Quantitative characterization, classification and reconstruction of oocyst shapes of *Eimeria* species from cattle

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SUMMARY

This study reports on morphological variability of *Eimeria* species, which may be given either by drawings or as quantitative data. The drawings may be used to facilitate identification by eye of 'unknown' *Eimeria* specimens, whereas quantitative data may serve as a reference set for identification by multivariate statistical techniques. The morphology of 810 *Eimeria* specimens was defined in binary (b/w) digital images by pixels of their oocyst outline. A Fourier transform of pixel positions yielded size and shape features. To classify coccidia, the quantitative data were employed in an agglomerative clustering by average linkage algorithm with equal weight assigned to size and shape. An inverse Fourier transform served to reconstruct oocyst outlines, i.e. outlines of average shape and size, from mean values of features in resulting clusters. Clusters were subsequently identified based on their average morphology by comparison with drawings of species in an earlier taxonomical work. Five hundred oocyst outlines were simulated for each cluster representing a species, and shape/size variability was presented in contour diagrams. Differences in species shapes, and correspondence in length and width, were seen after reconstruction by inverse Fourier transform and comparison with earlier studies.

Key words: classification, image analysis, Fourier transform, shape, numerical taxonomy, *Eimeria*.

INTRODUCTION

Analysis of morphology by digital image processing may provide a wealth of information which can be used to characterize biological organisms. Accurate and reproducible measurements, not only of size, but also of form and texture may be defined by algorithms applied to digital images. Although such new morphological features are seldom intuitive, their use may replace subjective and non-quantitative descriptions. This implies that new detailed, highly descriptive, and precise data may serve in quantitative classification.

Two categories of quantitative classifications are distinguished (Panel on Discriminant Analysis and Clustering, 1989). The first category comprises classification by criterions derived from reference samples. These problems are included under the heading of 'discrimination analysis' in the statistical literature. In a second and less trivial type of classification, covered by 'cluster analysis', no reference material is available. The classifications require selection of a 'suitable' procedure (algorithm) and a weighing of features.

The everyday identification of specimen shape/size rarely, if ever, addresses in a cogent manner the questions of how characters are measured (with a possible exception of simple size measurements), weighted, and classification performed. The empirical procedure contrasts with the use of image analysis in classifications, because precise definition of features is a pre-requisite, and rigorous data analysis necessitates weighting of features and a choice of classification procedure. It follows that such a quantitative classification has a desirable stringency and objectiveness, and does not rely on experience.

Objects with a 'simple' shape morphology without distinct qualitative characters are ubiquitous in nature, and prominent examples may be found amongst protozoa. Size and shape of the outline of the oocysts are the most distinctive, although not highly discriminative, features for a majority of species in the family Eimeriidae. Overlap in size and shapes of the oocysts constitutes a diagnostic problem since many, but not all, species are of veterinary and medical importance. Unfortunately, type specimens are not available for description of size and shape variability because long-term preservation deforms the oocysts (personal observation). Without reference material the classification of *Eimeria* spp. is of the second category mentioned, that is, the specimens must be grouped according to morphological similarity, and the resulting groups characterized. Some guidance for a subsequent identification of groups may be found from the illustrations of 'typical' species morphology, e.g. in

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Nyberg & Hammond (1965), Joyner *et al*. (1966), Levine & Ivens (1967), Courtney, Ernst & Benz (1976), and Levine (1985). Most species in these earlier studies are defined empirically because qualitative characters are absent.

I have quantified the morphology of unsporulated oocysts of *Eimeria* spp. in digital images by Fourier transform of the pixels constituting the oocyst outline. An agglomerative cluster analysis was used to group coccidia based on similarity in size and shape expressed by the amplitudes in the Fourier spectrum. Average shapes were reconstructed by inverse Fourier transformation of average amplitudes. To identify resulting clusters to species level, drawings in Joyner *et al*. (1966) were used for comparisons of shapes and amplitudes. Shape variability was quantified and presented in drawings.

MATERIALS AND METHODS

Sample preparation, image recording

Faecal samples, containing up to 700000 *Eimeria* oocysts per gram, were collected from 14 calves aged 1·5–14 months. To isolate the oocysts, subsamples were first diluted with water, centrifuged, and the supernatant removed. The sediment was mixed with a saturated sodium chloride and glucose solution and transferred to a modified McMaster counting chamber (Henriksen & Korsholm, 1984). Images of oocysts were then recorded from the transparent chamber (oil-immersion, $\times 100$ magnification) with a CCD (charge coupled device) camera attached to a light microscope. The images were digitized in a 512×512 pixel format.

A manual threshold operation was used to separate oocysts from their image background. Each grey level monochrome image was transformed into a binary image (black and white pixels only) by interactively choosing a grey level threshold, and automatically transforming pixels brighter and darker than the threshold into black and white pixels, respectively. In each image a threshold was chosen so that the transformation resulted in an array of white pixels representing the continuous outline of the oocyst wall. White pixels other than those of the outline were removed. Pixel positions of the boundary of the array were found by an edge tracking routine (Petersen, 1992). This was done in a total of 810 binary images. An additional 12 images were recorded from drawings of *Eimeria* spp. reported by Joyner *et al*. (1966). The species were *E. subspherica*, *E. ellipsoidalis*, *E. zuernii*, *E. cylindrica*, *E. alabamensis*, *E. bovis*, *E. canadensis*, *E. auburnensis*, *E. brasiliensis*, *E. wyomingensis*, *E. pellita* and *E. bukidnonensis*. All images were recorded in the same scale. GIPS-TAR-S 4.11 (Image House, Copenhagen, Denmark) was used for digitization and segmentation.

With the exception of the distinctive *E. bukidnonensis*, *E. pellita*, *E. brasiliensis*, Joyner *et al*. (1966) found any of the remaining 9 *Eimeria* species to occur in more than 12 % of bovine faecal samples. Hence the 9 species or a majority thereof may be expected to be represented by the 810 oocysts, because samples in this study were collected from 14 calves.

Morphology analysis

The x, y coordinates of the boundary pixels may be regarded as placed in the complex plane and position given by the complex number $x+iy$. The time-series obtained from sampling of 512 equidistant boundary pixels represented by $f(t) = x(t)+iy(t)$ were transformed by the corresponding discrete Fourier transform

$$F(u) = \frac{1}{N} \sum_{t=0}^{N-1} f(t)\, e^{\frac{-i2\pi ut}{N}}$$

where $i = \sqrt{-1}$, and $N = 512$. Thus, the transform defines for each of the frequencies,

$$u \in [0, 1, \ldots, N-1],$$

a harmonic, $F(u)$. The Fourier transform generates large values of $F(u)$ for u close to $N/2$. To obtain large values close to $u = 0$, the interval of u was simply shifted to $[-N/2+1;\ N/2]$.

The Fourier transform was normalized in order for the harmonics to be independent of boundary pixel position and start of sampling (Wallace & Wintz, 1980; Petersen, 1991).

Values of the harmonics consisted of a real and an imaginary component, i.e. $\mathrm{Re}[F(u)]$ and $\mathrm{Im}[F(u)]$, respectively. Corresponding amplitudes, defined as

$$|F(u)| = \sqrt{\{\mathrm{Re}[F(u)]^2 + \mathrm{Im}[F(u)]\}^2}$$

were used as descriptive features. The amplitude corresponding to $u = 1$, $|F(1)|$, is proportional to the area and was used as a size feature. Normalization yielded $F(0) = 0$. The remaining amplitudes designate shape of the outline.

Amplitudes for

$$u \in [-13, \ldots, -1, 1, \ldots, 13]$$

were used for classification. The frequency interval was chosen because later reconstruction of the most complex outline (that of *E. bukidnonensis*) proved satisfactory when 26 frequencies were used (cf. reconstruction of *E. bukidnonensis* in Fig. 1 lower right with Joyner *et al*. 1966).

A fast Fourier transformation (FFT) algorithm was used (Press *et al*. 1987) in the Image Analysis software (Vision Research, Copenhagen, Denmark).

Data analysis

The following agglomerative clustering was based on Euclidian distances between observations in the

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hyperspace spanned by the 26 amplitudes. Size and shape of the coccidia were regarded as being of equal importance in the classification. The size descriptor, $|F(1)|$, was therefore standardized to zero mean and unit standard deviation, whereas all other amplitudes, being shape descriptors, were standardized by subtraction of the mean and division by standard deviation, where mean and standard deviation were calculated for all values of shape descriptors for all observations.

Each observation was initially considered to be a single cluster. Clusters were then joined sequentially in pairs if the Euclidean distance was the minimum of distances between all cluster pairs. This was done until a single cluster contained all observations. The clustering was performed by an average linkage algorithm, that is, the distance was calculated as the average distance between all pairs of observations, with 1 observation in each cluster. Clustering by the average linkage algorithm has the advantage of simplicity and it is not biased towards producing clusters of equal size.

Clusters were given a provisional identity by assignment to a species based on the minimum squared Euclidean distance from the cluster mean to measurements in any of the species drawings by Joyner *et al*. (1966). In addition, average of cluster morphology was identified by eye to species based on the relative size and shape. It should be emphasized that the drawings by Joyner *et al*. (1966) may only provide tentative guidance for identification because species variability cannot be presented by 1 drawing per species.

Statistical analysis was performed with the use of SAS System for Windows 6.08 (SAS Institute Inc., Cary, NC, USA).

Reconstruction of species

A Fourier transform is characterized by having a corresponding inverse. After the shifting of the frequency interval the inverse Fourier transform, $f(t)$, was given by

$$f(t) = \sum_{u=\frac{N}{2}+1}^{\frac{N}{2}} F(u)\, e^{\frac{i2\pi ut}{N}}$$

Thus, by the inverse transformation morphology (*in casu* the oocyst outline) may be reconstructed from its harmonics, $F(u)$. The coordinates in the complex plane are given by the x and y values from, $f(t) = x(t) + iy(t)$. After normalization the origin is given by $F(0) = 0 + i0$. The normalization ensured specimens were oriented horizontally with the more narrow pole oriented to the right.

Using the inverse Fourier transform for values of $F(u)$ where $u \in [0, 1]$ and setting $F(u)$ to zero for all other frequencies, a circle will result with a centre in $0, 0^i$ and an area equivalent to that of the original object. By inclusion of values of higher order frequencies the shape will approach that of the object originally transformed. The exact size and form (given by the N boundary pixels) may be reconstructed when all N frequencies are employed.

In the clusters, mean values and standard deviations were calculated for the real and imaginary part of the harmonics. The mean values could then be used for reconstruction of typical morphology for selected cluster.

Given the mean, standard variation and normal distribution of the harmonics, random numbers of the real and imaginary part could be generated and used for simulating outlines. The outlines were represented in a frequency matrix where rows and columns represented x and y coordinates in the complex plane. Matrices were depicted by contour diagrams representing the frequency distribution of 500 outlines for each cluster interpreted as being a species cluster. The harmonics, $F(u)$, for

$$u \in [-13, \ldots, -1, 1, \ldots, 13],$$

were used to reconstruct typical specimens and $F(u)$ was set to zero for

$$u \in [-200, \ldots, -14, 0, 14, \ldots, 200]$$

for reconstruction of morphology. Matrices for the representation of simulated oocysts were 211×211.

The outlines of the *Eimeria* spp. reported by Joyner *et al*. (1966) were reconstructed based on the 26 coefficients from Fourier transformation of digital images of the original drawings. As for the clusters, a total of 401 coefficients were used with $F(u)$ for

$$u \in [-200, \ldots, -14, 0, 14, \ldots, 200]$$

set to a value of zero.

Mathcad PLUS 6.0 (MathSoft, Inc., Cambridge, MA, USA) was used for the inverse Fourier transform and graphical presentation.

RESULTS

The result from average linkage of 810 specimens is presented in a phenogram (Fig. 1), which shows the last 24 cluster joinings. (Ten clusters of 6 or less specimens, with a total of 26 specimens, have been omitted for clarity.) The clustering resulted in 3 major groups. Within these groups selected (numbered) clusters were identified by minimum distance to any of the relevant (see below) drawings in Joyner *et al*. (1966). This resulted in the following identifications (clusters in parentheses): *E. wyomingensis* (1–7, 19, 20, 22, 23), *E. cylindrica* (8, 11, 14, 15), *E. canadensis* (9, 10), *E. ellipsoidalis* (12, 13, 16, 17), *E. subspherica* (18), *E. auburnensis* (21), *E. bukidnonensis* (24).

Most of the 12 *Eimeria* species were expected to occur in the sample of 810 oocysts (see Materials and Methods section). The identification of all 24 clusters

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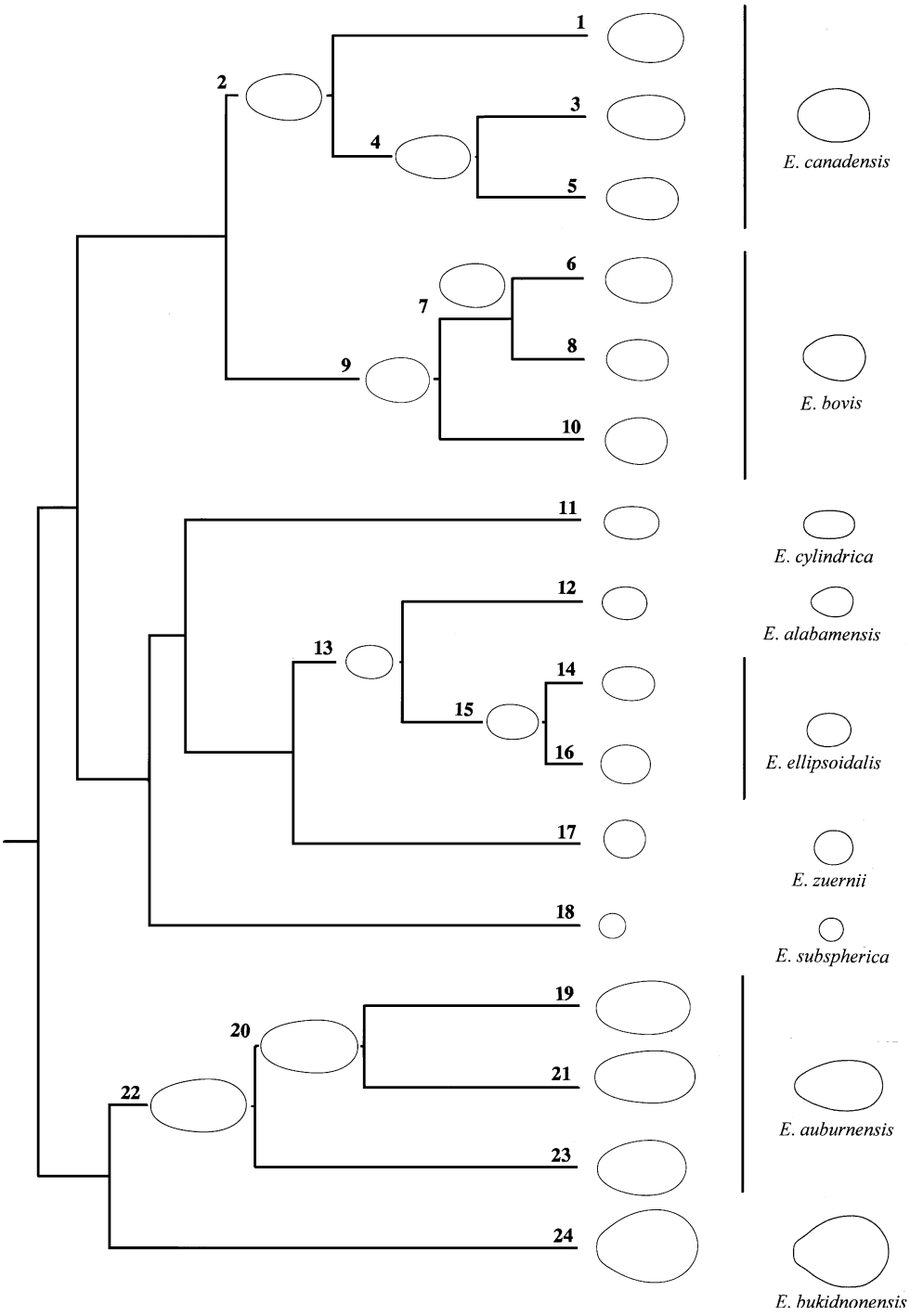

Fig. 1. Phenogram showing the clusterings of 26 amplitudes by the average linkage method. The reconstructed average morphology in clusters is presented at selected mergings. Reconstructed shapes from Joyner *et al*. (1966) are shown to the right of the corresponding clusters in the phenogram.

as belonging to only 7 species, and the identification of 15 out of 24 clusters as *E. wyomingensis* and *E. ellipsoidalis*, therefore indicate that drawings in Joyner *et al*. (1966) were not truly representative of the clusters.

Consequently, the clusters, based on reconstructed morphology, had to be identified by eye to species, i.e. the reconstructed illustrations from Joyner *et al*. (1966). These are shown to the right of the phenogram. Two distinct species could be excluded a priori; *E. brasiliensis*, which is recognizable by a prominent micropylar cap, and *E. pellita*, with a marked velvety surface, were not observed in the samples. The uncommon *E. illinoisensis* (Levine & Ivens, 1967; Levine, 1985) was not considered as it has not been reported in 2 large studies from Sweden and England (Svensson, 1994; Joyner *et al*. 1966). The remaining 10 species may be arranged in the following species groups according to similarity in size and shape: (1) *E. subspherica*, (2) *E. ellipsoidalis*,

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Table 1. Number of specimens found in each species cluster (N), corresponding cluster number in Fig. 1 and average length and width ($\pm$S.D.) in μm

Species	N	Cluster number	Character	
			Length	Width
E. subspherica	21	18	11·80 ($\pm$0·78)	11·09 ($\pm$0·81)
E. ellipsoidalis	77	15	22·81 ($\pm$1·06)	15·41 ($\pm$1·36)
E. zuernii	122	17	18·39 ($\pm$1·37)	16·87 ($\pm$1·35)
E. cylindrica	65	11	24·26 ($\pm$1·44)	14·47 ($\pm$0·98)
E. alabamensis	172	12	19·79 ($\pm$1·36)	14·55 ($\pm$0·97)
E. bovis	189	9	28·17 ($\pm$1·71)	19·96 ($\pm$1·36)
E. canadensis	67	2	33·30 ($\pm$1·58)	20·46 ($\pm$1·60)
E. auburnensis	53	22	42·21 ($\pm$2·09)	23·59 ($\pm$0·76)
E. bukidnonensis	18	24	44·63 ($\pm$1·49)	32·15 ($\pm$0·65)

E. zuernii, *E. cylindrica*, *E. alabamensis*, (3) *E. bovis*, *E. canadensis*, (4) *E. auburnensis*, *E. wyomingensis* and *E. bukidnonensis*. These distinctive species groups were easily identified in Fig. 1, and the clusters could then be identified based on the relative morphology within groups (see below).

Three clusters were distinguished by their late merging with other clusters (Fig. 1). These were cluster 11 identified as *E. cylindrica*, cluster 18 as *E. subspherica*, and cluster 24 as *E. bukidnonensis*. The clusters 1–10 comprised medium-sized ovoid oocysts, compatible with the *E. canadensis*/*E. bovis* group. The larger and more ovoid of the 2 subgroups, i.e. cluster 2, was interpreted as being representative of *E. canadensis*, and cluster 9 consequently to be *E. bovis*. The smaller oocysts of various shapes in clusters 11–17 correspond to the *E. ellipsoidalis*, *E. zuernii*, *E. cylindrica*/*E. alabamensis* group. Cluster 11 was identified as *E. cylindrica*. When cluster 17 is identified as the round *E. zuernii*, the smaller and slightly ovoid average morphology in cluster 12 may represent *E. alabamensis*, and cluster 15 consequently *E. ellipsoidalis*. This interpretation was substantiated by analysis of size distribution within clusters (see below). The remaining clusters 19 through 24 represent a group of very large oocysts belonging to the *E. aubernensis*, *E. wyomingensis*/*E. bukidnonensis* group. From the average morphology it is evident that cluster 20 represents the more slender *E. aubernensis*. Cluster 23 may be interpreted as *E. wyomingensis*. However, a more conservative interpretation would be *E. auburnensis*, since lengths and widths in the cluster 23 correspond more closely with those earlier reported for this species. Cluster 24 was identified as *E. bukidnonensis*.

The number of specimens in each species cluster is given in Table 1. *E. bukidnonensis*, with striated oocyst wall and very large size, could be readily identified. The number of oocysts in the *E. bukidnonensis* species cluster is identical to the number identified prior to the analysis.

Normal distribution of the size descriptor, $|F(1)|$, was tested by the Kolmogorov–Schmirnov D-statistic for each species cluster identified. The size features were normally distributed in all cases ($P > 0{\cdot}05$) with the exception of cluster 17 ($0{\cdot}02 < P < 0{\cdot}05$). This indicates that species clusters, with a possible exception of cluster 17, do not represent mergers of species. Heterogeneity in cluster 13 ($P < 0{\cdot}01$) supports the view that cluster 12 and cluster 15 represents 2 species cluster groups with different size distributions.

A contour plot of 500 simulated shapes for each of the identified species is shown in Fig. 2A and B. In all modelled outlines the orientation is identical due to normalization. The contour plots may be thought of as topographical maps. This means that close contour lines near the middle of the band indicate a well-defined morphology. Conversely, morphological variability is reflected by lines widely separated from the middle of the band.

The contours described in all cases a monotonic increase in frequencies towards the middle part of the band enclosed by contour lines. The shape of *E. subspherica* was extremely well defined as indicated by the concentric contour lines (Fig. 2A). Size variability was high relative to the size. The sides of the *E. cylindrica* oocysts curve more than is evidenced by the drawing in Joyner *et al*. (1966) (Fig. 1). Reconstruction of *E. alabamensis* morphology revealed a slightly ovoid shape with some variability in the apical region. The *E. ellipsoidalis* was comparably larger, less ovoid, more elliptical and showed some variability in the shape of the oocyst sides. The *E. zuernii* was variable in size and shape but its average outline was near circular. *E. bovis*, being the smallest in the *E. bovis*/*E. canadensis* group was clearly larger than any of the aforementioned species. The broad ovoid shape was distinct. *E. canadensis* was relatively larger, and more slender but with considerable shape variability of the sides. Reconstruction of *E. auburnensis* showed an

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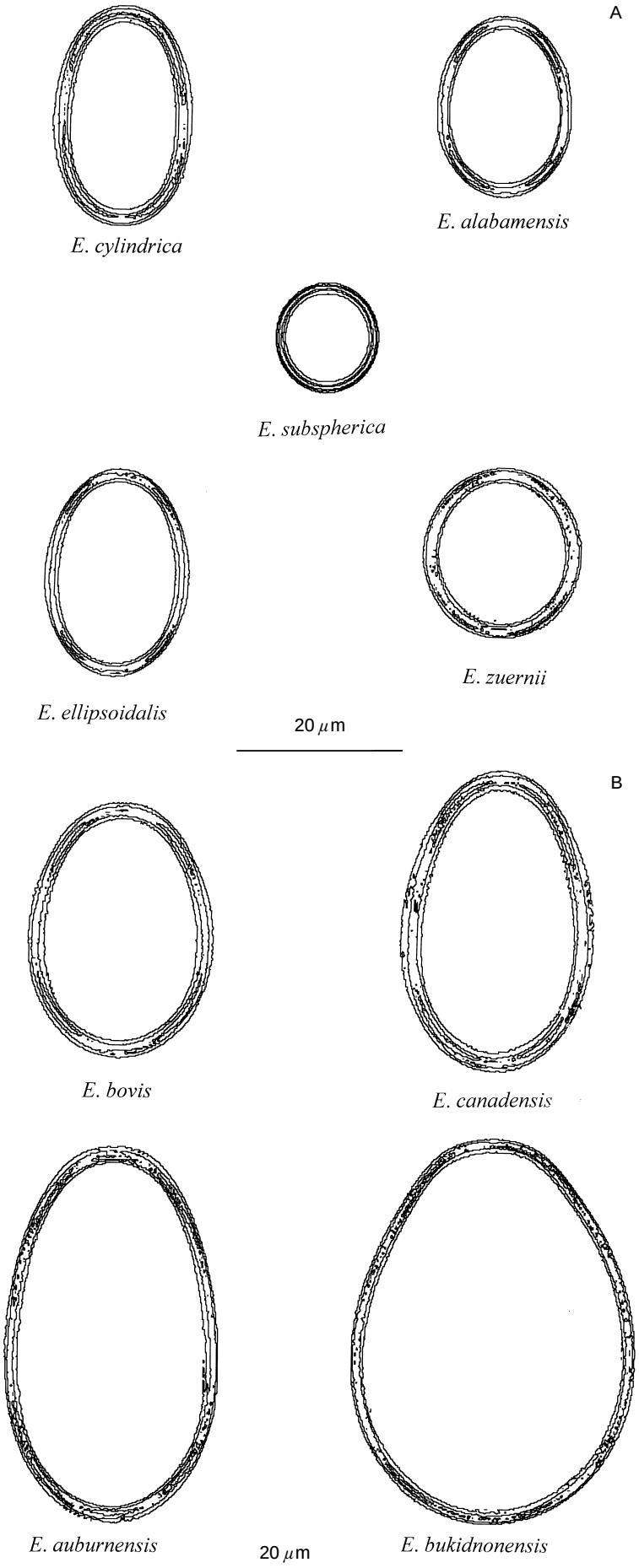

Fig. 2. For legend see opposite.

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elongated ovoid shape with an only marginally pointed apex when compared with Joyner *et al.* (1966) (drawing in Fig. 1). The size was considerably larger than that of *E. canadensis*. *E. bukidnonensis* was, relative to its size, of uniform shape with an almost flat apex.

Average length and width (±S.D.) are given for each species in Table 1. Average and standard variation of Fourier coefficients for species clusters are available from the author upon request.

DISCUSSION

This study contrasts with the empirical classification of subjectively perceived shapes; morphology was characterized quantitatively allowing a stringent and objective classification with a chosen weighing of descriptive features. Shape/size average and variability could consequently be given in both frequency (as quantitative data) and spatial (as drawings) domain for identified groups.

It is axiomatic that quantitative classification by agglomerative clustering should merge all subclusters representing 1, and only 1, species before other clusters are merged. The merger of subclusters then represents a 'monophenetic' species cluster (*cf.* the definition of a strictly monophyletic group (e.g. Futuyma, 1986)). The compliance of classification procedure to this is expected from a lower variability within species than between species. It should, however, be stressed that no one optimal algorithm exists although the average linkage, used in this study, may generally be preferred (Massart & Kaufman, 1983).

Precise descriptions of monophenetic groups of *Eimeria* are warranted because few qualitative characters are of potential value for an identification. The characters include layers of the (crushed) oocyst wall, micropyle, and residual bodies (which may only be observed after sporocyst formation in live coccidia) (Joyner *et al.* 1966; Levine & Ivens, 1967; Levine, 1985). Absence of a sporocyst residuum has been reported for *E. alabamensis* (Eckert *et al.* 1995; Levine & Ivens, 1967) as well as for *E. ellipsoidalis* (Becker & Frye, 1929; Nyberg & Hammond, 1965; Levine & Ivens, 1967). In *E. zurnii*, however, sporocyst residuum has been reported as absent by Nyberg & Hammond (1965), and present as well as absent by Levine & Ivens (1967). Hence the value of this, the purportedly only relevant qualitative character for separation of *E. zurnii* from *E. ellipsoidalis* or *E. alabamensis* may be questionable. Likewise, a micropyle has been reported as absent (Bruce, 1921), as present (Levine & Ivens, 1967) or as inconspicuous (Christensen, 1941) in *E. canadensis*. In *E. bovis* this character has been described as inconspicuous (Levine & Ivens, 1967), as a lightened area rather than an opening (Christensen, 1941), and as absent (Nyberg & Hammond, 1965). Such inconsistencies, due to indistinctness of characters or variability in their occurrence, may have prompted Joyner *et al.* (1966) to state that 'identification of species of *Eimeria* based upon characteristics of the oocysts is never an easy procedure'. Consequently, for all practical purposes, quantitative features of size and shape may be the only morphological features available for identification of the species currently recognized.

Species may show different degrees of morphological variability. This will be reflected in a phenogram, so that subclusters belonging to a species showing little variability will be merged at an early stage. Resulting species cluster may also be joined with clusters of other morphologically uniform species, before subclusters of more variable species are merged in their respective species clusters. In consequence, say, 10 species clusters are unlikely to be represented by the last 10 clusters formed (the last 10 clusters in Fig. 1 represents 2 subclusters of *E. canadensis*, 2 subclusters of *E. auburnensis*, 1 species cluster of each of *E. bovis*, *E. cylindrica*, *E. zuernii*, *E. subspherica* and *E. bukidnonensis*, in addition to a cluster with species cluster of *E. alabamensis* and *E. ellipsoidalis*). In order to represent all stages where species clusters are formed by mergings, a phenogram should therefore represent more cluster joinings than the expected maximum number of species.

A subsequent identification of clusters was facilitated by apportioning species membership to an exact average morphology. Species membership was obtained by comparison with the conventional species morphology reported as drawings in an earlier work (Joyner *et al.* 1966). This was done by use of the average quantitative characteristics (Fourier amplitudes) or simple comparison with reconstructed average shape and size. The assumption underlying the former procedure is that data points from each reference species lie, in terms of Euclidean distance, closest to the mean of its corresponding species clusters. If this is not so reference drawings are not representative for the sample studied. As seen from Fig. 1 and detailed in the Results section, the reference drawings in Joyner *et al.* (1966) were not always typical of the specimens studied. It should be emphasized, however, that length and width of species identified in this present study correspond with those given in earlier studies (Joyner *et al.* 1966; Levine & Ivens, 1967). Identification, facilitated by the reconstruction of average

Fig. 2. (A, B) Morphological variability of the oocyst outlines presented by contour plots of 500 simulations for each identified species in Fig. 1 (details in Results section).

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morphology of the clusters, therefore had to be carried out subjectively based on an expected representation of all or most of the relevant species in the sample (see Results section).

Generally, when clusters are identified by their Euclidean distance from cluster centre to the closest reference drawing, one may find aberrant subcluster(s) not to be assigned the identity of their expected true species cluster. This may apply for species of highly variable morphology and is expected regardless of whether the reference drawings represent the exact mean of the species cluster. Nevertheless, species clusters may be identified because they comprise all subclusters of one species and the majority of subclusters is expected to be assigned to the true species.

The species clusters may be regarded as constituting a reference set with known means and variations of descriptive features for each species. Hence, a classification criterion may be derived similar to that described by Sommer (1996) and employed in an identification procedure yielding probabilities for species membership for 'unknown' specimens.

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