Brief Report

Plasmodium (Novyella) nucleophilum from an Egyptian Goose in São Paulo Zoo, Brazil: microscopic confirmation and molecular characterization ☆

Carolina Romeiro Fernandes Chagas [a], Gediminas Valkiūnas [b], Carolina Vaz Cabral Nery [a], Paloma Canedo Henrique [a], Irys Hany Lima Gonzalez [a], Eliana Ferreira Monteiro [c], Lilian de Oliveira Guimarães [c], Camila Malta Romano [d], Karin Kirchgatter [c,*]

[a] Zoological Park Foundation, São Paulo, Brazil
[b] Institute of Ecology, Nature Research Centre, Lithuania
[c] Malaria Research Center, Superintendence for Endemic Disease Control, São Paulo Institute of Tropical Medicine, University of São Paulo, Brazil
[d] Laboratory of Virology, São Paulo Institute of Tropical Medicine, University of São Paulo, Brazil

ARTICLE INFO

ABSTRACT

Plasmodium (Novyella) nucleophilum was identified using microscopy and PCR, in an Egyptian Goose (Alopochen aegyptiacus) that died in São Paulo Zoo, Brazil. This parasite is characterized by elongated gametocytes, small meronts with scant cytoplasm, less than eight merozoites and mainly for having all the stages appressed to the nuclei of infected erythrocytes. Additionally, Plasmodium (Haemamoeba) sp. was identified by microscopy in the same blood sample. The latter parasite lacks nucleophilic blood stages and is characterized by large roundish trophozoites, each with a large prominent centrally collated vacuole. This co-infection was not confirmed by PCR amplification of the mitochondrial cytochrome b (cytb) gene and sequencing; only one Plasmodium sp. cytb sequence was detected in the blood sample. Since parasitemia of P. nucleophilum (2.4%) was much higher than that of P. (Haemamoeba) sp. (0.2%), PCR may have favored the amplification of the cytb sequence of the former. Phylogenetic analysis is in agreement with this conclusion because the reported cytb sequence was positioned in the same branch of sequences of several Novyella species. This is the first assignment of the mitochondrial cytb gene sequence to P. nucleophilum. The P. (Haemamoeba) parasite is particularly similar to Plasmodium (Haemamoeba) tejerai, because its advanced trophozoites and young erythrocytic meronts possess a large vacuole with prominent pigment granules arranged around it, the characteristic features of development in this species. For definitive identification of P. (Haemamoeba) species, mature meronts and gametocytes are required; however, these were absent from the thin blood smear. Representative images of the blood stages of P. nucleophilum and P. (Haemamoeba) sp. are provided. Together with microscopy data, the P. nucleophilum cytb sequence will assist in molecular identification (barcoding) of this Plasmodium species in other birds.

1. Introduction

Haemosporidian parasites (Sporozoa: Haemosporida) are cosmopolitan protists that parasitize birds and other animals and use blood-sucking dipteran insects as vectors. More than 200 species of avian haemosporidians from hundreds of bird species have been described including over 50 morphologically distinct species of the genus Plasmodium belonging to five subgenera (Valkiūnas, 2005; Zehtindjiev et al., 2012). Brazil has one of the richest bird faunas on the planet and some species of captive and wild birds have been shown to be infected with blood protists, including parasites belonging to all subgenera of Plasmodium, but only a few studies have been conducted on biology of these parasites (rev. in Braga et al., 2011).

In zoological parks, the animals live in a high density, close to animals from different zoogeographical regions, which can favor parasite infections (Primarck and Rodrigues, 2001; Silva and Corrêa, 2006). Many zoos have rich vegetation and are located in bush lands, which are visited by migrant birds every year. Infected migrant birds are potential donors of parasitic infections, which can be transmitted to zoo birds, particularly Plasmodium infections, since the relationship between the parasite and host is of low specificity (Bensch et al., 2000). Thus, studies involving parasites of migrating birds and the zoo animals that are in proximity to them are important for wildlife conservation.

The Atlantic forest is considered as one of world's biodiversity hot spots and some of the best and most extensive examples of this biome are in São Paulo (Ribeiro et al., 2009). São Paulo Zoo, established in 1958, is located in one of the last forest remnants within the City of São Paulo, the largest city in Brazil, with a high density and diversity of mosquitoes transmitting avian malaria (Guimarães et al., 2000; Bueno et al., 2010; Ribeiro et al., 2012). The zoo is located in an area of 824,529 m^2 of original Atlantic Forest with the headwaters of the historical Ipiranga stream, whose waters form a lake that receives several wild species of birds including migratory species. Around the lake, the forest shelters wild native animals, forming diverse wildlife parallel to the zoo animal collection. More than 3000 animals are on display, representing species of mammals, birds, reptiles, amphibians and invertebrates. During migration and the wintering of birds from various American countries, as well as from other Brazilian regions, the space destined for zoo animals is shared by migratory species, including opportunistic species. The latter are behaviorally flexible birds living in variable environmental conditions that are sustained by different food sources. Thus, living freely in the area of the zoo, such birds can rapidly take advantage of favorable conditions when they arise. Because blood-sucking mosquitoes that can transmit avian malaria are widespread in the zoo (Bueno et al., 2010) and favorable abiotic conditions for malaria transmission also exist, we initiated an investigation in São Paulo Zoo in order to estimate the prevalence of *Plasmodium* spp. in birds using both PCR-based and microscopy methods. In this paper, we report a malaria infection in an Egyptian Goose (*Alopochen aegyptiacus*) that was born in São Paulo Zoo and lived in one of the lakes where migratory birds are present during their migration and wintering. Two *Plasmodium* species were identified and a mitochondrial cytochrome b (*cytb*) gene sequence was determined, enabling the molecular characterization of one these parasites. Illustrations of blood stages of both parasites are provided, and phylogenetic analysis identified DNA lineages closely related to them.

2. Material and methods

2.1. Case history

A 10-year-old adult male goose, born at the São Paulo Zoo, was referred to the Veterinary Division with clinical signs of incoordination, in October 2011. During clinical examination, it was apathetic, skinny, dehydrated and presented pale mucosa. The goose had a small injury in the cloacal region, wet feathers and a lot of dipteran larvae all over its body. It was physically restrained for blood sample collection, venous access to administration of fluid with glucose and antibiotics (enrofloxacin), larvae removal and cleaning and drying of the feathers. During the procedure, the goose had a cardiorespiratory arrest, reanimation was unsuccessful and it died. The blood examination indicated that it was anemic with hematocrit levels of 31% (reference values are 41–54%). It presented severe leukocytosis, 88×10^3 µL ($6.39–21.73 \times 10^3$ µL), and heterophilia, 81.84×10^3 µL ($2.62–13.41 \times 10^3$ µL), indicating the presence of an infectious agent. Blood biochemistry results were abnormal: uric acid and aspartate aminotransferase (AST) were very high, 28.5 mg/dL (3.0–8.6 mg/dL) and 385 UI/L (9–75 UI/L), respectively. The reference values (in parenthesis) were obtained from ISIS 2002 (http://www.isis.org). During necropsy, thoracic air sac presented with a yellowish color and fungal colonies, suggesting *Aspergillus* sp. infection, which was confirmed by histopathology. *Plasmodium* sp. parasitemia, splenomegaly, a severe pulmonary edema and the presence of signs of multifocal necrosis on the liver were also observed. However, the hepatitis may have been caused by the fungal toxin released by *Aspergillus* sp. and could have contributed to the goose's death (Quist et al., 2007). The *causa mortis* was likely respiratory insufficiency.

2.2. Collection of blood samples and representative material

Venous blood was collected from median metatarsal vein of the goose during clinical examination for haemogram and biochemistry investigation. A thin blood smear was prepared without anticoagulant and the remaining blood was placed in a lithium heparin tube and frozen for DNA extraction. The thin blood smear was fixed by methanol, stained by Giemsa and 100 microscopic fields were examined under light microscopy (1000×) (Valkiūnas, 2005). The intensity of parasitemia was determined, as recommended by Godfrey et al. (1987). The voucher thin blood smears from the infected goose were deposited in the Clinical Laboratory of the Zoological Park Foundation, São Paulo, Brazil (accession numbers 26679A and 26679B); digital images of the parasites are also available in the Clinical Laboratory and in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania. The sample of gDNA from the Egyptian Goose (original field number is 7885) is deposited in the Clinical Laboratory of the Zoological Park Foundation, São Paulo, Brazil.

Morphology of *Plasmodium nucleophilum* from the Egyptian Goose was compared with the neohapantotype preparations of the same parasite from experimentally infected domestic canary *Serinus canaria domestica* (R. D. Manwell's original strain, accession nos. 640, 641) in the Garnham Collection at the Natural History Museum, London.

2.3. Goose genomic DNA (gDNA) extraction, PCR amplification of *Plasmodium* sp. cytb fragments, sequencing and sequence data analysis

After thawing, the blood sample was centrifuged and 100–150 µl of red blood pellets were used for the DNA extraction. First, an initial lyse was performed with 1% saponin. Next, the pellet was washed twice in ultrapure water and submitted to the extraction protocol with the GFX™ Genomic Blood DNA Purification Kit (Amersham Biosciences, GE Healthcare), following the manufacturer's instructions. The gDNA was eluted in 100 µl of ultrapure water and stored at −20 °C. A fragment of 1.1 kb (approximately 92% of the gene) from the mitochondrial cytochrome b gene (*cytb*) was amplified using a nested PCR, taking standard precautions to prevent cross-contamination of samples. The PCR reactions were conducted as previously described (Perkins and Schall, 2002) using primers DW2 and DW4 and 5 µl of gDNA in the first reaction and 1 µl aliquot of this product was used as a template for a nested reaction with primers DW1 and DW6. All these primers are specific to malarial parasites and do not amplify host DNA or that of other apicomplexan species.

The PCR product of 1 kb was sequenced by Big Dye Terminator v3.0 Cycle Sequencing Kit in ABI Genetic Analyzer (ABI, USA), using PCR oligonucleotides (DW1 and DW6) and internal primers DW3 and DW8 (Perkins and Schall, 2002). The sequence obtained was aligned using BLASTN (Basic Local Alignment Search Tool) with sequences from the GenBank available at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi (Altschul et al., 1997) and MalAvi available at http://mbio-serv2.mbioekol.lu.se/Malavi/blast.html (Zhang et al., 2000). The *P. nucleophilum* sequence was deposited in the GenBank database with accession number JX467689.

2.4. Phylogenetic analysis

The phylogenetic relationship of the *P. nucleophilum* lineage from the Egyptian Goose and other *Plasmodium* species described

in birds was inferred using *cytb* gene sequences. The sequences were selected from the MalAvi database, a public database of malaria parasites and related haemosporidians in avian hosts (Bensch et al., 2009). The phylogenetic tree was constructed using the Bayesian inference method implemented in MrBayes v3.2.0 (Huelsenbeck and Ronquist, 2001). Bayesian inference was performed with two Markov Chain Monte Carlo searches of 3 million generations each with sampling of 1 in 300 trees. After a burn-in of 25%, the remaining 15,002 trees were used to calculate the 50% majority-rule consensus tree.

3. Results and discussion

Co-infection of *P. (Novyella) nucleophilum* Manwell, 1935 (Fig. 1a–f) and *Plasmodium (Haemamoeba)* sp. (Fig. 1g–i) was detected by microscopy. The intensity of parasitemia of these parasites was 2.4% and 0.2%, respectively. These two species can be readily distinguished from each other due to the presence or absence of nucleophilic parasites and of large roundish trophozoites. *P. nucleophilum* is characterized by (i) small (less than erythrocyte nuclei) meronts with scanty cytoplasm; (ii) ⩽8 merozoites in meronts; (iii) small (length <10 µm on average) elongated gametocytes possessing <10 pigment granules; (iv) the presence of vacuoles and refractive globules in some trophozoites and meronts; (v) trophozoites, meronts and gametocytes appressed to erythrocyte nuclei (nucleophilic feature) (Fig. 1a–f). The parasites observed in the Egyptian Goose were similar to the blood stages present in the type material of *P. nucleophilum*. However, (i) small vacuoles were frequently observed in trophozoites and meronts in the blood of the goose, and (ii) refractive globules were observed in many erythrocytic meronts. These feature are also visible in the type material of *P. nucleophilum*, but were observed less frequently; this could be a peculiarity of the parasite development

in the goose. In contrast, the second species identified, *P. (Haemamoeba)* sp., lacks nucleophilic blood stages and is characterized by large roundish trophozoites, each with a prominent centrally collated vacuole (Fig. 1g–i). Because large trophozoites (size close to erythrocyte nuclei), which displace the erythrocyte nuclei were present, this parasite belongs to the subgenus *Haemamoeba* (Fig. 1g). For definitive identification of *P. (Haemamoeba)* species, mature meronts and gametocytes are required; however, these blood stages were absent from the thin blood film.

P. (Novyella) nucleophilum has been recorded in all zoogeographical regions, except the Australian and Antarctic, but is particularly frequently reported in the Americas. This parasite is particularly common in birds belonging to the Passeriformes, but has also been identified in species of Anseriformes, Columbiformes and Piciformes. Originally, *P. nucleophilum* was discovered in North America and has subsequently been reported in many bird species in South America (Manwell, 1935; Garnham, 1966). A sub-species, *P. nucleophilum toucani*, has been reported in Brazil in the Chestnut-mandibled Toucan (Piciformes), but its distribution has been insufficiently investigated (Manwell and Sessler, 1971). It is worth mentioning that Lucena (1939) identified *P. nucleophilum* in house sparrows (*Passer domesticus*) examined in Sao Paulo. It is probable that this bird serves as a natural reservoir host of *P. nucleophilum*-malaria infection, a fact that warrants further investigation. Blood stages of the reported parasite are morphologically similar to previous descriptions (Manwell, 1935; Valkiūnas, 2005), so their detailed morphological description was not provided. Virulence of *P. nucleophilum* varies markedly. Certain subspecies are relatively benign in experimentally infected canaries, but can be highly virulent in experimentally infected ducklings, and are fatal in many cases (Valkiūnas, 2005).

The *Haemamoeba* subgenus contains 11 malaria parasite species, and *P. relictum*, the type species, has been reported from São
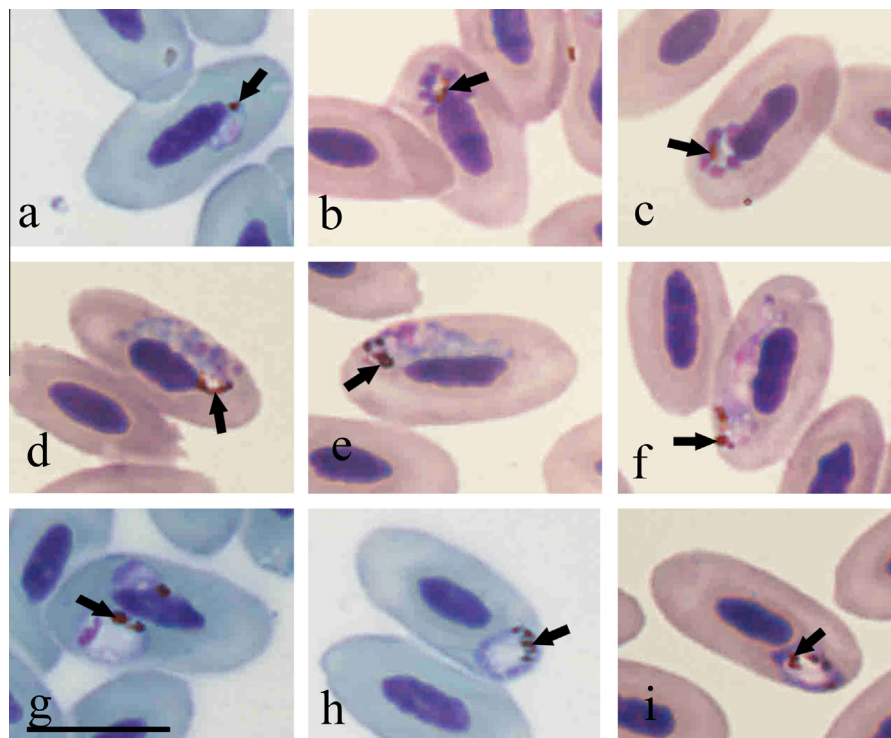

Fig. 1. Photomicrographs of *Plasmodium* parasites visualized from thin blood smears obtained from an Egyptian Goose (*Alopochen aegyptiacus*) in São Paulo Zoo, Brazil. Characteristic of *Plasmodium (Novyella) nucleophilum* (lineage EG01, GenBank JX467689) the trophozoite (a), meronts (b–c), macrogametocytes (d, e), and microgametocyte (f) are appressed to erythrocyte nuclei (nucleophilic features). *Plasmodium (Haemamoeba)* sp. (g–i) lacks nucleophilic blood stages and possesses large roundish trophozoites, each with a prominent centrally located vacuole; pigment granules are gathered around the vacuoles. Note that early *Plasmodium (H.)* sp. trophozoites markedly displace erythrocyte nuclei (g). Arrows, pigment granules. Scale bar = 10 µm.

Table 1
Avian hosts and countries where the DENPET03 lineage of *Plasmodium nucleophilum* has been reported.

| Order	Family	Species	Reference	Country
Passeriformes	Fringillidae	*Basileuterus culicivorus*	Durrant et al. (2006)	Uruguay
		Basileuterus leucoblepharus	Durrant et al. (2006)	Uruguay
		Cacicus cela	Durrant et al. (2006)	Guyana
		Cacicus haemorrhous	Durrant et al. (2006)	Guyana
		Cranioleuca pyrrhophia	Durrant et al. (2006)	Uruguay
		Geothlypis trichas	Pagenkopp et al. (2008)	USA
		Gnorimopsar chopi	Durrant et al. (2006)	Uruguay
		Setophaga petechia	Szymanski and Lovette (2005)	USA
		Volatinia jacarina	Durrant et al. (2006)	Guyana
		Zonotrichia capensis	Durrant et al. (2006)	Uruguay
	Passeridae	*Passer domesticus*	Marzal et al. (2011)	Brazil[a]
	Vireonidae	*Vireo griseus*	Ricklefs and Fallon (2002)	USA
Psittaciformes	Psittacidae	*Diopsittaca nobilis*	Durrant et al. (2006)	Guyana

[a] Midwest and North.
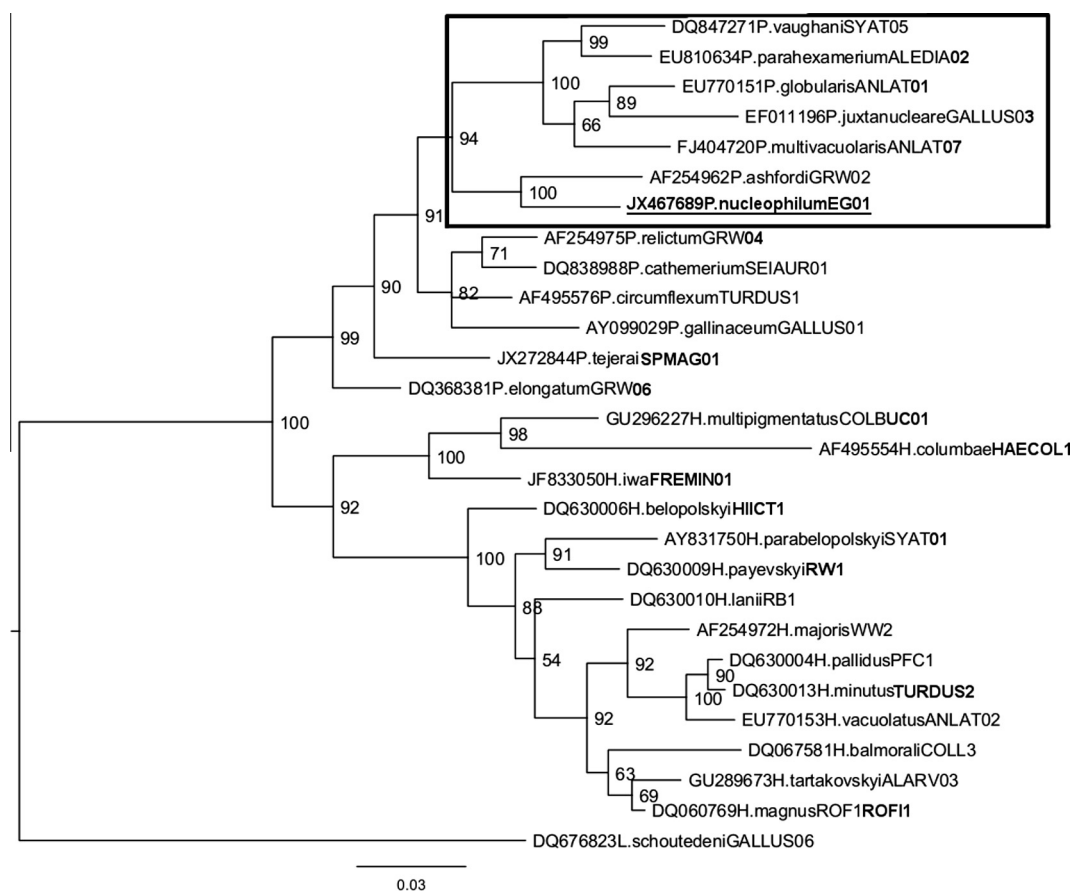
Fig. 2. Bayesian phylogeny of cytochrome *b* gene lineages of species of avian haemosporidian parasites. A lineage recorded in the Egyptian Goose *Alopochen aegyptiacus* is provided underlined. Names of the lineages are given after the species names of parasites. GenBank accession numbers of the lineages are provided before the parasite species names. Nodal support values (in percentage) indicate posterior clade probabilities. *Plasmodium* species from *Novyella* subgenus are boxed.

Paulo Zoo (Bueno et al., 2010). However, the *P.* (*Haemamoeba*) parasite recorded in this study is more similar to *Plasmodium* (*Haemamoeba*) *tejerai* because each of its advanced trophozoites and young erythrocytic meronts possessed a large vacuole with pigment granules arranged around it, the characteristic features of this species. Other described *P.* (*Haemamoeba*) parasites do not possess these characters (Valkiūnas, 2005). *Plasmodium tejerai* was first recorded in domestic turkey, *Meleagris gallopavo*, in Venezuela and some experimentally infected birds, including several Anatidae species (Gabaldon and Ulloa, 1977). Recently, it was identified in penguins from a rehabilitation centre in South Brazil

(Silveira et al., 2013). It is the only *Plasmodium* parasite with large vacuoles and pigment granules arranged around the vacuoles in trophozoites (Fig. 1g–i) found in South America (Gabaldon and Ulloa, 1977). Because mature meronts and gametocytes of *P.* (*Haemamoeba*) sp. were absent from our blood sample and we were unable to detect sequence data, the definitive identification of the parasite is currently impossible.

The PCR protocol did not confirm co-infection and amplify only one *cytb* sequence, since no "double bases" were present in electropherograms. Previous studies have demonstrated that mixed infections are not efficiently detected using general primers (Valkiūnas

et al., 2006; Martínez et al., 2009). Moreover, *Plasmodium* spp. detection depends on the parasite lineage composition during co-infections and the intensity of the infections. For haemosporidians, the PCR assays currently used can underestimate the occurrence of co-infections in the majority of natural infections (Valkiūnas et al, 2008a). In a recent study that used a PCR methodology to detect haemosporidians in 214 Brazilian birds, 14% of *Plasmodium* spp. infections were verified, but no co-infections were observed (Marzal et al., 2011). Failure to amplify the *cytb* gene of *Plasmodium polymorphum* in a mixed infection with *P. relictum*, even with a significantly greater parasitemia in the former, has also been demonstrated (Zehtindjiev et al., 2012). Thus, the combination of microscopy and PCR-based tools is essential for correct identification of *Plasmodium* spp. and investigation of the biodiversity of haemosporidian parasites in wildlife.

The sequence obtained (GenBank #JX467689, 1122 bp) was compared by BLASTN with GenBank and MalAvi sequences. In the GenBank database, the most similar *Plasmodium* sequences were GQ395660 and GQ141580 (both from *Plasmodium* sp.) with 97% identity (1006 of 1036 bp). The most similar sequence with species identification was a sequence from *P. ashfordi* (#AF254962) belonging to the subgenus *Novyella*, with 94% identity (451 of 478 bp). This sequence was formerly incorrectly attributed to *P. nucleophilum* (see review in Valkiūnas et al., 2007). In the GenBank, the presence of this and other misidentified sequences of avian haemosporidians has been proven (Valkiūnas et al., 2008b). The great majority of blood stages of *P. ashfordi* are non-nucleophilic, thus it can be readily distinguished from *P. nucleophilum* (Fig. 1).

In the MalAvi database, which comprises 1323 sequences and 590,601 total base pairs, we identified a sequence (AY640137), DENPET03 lineage of *P. nucleophilum*, with 100% identity in 479 bp (nucleotides 235–713 from our sequence). This sequence has been reported in 13 avian hosts belonging to 11 genera, 5 families and 2 orders (including passerines) in North and South America (Table 1). This is in agreement with previous microscopic studies (Garnham, 1966; Valkiūnas, 2005) and demonstrates that *P. nucleophilum* is likely a host generalist, as has been demonstrated for many other avian *Plasmodium* species (Szymanski and Lovette, 2005; Palinauskas et al., 2007). However, in the MalAvi database, there is no record of occurrence of any haemosporidian parasites in the Egyptian goose (http://mbio-serv2.mbioekol.lu.se/Malavi/index.html).

The phylogenetic analysis is in agreement with our morphological identification since the reported *cytb* sequence clustered with sequences of several *Novyella* species, forming a well-supported clade with high posterior probability (Fig. 2). This record was included in the MalAvi database as EG01 (acronym of Egyptian goose lineage 1).

Although in most organisms the gene of choice for barcoding is cytochrome oxidase subunit I (COI), a 479 bp fragment of the *cytb* gene has been successfully used as a barcode of avian haemosporidian species (Bensch et al., 2009). This study provides the first assignment of a mitochondrial *cytb* gene sequence to *P. nucleophilum* deposited in the GenBank and can be used for molecular identification (barcoding) of this infection in other birds. The same sequence has been reported in numerous bird species in the Americas (Table 1). The identification of barcodes to recognize avian haemosporidians species is an important step, since morphological identification of these parasites requires extensive taxonomic experience and is not always possible due to predominance of light parasitemias in wildlife (Hajibabaei et al., 2007). Currently, there are few experts with such knowledge and few people in the next generation of scientists are learning these taxonomic skills (Valkiūnas et al., 2008b); thus, the development of barcodes to identify avian malaria and related parasites is an urgent task.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSIBLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Bensch, S., Stjernman, M., Hasselquist, D., Östman, Ö., Hansson, B., Westerdahl, H., Pinheiro, R.T., 2000. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc. Biol. Sci. 267, 1583–1589.

Bensch, S., Hellgren, O., Pérez-Tris, J., 2009. MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. Mol. Ecol. Resour. 9, 1353–1358.

Braga, E.M., Silveira, P., Belo, N.O., Valkiūnas, G., 2011. Recent advances in the study of avian malaria: an overview with an emphasis on the distribution of *Plasmodium* spp. in Brazil. Mem. Inst. Oswaldo Cruz. 106 (Suppl. I), 3–11.

Bueno, M.G., Lopez, R.P.G., Menezes, R.M.T., Costa-Nascimento, M.J., Lima, G.F.M.C., Araújo, R.A.S., Guida, F.J.V., Kirchgatter, K., 2010. Identification of *Plasmodium relictum* causing mortality in penguins (*Spheniscus magellanicus*) from Sao Paulo Zoo. Brazil. Vet. Parasitol. 173, 123–127.

Durrant, K.L., Beadell, J.S., Ishtiaq, F., Graves, G.R., Olson, S.L., Gering, E., Peirce, M.A., Milensky, C.M., Schmidt, B.K., Gebhard, C., Fleischer, R.C., 2006. Avian haematozoa in South America: a comparison of temperate and tropical zones. Ornithol. Monogr. 60, 98–111.

Gabaldon, A., Ulloa, G., 1977. *Plasmodium* (*Haemamoeba*) *tejerai* sp. n. en pavo domestico (*Melleagris gallopavo*) del Venezuela. Bol. Dir. Malariol. Saneam. Ambient. 17, 255–273.

Garnham, P.C.C., 1966. Malaria Parasites and Other Haemosporidia. Blackwell Scientific Publications, Oxford, p. 1114.

Godfrey, R.D., Fedynich, A.M., Pence, D.B., 1987. Quantification of hematozoa in blood smears. J. Wildl. Dis. 23, 558–565.

Guimarães, A.E., Mello, R.P., Lopes, C.M., Gentile, C., 2000. Ecology of mosquitoes (Diptera: culicidae) in areas of Serra do Mar State Park, State of São Paulo, Brazil. I – monthly frequency and climatic factors. Mem. Inst. Oswaldo Cruz 95, 1–16.

Hajibabaei, M., Singer, G.A., Hebert, P.D., Hickey, D.A., 2007. DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends Genet. 23, 167–172.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17, 754–755.

Lucena, D.T., 1939. Malaria aviaria: subsidios para sua sistematica e transmissão. Officinas graphicos do Jornal do Commercio, Recife, p. 126.

Manwell, R.D., 1935. How many species of avian malaria parasites are there? Am. J. Trop. Med. 15, 265–283.

Manwell, R.D., Sessler, G.J., 1971. Malaria parasites of toucans. J. Protozool. 18, 570–574.

Martínez, J., Martínez-De La Puente, J., Herrero, J., Del Cerro, S., Lobato, E., Rivero-De Aguilar, J., Vásquez, R.A., Merino, S., 2009. A restriction site to differentiate *Plasmodium* and *Haemoproteus* infections in birds: on the inefficiency of general primers for detection of mixed infections. Parasitology 136, 713–722.

Marzal, A., Ricklefs, R.E., Valkiūnas, G., Albayrak, T., Arriero, E., Bonneaud, C., Czirják, G.A., Ewen, J., Hellgren, O., Hořáková, D., Iezhova, T.A., Jensen, H., Križanauskienė, A., Lima, M.R., de Lope, F., Magnussen, E., Martin, L.B., Møller, A.P., Palinauskas, V., Pap, P.L., Pérez-Tris, J., Sehgal, R.N., Soler, M., Szöllosi, E., Westerdahl, H., Zetindjiev, P., Bensch, S., 2011. Diversity, loss, and gain of malaria parasites in a globally invasive bird. PLoS One 6, e21905.

Pagenkopp, K., Klicka, J., Durrant, K., Garvin, J., Fleischer, R., 2008. Geographic variation in malarial parasite lineages in the common yellow throat (*Geothlypis trichas*). Conserv. Genet. 9, 1577–1588.

Palinauskas, V., Kosarev, V., Shapoval, A., Bensch, S., Valkiūnas, G., 2007. Comparison of mitochondrial cytochrome b lineages and morphospecies of two avian malaria parasites of the subgenera *Haemamoeba* and *Giovannolaia* (Haemosporida: Plasmodiidae). Zootaxa 1626, 39–50.

Perkins, S.L., Schall, J.J., 2002. A molecular phylogeny of malarial parasites recovered from cytochrome b gene sequences. J. Parasitol. 88, 972–978.

Primarck, B.R., Rodrigues, E., 2001. Biologia da Conservação. Planta, Londrina, p. 328.

Quist, C.F., Cornish, T., Wyatt, R.D., 2007. Mycotoxicosis. In: Thomas, N.J., Hunter, D.B., Atkinson, C.T. (Eds.), Infectious Diseases of Wild Birds, Blackwell Publishing, Oxford, pp. 417–430.

Ribeiro, M.C., Metzger, J.P., Martensen, A.C., Ponzoni, F.J., Hirota, M.M., 2009. The Brazilian Atlantic Forest: How much is left, and how is the remaining forest distributed? Implications for conservation. Biol. Conserv. 142, 1141–1153.

Ribeiro, A.F., Urbinatti, P.R., de Castro Duarte, A.M., de Paula, M.B., Pereira, D.M., Mucci, L.F., Fernandes, A., de Mello, M.H., de Matos Júnior, M.O., de Oliveira, R.C., Natal, D., dos Santos Malafronte, R., 2012. Mosquitoes in degraded and preserved areas of the Atlantic Forest and potential for vector-borne disease risk in the municipality of São Paulo. Brazil. J. Vector Ecol. 37, 316–324.

Ricklefs, R.E., Fallon, S.M., 2002. Diversification and host switching in avian malaria parasites. Proc. Biol. Sci. 269, 885–892.

Silva, J.C.R., Corrêa, S.H.R., 2006. Manejo Sanitário e Biosseguridade. In: Cubas, Z.S., Silva, J.C.R., Catão-Dias, J.L. (Eds.), Tratado de Animais Selvagens – Medicina Veterinária, Roca, São Paulo, pp. 1226–1244.

Silveira, P., Belo, N.O., Lacorte, G.A., Kolesnikovas, C.K., Vanstreels, R.E., Steindel, M., Catão-Dias, J.L., Valkiūnas, G., Braga, E.M., 2013. Parasitological and new molecular-phylogenetic characterization of the malaria parasite *Plasmodium tejerai* in South American penguins. Parasitol. Int. 62, 165–171.

Szymanski, M.M., Lovette, I.J., 2005. High lineage diversity and host sharing of malarial parasites in a local avian assemblage. J. Parasitol. 91, 768–774.

Valkiūnas, G., 2005. Avian Malaria Parasites and Other Haemosporidia. CRC Press, New York, p. 935.

Valkiūnas, G., Bensch, S., Iezhova, T.A., Krizanauskiene, A., Hellgren, O., Bolshakov, C., 2006. Nested cytochrome *b* polymerase chain reaction diagnostics underestimate mixed infections of avian blood haemosporidian parasites: microscopy is still essential. J. Parasitol. 92, 418–422.

Valkiūnas, G., Zehtindjiev, P., Hellgren, O., Ilieva, M., Iezhova, T.A., Bensch, S., 2007. Linkage between mitochondrial cytochrome *b* lineages and morphospecies of two avian malaria parasites, with a description of *Plasmodium (Novyella) ashfordi* sp. nov. Parasitol. Res. 100, 1311–1322.

Valkiūnas, G., Iezhova, T.A., Krizanauskiene, A., Palinauskas, V., Sehgal, R.N.M., Bensch, S., 2008a. A comparative analysis of microscopy and PCR-based detection methods for blood parasites. J. Parasitol. 94, 1395–1401.

Valkiūnas, G., Atkinson, C.T., Bensch, S., Sehgal, R.N., Ricklefs, R.E., 2008b. Parasite misidentifications in genbank: how to minimize their number? Trends Parasitol. 24, 247–248.

Zehtindjiev, P., Krizanauskiene, A., Bensch, S., Palinauskas, V., Asghar, M., Dimitrov, D., Scebba, S., Valkiūnas, G., 2012. A new morphologically distinct avian malaria parasite that fails detection by established polymerase chain reaction-based protocols for amplification of the cytochrome b gene. J. Parasitol. 98, 657–665.

Zhang, Z., Schwartz, S., Wagner, L., Miller, W., 2000. A greedy algorithm for aligning DNA sequences. J. Comput. Biol. 7, 203–214.