the final shape variables. The final shape variables were analyzed using discriminant analysis to examine their grouping, which was illustrated using a factor map, and the Mahalanobis distances were computed. For statistical comparisons of egg shapes based on pairwise Mahalanobis distances between parasite species, a non-parametric permutation test (1000 permutations) with Bonferroni *post hoc* correction was used (p-values < 0.05). A hierarchical clustering tree based on the Mahalanobis distances between group shapes was created to estimate the shape similarity. To estimate the potential of shape GM analysis for species identification, the validated (cross-checked) classification test based on Mahalanobis distances was performed to verify the correct assignments.

2.8. Software

The online XYOM application, freely available at https://xyom.io/ was used in this experiment (Dujardin and Dujardin, 2019). Four sections of XYOM—Digitization, Miscellaneous, Characterization, and Classification—were used. Firstly, Digitization was used for the digitization of egg contours based on plotting pseudo-landmarks. Then, Miscellaneous was used for the repeatability testing. After that, Characterization was performed for the generation of size and shape variables from the coordinates of pseudo-landmarks generated by analysis using elliptical Fourier analysis, principal components analysis, and discriminant analysis, and allometric testing. Finally, Classification was performed for the estimation of size and shape variables, including calculating the statistical significance, computing a validated classification, and building a hierarchical clustering tree.

Table 1
Parasite species and number of eggs analyzed.

Species	Number of eggs analyzed	Sample sources
Ascaris lumbricoides	50	Screening patients in Nakhon Phanom Province
Trichuris trichiura	50	Screening patients in Nakhon Phanom Province
Enterobius vermicularis	50	Permanent slides[b]
Hookworm	50	Permanent slides[a]
Capillaria philippinensis	50	Permanent slides[b]
Opisthorchis spp.	50	Screening patients in Nakhon Phanom Province
Fasciola spp.	30	Permanent slides[a]
Paragonimus spp.	50	Permanent slides[a]
Schistosoma mekongi	30	Permanent slides[b]
Taenia spp.	50	Permanent slides[a]
Hymenolepis diminuta	50	Permanent slides[b]
Hymenolepis nana	50	Permanent slides[b]

[a] Samples obtained from the Department of Parasitology, Phramongkutklao College of Medicine, Thailand.
[b] Samples obtained from the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Thailand.

3. Results

In this study, 12 species of parasite eggs were collected and analyzed. The information about these samples are shown in Table 1. However, five groups of parasites, including hookworm, *Opisthorchis* spp., *Fasciola* spp., *Paragonimus* spp. and *Taenia* spp. were not clearly identified at the species level, because the morphology of the eggs was not distinctly different between the members of each group. To ensure the accuracy of the samples in this study, five types of parasites were classified only at group or genus levels.

3.1. Repeatability

The results of measurement error of the image set showed less than 12% error. The repeatability of egg shape based on normalized elliptic Fourier coefficients was 88%.

3.2. Allometry

The prediction of the contribution of size to shape is shown in Fig. 2. A scatter diagram reveals a positive correlation between size and shape, based on the slopes of the line upwards from left to right. This result indicated that the egg size affected the egg shape.

3.3. Discrimination by size

The global size variation of the complete contours of the eggs of 12 parasite species is shown in Fig. 3. *Fasciola* eggs were the largest (0.054 mm), followed by *H. diminuta* (0.030 mm), *Paragonimus* spp. (0.029 mm), hookworm (0.029 mm), *S. mekongi* (0.028 mm), *A. lumbricoides* (0.026 mm), *E. vermicularis* (0.024 mm), *T. trichiura* (0.023 mm), *H. nana* (0.019 mm), *C. philippinensis* (0.018 mm), *Taenia* spp. (0.016 mm), and *Opisthorchis* spp. (0.010 mm) (Table 2). Pairwise comparisons of the mean global size between parasite groups showed significant differences in 49 pairs out of 66 pairs (Table 3). However, validated classification based maximum likelihood among 12 parasite species revealed unsatisfactory results (30.18% of totally correct assignment), ranging from a

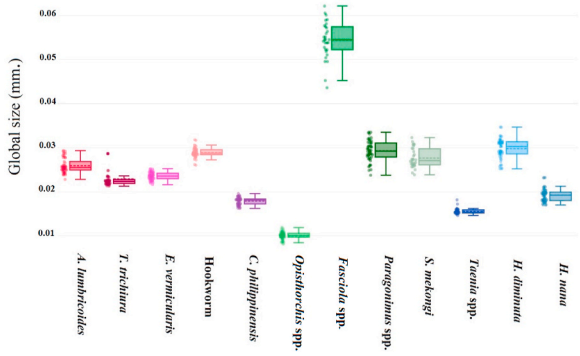

Fig. 3. Quantile boxes (25th and 75th quartiles) showing global size variation among the egg samples of 12 species. In each quantile box, the horizontal line indicates the group median and the dotted line indicates the group mean global size.

Table 2
Mean global size of egg contour among 12 parasite species.

Parasite eggs	n	Mean (mm.)	Standard Deviation
Ascaris lumbricoides	50	0.026	0.0017
Trichuris trichiura	50	0.023	0.0019
Enterobius vermicularis	50	0.024	0.0009
Hookworm	50	0.029	0.0011
Capillaria philippinensis	50	0.018	0.0008
Opisthorchis spp.	50	0.010	0.0008
Fasciola spp.	30	0.054	0.0042
Paragonimus spp.	50	0.029	0.0023
Schistosoma mekongi	30	0.028	0.0022
Taenia spp.	50	0.016	0.0006
Hymenolepis diminuta	50	0.030	0.0023
Hymenolepis nana	50	0.019	0.0016

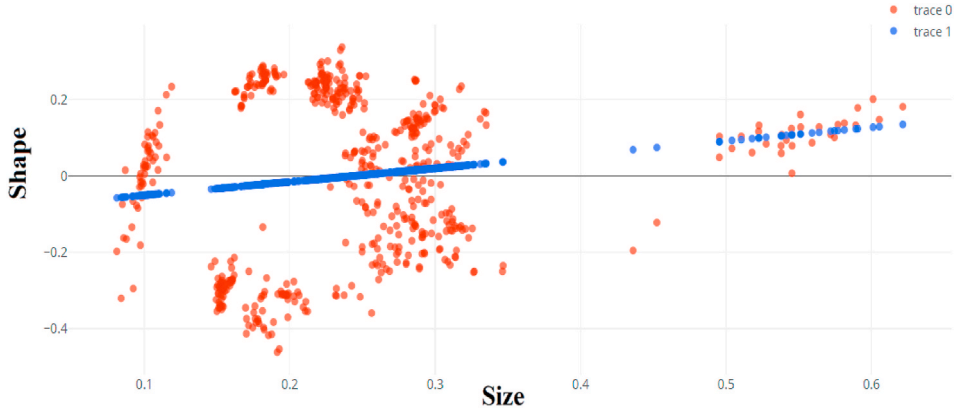

Fig. 2. Scatter diagram of linear regression prediction (blue dots) of the contribution of size to shape of parasite eggs in this study.

Table 3
Significant differences in the mean global size of eggs between pairs of parasite species.

	AL	TT	EV	HW	CP	OS	FS	PS	SK	TS	HD	HN
AL												
TT	0.107											
EV	0.209	0.750										
HW	0.122	0.003*	0.004*									
CP	0.000*	0.012*	0.000*	0.000*								
OS	0.000*	0.000*	0.000*	0.000*	0.000*							
FS	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*						
PS	0.065	0.001*	0.001*	0.823	0.000*	0.000*	0.000*					
SK	0.429	0.023*	0.067	0.606	0.000*	0.000*	0.000*	0.462				
TS	0.000*	0.000*	0.000*	0.000*	0.213	0.005*	0.000*	0.000*	0.000*			
HD	0.045*	0.001*	0.001*	0.681	0.000*	0.000*	0.000*	0.798	0.326	0.000*		
HN	0.001*	0.061	0.018*	0.000*	0.489	0.000*	0.000*	0.000*	0.000*	0.055	0.000*	

Abbreviations: AL = *A. lumbricoides*, TT = *T. trichiura,* EV = *E. vermicularis*, HW = hookworm, CP = *C. philippinensis*, OS = *Opisthorchis* spp., FS = *Fasciola* spp., PS = *Paragonimus* spp., SK = *S. mekongi*, TS = *Taenia* spp., HD = *H. diminuta*, HN = *H. nana*. An asterisk (*) after a value represents the statistical difference at $p < 0.05$.

Table 4
Correct reclassification scores (%) based on the global size of egg contours among 12 species derived from maximum likelihood analysis.

Parasite eggs	Percentage of correctly assigned individuals (assigned/observed)	Level of strength
Ascaris lumbricoides	52% (26/50)	Moderate
Trichuris trichiura	78% (39/50)	Substantial
Enterobius vermicularis	24% (12/50)	Low
Hookworm	26% (13/50)	Low
Capillaria philippinensis	6% (3/50)	Low
Opisthorchis spp.	0% (0/50)	Low
Fasciola spp.	100% (30/30)	Perfect
Paragonimus spp.	10% (5/50)	Low
Schistosoma mekongi	30% (9/30)	Low
Taenia spp.	0% (0/50)	Low
Hymenolepis diminuta	50% (25/50)	Moderate
Hymenolepis nana	14% (7/50)	Low
Total score	30.18% (169/560)	Low

Level of strength was modified from the scale proposed by Landis and Koch (1977), and Gómez and Correa (2017) which is divided into Low (less than 41%), Moderate (41%–60%), Substantial (61%–80%), Almost perfect (81%–99%), and Perfect (100%).

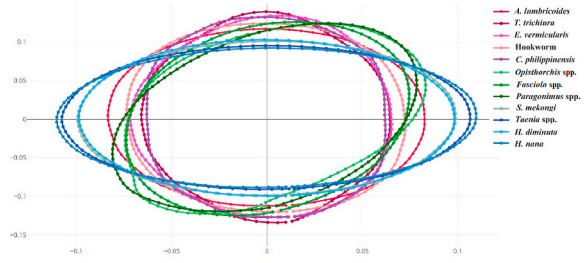

Fig. 4. Superposition of mean egg contours showing shape differences between the 12 parasite species.

minimum of 0% (*Opisthorchis* spp. and *Taenia* spp.) to a maximum of 100% (*Fasciola* spp.) (Table 4).

3.4. Discrimination by shape

After inverse Fourier analysis, Procrustes superposition was performed, to illustrate the estimated contours which showed shape differences in the 12 parasite species (Fig. 4). Discriminant analysis revealed variation in egg shapes among parasite species as presented in the factor map in Fig. 5. The factor map displays inter-group overlap, indicating that helminth eggs had similar external contours between parasites/genus/species (Fig. 5). The similarity in egg shape between groups was checked using the Mahalanobis values shown in Table 5. Comparisons of Mahalanobis distances between the pairs of parasite species showed significant differences in all pairs ($p < 0.05$, Table 5). A hierarchical clustering tree derived from the shape of the parasite eggs based on Mahalanobis distances identified three clusters of shape similarities (Fig. 6): cluster 1 including *A. lumbricoides*, hookworm, *E. vermicularis, C. philippinensis,* and *T. trichiura,* cluster 2 including *Fasciola* spp., *Paragonimus* spp., and *Opisthorchis* spp., and cluster 3 including *S. mekongi, H. diminuta, Taenia* spp., and *H. nana.* Correct reclassification scores based on the Mahalanobis distances of egg shape between species revealed satisfactory results (84.29% of total correct assignment), ranging from a minimum of 60% (*S. mekongi*) to a maximum of 100% (*E. vermicularis*) (Table 6).

4. Discussion

The identification of helminth eggs from feces is important to assess infection by many parasites in humans, especially soil-transmitted helminths. Copro-microscopy is the gold standard method for screening patients with parasitic infections in remote areas (Nikolay et al., 2014). However, it is difficult for inexperienced practitioners to identify the species of helminth eggs from patient stool specimens. This study provides the first GM analysis to distinguish between eggs of 12 parasites that are commonly found in human infections: *A. lumbricoides, T. trichiura, E. vermicularis*, hookworm, *C. philippinensis, Opisthorchis* spp., *Fasciola* spp., *Paragonimus* spp., *S. mekongi, Taenia* spp., *H. diminuta*, and *H. nana*. Although *Paragonimus* eggs are usually detected in sputum. However, their eggs are sometimes found in feces because coughed-up eggs are swallowed (Roy et al., 2015). Thus, this parasite is not excluded from the test. Generally, helminth eggs do not have a specific location for analytical coordinates, or are deficient in exact landmarks, and so cannot be analyzed using the landmark-based GM approach (Dujardin et al., 2014). Thus, the outline-based GM approach was used

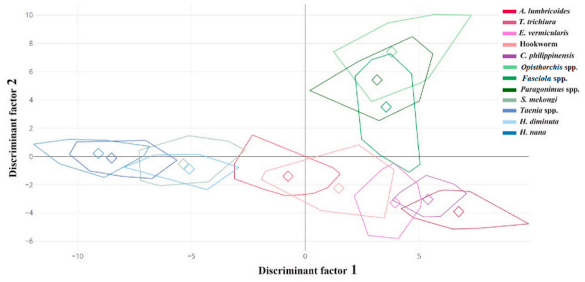

Fig. 5. Factor map of two discriminant factors based on discriminant analysis, showing shape variation among the 12 parasite species. Each color group is represented by its perimeter. The small squares in the perimeters represent the centroid of each population group.

Table 5
Mahalanobis distances based on egg shape between pairs of parasite species.

	AL	TT	EV	HW	CP	OS	FS	PS	SK	TS	HD	HN
AL	0.000											
TT	10.76	0.000										
EV	7.207	7.349	0.000									
HW	3.400	10.35	5.853	0.000								
CP	7.452	7.535	6.221	5.871	0.000							
OS	10.89	13.10	11.84	11.30	11.57	0.000						
FS	7.887	10.42	8.474	7.831	8.660	7.667	0.000					
PS	9.331	11.86	10.07	9.667	10.25	7.538	4.406	0.000				
SK	5.559	14.12	10.88	7.968	11.69	13.23	10.34	10.99	0.000			
TS	9.038	16.30	13.52	11.56	14.78	14.81	13.57	13.78	5.515	0.000		
HD	5.047	13.48	10.43	7.729	11.34	12.95	10.30	11.09	1.579	5.104	0.000	
HN	9.796	16.82	13.98	12.29	15.56	15.32	13.75	13.99	5.620	2.332	5.448	0.000

Abbreviations: AL = *A. lumbricoides*, TT = *T. trichiura*, EV = *E. vermicularis*, HW = hookworm, CP = *C. philippinensis*, OS = *Opisthorchis* spp., FS = *Fasciola* spp., PS = *Paragonimus* spp., SK = *S. mekongi*, TS = *Taenia* spp., HD = *H. diminuta*, HN = *H. nana*. Mahalanobis distance values close to zero indicate high similarity. All pairwise comparisons of Mahalanobis distances between parasite species were statistically significantly different ($p < 0.05$).

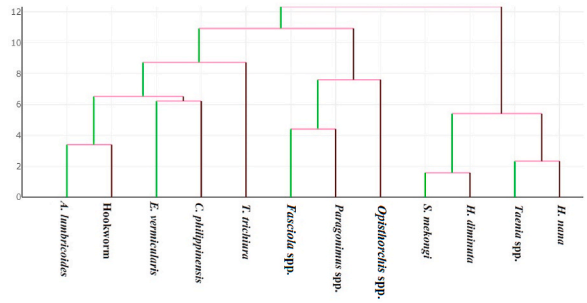

Fig. 6. Hierarchical clustering tree based on Mahalanobis distances between average egg shapes of the 12 parasite species.

Table 6
Correct reclassification scores (%) derived from Mahalanobis distances of egg shape among 12 species.

Parasite eggs	Percentage of correctly assigned individuals (assigned/observed)	Level of strength
Ascaris lumbricoides	90% (45/50)	Almost perfect
Trichuris trichiura	96% (48/50)	Almost perfect
Enterobius vermicularis	100% (50/50)	Perfect
Hookworm	90% (45/50)	Almost perfect
Capillaria philippinensis	92% (46/50)	Almost perfect
Opisthorchis spp.	94% (47/50)	Almost perfect
Fasciola spp.	83.33% (25/30)	Almost perfect
Paragonimus spp.	82% (41/50)	Almost perfect
Schistosoma mekongi	60% (18/30)	Moderate
Taenia spp.	70% (35/50)	Substantial
Hymenolepis diminuta	70% (35/50)	Substantial
Hymenolepis nana	74% (37/50)	Substantial
Total score	84.29% (472/560)	Almost perfect

Level of strength was modified from the scale proposed by Landis and Koch (1977), and Gómez and Correa (2017) which is divided into Low (less than 41%), Moderate (41%–60%), Substantial (61%–80%), Almost perfect (81%–99%), and Perfect (100%).

in this study.

An analysis of the contribution of size to species variation (or allometry) revealed a relationship between the size and shape variables of parasite eggs in this study. This relationship is common in living organisms, because size and shape are not independent attributes, but the correlation does not have enough power to cause an identification error based on the interspecific variation (Lorenz et al., 2017).

The outline-based GM analysis used in this study to identify parasite eggs was divided into two analytical parts: size and shape. The results of size analysis showed that this feature cannot be used as the main variable for the identification of parasite species at the egg stage (<31% overall accuracy indicated that the performance was poor). However, size investigations in this study indicated that only *Fasciola* eggs could be effectively distinguished from other parasite eggs (100% correct assignments) based on the validated reclassification. Therefore, the size variable was unsuitable for use in practice. This analytical outcome is consistent with research into other animals, which has found that size cannot be used for the classification of mosquitoes (Chaiphongpachara et al., 2018, 2019; Chaiphongpachara and Laojun, 2019, 2020), Triatomine bugs (Santillán-Guayasamín et al., 2017), fireflies (Sumruayphol and Chaiphongpachara, 2019), blow flies (Sontigun et al., 2017), *Stomoxys* flies (Changbunjong et al., 2016), and *Fasciola* flukes (Sumruayphol et al., 2020). The main reason for the unsatisfactory results of size GM analyses is size differences between species. The global size variation of egg contours (Fig. 3 and Table 2) and the comparisons of the statistical differences (Table 3) indicated that the eggs of many helminth species were very similar in size, such as hookworm (0.029 mm) versus *Paragonimus* spp. (0.029 mm, $p = 0.823$), *Paragonimus* spp. (0.029 mm) versus *H. diminuta* (0.030 mm, $p = 0.798$), *T. trichiura* (0.023 mm) versus *E. vermicularis* (0.024 mm, $p = 0.750$), and *H. diminuta* (0.030 mm) versus hookworm (0.029 mm, $p = 0.681$).

Conversely, the shape GM analysis produced more promising results, with more than 80% overall accuracy based on the validated reclassification, indicating that the performance was almost perfect. The complete external contour of the egg, even though oval in shape, is species-specific, and differences (shown in Fig. 4) were detected in all pairs of the 12 parasite species, using comparisons of the Mahalanobis distances ($p < 0.05$, Table 5). In previous research, the outline-based GM approach has been used to analyze the shape of two fluke species (*Fasciola gigantica* and *F. hepatica*) at the adult stage, and it was found that the body structure can be used for species detection (Sumruayphol et al., 2020). The shape analysis in this study suggested that not only the adult stage, but also egg shape can assist in species identification. As in previous investigations, García-Sánchez et al. (2020) found that the shape of the helminth eggs (*Trichuris* species) could be distinguished even if the eggs belonged to the same species, but were found in different hosts. Previous studies have successfully applied the outline-based GM technique to identify animal species from the shape of some parts of organs, for example, the scutum of Trombiculid mites (Sungvornyothin et al., 2019), the outline of adult fireflies (Sumruayphol and Chaiphongpachara, 2019), the outer edge of the fly pupa (Chaiphongpachara and Tubsamut, 2019), the shape of the carapace of turtles (Vitek, 2018), the head and pronotum of *Odontomachus* ants (Samung et al., 2022), and the shape of birds' eggs (Attard et al., 2018).

However, outline-based GM analysis of shape was not very effective in some parasite eggs, especially *S. mekongi*, which had 60% of

individuals correctly assigned based on the validated reclassification. The shape of an embryonated *S. mekongi* egg is nearly spherical (Mosimann et al., 1978), and is therefore similar to the eggs of many helminth species that are round, including *H. diminuta, Taenia* spp., and *H. nana*. Although *S. mekongi* eggs have a diminutive spine on the lateral axis, which is unique to the species, they are hard to digitize for GM analysis because this character may not be evident in a two-dimensional image. These results indicated that the effectiveness of GM varies by species of parasite, depending on the shape of the eggs.

A hierarchical clustering tree based on Mahalanobis distances in this study divided parasite species into three clusters based on egg shape similarities. This result of a clustering tree from GM analysis was consistent with the external morphology of the egg of parasite species. Three clusters of the classification tree based on shape differences included nematode egg cluster, fluke egg cluster, and cestode and *S. mekongi* egg cluster. A cluster of nematode eggs, which are round (*A. lumbricoides* and hookworm), barrel (*C. philippinensis* and *T. trichiura*) (Fischer et al., 2018) and asymmetric-shaped (*E. vermicularis*) eggs (Camacho and Reinhard, 2019), were similar to a cluster of fluke eggs (*Fasciola* spp., *Paragonimus* spp., and *Opisthorchis* spp.), which are ovoid and pyriform in shape (Lee et al., 2012). While a cluster of cestode and *S. mekongi* eggs was separated from the other two clusters.

In this way, treatment decisions can be made quickly, and can contribute to controlling the disease. However, further studies of the eggs of other helminth species should be carried out, because some eggs may be too similar to permit the detection of differences using the outline-based GM approach. In this study, only one form of *A. lumbricoides* was included: embryonated egg owing to mainly found in our field. However, there is a concern with another two forms of egg (unfertilized and decorticated) as well which will be investigated later. In addition, a major obstacle in the diagnosis of soil-transmitted and other intestinal helminths is the confusion between artificial contents (such as grain, pollen and medicine crystal) and helminth eggs (Pampiglione et al., 1987). Therefore, further studies using this technique to separate helminth eggs from pseudoparasites are needed.

5. Conclusions

The finding presented in this study demonstrated that outline-based GM analysis of shape could be applied to the identification of 12 pathogen parasites in the egg stage. This is the first time that outline-based GM has been preliminarily confirmed as a valuable approach to support copro-microscopic analysis, in order to effectively screen helminth eggs. However, this study is intended to describe GM technique and to show preliminary results. The use of this technique should be revalidated each time in every other setting, especially when other (similar) eggs are endemic in these settings and may cause erroneous results. Additionally, further studies with a larger set of helminth eggs and artefacts should be carried out to increase confidence in the identification of parasite species in the absence of local experts.

Authors' contributions

NS: planned the experiments, collected the specimen, analysis, discussion and preparing manuscript; MM: preparing samples, giving advice, preparing manuscript; KK: collected the specimen and analysis; SL: collected the specimens, analysis and preparing manuscript; TC: planned the experiments, collected the specimen, analysis, discussion, preparing manuscript and editing manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The author declares no conflict of interest.

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