

## Shape and size of red blood cells from the Pygoscelid penguins of Antarctica using atomic force microscopy

Cynthia C. Bonatto · Beatriz S. Magalhães ·  
Joaquim O. Branco · Edison L. Durigon ·  
Luciano P. Silva

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**Abstract** Antarctic biodiversity is evolutionarily complex, reflecting the extreme ambient conditions. Therefore, Antarctic organisms exhibit sophisticated adaptations in all organization levels, including organs, tissues, and cells. Since red blood cells (RBCs) travel through the vertebrates blood delivering O<sub>2</sub> to all tissues and organs and purging the unwanted CO<sub>2</sub>, they represent an interesting model to investigate biological adaptations. We have used atomic force microscopy (AFM) to compare the shape and size of RBCs of the Pygoscelid penguins. A total of 18 landmarks were measured in AFM images. When analyzed individually, the parameters were not capable of discriminating the RBCs of each species. However, the simultaneous use of multiple parameters discriminated (74%) among the RBCs. In addition, the use of RBC measurements was sufficient to hierarchically cluster the species in accordance to other common and reliable phylogenetic strategies. In light of

these results, the use of RBC characters could effectively benefit taxonomic inferences.

**Keywords** Antarctica · Atomic force microscopy · *Pygoscelis adeliae* · *Pygoscelis antarcticus* · *Pygoscelis papua* · Red blood cells

### Introduction

Penguins (Sphenisciformes) are flightless birds adapted to movement in water and distributed in the southern hemisphere. The Adélie (*Pygoscelis adeliae*), Chinstrap (*P. antarcticus*), and Gentoo (*P. papua*) cogenetic penguin species (Pygoscelids) overlap in the area of the Antarctic Peninsula. Hematologic data on Pygoscelids include white and red blood cells (WBCs and RBCs) total and differential counts (Zinsmeister and VanDerHeyden 1987; Hawkey et al. 1989) and blood viscosity (Clarke and Nicol 1993). There is an increasing interest in applying high-throughput approaches to the study of blood samples of penguin species due to: (1) basic hematological research interest; (2) their role as natural reservoirs of infective agents. In this sense, the unique ability of atomic force microscopy (AFM) to record the topography of samples at the nanometric scale allows unprecedented characterization of cells size and shape. When applied to the study of cell morphology, AFM offers detailed tridimensional information on cell biophysics and structure providing insight into both the physiological and pathological shifts of the cells. In addition, cell measurements can be associated with the resting metabolic rate (RMR) and genome size (Gregory 2002) of vertebrates, and can even be used to establish phylogenetic relationships. In the present study, AFM has been applied to examine the RBC morphology of the three extant species of

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C. C. Bonatto · B. S. Magalhães · L. P. Silva (✉)  
Laboratório de Espectrometria de Massa,  
Embrapa Recursos Genéticos e Biotecnologia,  
Núcleo Temático de Biotecnologia (NTBio),  
Parque Estação Biológica, Brasília, DF 70770-900, Brazil  
e-mail: paulinol@cenargen.embrapa.br;  
lucianopaulinosilva@gmail.com

J. O. Branco  
Laboratório de Biologia,  
Centro de Ciências Tecnológicas da Terra e do Mar,  
Universidade do Vale do Itajaí, Rua Uruguai 458,  
Itajaí, SC 88302-202, Brazil

E. L. Durigon  
Laboratório de Virologia, Departamento de Microbiologia,  
Instituto de Ciências Biomédicas, Universidade de São Paulo,  
Av. Professor Lineu Prestes, 1374, Prédio ICB II,  
São Paulo, SP 05508-900, Brazil

Pygoscelid penguins and the data were further analyzed to support the phylogenetic relationships among them and with other flightless and flying bird orders.

## Materials and methods

### Blood sample

Adult penguins ( $n = 3$ ) with an average body weight ( $\pm$ standard error of the mean, SEM) of  $4.7 \pm 0.3$  kg for *P. adeliae*,  $3.9 \pm 0.2$  kg for *P. antarcticus*, and  $5.5 \pm 0.2$  kg for *P. papua* were captured at Almirantado Bay, King George Island, Antarctic Peninsula from 1st December–20th December 2007. A blood sample of each specimen was collected from the foot vein with a 1-mM ethylenediaminetetraacetic acid rinsed syringe. One microliter of the sample was spread over a glass cover slip surface, air-dried for 5 min, and fixed with methanol for 5 min.

### Atomic force microscopy analysis

Atomic force microscopy was performed in air on the blood films using a SPM-9600 equipment (Shimadzu, Japan). The images were acquired in constant force contact mode using 200- $\mu$ m-length V-shaped cantilevers (nominal spring constant of  $\sim 0.15$  N/m, resonant frequency of  $\sim 24$  kHz) with integrated pyramidal tips (curvature radius  $< 20$  nm). The scanner used has a travel of 125  $\mu$ m in XY-directions and 7  $\mu$ m in the Z-direction. All AFM images were acquired as  $512 \times 512$  pixels at scan rate of 1 Hz. The images obtained were processed by SPM-9600 off-line software. The processing consisted in an automatic plane fit leveling of the surface. One-hundred individual RBCs of each animal were manually half-height segmented from the background using the labeling function of the particle analysis software following cell measurements.

### Data analysis

A total of 18 characters were obtained and submitted to one-way ANOVA. The means of the characters were compared among species by Fisher's PLSD test. The performance of the 18 characters was also collectively evaluated by discriminant analysis (complete estimation and tolerance of  $10^{-4}$ ) and hierarchical cluster analysis (HCA) using the unweighted pair-group method (single linkage and Euclidean distances). Molecular (Baker et al. 2006), integumentary, breeding (Giannini and Bertelli 2004), myological, osteological (Ksepka et al. 2006), and WBC (Zinsmeister and VanDerHeyden 1987) characters available in the literature were also subjected to the HCA. DNA and protein sequences were submitted to HCA after obtain-

ing numerical data from DNA and protein maximum likelihood analyses. The results were used to cluster the species in which each sequence represents one character. The sequences used were cytochrome *b*, cytochrome *c* oxidase, RAG, and MHC antigen. Characters obtained in the literature were taken from tables of the papers. RBC sizes were obtained from <http://www.genomesize.com/cellsize/> including 385 and 3 species of flying and flightless birds, respectively. RMRs of 54 species were obtained from <http://www.genomics.senescence.info/species/index.html> including 51 and 3 species of flying and flightless birds, respectively.

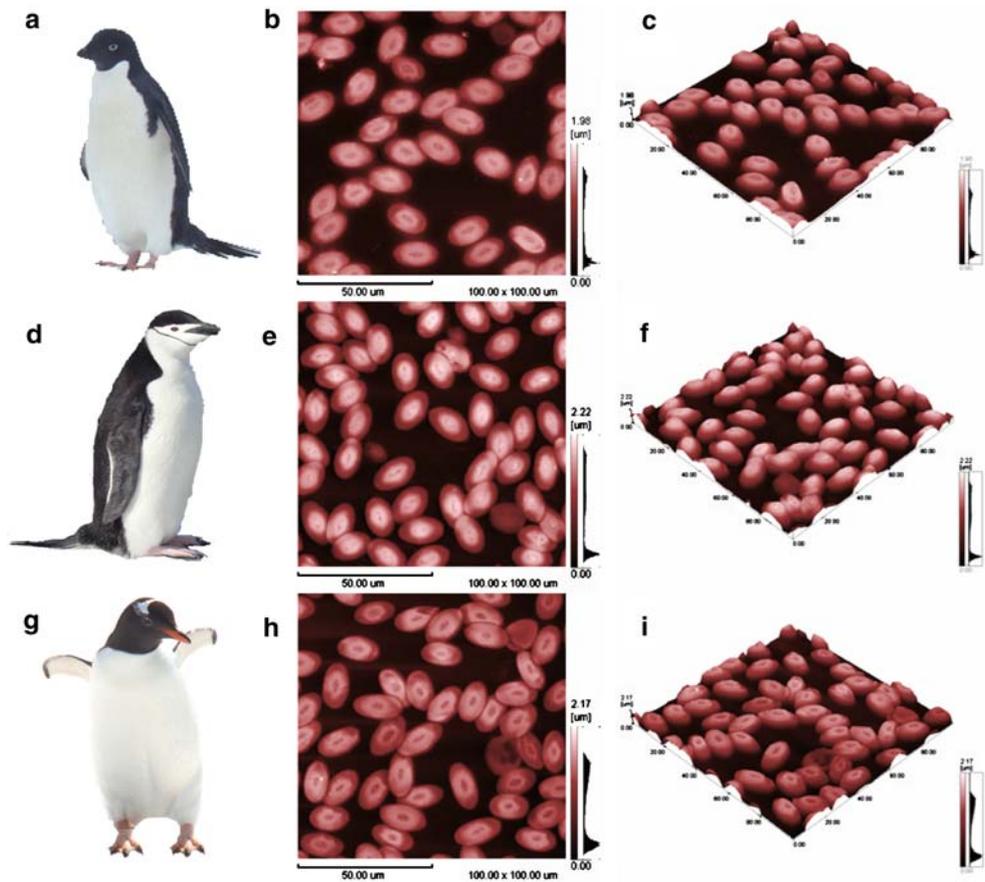
## Results

Pygoscelid penguins (Fig. 1a, d, g) showed ellipsoid RBCs as displayed by 2D (Fig. 1b, e, h) and 3D (Fig. 1c, f, i) images. Comparisons of the properties of the Pygoscelid RBCs are given in Tables 1. No significant differences among the *Pygoscelis* species RBC were noted when considering the individual average values ( $P < 0.05$ ). Compared to flying birds average dimensional values available in the literature (Fig. 2a–c), *Pygoscelis* penguins have significantly greater RBCs major axis (23%), minor axis (63%), and area (71%). In contrast, there was only a statistically significant increase for the minor axis (34%) of the RBCs of Pygoscelid penguins when compared to flightless birds. A negative correlation was observed between RBC size and RMR of the bird species which include Pygoscelid penguins, other flightless birds, and flying birds (Fig. 2d).

Discriminant analysis (DA) was performed on the data collected from 900 RBCs subdivided into *Pygoscelis* species (100 cells of each one of the three animals). Figure 2e displays all 900 RBCs in the bidimensional space defined by the first and second canonical discriminant functions and the ellipses surrounding 3/4 of the data set of each species. Casewise and cross-validation matrices from DA classified most RBCs to their correct species. *P. adeliae* RBCs were more likely to be correctly classified than *P. antarcticus* or *P. papua* RBCs. In summary, DA correctly classified 74% of RBCs to species.

Nine HCAs were conducted with our data and data obtained from the literature (see Sect. "Materials and methods"). The first analysis used only the RBCs measured data set of the present study (Fig. 3a). In this case, species were segregated into two subsets of Pygoscelid (*P. antarcticus* + *P. papua* and *P. adeliae*). The next seven analyses (Fig. 3b–h) used characters available in the literature. The only analyses congruent to RBCs clustering result was the one obtained from DNA data. The last analysis (Fig. 3i) combined the literature (192 characters) and present data (18 characters) to render a consensus hierarchical

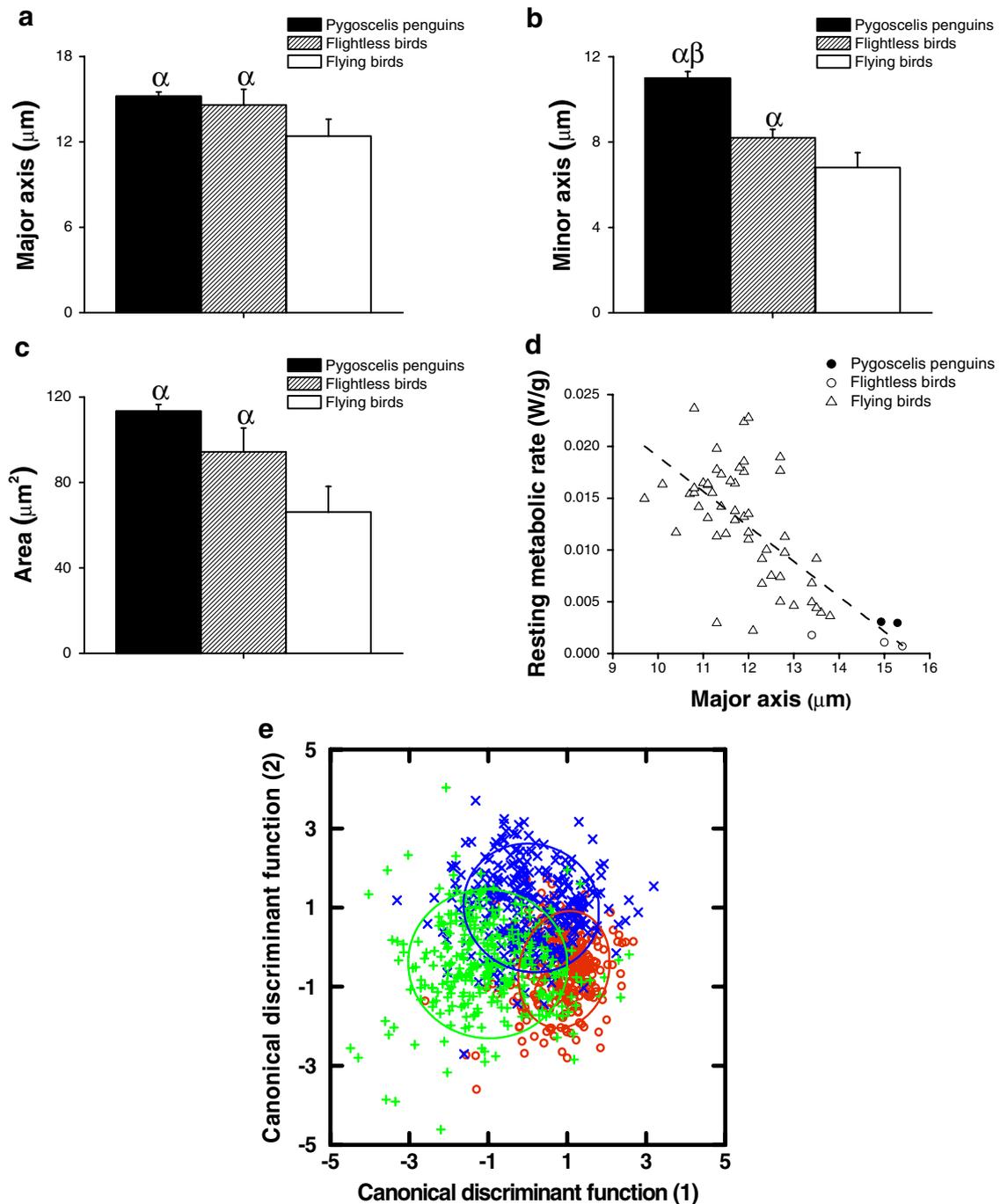
**Fig. 1** Photographs, AFM 2D views, and AFM 3D views of RBCs from adult specimens of *P. adeliae* (a, b, c, respectively), *P. antarcticus* (d, e, f, respectively); *P. papua* (g, h, i, respectively)



**Table 1** One-dimensional, bi-dimensional, tri-dimensional, and shape descriptor parameters of the pygoscelid RBCs ( $n = 100$ ) evaluated by atomic force microscopy

One-dimensional							
	Max. diameter (μm)	Pattern width (μm)	Mean radius (μm)	Max. Z (μm)	Min. Z (μm)	Average Z (μm)	Perimeter (μm)
<i>Pygoscelis adeliae</i>	14.93 ± 0.08	10.77 ± 0.46	5.92 ± 0.07	1.84 ± 0.15	1.02 ± 0.08	1.53 ± 0.12	40.82 ± 0.41
<i>Pygoscelis antarcticus</i>	15.47 ± 0.20	11.39 ± 0.12	6.11 ± 0.08	1.94 ± 0.09	1.03 ± 0.03	1.52 ± 0.04	42.37 ± 0.68
<i>Pygoscelis papua</i>	15.29 ± 0.40	10.91 ± 0.28	6.02 ± 0.15	1.95 ± 0.13	1.07 ± 0.07	1.57 ± 0.08	41.79 ± 1.14
Bi-dimensional and tri-dimensional							
	Area excluding hole (μm <sup>2</sup> )	Area including hole (μm <sup>2</sup> )	Surface area (μm <sup>2</sup> )	Volume (μm <sup>3</sup> )	Surface/volume ratio		
<i>Pygoscelis adeliae</i>	110.08 ± 2.78	110.13 ± 2.80	114.29 ± 2.52	168.17 ± 8.52	0.68 ± 0.05		
<i>Pygoscelis antarcticus</i>	116.49 ± 2.95	116.81 ± 2.80	120.68 ± 3.12	176.62 ± 3.63	0.68 ± 0.01		
<i>Pygoscelis papua</i>	113.40 ± 5.59	113.71 ± 5.48	118.93 ± 5.20	177.33 ± 9.77	0.67 ± 0.03		
Shape descriptors							
	Form factor	Roundness	Compactness	Aspect ratio	Elongation	Roughness	
<i>Pygoscelis adeliae</i>	0.83 ± 0.00	0.63 ± 0.01	0.79 ± 0.01	1.43 ± 0.06	15.18 ± 0.09	1.21 ± 0.01	
<i>Pygoscelis antarcticus</i>	0.81 ± 0.01	0.62 ± 0.00	0.79 ± 0.00	1.40 ± 0.02	15.42 ± 0.20	1.23 ± 0.02	
<i>Pygoscelis papua</i>	0.81 ± 0.01	0.62 ± 0.00	0.79 ± 0.00	1.44 ± 0.06	15.42 ± 0.13	1.23 ± 0.01	

The values are the mean ± SEM of three animals



**Fig. 2** Histograms of the (a) major axis, (b) minor axis, and (c) area of *Pygoscelis* RBCs compared with the dimensions of flightless and flying bird RBCs. Plotted values are means  $\pm$  SEM  $^{\alpha}$ Values statistically different from flying birds at  $P < 0.05$ ;  $^{\beta}$ Values statistically different from flightless birds at  $P < 0.05$ ; **d** scatter plot of the RMR versus RBCs major axis ( $r = -0.70$ ,  $P < 0.0001$ ) of *Pygoscelis*, flightless, and

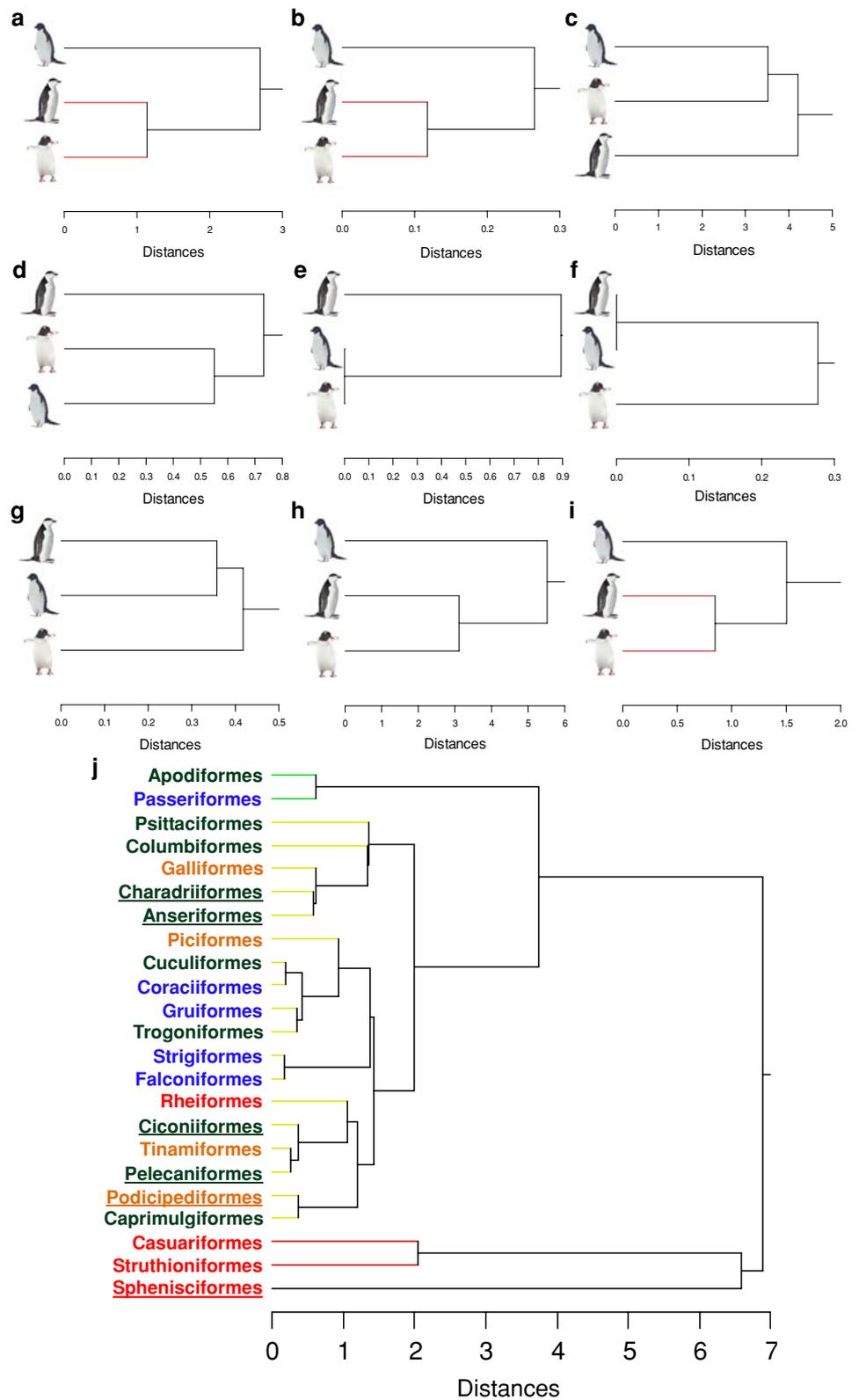
flying birds; **e** canonical discriminant functions of the *Pygoscelis* species using all RBC measurements and shape descriptors. Each point displays an individual cell of each species ( $n = 300$ ) together with 75% confidence ellipses surrounding them. Red (empty circle) = *P. adeliae*; Blue (cross) = *P. antarcticus*; and Green (plus) = *P. papua*

clustering that reedited RBCs- and DNA-based clusters. HCA of avian orders also showed that RBC parameters were able to group the *Pygoscelis* (Fig. 3j) with two orders of flightless birds, the Struthioniformes (ostrich) and Casuariformes (emu).

## Discussion

Despite the large range of RBC size and shape described over one century ago (Gulliver 1875), few studies have attempted to survey the size and shape of RBCs of Antarctic

**Fig. 3** Dendrograms representing the alternative HCAs of the *Pygoscelis* species according to RBCs (a), DNA sequences (b), protein sequences (c), integumentary (d), breeding (e), myological (f), osteological (g), white blood cells counts (h), and consensus of the combined data set (i). The red color of the terminal nodes changes down the cluster tree to reflect changes in node length on the distance scale between less than and greater than 0.5. **j** HCA of the Avian orders according to RBC average cell length (*major axis*), cell width (*minor axis*), and cell area of the individual species of the present study (Sphenisciformes) and cell size database (other orders). The representation includes information on flight ability across orders as indicated by colored fonts: flightless (*red*), weak (*orange*), moderate, variable, or strong but brief (*blue*), and strong (*green*). The underlined names indicate aquatic bird orders. The color coding of the terminal nodes changes down the cluster tree to reflect changes in node length on the distance scale between less than and greater than 0.5



vertebrates (Hawkey et al. 1989). When comparing the data set of RBC parameters of *Pygoscelis* penguins to those available for other vertebrates, it was not possible to identify any variation in their size values that could be interpreted as an

adaptation to extreme environmental conditions or penguins lifestyle. However, the RBC sizes of *Pygoscelis* were similar to those described in other flightless birds, in contrast to RBC sizes of flying birds. Furthermore, it has been

demonstrated that not only does a strong positive correlation exist between birds RBC size and genome size (C-value), but a general inverse association between flight skill and genome size also exists among birds (Gregory 2002; Hughes and Hughes 1995). This association has been causally associated with metabolic cost reduction. However, a recent study with extinct saurischian dinosaur lineage indicated that flight and genome/cell size may be functionally related instead of causally coupled (Organ et al. 2007), since a small genome size, presumably was not directly linked to improved flight skills or metabolic cost reduction. No general consensus has been reached thus far and the large *Pygoscelis* RBCs size (and presumably genome size) reported in the present study cannot be interpreted without references to the particular physiology (e.g., RMR) and lifestyle (e.g., flightless) of the group. The strong negative correlation between the RBCs size and RMR among flying, flightless, and *Pygoscelids* could at least in part elicit the physiological relevance of large RBC in *Pygoscelis*. In addition, all other flightless birds were scattered closely the *Pygoscelids*.

Furthermore a comprehensive set of RBC measurements obtained by AFM was used for the first time in a species clustering. When interpreted individually, the RBC parameters obtained by AFM were not capable to statistically discriminate the *Pygoscelis* species. In fact, a common drawback in phylogenetic approaches is the inability to distinguish organisms using single characters. Hence, several non-redundant characters are generally necessary to distinguish closely related species (Livezey and Zusi 2007). However, even when all AFM parameters were simultaneously used in DA, 74% of the RBCs were identified to the correct species of the *Pygoscelis* genus. In fact, it has been shown that the RBCs of close-related species share similar and even undistinguishable shapes and sizes due in part to their similar genome sizes (Gregory 2002). Therefore, it is possible that the similarities among *Pygoscelis* RBCs represent an ancestral/conserved feature of this genus. These findings may reflect lifestyle similarities among the species that ultimately could be associated to phylogenetic relationships. In order to test this hypothesis, species belonging 23 avian orders were compared and supported in a flightless bird clade, which include Sphenisciformes, Struthioniformes, and Casuariformes. This result challenges a recent DNA-based phylogeny of avian orders in which aquatic birds belonging to Pelecaniformes, Ciconiiformes, Procellariiformes, and Gaviiformes share the same clade with Sphenisciformes (Hackett et al. 2008). In this regard, the RBC-based classification proposed here may represent a suitable alternative to reconstruct the flightless habits of the species more than simply infer phylogenetic relationships.

Various methods exist in order to generate a fingerprint (or barcode) of an organism that could cluster it as belonging to a species. However, such strategies commonly result

in different phylogenetic scenarios. This conflicting situation has led to recurring debates in taxonomy and reinforces the notion that this ancient field of Biology needs re-examining (Godfray 2002). The relationship of the living *Pygoscelis* species according to RBCs data agree with those recovered by the DNA analysis. The use of DNA sequences is considered to be one of the best techniques to revisit the taxonomic classification due to the range of different combinations in a few hundred of base pairs (Hebert et al. 2003). Interestingly, the protein data set originated from translated DNA sequences failed to reproduce the phylogeny of *Pygoscelis* inferred by DNA and RBC parameters. It has been demonstrated in some cases that evolutionary relationships among species derived from DNA versus protein sequence comparisons differ to some extent (Schmidt et al. 2003). It is possible that molecular constraints of Antarctic penguins take part in these differences. Likewise, the cladistic inferences obtained using integumentary, breeding, myological, osteological, and WBC parameters differed markedly from each other and from RBC- and DNA-based methods of phylogenetic classification. As divergence among results haunts all taxonomists (Livezey and Zusi 2007), a consensus of multiple strategies has been the recommended solution (Hugot 1998) and the RBC-based method proposed herein was perfectly fitted.

There are many possible reasons why RBCs may recover *Pygoscelis* phylogenetic relationships: (1) The RBCs role in a vital system such as oxygen transport may enhance the significance of AFM-obtained structural characters in phylogenetic analysis; (2) Euclidean distance-based methods of phylogenetic inference may be more suitable for the analysis of continuous data rather than discrete data sets usually obtained from typical anatomical characters; (3) The geometry of RBCs may have originated early in the *Pygoscelid* lineage with extant species exhibiting changes able to correctly cluster the species according to their habits when compared to other extant orders. These hypotheses are not mutually exclusive, and their relevance needs further investigation.

In conclusion, AFM high-resolution associated with its ability to determine the shape and size of single cells has shown to be valuable for comparative hematologic studies and even achieve phylogenetic inferences. Future studies on the phylogeny of other penguins and vertebrates could benefit from including high-throughput RBC measurements obtained by AFM. In a visionary sense, these measurements can be regarded as barcodes that are embedded in RBC geometry, representing a novel outlook of biological diversity and evolution.

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