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Differentiation of *Trichuris* species eggs from non-human primates by geometric morphometric analysis

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ABSTRACT

Human trichuriasis is a neglected tropical disease which affects millions of people worldwide, mostly living in low socio-economic conditions. Numerous studies have been conducted over the past 10 years to compare the different techniques for *T. trichiura* eggs detection. Our study provides the first geometric morphometric analysis for the specific detection of eggs of *Trichuris* sp. isolated from stools of macaque (*M. sylvanus)*, colobus (*C. g. kikuyensis)*, grivets (*C. aethiops)* and the Brazza's monkey (*C. neglectus*) from zoos in Spain. Principal Component Analysis (PCA) arises as an efficient method to determine *Trichuris* spp. eggs. The selected measurements to be included in the PCA were proposed for the first time in the present work, as far as we know, as we could not find previous studies reporting standardized parameters.

1. Introduction

Soil-Transmitted-Helminth (STH) diseases are caused by *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms. *T. trichiura*, also known as "whipworm" due to its characteristic body shape, is responsible of human trichuriasis, a neglected tropical disease which affects between 604 and 795 million of people worldwide, mostly living in low socio-economic conditions (Fenwick, 2012; Tahseen, 2018). It constitutes the major cause of childhood diarrhea and stunting of growth (Stephenson et al., 2000).

Trichuriasis, alongside the other soil-transmitted helminthiasis, is a public health issue, given massive scale of the problem. Within the broad tropical and subtropical belt, infection and re-infection are apparently unhindered despite large-scale public health programs (Motarjemi et al., 2014). However, trichuriasis is amenable to control in some cases through mass administration of anthelminthic drugs, often administered annually or twice a year (Webster et al., 2014). Large-scale deworming is necessary to reduce the worldwide morbidity of these infections, but without improved water supplies and sanitation, this approach cannot be relied on for sustainable reductions in parasite frequency or intensity of infection.

Coprodiagnoses to detect *T. trichiura* eggs is the most widely used approach for the detection of whipworm infection. Several examination techniques are available to detect the presence of *Trichuris* spp. eggs in faecal samples including direct faecal smear, formalin-ether concentration (FECM) (Ridley and Hawgood, 1956; Allen and Ridley, 1970), sedimentation after fixation with sodium acetate-acetic acid-formalin (SAF) (Gonçalves et al., 2014), Kato-Katz (Katz et al., 1972; Peters et al., 1980), McMaster (WHO, 2008), FLOTAC® (Cringoli, 2006), Mini FLOTAC® and FekPac® (Bosco et al., 2014; Godber et al., 2015).

All these techniques are, unfortunately, not able to differentiate eggs of different *Trichuris* spp. Then, when eggs in human or non-human primate stool are detected, they are identified as *T. trichiura*. When eggs are present in samples from dogs or swine, they are identified as *Trichuris vulpis* or *Trichuris suis*, respectively. Furthermore, there is evidence of human parasitism with species of *Trichuris* from dogs (*T. vulpis*) based on the presence of large eggs in human faecal samples (Corrêa et al., 1980). Nevertheless, Yoshikawa et al. (1989) observed that eggs obtained from the uteri of *T. trichiura* were of two sizes, so conclusions based on egg size alone should be treated with caution (Betson et al., 2015).

This scenario is further complicated by the latest discoveries concerning the *Trichuris* spp. involved in humans and primates. Thus, several authors (Ravasi et al., 2012; Liu et al., 2013; Cutillas et al., 2014; Callejón et al., 2017; Cavallero et al., 2019) reported the existence of various species circulating in the human population and a complex of

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species present in human and non-human primates (NHP). Thus, Bölükbas et al. (2014) concluded that the presence of *T. trichiura* in monkeys in zoos possess a high risk to zookeepers and also to visitors' welfare due to its zoonotic character, suggesting that an effective parasite control program should be established and stool control should be done regularly for primates.

Morphological studies of *Trichuris* isolated from primates and humans have concluded that the species infecting these hosts is the same, despite slight morphological variations that are distinguishable when scanning electron microscopy is used (Ooi et al., 1993).

García-Sánchez et al. (2019) found strong support for geometric morphometrics as a useful tool to differentiate male *Trichuris* populations parasitizing *Colobus guereza kikuyensis*, *Papio ursinus*, *Macaca sylvanus*, *Pan troglodytes* and *Sus scrofa domestica*. Nevertheless, this kind of analysis has never been applied to *Trichuris* eggs. In the present work, a morphometric study of the eggs of species of *Trichuris* Roederer, 1761 (Nematoda: Trichuridae) parasitizing Non-Human Primates (NHP) (*Macaca sylvanus*, *C. g. kikuyensis*, *Chlorocebus aethiops* and *Cercopithecus neglectus*) has been carried out using, for the first time, geometric morphometric tools.

2. Material and methods

2.1. Sample collection

Trichuris spp. eggs were obtained from four faecal samples from 4 individual primates species kept in captivity. The samples were retrieved from macaque (*Macaca sylvanus*) from Castellar Zoo (Cádiz, Spain), colobus (*Colobus guereza kikuyensis*) from Fuengirola Zoo (Málaga, Spain), grivet (*Chlorocebus aethiops*) from Selwo Aventura Park (Málaga, Spain) and Brazza's monkey (*Cercopithecus neglectus*) from the Córdoba Zoo (Córdoba, Spain). Previously, molecular analysis was carried out to identify the *Trichuris* species parasitizing macaque, colobus, Brazza's monkey and grivet (unpublished).

Coproanalyses were performed in two steps, starting with a direct examination of aliquots diluted in saline solution 0.9% and posteriorly a Telemann concentration technique (saline solution-ether-centrifugation) to sediment *Trichuris* spp. eggs. This technique, initially developed by Telemann (1908), is useful for concentrating helminths eggs, which helps to release them from the debris in which they are buried. The sediments obtained were stored in Eppendorf tubes with distilled water and examined afterwards under a stereoscopic microscope.

Initially, each identified egg was isolated with a micropipette, thoroughly washed with distilled water and immediately placed between microscope slides and coverslips with a water-based mounting medium to improve their image and preservation. In order to photograph the eggs without deforming them, they were dried for 24–48 h.

The measurements were made with a microscope (Leitz Dialux 20 EB) at 100x magnification and using image analysis software (ImagePro Plus, version 5.1 for Windows, Media cybernetics, Silver Spring, USA) for images captured by a digital camera (Nikon Coolpix 5400).

2.2. Morphological studies and metric data processing

88 eggs of *Trichuris* sp. were collected from the stool samples (Fig. 1):

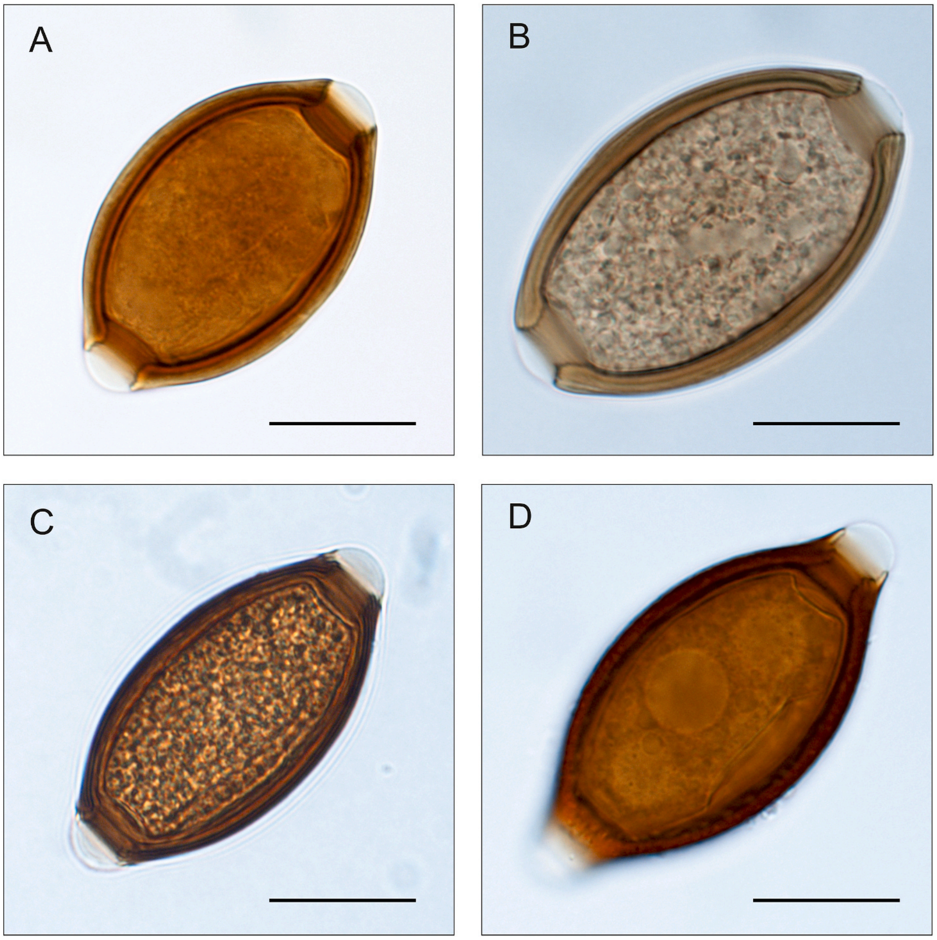

Fig. 1. *Trichuris* sp. eggs collected from the samples. A. Macaque (*Macaca sylvanus*) B. Colobus (*Colobus guereza kikuyensis*). C. Grivet (*Chlorocebus aethiops*. D. Brazza's monkey (*Cercopithecus neglectus*). The bar represents 20 μm.

31 from macaque, 26 from colobus, 24 from grivet and 7 from Brazza's monkey.

With the image analysis software, lineal biometric characters, areas and ratios of the eggs were obtained. The principal measurements were:

- Area (μ^2): egg area (A).
- Lineal biometric characters (μ): egg perimeter (P) and egg roundness (R).
- Size ratio (SR): size length over size width.

Egg roundness ($R = P2/4\pi A$) was used to measure the egg shape. Circular objects always have a roundness of 1.00, while objects that are irregular have larger values, indicating how circular an object is (Anonymous, 2001).

Other measurements obtained from each egg were available in the options of the image analysis software: Area/Box, Box X/Y, Center-X, Center-Y, Axis (major), Axis (minor), Diameter (max), Diameter (min), Diameter (mean), Radius (max), Radius (min), Radius Ratio, Perimeter2, Perimeter (convex), Perimeter (ellipse), Perimeter (ratio), Area (polygon), Center-X (mass), Center-Y (mass), Box Width, Box Height and Perimeter3.

In addition, other lineal measurements (Fig. 2) were specifically assayed in this study due to the lack of standardized parameters. These measurements consider the characteristic shape of these eggs, with their two distinct mucoid polar egg opercula (Fig. 2).

To guarantee the reliability of the study, the measurements of the eggs were always taken by the same researcher.

Morphological variation is quantified by geometric morphometrics (Rohlf and Marcus, 1993), a technique offering an estimate of size where different axes of growth are integrated into a single variable (the "centroid size'') (Bookstein, 1989). The estimate of size is contained in a single variable reflecting variation in many directions, as many as there are landmarks under study, and shape is defined as their relative positions after correction for size, position and orientation. With these informative data, and the corresponding software freely available to conduct complex analyses, significant biological and epidemiological features can be quantified more accurately (Dujardin, 2008). Current statistical techniques in morphometrics make it possible to test the null hypothesis of conspecific populations being simply the allometric extension of each other, provided a common allometric trend is identifiable (Rohlf and Marcus, 1993). Multivariate analyses were applied to calculate the phenotypic variations among whipworm eggs, using size-free canonical discriminant analysis on the covariance of log-transformed measurements to assess phenotypic variations between the samples. These analyses are applied to exclude the effect of within-group ontogenetic variations by reducing the effect of each character on the first pooled within-group principal component (a multivariate size estimator) (Dos Reis et al., 1990). The principal component analysis (PCA) is used to summarize most of the variations in a multivariate dataset in a few dimensions (Dujardin and Le Pont, 2004). The resulting "allometry-free", or size free, variables were submitted to a canonical variate analysis (CVA), and Mahalanobis distances were derived (Mahalanobis, 1936). The Mahalanobis distance is a statistical technique that can be used to measure how distant a point is from the center of a multivariate normal distribution. The degree of similarity between egg populations was assessed through pairwise Mahalanobis distances.

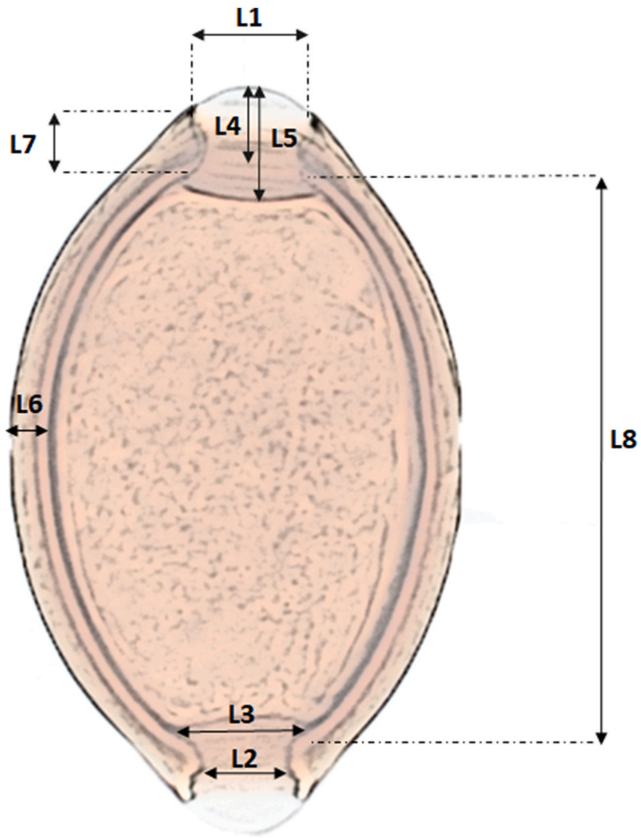

Fig. 2. ***Trichuris* sp. egg lineal measurements.** L1: maximum width of polar opercula, L2: minimum width of polar opercula, L3: base width of polar opercula, L4: length of polar opercula, measured from exterior midpoint to the narrow midpoint, L5: total length of polar opercula, measured from the exterior midpoint to the base midpoint, L6: wall thickness at its midpoint, L7: wall thickness in contact with polar opercula, L8: interior length of the egg.

Phenotypic analysis of *Trichuris* eggs was carried out by using several modules of the CLIC package version 97 (Dujardin and Slice, 2007), which is freely available at http://xyom-clic.eu/the-clic-package/and BAC v.2 software (Dujardin, 2002; Valero et al., 2009; García-Sánchez et al., 2019), and used for multivariate analyses of the morphometric data. Furthermore, Mahalanobis distances were calculated using CLIC software and tested by nonparametric permutation tests with 1000 iterations each.

A total of 45 measurements were evaluated initially. Considering that the results were statistically significant when $P < 0.05$, the following non-redundant measurements (one measurement is not included in another) for *Trichuris* eggs were used: L1, L2, L3, L4, L5, L6, L7 and L8, where at least one dimension was measured among the most remarkable morphological characters.

To investigate the statistical properties of the samples, a preliminary Shapiro-Wilk test for normality was carried out. If this test was not significant, the Student's *t* test was conducted; if the preliminary test rejects the null hypothesis of normality, the nonparametric Mann-Whitney *U* test was applied instead. All statistical analyses of the present study were performed with R commander (Rcmdr), the graphical user interface for R software, version 2.5-0.

3. Results

Previous molecular analysis determined the presence of *T. colobae* in colobus and *T. trichiura* in macaque and grivet (unpublished). No molecular identification was obtained for *Trichuris* sp. from Brazza's monkey.

Statistical tests showed several significant measurements for subsequent morphometric analyses. Therefore, twelve measurements for each population were used: area, perimeter, roundness, size ratio, L1, L2, L3, L4, L5, L6, L7 and L8 (Table 1).

The size of *Trichuris* eggs obtained from NHP samples is shown in Table 1. The study of the influence of the host species on egg phenotypic characteristics was carried out by PCA. *Trichuris* spp. variables all correlated significantly with PC1, contributing 61% to the overall variation in eggs.

Table 1
Biometric data of *Trichuris* eggs groups: macaque (*Macaca sylvanus*), colobus (*Colobus guereza kikuyensis*), grivets (*Chlorocebus aethiops*), the Brazza's monkey (*Cercopithecus neglectus*). Measurements are presented in µm.

	Trichuris sp. from macaque						*Trichuris sp.* from colobus					
	MAX	MIN	Median	B ± SD	CV	CI (95%)	MAX	MIN	Median	B ± SD	CV	CI (95%)
A	1675	934	1087	1128 ± 180	15.9%	1064.6–1191.4	2457	1294	1695	1770 ± 260[†]	14.7%	1664.9–1875.1
P	150	122	130	132 ± 6.90	5.2%	129.6–134.4	185	138	155	158 ± 9.69[†]	6.1%	154.1–161.9
R	1.40	1.07	1.26	1.25 ± 0.08	6.4%	1.22–1.28	1.21	1.08	1.14	1.15 ± 0.04[†]	3.5%	1.13–1.17
SR	2.37	1.49	2.07	2.02 ± 0.23	11.4%	1.94–2.10	1.98	1.46	1.68	1.72 ± 0.16[†]	9.3%	1.66–1.78
L1	9.54	7.29	8.10	8.14 ± 0.47	5.8%	7.97–8.31	12.02	8.50	10.45	10.36 ± 1.03[†]	9.9%	10.20–10.52
L2	7.84	4.87	5.91	5.96 ± 0.75	12.6%	5.70–6.22	10.44	6.35	7.86	7.90 ± 1.02[†]	12.9%	7.90–8.31
L3	10.59	7.16	8.22	8.55 ± 0.91	10.6%	8.23–8.87	15.15	9.62	11.79	11.99 ± 1.39[†]	11.6%	11.43–12.55
L4	5.95	3.89	5.10	5.07 ± 0.51	10.1%	4.89–5.25	5.81	1.96	4.08	3.97 ± 1.07[†]	26.9%	3.54–4.40
L5	10.11	5.91	9.06	8.77 ± 0.99	11.3%	8.42–9.12	10.08	5.21	8.41	7.99 ± 1.32[†]	16.5%	7.46–8.52
L6	3.53	1.56	2.67	2.67 ± 0.47	17.6%	2.50–2.84	4.12	2.03	2.79	2.90 ± 0.54	18.6%	2.68–3.12
L7	5.95	3.55	4.11	4.23 ± 0.49	11.6%	4.06–4.40	5.46	3.09	4.50	4.46 ± 0.49[†]	10.9%	4.26–4.66
L8	48.27	38.48	41.77	42.35 ± 2.59	6.1%	41.44–43.26	59.66	45.62	50.37	51.06 ± 3.14[†]	6.1%	49.79–52.33

	Trichuris sp. from grivet						*Trichuris sp.* from Brazza's monkey					
	MAX	MIN	Median	B ± SD	CV	CI (95%)	MAX	MIN	Median	B ± SD	CV	CI (95%)
A	1708	1023	1153	1208 ± 152[a]	12.6%	1143.8–1272.2	1488	1268	1364	1361 ± 76[a]	5.6%	1290.7–1431.3
P	152	126	133	135 ± 5.96[a]	4.4%	132.5–137.5	154	142	147	148 ± 4.56[a]	3.1%	143.78–152.2
R	1.31	1.10	1.24	1.23 ± 0.05	4.1%	1.21–1.25	1.42	1.22	1.31	1.31 ± 0.07	5.3%	1.25–1.37
SR	2.22	1.58	2.01	1.98 ± 0.16	8.1%	1.91–2.05	2.23	1.81	2.09	2.02 ± 0.15	7.4%	1.88–2.16
L1	9.20	7.71	8.41	8.36 ± 0.39	4.7%	8.20–8.52	8.47	7.16	8.11	7.98 ± 0.45	5.6%	7.56–8.40
L2	7.53	4.46	6.04	6.01 ± 0.64	10,6%	5.74–6.28	6.87	5.22	5.54	5.77 ± 0.56	9.7%	5.25–6.29
L3	10.59	7.10	8.64	8.79 ± 0.84	9.5%	8.44–9.14	10.84	5.86	8.77	8.40 ± 1.75	20.8%	6.78–10.02
L4	6.05	3.79	5.50	5.30 ± 0.60	11.3%	5.05–5.55	8.84	4.83	7.41	7.06 ± 1.38[a]	5.4%	5.78–8.34
L5	9.95	6.10	8.70	8.42 ± 0.86	10.2%	8.06–8.78	11.65	6.11	10.61	10.02 ± 1.85[a]	18.5%	8.31–11.73
L6	2.70	1.85	2.35	2.33 ± 0.23[a]	9.9%	2.23–2.43	3.52	2.08	2.55	2.61 ± 0.51	19.5%	2.14–3.08
L7	5.35	3.51	4.18	4.21 ± 0.43	10.2%	4.03–4.39	6.39	5.46	6.22	6.13 ± 0.33[a]	5.38%	5.82–6.44
L8	48.84	40.46	42.99	43.49 ± 2.03	4.7%	42.63–44.35	46.99	41.79	44.92	44.87 ± 1.78[a]	3.9%	43.22–46.52

[a] Significant differences between *Trichuris* sp. from *colobus*, *Trichuris* sp. from grivet and *Trichuris* sp. from Brazza's monkey compared to *Trichuris* sp. from macaque ($P < 0.05$). A: Area, P: Perimeter, R: Roundness, SR: Size ratio, L1-L8: Egg measures, B: Arithmetic mean, SD: Standard deviation, CV: Coefficient of variation, CI (95%): Confidence interval at a 95% confidence level.

The resulting factor map (Fig. 3) clearly illustrates global size differences in the colobus population versus the other NHP populations, presenting a bigger size in the former. This factor map patently illustrates three zones, corresponding to: a) an area covering eggs from colobus and grivet, which confirms a similar morphological identity; b) an area covering eggs from Brazza's monkey, with only a partial overlap with the previous area; and c) an area corresponding to colobus.

The degree of similarity between egg populations was assessed through pairwise Mahalanobis distances. These distances were calculated comparing eggs from the host species with each other (Table 2). When comparing eggs of *Trichuris* spp., larger distances were detected in the case of macaque vs Brazza's monkey, macaque vs colobus, colobus vs Brazza's monkey and grivet vs Brazza's monkey than in macaque vs grivet and colobus vs grivet. This could mean that grivet is the least divergent community. These results agree with the analysis observed in Fig. 3.

4. Discussion

Trichuriasis has always been described as tropical or subtropical

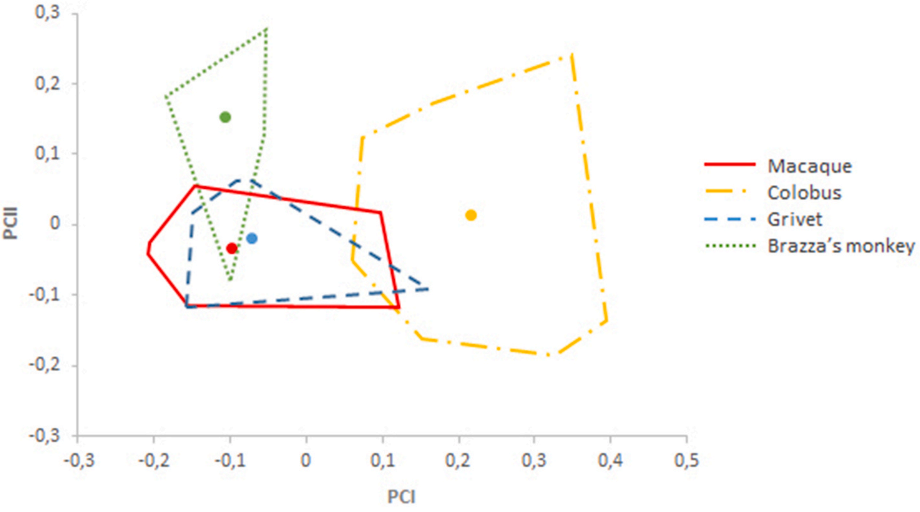

Fig. 3. Factor map corresponding to *Trichuris* sp. eggs derived from different host primate species: macaque (*M. sylvanus*), colobus (*C. g. kikuyensis*), grivets (*C. aethiops*) and the Brazza's monkey (*C. neglectus*) from zoos in Spain. Samples are projected onto the first (PC1, 61%) and second (PC2, 18%) principal components. Each group is represented by its perimeter. Circles represent the centroid in each community.

Table 2
Mahalanobis distances between *Trichuris* sp. egg groups: *Trichuris* sp. isolated from macaque (*M. sylvanus*), *Trichuris* sp. from colobus (*C. g. kikuyensis*), *Trichuris* sp. from grivet (*C. aethiops*) and *Trichuris* sp. from Brazza's monkey (*C. neglectus*).

	Macaque	Colobus	Grivet	Brazza's monkey
Macaque	0.00			
Colobus	3.57	0.00		
Grivet	1.03	3.49	0.00	
Brazza's monkey	4.33	5.36	3.78	0.00

disease. However, the increase in international travel as well as the arrival of new immigrants have made some tropical diseases realities in developed countries as well. Quick diagnosis has always been a priority to determine the appropriate treatment and prevent fatalities. In addition, now more than ever, advances in diagnostics can help prevent transmission and provide active surveillance. Unfortunately, there have been few major advances in specific diagnostic methods for parasitic infections (Ricciardi and Ndao, 2015).

The differentiation of *Trichuris* spp. is an arduous task. For decades, this identification was based on host the specificity and/or on the morphological characteristics (typical "whip" shape) of the adults of this genus. However, in many cases, these morphological values overlap, and it is difficult to differentiate these species. In fact, several cryptic species (Callejón et al., 2012), synonymies (Oliveros et al., 2000), or new species (Cutillas et al., 2014; Callejón et al., 2017) have been defined in *Trichuris*. The more discriminating molecular methods have shown that not all species previously described by morphometrics will remain truly defined species (Salaba et al., 2013).

Our study provides the first geometric morphometric analysis of eggs of *Trichuris* sp. isolated from stools of macaque (*M. sylvanus)*, colobus (*C. g. kikuyensis)*, grivets (*C. aethiops)* and the Brazza's monkey (*C. neglectus*) from zoos in Spain. PCA arises as an efficient method to analyze *Trichuris* sp. eggs. The selected measurements to be included in the PCA were proposed for the first time in the present work, as far as we know, as we could not find previous studies reporting standardized parameters. Through PCA, we achieved the differentiation of *T. colobae*, obtaining a well-defined area that allows its identification, presenting only a partial overlap zone with *Trichuris* sp. from macaques and grivets. Furthermore, molecular studies corroborated these results. Thus, Cutillas et al. (2014) proposed the existence of a new species of *Trichuris* parasitizing *C. g. kikuyensis*: *Trichuris colobae*. Furthermore, *Trichuris* sp. from grivet and macaque was demonstrated to be *T. trichiura* by molecular analysis based on ribosomal and mitochondrial markers (unpublished data).

In addition, *Trichuris* sp. from Brazza's monkey appeared distant from that from colobus, with a partial overlapping area with *Trichuris* sp. from macaque and grivet, but again with a safe assumption of the differentiation of this whipworm from the others. A difficulty in the analysis of this group was that the stool sample was poorly loaded with *Trichuris* eggs and, therefore, only few eggs could be obtained. This fact could reflect climatic and ecological influences on the lifecycle of parasites, as well as potential changes in diet (McLennan et al., 2017) since primates were in different Zoos of Spain. Thus, Rothman et al. (2009) reported that a pinworm, was negatively affected by the level of condensed tannins in the diet, suggesting that the level of tannins may impact this parasite of the gorillas. Given that *Trichuris* sp. from Brazza's monkey clearly separates in this case, it would be interesting in the future to obtain more eggs of this species to carry out a more consistent PCA analysis followed by molecular studies. Furthermore, we could suggest a possible different species of *Trichuris* parasitizing to the Brazza's monkey.

On the other hand, the factor map did not show global size differences between macaques and grivets' *Trichuris* spp., with complete overlapping areas that did not allow the differentiation of these eggs. Both *Trichuris* populations have been molecularly identified as *Trichuris trichiura* (unpublished data), which is the reason that explains this superposition. This fact also clarifies why *Trichuris* eggs from grivet is the least divergent community, considering the values of Mahalanobis distances between *Trichuris* egg groups.

This new identification method allows determining specifically, as molecular studies, which species of *Trichuris* is involved in the transmission of Trichuriasis to Non-Human Primates. Therefore, it remarks its importance in epidemiological studies, since it will assist in the identification and control of *Trichuris* in endemic settings. The potential transmission of infectious agents from monkeys and apes to humans is why the study of primate parasites is so significant.

5. Conclusions

This is the first approximation to distinguish *Trichuris* spp. studying the eggs with morphometric analysis. This method is presented as a useful tool to be applied on this field, since it allows to safely differentiate *Trichuris* spp. eggs isolated from different hosts stools. The method also confirmed its usability and utility since the same species appear overlapped in the factor map. Geometric morphometric analysis represents a new methodology that could be applied to future studies to deepen into the relationship between *Trichuris* and their host species.

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Declaration of competing interest

No competing interests exist.

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