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Demonstrating morphometric protocols using polystome marginal hooklet measurements

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Abstract

The flexible body structure of polystomes (Monogenea: Polystomatidae) encumbers taxonomic classification and species identification. Large intraspecific and limited interspecific variation in the morphology of polystomes further complicates the identification of species. Apart from employing the host-specific nature of the polystomes, taxonomic characterisation relies heavily on the sclerotised skeletal structures, such as the hamuli and the marginal hooklets. The currently accepted measurement system for marginal hooklets appears to be non-optimal and an improved protocol is needed. This paper describes in detail how such a protocol is found by evaluating various sets of measurements statistically in order to identify the most informative combination of parameters. Thirteen measurements of marginal hooklets from 11 different Southern African *Polystoma* species were taken and evaluated. A new protocol for discriminating between species of *Polystoma* Zeder, 1800, that employs only three measurements, is proposed. The value of the processes to derive morphometric protocols, as described, is that it is not restricted to a specific taxon, but that it can be amended and applied to any taxonomic grouping.

Introduction

Within the biological sciences morphometrics forms the basis of taxonomy. Organisms are frequently described based on measurements or combinations of measurements and ratios between measurements. However, far too often measurements are taken simply because they are measurable or because previous workers have measured certain features. Various statistical classification techniques were used to build classification rules and to assess their likely performance in the classification of new species (McLachlan, 1992). Furthermore, boundaries between species are often poorly defined because of: (1) few distinguishable morphometric features; (2) the simplicity of the forms under study; (3) overlaps between the species in morphometric space; and (4) large variation in morphometric features within a species (Anton & Duthie, 1981).

Flatworms are known for their soft, flexible body structure. The degree to which a specimen is flattened during the fixation process has a definite effect on the body measurements. As a result, body measurements are not always very informative for distinguishing between species. Flatworms do, however, possess skeletal structures consisting of scleroproteins (Hyman, 1951), and these structures are frequently used as taxonomic characters. The flatworm class Monogenea is one such group where measurements of skeletal structures are often used to distinguish between species.

Monogeneans are primarily fish parasites but one family, the Polystomatidae Carus, 1863, is known to mainly infect anurans and freshwater turtles. They are also known from salamanders, the Australian lungfish and the African hippopotamus. Polystomes are known for intraspecific variation

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and limited interspecific variation in morphological characters (Tinsley, 1973). As a result, high emphasis is placed on the sclerotised skeletal structures of parasites as taxonomic characters. These sclerotised parts include the penial spines used in copulation, the pair of hamuli or large hooks that are present in the majority of polystome genera which are used for attachment to the host, and the marginal hooklets. These 16 marginal hooklets enable the oncomiracidium to get a firm grip on the host. Although the marginal hooklets appear to be non-functional in mature parasites, they are retained in the tissue and can often still be measured in flattened specimens. Since the earliest descriptions of this group, the number, position, shape and length of especially the posterior-most pair of marginal hooklets were noted.

Marginal hooklets of polystomes are important taxonomic characters as they stay constant throughout the development of the parasite and their morphology is stable within a species, although there may be differences between species (Murith, Miremad-Gassmann & Vaucher, 1978). During the 1970s and early 1980s various French researchers were active in Africa studying polystomes. However, probably the most productive single contributor to the knowledge on polystomatids of anurans in Africa was Tinsley, who published with co-authors on various aspects of the taxonomy and biology of *Eupolystoma* Kaw, 1950, *Polystoma* Zeder, 1800 and *Protopolystoma* Bychowsky, 1957. It has now reached a stage where there are currently more than 30 species of *Polystoma* known from African anurans. Consequently, it is becoming increasingly difficult to differentiate between species and this places an even higher demand on species-specific taxonomic parameters.

Murith made a major contribution to the systematics of *Polystoma*. She recognised the potential of using the stability of the marginal hooklets as a specific taxonomic character. Murith (1979) studied the posterior-most pair of marginal hooklets referred to as the C1 hooklets of *Metapolystoma cachani* (Gallien, 1956) Combes, 1976 as well as *Polystoma baeri* Maeder, Euzet & Combes, 1970, *P. dorsale* Maeder, Euzet & Combes, 1970, *P. ebriense* Maeder, 1973 and *P. mangenoti* Gallien, 1956 in a study of polystome systematics and proposed a set of measurements to be taken when comparing different polystome species (Figure 1). Based on marginal hooklet measurements, Murith (1979) was able to point out that various polystome species infect the same host species, *Dicroglossus occipitalis*. This was surprising, as polystome parasites are known to be host specific (Bourgat & Salami-Cadoux, 1976; Combes, 1966; Combes & Channing, 1979; Du Preez & Kok, 1997; Euzet, Combes & Batchvarov, 1974; Kok & Du Preez, 1987; Kok & Van Wyk, 1986; Maeder, 1973; Maeder, Euzet & Combes, 1970; Murith, 1982; Tinsley, 1974). Bourgat & Murith (1980) measured the total length and handle length of marginal hooklet C1 for a number of species. Murith (1981) took this a step further by calculating the ratio of total length against handle length for marginal hooklet C1 (a/b ratio). She compared the proposed set of measurements for 12 polystome species in the Ivory Coast and showed that marginal hooklet morphometrics can be a very useful tool to identify or separate species. In South Africa, Kok, Du Preez and coworkers mainly followed Murith (1981) in accepting the importance of the larval and adult haptoral sclerites as structures which should be quantified (see Du Preez & Kok, 1992, 1993, 1995; Du Preez & Lim, 2000; Du Preez, Vaucher & Mariaux, 2002; Du Preez, Tinsley & De Sa, 2003; Kok & Seaman, 1987; Kok & Van Wyk, 1986; Lim & Du Preez, 2001; Van Niekerk, Kok & Seaman, 1993). There are, however, a few problems with the measurement system as proposed by Murith. Measurement b (Figure 1) has a low repeatability and is not objectively measurable, as it is a measurement from a fixed point to an estimated position at the centre of the base of the guard. The same could be said for measurements e+ and e− (Figure 1).

The research objective of this paper was to search for an improvement on the set of five measurements required by Murith, in order to provide an improved classification protocol based

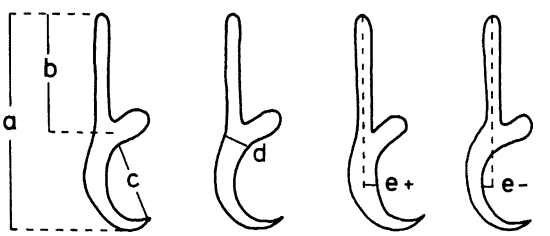

Figure 1. Diagram illustrating the parameters as suggested by Murith (1979). Figure taken from Murith (1979).

on the measurements of the marginal hooklet sclerites. It will be considered an improvement over Murith's protocol, if (1) fewer than five measurements are needed, (2) the measurements have higher *measurability*, in a sense to be described below, and if (3) the proposed set has a higher *classification potential* (to be described in detail below). The search method was applied to eleven species of southern African *Polystoma* species only.

Describing an improved classification protocol for polystomes is only part of the purpose of this paper. More importantly, the search method for obtaining a set of useful characters to be employed in a discrimination scheme is described, so that this method may be adapted and used for other taxonomic classifications based upon morphometric features.

Classification based on measurements is in essence a multivariate problem and may be approached using various multivariate techniques. However, we have in mind a simple classification protocol (in the style of Murith) intended for use by zoologists with only moderate computing facilities available (i.e. we assume no sophisticated statistical or numerical software packages). The suggested protocol is therefore based on a bivariate approach and utilises only simple mathematical expressions, which could possibly be calculated with a handheld pocket calculator only, if necessary. However, while application of the protocol does not require intensive computation, devising the protocol still requires some computation and suitable software.

Materials and methods

Parasite material

Lactophenol preparations were prepared of oncomiracidia of 11 Southern African *Polystoma* species. These included *Polystoma australe* Kok & van Wyk, 1986, *P. claudecombesi* Du Preez & Kok, 1995, *P. dawiekoki* Du Preez, Vaucher & Mariaux, 2002, *P. marmorati* Van Niekerk, Kok & Seaman, 1993 *P. natalense* Combes & Channing, 1979, *P. sodwanense* Du Preez & Kok, 1992, *P. testimagna* Du Preez & Kok, 1993, *P. umthakathi* Kok & Seaman, 1987, and three undescribed species referred to as spp. A, B and C, respectively, from the anuran hosts *Hyperolius tuberilinguis* Smith, *Cacosternum nanum* Boulenger and *Ptychadena oxyrhynchus* (Smith).

Digital images

One hooklet of the posterior-most pair of marginal hooklets (C1) of 10 specimens per species were digitally photographed using a Nikon Coolpix 4500 camera mounted on a Nikon E800 compound microscope. For each set of photographs a stage micrometer with calibration scale was photographed and used to calibrate measurements. Digital images were examined and measured using the Scion-Image image analyser program. A total of 107 hooklets were photographed and measured. Only hooklets that were photographed in a flat orientation were used. As a point of departure in the exercise of finding good parameters, 13 measurements were taken from each hooklet (Figure 2). Measurements 1 and 6 were taken from Murith (1979), while the remainder are novel measurements.

Results

Developing a classification protocol

The deciding factors determining which measurements should be used may be divided into two main categories: (1) their *measurability* and (2) their *classification potential*. Each of these categories will be discussed below.

Measurability of the parameters

As far as measurability is concerned, we propose that the following considerations need to influence the choice of measurements. Good parameters should be:

(1) Easily measurable: It is advisable to include only length measurements that can be taken under a microscope using a standard eyepiece graticule. Angles or other sophisticated quantifiers should be avoided. These length measurements should be either the distance between clearly identifiable points on the hooklet (e.g. parameter p_2 in Figure 2) or a perpendicular distance from a reference line (e.g. parameter p_4 in Figure 2).

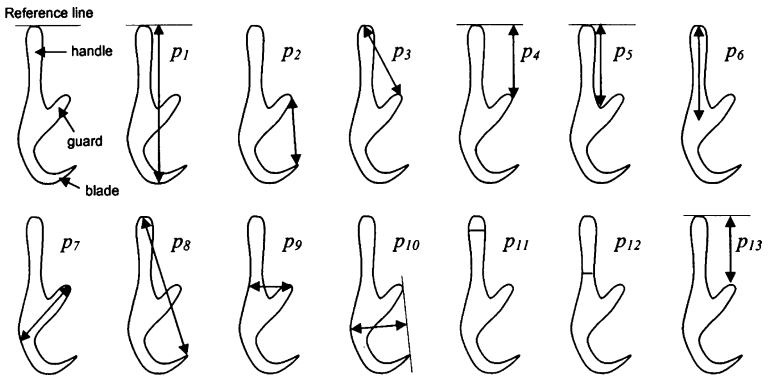

Figure 2. Diagrammatic illustration of the 13 marginal hooklet measurements evaluated. Parameters are labelled: p_1, p_2 ,...,p_{13}. p_1 represents the length of the hooklet; $p2$ the tangent from the tip of the blade to the guard; $p3$ the distance from the tip of the guard to the tip of the handle; $p4$ the distance from a position where a tangent between the tip of the blade and the guard would touch the guard to the reference line; $p5$ the distance from the corner between the handle and guard to the reference line; $p6$ the distance from the tip of the handle to the mid-point of the guard base; $p7$ the distance through the centre of the guard to the edge of the hooklet; $p8$ the distance from the tip of the blade to the tip of the handle; $p9$ the width of the hooklet at the level of the guard measured along a line perpendicular to the long axis of the hooklet; $p10$ the distance from a tangent to the back of the blade curve; $p11$ the widest point of the handle; $p12$ the narrowest point of the handle; $p13$ the shortest distance between the reference line and the guard.

(2) Repeatable: Parameters where a point to which a length is measured is subject to interpretation by the researcher should be avoided. Parameter p_6 in Figure 2 is an example where the choice of where the centroid of the guard base lies may be chosen differently by different researchers. Parameter p_7 is another example where the repeatability of the measurement may be low.

(3) Not be geometrically redundant: Figure 3 shows an example that illustrates this point. If a and b have been measured, it is unnecessary to measure c, as well, since a, b and c are related by $b^2 + c^2 = a^2$. This means that, by also measuring c, no extra information is added. It may not be possible to avoid more complicated geometric relationships, but obvious relationships should be avoided.

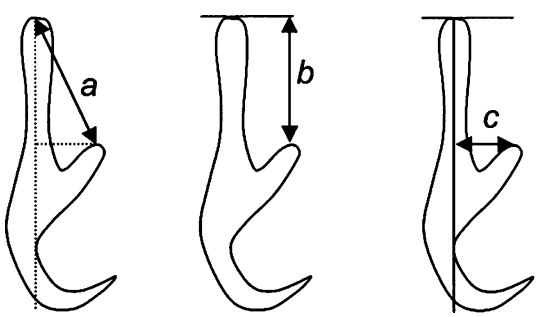

Figure 3. Diagram to illustrate a geometric interrelationship of a, b and c.

(4) Representative: Representativeness is a measure of how well the shape of the hooklet can be reconstructed from the chosen set of measured parameters. As an example, a set consisting of only parameters p_4, p_5 and p_6 is not very representative, since they are all approximately vertical measurements relating the top of the hooklet to a point somewhere in the middle. It would be impossible to reconstruct a reasonable shape from only these three parameters, since this set does not include the overall length or any parameter that gives some indication of how wide the hooklet is. A more representative set would be, for example, parameters p_1, p_3 and p_8.

(5) Non-negative: Parameter e, as proposed by Murith (1979) (Figure 1), may become negative. Mathematical classification systems do not mind negative parameters. However, since the measurement is taken by a human researcher, interpretation of which side is negative may cause confusion. Usually, the sign of a measurement indicates whether a point lies to the right or to the left of (or alternatively above or below) a certain reference line. Interpretation of which side is right or left may be confusing, especially when some images are upside down. It is not suggested here that a very useful measurement, which may become negative and cannot be represented in any other way, must be omitted. However, if there is some room to choose, it is best not to allow any

parameter to become negative. It is therefore recommended that one should choose simple distances between well-defined points and avoid perpendicular distances from reference lines that run through the hooklet.

It should be clear that many of the 13 parameters shown in Figure 2 could be discarded immediately on account of their low measurability. We have, however, used all in this study and attempted to discard them in a systematic way based on how well they can be used to separate the various species. In the end, when two parameters are found to be approximately equally useful for separation, the one with the better measurability has been retained and the other has been discarded.

Classification methods

The aim of this section is to devise a suitable representation of the parameters such that some form of grouping appears in the representation. Naturally, a good representation will be one where the parameters falling in each group belong to the same species, i.e. the misclassification rate should be as low as possible. This is equivalent to requiring that the individual groups are tight and that different groups lie far apart.

In view of our objective of providing a simple bivariate discrimination scheme, we shall employ the method here of plotting two parameters as x- and y- co-ordinates against each other on vertical and horizontal axes, respectively. Alternatively the x- and y- co-ordinates need not be parameters themselves, but may consist of mathematical expressions involving all or some of the parameters; for example, it may very well be that $p_1 \times p_4$ against $p_9 + p_3$ provides a far better separation of the groups than plotting any two individual parameters against each other.

It must be emphasised that we are not doing a statistical cluster analysis, since the species are already classified and it is *a priori* known to which species each given parameter belongs.

Discrimination analysis

Discrimination analysis (in particular the method of minimising the expected cost of misclassification – see Johnson & Wichern, 1988) has been attempted. However, because of the small sample sizes, the method does not appear to give acceptable results, and a different method is proposed. We shall discuss the results briefly.

Figure 4 shows the results of discrimination analysis for the 11 species, using parameters p_9

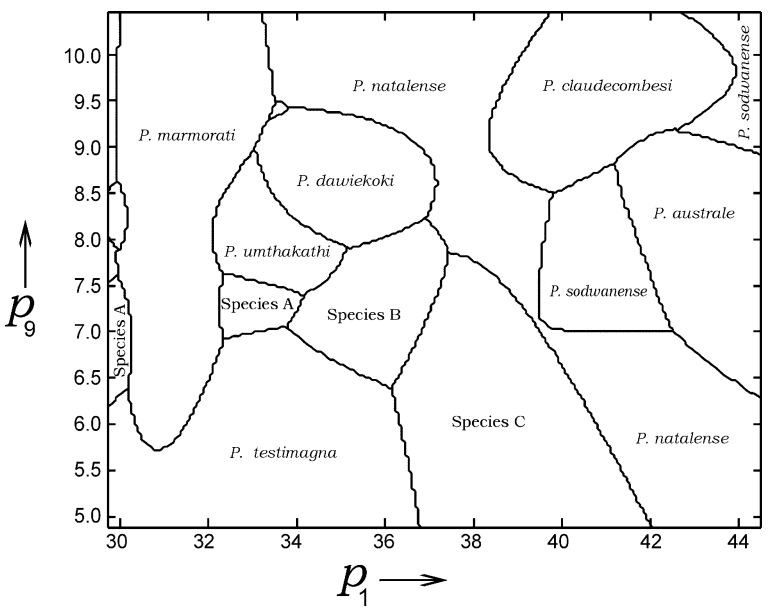

Figure 4. Resultant plot of discriminant analysis of all 11 species based on p_9 against p_1. Each patch shows a region where the species, whose name is printed in the region, has the highest discriminant score.

and p_1. For simplicity, we have assumed that the cost of misclassification is the same for each species and that the prior probability that a point belongs to a species is proportional to the sample size of that species. Each specimen in the ranges for p_1 from 29.8 to 44.5 and for p_9 from 4.8 to 10.5 has been assigned to a particular class. The results are interesting but disappointing.

The method of obtaining separation of the species by this method using only two parameters (p_9 and p_1 in the example) suffers from a number of weaknesses:

(1) The calculation of the regions requires intensive computation.
(2) The normal distributions were calculated from very small samples (no more than 10 specimens per species) and therefore any additional data may alter the regions significantly (i.e. the boundaries of the regions are not very fixed).
(3) The regions are geometrically irregular patches.
(4) Some regions split into two or more disjointed patches, where one patch is obviously not representative of the specimens of the species even though its discriminant score is the greatest of all species in that region. (For example *P. sodwanensis* lies in the vicinity of $p_1 = 41$ and $p_9 = 8$, while points in the upper right corner of the bounding rectangle, which would obviously belong either to *P. claudecombesi* or *P. australis*, are also assigned to *P. sodwanensis* by this method.) This is perhaps the most undesirable feature of this method. It is not clear how one can assign a measure of goodness of classification to this separation. In view of the fact that our prime purpose is to decide which parameters separate the species best, this method does not help much.

We therefore propose a simplified method based only on counting points inside confidence ellipses.

Classifying potential based on confidence ellipses

The calculation of a confidence ellipse is a standard procedure in multivariate statistics. However, a short explanation of what it means and how it is obtained may be appropriate here. Consider two parameters of a single species, call it x and y, and assume the points are plotted on a system of axes. Let $\bar{x}$ and $\bar{y}$ be the sample mean values of the x- and y- parameters respectively. When x and y for a single species are plotted against each other, it is expected that the points will fall in a group centred about the centre point $(\bar{x}, \bar{y})$. Figure 5 shows an example where p_2 (as y) is plotted versus p_1 (as x) for the 10 measurements obtained from the *P. claudecombesi* specimens. The centre point, given by (40.51,17.20) in this case, is indicated by a $\oplus$ symbol. Also shown is an ellipse that is centred on the point $(\bar{x}, \bar{y})$ and that circumscribes all points. The assumption is made that the points have a bivariate normal distribution. This means that, as more and more points are sampled and plotted, the density of points will approach the shape of a mound described by the normal distribution function for two variables. Figure 6(a) shows the expected density for p_2 versus p_1 of *P. claudecombesi* as derived from the 10 data points available for this species.

The contours (of equal probability) of the probability function are concentric ellipses all having $(\bar{x}, \bar{y})$ as their centre. These concentric ellipses are also shown in Figure 6(a). These ellipses are referred to as *confidence ellipses*. Figure 6(b) shows an example of simulated points plotted on a system of axes together with a set of confidence ellipses of various sizes. These ellipses are all scaled versions of a standard ellipse whose shape and orientation are determined by the data.

Each confidence ellipse is scaled by a factor called the chi-value, denoted by χ. For the standard ellipse, $\chi = 1$. The percentage of points that is expected to lie inside the ellipse is determined by the chi-square function. For the sake of completeness we give the function here. If p is the percentage, expressed as a fraction, then $p = 1 - e^{-\frac{1}{2}\chi^2}$. For example, for an ellipse with $\chi = 1$, it is expected that 39.35% of the points will lie inside it. For an ellipse stretched by a factor two, i.e. $\chi = 2$, it is expected that 86.47% of the points will lie inside it. If one wants to know the factor for which it is expected that a certain percentage of the points lie inside it, then the inverse function, given by $\chi = \sqrt{2 \ln\left(\frac{1}{1-p}\right)}$, may be

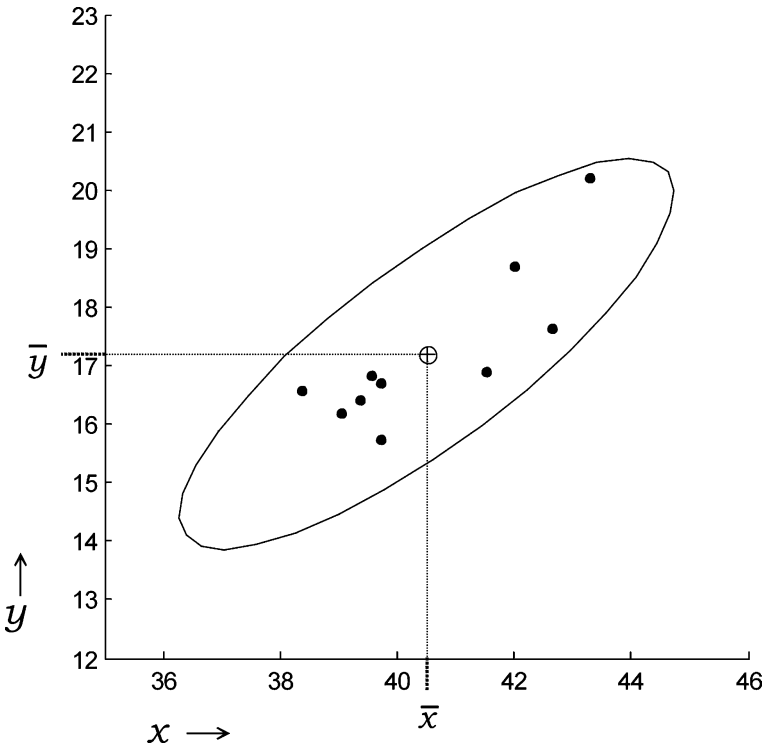

Figure 5. An example where p_1 (as x) is plotted versus p_2 (as y) for the 10 measurements of the species *Polystoma claudecombesi*.

used. For example, in order to have 95% of the points lie inside the ellipse, $\chi = 2.4477$.

Although it is customary to label the ellipse by the percentage of points that is expected to lie inside it, we shall for the purpose of this paper not use the percentages to label ellipses, but rather the chi-value itself. One may therefore simply consider χ to be a scaling factor that scales the size of the ellipse.

When the x and y parameters of two species are plotted on the same axes, confidence ellipses for each species may be drawn centred on the mean points for each species, as shown in Figure 7. Although different scaling factors may be used for different species, we have used the same value of χ for both ellipses. Figure 7 shows the points (x,y) of an imaginary species A together with its confidence ellipse (in a thick, solid line). The confidence ellipse of another imaginary species B is plotted (in a dashed line) without its points. The scaling constant was $\chi = 1.1$ for both species.

The points of species A may be classified into four categories:

(1) points only inside the confidence ellipse of A (points 1 and 2), i.e. points correctly classified,
(2) points only inside the confidence ellipse of B (points 3 and 4), i.e. points misclassified,
(3) points which lie inside both ellipses (points 5 and 6), i.e. points with double classification (or more generally if there are more ellipses, points with multiple classification), and
(4) points which lie outside both ellipses (points 7, 8 and 9) i.e. points not classified.

For each species the number of points correctly classified, incorrectly classified and not classified may be counted. Let N_{cor} be the total number of points correctly classified, let N_{mis} be the total number of points misclassified, let N_{mult} be the total number of points with multiple classification, and let N_{not} be the total number of points not classified. Obviously, a good classification will have N_{cor} as large as possible and the other counts as small as possible.

In order to find a good classification, we construct a *classification potential* function, which we shall label $P(N_{cor}, N_{mis}, N_{mult}, N_{not})$, such that it increases when N_{cor} increases, and decreases when

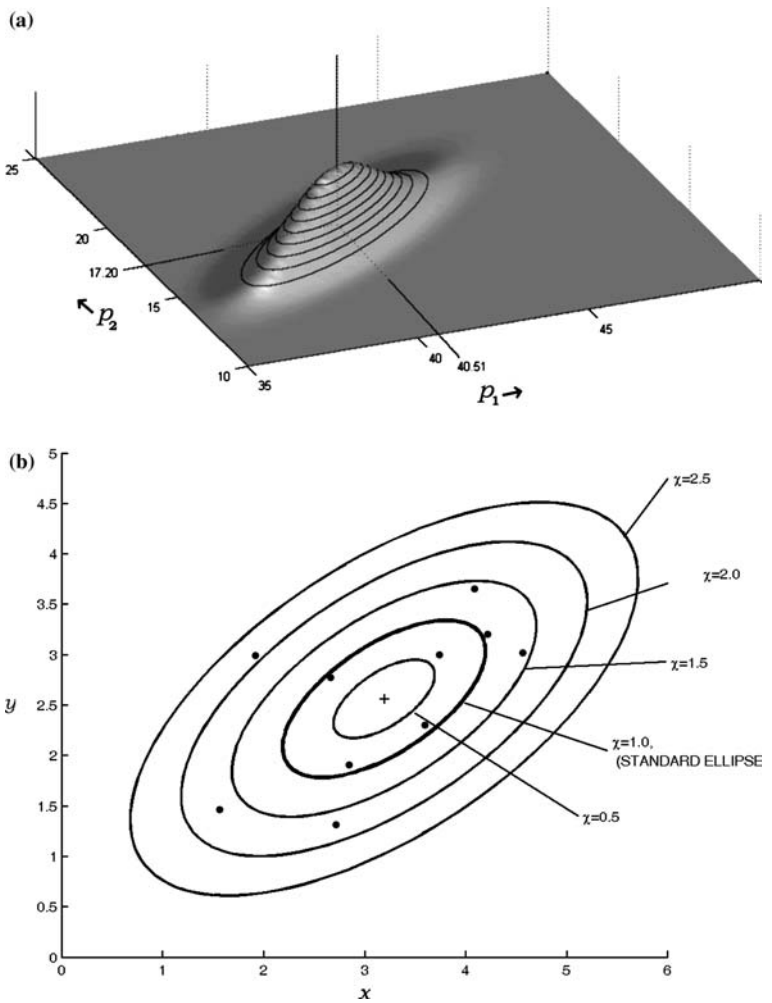

Figure 6. (a) Three-dimensional surface plot showing the expected bivariate normal density for p_2 against p_1 of *P. claudecombesi* as derived from the ten data points available for this species. (b) An example of points plotted on a system of axes together with a set of confidence ellipses.

any of N_{mis}, N_{mult} or N_{not} increases. One possibility is to construct P simply as $P(N_{cor}, N_{mis}, N_{mult}, N_{not}) = N_{cor} - N_{mis} - N_{mult} - N_{not}$. One may also add weights to each of these counts, so that P is given by $P = N_{cor} - c_{mis}\ N_{mis} - c_{mult}\ N_{mult} - c_{not}\ N_{not}$ where c_{mis}, c_{mult} and c_{not} are the relative costs of obtaining misclassification, multiple classification or no classification. In this case the researcher must then supply the values of c_{mis}, c_{mult}, and c_{not} based on how serious he/she rates a misclassification, multiple classification or not classifying in terms of correct classification.

In this paper we have used the simple function $P = N_{cor} - N_{mis} - \frac{1}{2}N_{mult}$, i.e. we assume that the cost of misclassification is the same as the gain in correct classification and the cost of multiple classification is only half as bad as misclassification. We ignore the number of points not classified. Many choices for the costs are possible and we do not claim that the current choice is necessarily more sensible than others.

The classification potential P also depends on the scaling factor χ. When χ is very small, the confidence ellipses are all small and all points may be unclassified, i.e. $P = 0$. As χ increases, more and more points become correctly classified, so that we expect P to increase with increasing χ.

However, as the sizes of ellipses grow, more and more points become misclassified as well, so that P will eventually start decreasing again. It is

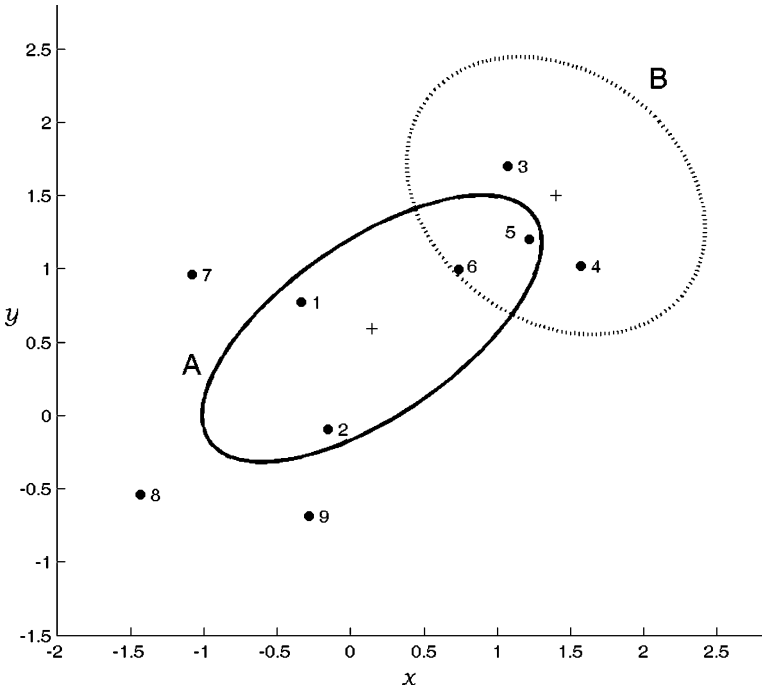

Figure 7. A diagram for illustrating the four categories of points. Nine points of an imaginary species A together with its confidence ellipse (in thick solid line) are shown. The confidence ellipse of another imaginary species B is plotted (in the dashed line) without its points. The four classes of points are: correctly classified (1 and 2), misclassified (3 and 4), multiply classified (5 and 6) and not classified (7, 8 and 9).

therefore expected that P will attain a maximum for some value of χ. This effect is illustrated in Figure 8 for parameters p_7 against p_1, where the maximum is attained at approximately $\chi = 0.54$. If χ is increased from 0, the classification potential P increases from 0 to its maximum value and then decreases again. For some cases, however, P simply starts to decrease into negative numbers immediately as χ is increased from zero. This is also illustrated in Figure 8 for p_6 against p_7.

A computer program has been written that takes all pair-wise combinations of the 13 parameters as input and calculates the maximum classification potential together with the particular value of χ which returned the maximum value. (For each pair of parameters the program actually varies χ automatically starting from 0 and incremented in increments of 0.05. It then selects the case with greatest value of P.) Table 1 lists the 20 best performers out of the 78 combinations.

Since all 107 specimens were used in this example, the theoretical maximum value of P is 107 (when all specimens would have been correctly classified) and the theoretical minimum of P is 0. This minimum occurs at those cases where P becomes negative immediately with increasing χ. For all cases investigated, the maximum P never occurred at χ values greater than 1.3. Our program therefore varied χ from 0 to 1.6 (well above 1.3) and selected the largest value of P found.

If our investigation would have stopped here, the best choice according to these results would be to measure only p_7 and p_8. The program was also adapted to test various products and quotients of parameters. The results of the top 20 performers out of 3003 of pairs of products of parameters are listed in Table 2. The best performer is $p_1 \times p_2$ against $p_8 \times p_9$ with a classification potential of 18.0.

This requires four measurements to be made. Since p_1 and p_8 are rather similar measurements, one could argue that taking both measurements is unnecessary. If we want to economise on the number of measurements, and require only three measurements, the next best two cases using only three measurements are $p_2 \times p_8$ against $p_8 \times p_9$ with a classification potential of 14.5 and $p_1 \times p_2$ against $p_1 \times p_9$ with a classification potential of 13.5.

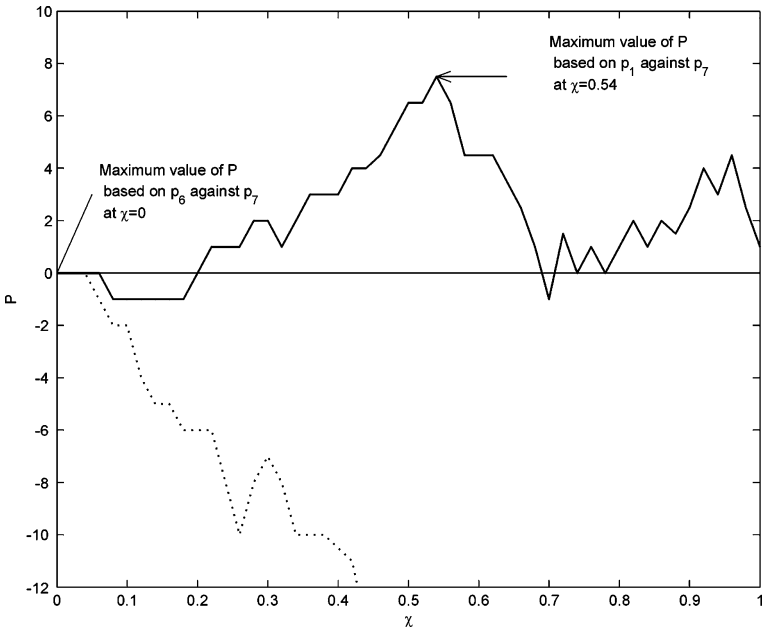

Figure 8. A graph to illustrate the variation of classification potential, P with χ. For some pairs of parameters (such as p_1 against p_7) a maximum is attained, while for other pairs (such as p_6 against p_7) P simply decreases immediately and the maximum for P is taken as zero.

Figure 9 shows a scatter plot of p_1 p_2 against p_1 p_9. Here the ellipses of all eleven species were all drawn at size $\chi = 0.90$. It is clear from Figure 9 that there are two pairs of species that still overlap significantly in this scatter plot, i.e. *P. australis* and *P. marmorati* in the upper right part of the plot

Table 1. Classification potential as determined for pair-wise combinations of the 13 parameters.

Performance grade	First parameter	Second parameter	Scaling constant (χ)	Classification potential ($P = N_{cor} - N_{mis} - 1/2\ N_{mult}$)
1	p_7	p_8	0.90	13.5
2	p_1	p_7	0.55	7.5
3	p_8	p_9	0.85	7.0
4	p_1	p_9	0.95	7.0
5	p_2	p_7	0.80	5.5
6	p_4	p_7	0.15	5.0
7	p_2	p_9	0.55	5.0
8	p_6	p_8	1.30	4.5
9	p_8	p_{12}	0.40	4.0
10	p_1	p_{10}	0.50	4.0
11	p_8	p_{10}	0.50	3.0
12	p_2	p_{10}	0.65	3.0
13	p_8	p_{13}	0.20	2.0
14	p_5	p_{10}	0.25	2.0
15	p_3	p_{10}	0.15	2.0
16	p_3	p_8	0.25	2.0
17	p_3	p_5	0.15	2.0
18	p_1	p_8	0.15	2.0
19	p_5	p_8	0.55	1.5
20	p_1	p_5	0.35	1.5

Table 2. Classification potential as determined for products of the 13 parameters.

Performance grade	First parameter	Second parameter	Scaling constant (χ)	Classification potential ($P = N_{cor} - N_{mis}\ 1/2\ N_{mult}$)
1	$p_1 \times p_2$	$p_8 \times p_9$	0.85	18.0
2	$p_2 \times p_8$	$p_8 \times p_9$	1.10	14.5
3	$p_1 \times p_8$	$p_6 \times p_7$	0.85	14.5
4	$p_1 \times p_8$	$p_5 \times p_7$	1.10	14.0
5	$p_1 \times p_2$	$p_4 \times p_9$	0.85	14.0
6	$p_1 \times p_2$	$p_1 \times p_9$	0.90	13.5
7	$p_2 \times p_8$	$p_5 \times p_7$	0.80	13.0
8	$p_1 \times p_2$	$p_9 \times p_{13}$	0.70	13.0
9	$p_1 \times p_2$	$p_6 \times p_{10}$	0.95	13.0
10	$p_2 \times p_8$	$p_9 \times p_{13}$	0.70	12.5
11	$p_2 \times p_8$	$p_6 \times p_7$	0.95	12.5
12	$p_1 \times p_9$	$p_2 \times p_8$	1.05	12.5
13	$p_1 \times p_2$	$p_6 \times p_9$	0.85	12.5
14	$p_2 \times p_8$	$p_6 \times p_{10}$	0.50	12.0
15	$p_1 \times p_2$	$p_8 \times p_{10}$	0.90	12.0
16	$p_1 \times p_8$	$p_7 \times p_{13}$	0.90	11.0
17	$p_1 \times p_8$	$p_7 \times p_8$	0.90	11.0
18	$p_1 \times p_8$	$p_4 \times p_9$	1.05	11.0
19	$p_2 \times p_8$	$p_5 \times p_9$	0.70	10.5
20	$p_2 \times p_8$	$p_4 \times p_9$	0.90	10.5

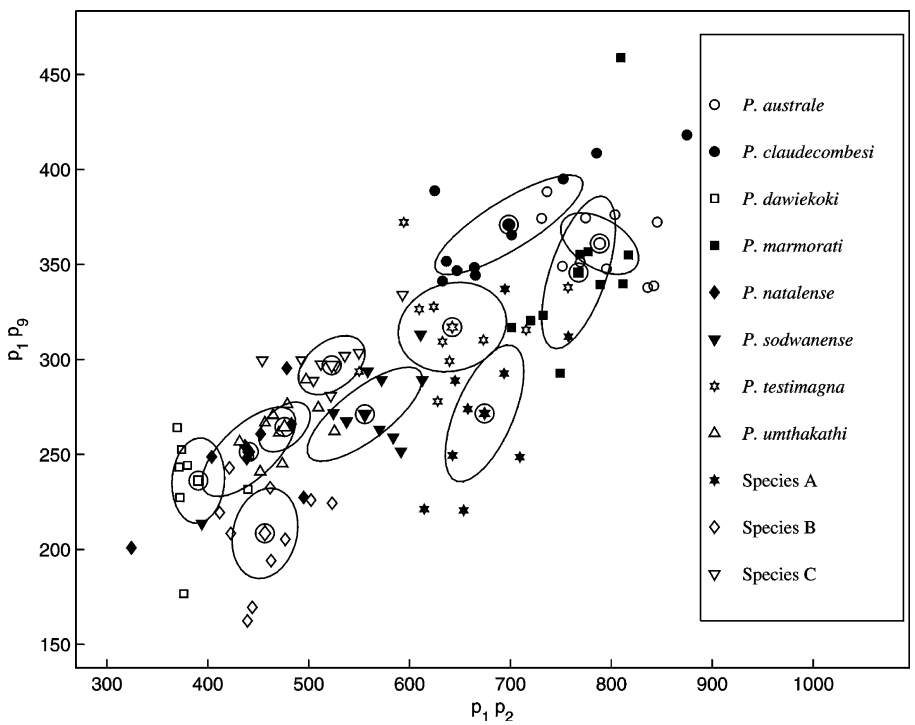

Figure 9. Scatter plot of $p_1 \times p_9$ against $p_1 \times p_2$ for 11 species, together with their confidence ellipses all drawn with a scaling factor $\chi = 0.9$.

Table 3. Classification potential as determined for products and quotients of the 13 parameters for pairs of overlapping species being grouped.

Performance grade	First parameter	Second parameter	Scaling constant (χ)	Classification potential ($P = N_{cor} - N_{mis} 1/2\ N_{mult}$)
1	$p_1 \times p_2$	$p_8 \times p_9$	0.95	42.5
2	$p_1 \times p_2$	$p_1 \times p_9$	1.05	40.5
3	$p_2 \times p_8$	$p_8 \times p_9$	1.05	39.5
4	$p_1 \times p_2$	$p_6 \times p_9$	1.00	37.0
5	$p_1 \times p_9$	$p_2 \times p_8$	1.05	36.5
6	$p_1 \times p_8$	$p_3 \times p_9$	1.00	35.5
7	$p_1 \times p_2$	$p_3 \times p_9$	0.95	35.5
8	$p_2 \times p_8$	$p_6 \times p_9$	0.95	34.5
9	$p_2 \times p_3$	$p_4 \times p_9$	1.15	34.5
10	$p_1 \times p_8$	$p_6 \times p_9$	1.05	32.5
11	$p_1 \times p_8$	$p_4 \times p_9$	1.05	32.5
12	$p_2 \times p_8$	$p_3 \times p_9$	0.95	32.0
13	$p_2 \times p_3$	$p_8 \times p_9$	0.95	31.5
14	$p_2 \times p_3$	$p_6 \times p_9$	1.10	31.5
15	$p_2 \times p_3$	$p_3 \times p_9$	1.05	31.5
16	$p_1 \times p_2$	$p_4 \times p_9$	1.20	31.5
17	$p_2 \times p_8$	$p_5 \times p_9$	0.85	31.0
18	$p_1 \times p_8$	$p_6 \times p_7$	1.10	31.0
19	$p_1 \times p_2$	$p_9 \times p_{13}$	1.05	31.0
20	$p_1 \times p_8$	$p_5 \times p_7$	1.20	30.5

and *P. umthakathi* and *P. natalensis* in the lower left patch just to the right of the ellipse for *P. dawiekoki*. The search program was run again, with these pairs of overlapping species being grouped into two single groups of two species each. The results are listed in Table 3. Again $p_1\ p_2$ against $p_8\ p_9$ appears as the overall winner with a classification potential of 42.5. Once again, if we want to economise on the number of measurements, and require only three measurements, the next best case using only three measurements is $p_1 \times p_2$ against $p_1 \times p_9$ with a classification potential of 40.5. A scatter plot of $p_1 \times p_2$ against $p_1 \times p_9$ is presented in Figure 10. It provides a satisfactory separation for the nine groups, two of which consist of pairs of species, with each pair pooled into a single group. It should also be noted that the scaling constant is $\chi = 1.05$, which implies that 42.4% of points are expected to lie inside these ellipses. We propose that $p_1 \times p_2$ against $p_1 \times p_9$ be used to separate members of the genus *Polystoma*.

In order to assist authors who would want to apply this method to other classifications based upon morphometric features a dedicated Excel spreadsheet was developed that will draw the confidence ellipses automatically. This can be found at the personal website of the first author or is available on request from either author.

Discussion

Taxonomists are always on the lookout for new characters or combinations thereof to help identify or describe taxa. The Monogenea is no exception and the measurements of sclerites, including the larval sclerites, are commonly used for this purpose. There is, however, the question as to how reliable and informative specific measurements in general are.

Among fish monogeneans, fluctuations in salinity and temperature have been shown to influence the morphological variation observed in gyrodactylid marginal hooklets (Malmberg, 1970; Mo, 1991a, b, 1991c). Despite this, the morphometrics of the haptoral sclerites still provide a valuable tool to separate species. Mo & Appleby

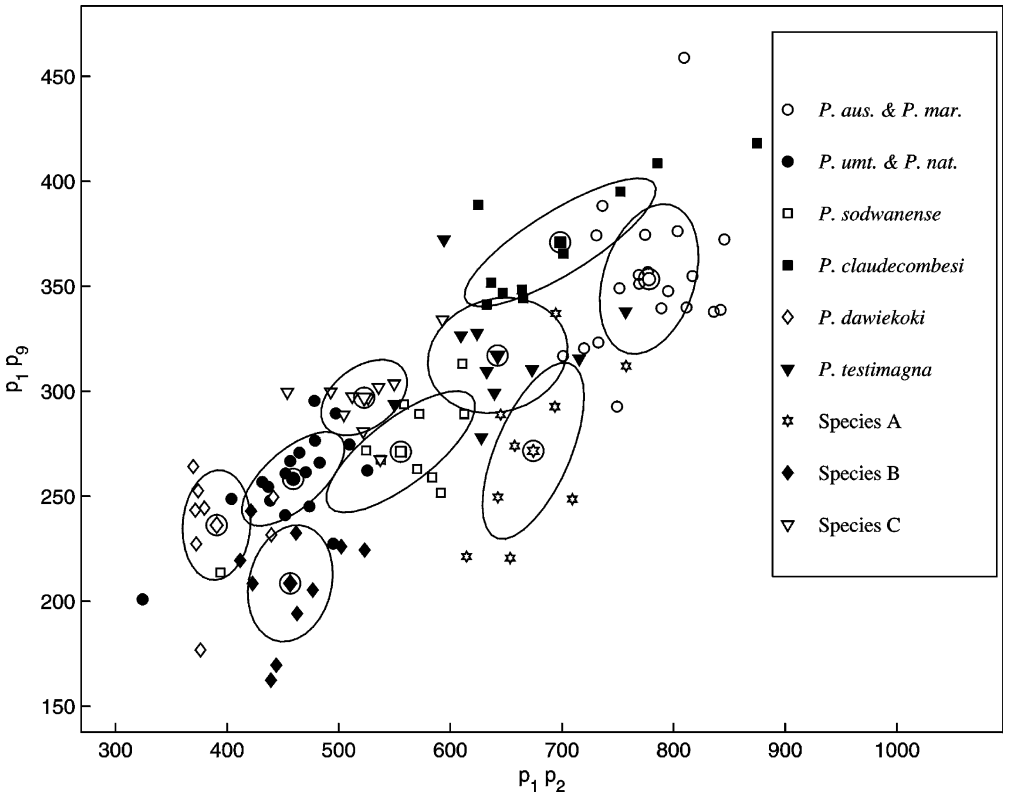

Figure 10. Scatter plot of $p_1 \times p_9$ against $p_1 \times p_2$ for nine groups (two pairs of species pooled into two groups), together with their confidence ellipses all drawn with a scaling factor $\chi = 1.05$.

(1990) described a technique whereby haptoral sclerites of monogeneans can be cleared and released from enclosing tissue for scanning electron microscopical studies using a digestion technique. Shinn, Kay & Sommerville (2000), using this technique, studied statistical classifiers for discrimination between species of *Gyrodactylus* Nordmann, 1832. They evaluated measurements of the hamulus, ventral bar and marginal hooklet and found that the marginal data subset of measurements gave the best classification of *G. salaries* Malmberg, 1957. The use of a statistical classification system, once optimised and validated, provides a rapid, reliable and cheap alternative to traditional methods (McHugh, Shinn & Kay, 2000).

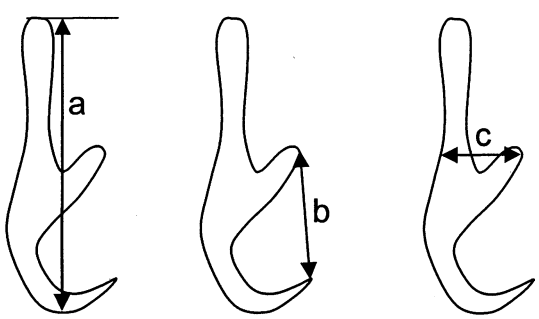

Figure 11. Proposed protocol for C1 marginal hooklet measurements for the genus *Polystoma*. Measurement a represents the length of the hooklet; b the distance from the tip of the hook to a position where a tangent line from the tip of the hook will touch the guard of the hooklet; and c the width of the marginal hooklet at the level of the guard.

Jackson & Tinsley (1995) reported for *Gyrdicotylus gallieni* Vercammen-Granjean, 1960, a viviparous gyrodactylid occurring in the oral cavity of *Xenopus laevis* (Daudin), that environmentally-induced variability, as well as genetically-based variation, are relatively minor and that sclerite characters should provide unambiguous information for studies on speciation.

Marginal hooklets of polystomes provide a valuable tool to separate species, as their shape and size stay constant throughout the development of the parasite (Murith et al., 1978; Du Preez,

unpublished data). The protocol proposed by Murith (1981) proved to be a valuable tool in separating species. The protocol proposed in the present study based on 11 South African *Polystoma* species (107 specimens), however, has a much higher classification potential of 13.5 compared to the classification potential of 6 for a plot of a against b by Murith (1981). The three parameters identified in the present study (Figure 11) describe three very different (and therefore also representative) length measurements of the marginal hooklet, where: p_1 (a) is the length of the hooklet, p_2 (b) describes the aperture distance of the hook and p_9 (c) is the width. Measuring only a, b and c and plotting a × b versus a × c provides a suitable first step for spreading the species as clearly as possible. It is important to note that this protocol will not separate all species equally well and that some overlaps will still occur as we have observed in this study. Also, we obviously do not propose that all the other morphological features should be ignored. The plots obtained from the marginal hooklets is merely an additional tool which will allow additional resolution and should be considered together with all other characters when studying polystomes. Reporting ratios and measurements of the total parasite and specific structures, position and nature of reproductive organs, number and length of genital spines, adult hamuli and meristic gut characters remain of the utmost importance.

We do, however, believe that the plot of the marginal hooklet measurements, as proposed here, represents a crucial protocol when comparing species. With the currently-proposed method it is important that the marginal hooklet must be flat within the same focal plane.

The protocol developed in the present study is based on 11 South African polystome species of *Polystoma*. It is, however, possible that there could be another set of parameters with a higher classification potential for other polystomatid genera. The protocol proposed in the present study should and most likely will give a high classification potential for all polystome genera, but this remains to be studied. The value of the processes to derive morphometric protocols, as described herein, is that it is not restricted to a specific taxon, but that it can be amended and applied to any taxonomic grouping.

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