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ORIGINAL PAPER

Prevalence and diversity of avian malaria parasites in migratory Black Skimmers (*Rynchops niger*, Laridae, Charadriiformes) from the Brazilian Amazon Basin

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Abstract The Medium Solimões River region in the Brazilian Amazon Basin is an area utilized for reproduction and nesting by a variety of species of migratory aquatic birds such as Black Skimmers (*Rynchops niger*). These migratory birds form mixed-species reproductive colonies with high population densities and exhibit a large range of migration routes. This study aimed to describe the prevalence and diversity of the avian malaria parasites *Plasmodium* and *Haemoproteus* in Black Skimmers, on the basis of the association between microscopic observation of blood smears and amplification of the mitochondrial cytochrome b gene (mtDNA cyt-b). The overall prevalence rates of the parasites for juvenile and adult bird specimens were 16 % (5/31) and 22 % (15/68), respectively. Sequencing the mtDNA cyt-b marker revealed two *Plasmodium* lineages, which had been previously described in different regions of the American continent, including a Neotropical region in Southeast Brazil, and one *Haemoproteus* lineage. The fact that avian malarial parasites have been found infecting the Black Skimmers in the Brazilian Amazon ecosystem, which exhibits considerable diversity, highlights the importance of these migratory birds as a potential source of infection and dispersion of pathogens to other susceptible birds of the Nearctic and Neotropical regions.

Keywords *Plasmodium* · *Haemoproteus* · *Rynchops niger* · Migratory birds · Amazon

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Introduction

Brazil has one of the greatest bird diversities in the world (Sick 1997), and the most recent records estimate the presence of more than 1,800 species distributed in the Cerrado, Pampa, and Amazon biomes (CBRO 2011). Some studies demonstrated that biodiversity in the Amazon has suffered a strong and negative impact due to habitat fragmentation (Lees and Peres 2006). Thus, with the intention of preserving the environment and native species and promoting sustainable resource management based on community participation, protected areas were created since 2000, including the Mamirauá Sustainable Development Reserve (MSDR). This reserve is located in a region where sustainable forest management is the conservation focus. This characteristic was used by United Nations Ramsar Convention to identify MSDR as an area of global importance to "the conservation and sustainable use of wetlands" (RAMSAR 1997).

The MSDR located in Medium Solimões River (Amazon River) region in the Brazilian Amazon Basin is used for reproduction and nesting by a variety of species of migratory aquatic birds such as *Rynchops niger* (Laridae, Charadriiformes), also known as Black Skimmers. This migratory species has been found in bays, estuaries, lagoons, mudflats, beaches, shell banks, spoil islands, and coastal marshes, constituting reproductive mixed-species colonies with high population densities (Raeder, 2003). The breeding and nesting take place in large flocks usually located on shell banks. *R. niger* has a large range distribution and can be observed in the Caribbean islands, the Pacific, and the Atlantic coasts of the North American continent. In South America, they are found throughout the continent (Burger and Gochfeld 1990; Sick 1997) (Fig. 1).

The reproduction of black skimmers in an area which stimulates the sustainable use of natural resources may increase the

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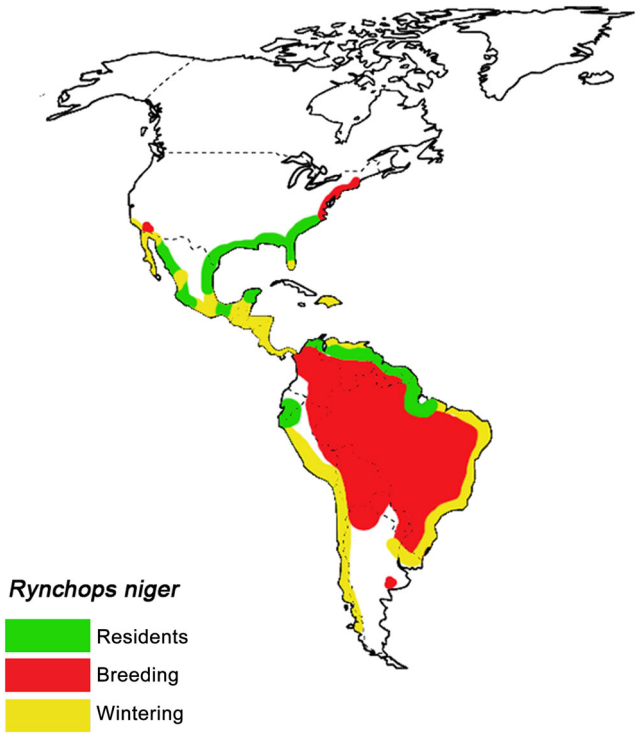

Fig. 1 Map of distribution of *Rynchops niger*

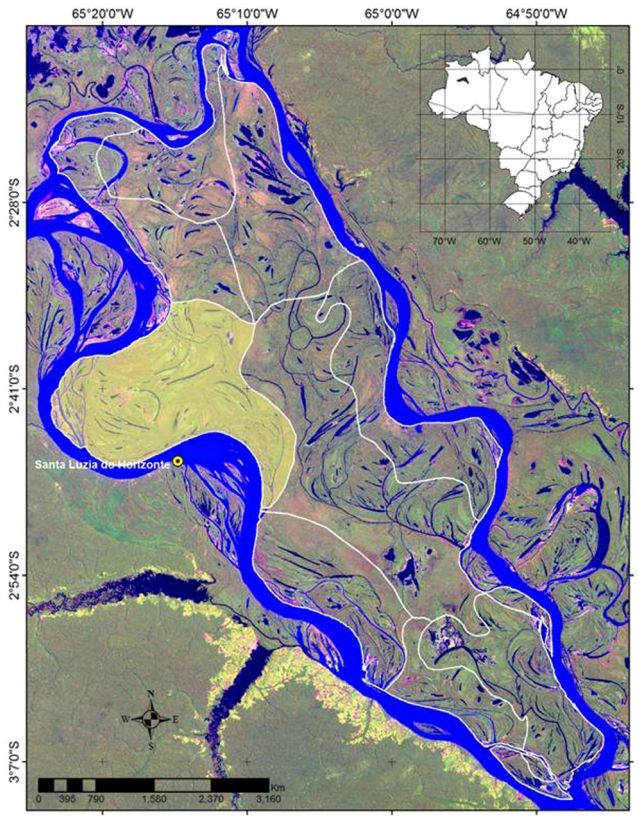

Fig. 2 Map of Mamirauá Sustainable Development Reserve (MSDR). The area studied is marked in *yellow*

risk of infection by blood parasites since its habitat alteration may favor the reproduction vectors. Besides, these waterfowl have a large range of the migration routes that could increase the potential risk for transmission of infectious and dispersal pathogenic agents to susceptible birds from different regions. This migratory behavior may also contribute to the introduction of new pathogens to the Brazilian Amazon ecosystem, which can have a considerable impact on resident communities of birds. The avian hemosporidian diversity in waterfowl of Amazon biome is not well known. Considering that hemosporidian prevalence varies according to the distribution of their hosts, investigating the occurrence of these parasites in wild birds from different biomes becomes an important step in monitoring programs for the conservation of biodiversity (Braga et al. 2011). This study aimed to understand the extent to which avian malarial parasites can infect migratory waterfowl in a habitat located in the Brazilian Amazon region. We believe that the characterization of prevalence and diversity of avian malarial parasites in waterfowl can be used to monitor the health of wild birds present in the MSDR. The data obtained can suggest a possible role of this migratory bird in the introduction and dispersal of several pathogens along American continent.

Materials and methods

Study area and sampling

The MSDR is located in the Central Amazon Corridor in the Brazilian Amazon basin (01° 49′ 0″ S and 65° 42′ 0″ W) and is considered a singular ecosystem, which includes human occupation. It covers 1,124,000 ha of seasonally flooded forest, surrounded by the Solimões, Japurá, and Auati-Paraná rivers (Fig. 2). This ecosystem represents around 2 % of the Amazon Forest area (Junk 1983), and, together with Jaú National Park and Amanã Sustainable Development Reserve, forms the largest contiguous tropical rainforest corridor with over 5,700,000 ha. The weather is tropical humid, with an average annual rainfall of 2.350 mm (Ayres 1993). We obtained blood samples from 99 birds of *R. niger* species from Praia do Meio in MSDR. Praia do Meio (2° 44′ 00″ S, 65° 14′ 00″ W) is a sandbar formed in the Solimões River during the dry season (August to November), when water levels drop and expose sandbanks and beaches, thereby providing a breeding and seasonal habitat for *R. niger*. According to monitoring data in Mamirauá, the *R. niger* population experiences accentuated fluctuations throughout the year, with the largest abundance in the dry season (September to December) and few individuals during the flood season (January to August) in the survey area (Bernardon and Nassar 2012; Pires and Bernardon 2014). Thus, we can consider the groups sampled mostly composed of migratory birds. Therefore, we chose the dry season for

sampling to involve the greater number of individuals and several age groups. Blood samples were collected between October (16th to 30th) and November (10th to 26th) 2012, and two thin blood smears were prepared immediately after blood collection, with the remaining blood used for the PCR procedure. During the first sampling, 32 migratory birds of different ages were captured. During the second sampling, 67 adult birds were captured (over 1 year of age). Young birds (between 15 and 90 days post-hatching, 98 % of birds were more than 4 weeks of age) were manually captured in the morning (6:00 am to 9:00 am) and during the afternoon (4:30 pm to 6:00 pm) period. The adult birds were captured in mist nets at night (7:00 pm to 12:00 pm). Each bird was banded with a metal ring from the Brazilian birding agency (CEMAVE/ICMBio), and 30 μL of blood was obtained from the brachial vein. An aliquot of the collected blood was used to prepare thin blood smears (two for each bird) that were air-dried and fixed in absolute methanol immediately after collection in the field. The smears were stained with Giemsa stain according to the protocol described by Valkiūnas (2005). The remaining blood was stored in lysis buffer for DNA analysis. This project was approved by the Sustainable Development Mamirauá Reserve Ethics Committee (CEP IDSM 005/2012).

Microscopic analysis of blood smears

Blood smears were examined for 10–15 min at ×400, and then 200 fields were studied at high magnification (×1,000) using an Olympus Microscope CX31 (Olympus Corporation, Tokyo, Japan). The parasites were identified by microscopy according to the morphological characteristics of different blood stages (Garnham 1966; Valkiūnas 2005). The Olympus CX31 light microscope equipped with Q color 5 digital Olympus camera and imaging Software Q-Capture Pro 7 was used to prepare images from the parasites.

DNA extraction

Approximately 20 μL blood sample was stored at room temperature (22–25 °C) in cell lysis solution (PROMEGA, Madison, WI, USA) for approximately 1 day prior to DNA extraction. DNA from blood samples was extracted with the Wizard Genomic DNA Purification Kit (PROMEGA) according to the manufacturer's protocol. The DNA pellet was re-suspended in 30 μL hydration solution and kept at −20 °C until use.

PCR screening

We used screening primers designed to amplify a 154-nucleotide segment of RNA-coding mitochondrial DNA according to Fallon et al. (2003): 343F (5′-GCTCACGCATCG CTTCT-3′) and 496R (5′-GACCGGTCATTTTCTTTG-3′). PCR reactions were run in 10 μL volumes that contained the following final concentrations: 0.4 mM of each primer, 200 mM of each dNTP (PROMEGA), 10 mM Tris–HCl, pH 8.5, 50 mM KCl, and 1 U of Taq DNA polymerase (PHONEUTRIA, Minas Gerais, Brazil). Thermal cycling conditions were as follows: initial denaturation for 2 min at 94 °C followed by 35 cycles with 1 min denaturation at 94 °C, 1 min annealing at 62 °C, and 1 min 10 s extension at 72 °C. This was followed by a final extension for 3 min at 72 °C. The amplified products were visualized in 6 % polyacrylamide gels stained with silver nitrate (Sanguinetti et al. 1994; Ribeiro et al. 2005). Samples that yielded negative results by microscopy and positive on DNA amplification were reexamined at 200 fields in order to confirm the presence of parasites.

Cytochrome b amplification

From samples that were positive by microscopy, mitochondrial DNA amplification, or both, a 480-bp fragment of the cyt *b* gene was amplified under the following conditions: first-round reaction using the primers HaemNFI (5′-AGACATGA AATATTATGGITAAG-3′) and HaemNR3 (5′-GAAATAAG ATAA GAAATACCATTC-3′) (Hellgren et al. 2004) with 1 μL of genomic DNA. A 1-μL aliquot of this product was used as a template for a nested reaction with the primers HaemF (5′-CTTATGGTGTCGATATATGCATG-3′) and HaemR2 (5′-CGCTTATCTGGAGATTGTAATGGTT-3′) (Bensch et al. 2000). PCR products were visualized using 6 % polyacrylamide gels stained with silver nitrate (Sanguinetti et al. 1994; Ribeiro et al. 2005).

Phylogenetic analysis

Positive PCR products were purified using polyethylene glycol (PEG) according to the protocol described by Sambrook et al. (2001). Bidirectional sequencing with dye-terminator fluorescent labeling was performed in an ABI Prism 3100 automated sequencer (Applied Biosystems, Inc.). We sequenced 480 base pairs of the cyt b gene for *Plasmodium* spp. and *Haemoproteus* spp. DNA sequences were aligned by CLUSTALW using MEGA version 5 (Tamura et al. 2011). Phylogenetic trees of parasite lineages were constructed using Bayesian Markov chain Monte Carlo (MCMC) analysis in the program BEAST (Drummond et al. 2012). Input files were generated using BEAUTi (included in the BEAST software package). We ran four Markov chain Monte Carlo chains for 10,000,000 generations sampling every hundredth generation and excluding the first 1,000 trees as burn-in. Tree Annotator (also part of the BEAST software package) was used to summarize the trees produced in BEAST (Drummond et al. 2012). We calculated sequence divergence between lineages in MEGA5 with a Jukes-Cantor substitution

model. We considered sequences as different cyt b lineages if the sequence divergence was greater than 2 % (Ricklefs and Outlaw 2010). In an attempt to assign the sequences to describe parasite lineages, we compared the sequences with records in the GenBank and MalAvi (http://mbio-serv2.mbioekol.lu.se/Malavi/) databases, which contain cyt-b data for most of published avian hemosporidian parasite lineages. The sequences are available through GenBank (accession numbers KJ469131-KJ469133). Lineages used in the phylogenetic analysis are available both in the GenBank (accession numbers JX467689, JN819335, AY640137, DQ241530) and MalAvi databases.

Results

The combination of PCR and microscopy revealed that 20 specimens (20 % prevalence; *N*=99) were infected with either *Haemoproteus* or *Plasmodium*. Of the 31 young birds captured, only five (16 %) were infected by hemosporidians. Among 68 adult birds, 15 were positive (22 %) for the infection. Sequencing allowed us to identify two distinct *Plasmodium* sp. lineages and one *Haemoproteus* sp. lineage from the *R. niger* specimens. The two *Plasmodium* lineages found in this study, *Plasmodium* sp. RYNI_RDSM1 and *Plasmodium* sp. RYNI_RDSM2, had previously been described in different regions of the American continent (Ishak et al. 2008; Merino et al. 2008; Pagenknopp et al. 2008; Levin et al. 2013), including a Neotropical region in Southeast Brazil (Lacorte et al. 2013) (Table 1).

The phylogenetic analysis revealed three highly supported clades (Fig. 3). In the first clade, one of the sequences recovered from *R. niger* showed similarity with different lineages identified as *Plasmodium nucleophilum* (JX467689), *Plasmodium* sp. HMA-2012 (JN819335), *Plasmodium* sp. DENPET03 (AY640137), and *Plasmodium* sp. B23 (DQ241530), which were found in different continents. A second lineage of *Plasmodium* recovered showed high similarity with PHPAT01 (JX025077) and ChP5 (EF153642) lineages, which were found only in South America. The *Haemoproteus* lineage was a 100 % sequence match with *Haemoproteus macrovacuolatus* from Colombia.

Figure 4 shows areas of *R. niger* occurrence and depicts overlapping distribution of the two *Plasmodium* lineages recovered from *R. niger*. Both young and mature gametocytes of this hemosporidian parasite were observed in stained thin blood smears (Fig. 5). However, parasitemia was light (<0.01 %) preventing the precise morphological characterization of the species involved in the infection.

Discussion

Although the Brazilian Amazon Basin contains a remarkable diversity of habitats and host species, there is a lack of information about the diversity of malarial parasites in Neotropical waterfowl communities. Characterization of the malarial parasites among migratory bird species is important to support studies that aim to address the possible dispersal of microorganisms in Neotropical habitats. Thus, we performed the first characterization of hemosporidian parasites in *R. niger* (Black Skimmer) populations, an important migratory aquatic bird in the Brazilian Amazon region. It is noteworthy to mention that our survey is also justified by the fact that information about ecological and biological aspects associated with this waterbird species is scarce despite its importance in the context of conservation medicine and ecosystem health (Mellink and Riojas-Lopez 2008; Ödeen et al. 2010).

This work was conducted in an area that presents peculiar characteristics: a defined seasonality, being submerged for most of the year, and it is a nesting site with high bird densities in association with low human activity (IDSM 2011). Thus, this region differs from other habitats where studies on avian malaria have been conducted so far. It is known that different habitats with differing levels of ecological interactions and environmental changes may provide different compositions influencing the dynamics of the parasite–vector–host triad (Shutler et al. 1996; Garvin and Remsen 1997; Piersma 1997; Mendes et al. 2005; Durrant et al. 2006; Arriero and Moller 2008; Belo et al. 2011; Szöllözi et al. 2011; Delgado-v and French 2012; Loiseau et al. 2012). An interesting point raised by our results is the proposal that transmission of hemosporidians occurs in loco. In fact, young birds that were born in the study area (Praia do Meio) and remained there during the reproduction period were infected by malarial parasites. Additionally, another factor that could reinforce the likelihood of primary infection by hemoparasites in loco is the detection of only young forms of *Haemoproteus* spp. in young birds after blood smear examination. This evidence suggests that primary infection occurs during the first few months of a bird's life.

Both young and mature gametocytes of *Haemoproteus* were found in adult birds, pointing to the possible relapse of the acute phase of disease associated with stress caused by hormonal changes during reproductive process, a period that can lead to depression of immunity (Zuk and Mckean 1996). The higher hemosporidian occurrence in adults can also be influenced by factors associated with the nesting behavior. The activity of birds decreases during the nesting period increasing exposure to vectors (Valkiūnas 2005).

The infection by *Haemoproteus* spp. among *R. niger* specimens could be explained by the behavior of the skimmers. This waterfowl has higher activity at night and remains resting on the ground near the water in the hottest time of the day.

Table 1 Host species and parasite lineages previously shown to be related in other regions

Parasite lineage	GenBank number identical lineages	Host	Geographic location
Plasmodium sp. RYNI_RDSM1	EU328169	*Geothlypis trinchas*	USA
	JX025077	*Phaeomyas murina*	Brazil
	EF153642	*Phrygilus patagonicus*	Chile
Plasmodium sp. RYNI_RDSM2	JX467689	*Alopochen aegypticus*	Brazil
	JX021474	*Brasileuterus flaveolus*	Brazil
	JX501898	*Cypsnagra hirundinacea*	Brazil
	JX501892	*Mimus saturninus*	Brazil
	JX501859	*Hemithraupis guira*	Brazil
	AY640137	*Dendroica petechia*	USA
	JN819335	*Tangara icterocephala*	Costa Rica
	EU328165	*Geothlypis trichas*	USA
	DQ241530	Numerous hosts	Guyana/Uruguai
	JN819517	*Mimus gilvus*	Venezuela

This sedentary time presumably coincides with the period of greatest activity of potential *Haemoproteus* spp. vectors. Indeed, it has been verified that different species of Culicoides (Diptera: Ceratopogonidae) have higher activity during the hottest hours of the day (Linley et al. 1983; Trindade and Gorayeb 2005). Moreover, exposure to *Plasmodium* transmission would be relatively lower, since mosquitoes usually bite at twilight (Forattini et al., 1986), a period that coincides with greater activity of adults of *R. niger*. Therefore, it is a consensus that further entomological studies are essential to understand the dynamics of hemosporidian transmission in such Neotropical environments.

The *Plasmodium* lineages recovered in this study match the lineages previously described in *Dolichonyx oryzivorus* (bobolinks), a migratory bird that includes Brazil within its migratory routes (Levin et al. 2013). Bobolinks also migrate through regions of high biological importance such as the Galapagos Archipelago. It is worth mentioning that a recent study reinforces the idea that these migratory songbirds could be considered an important link for the introduction and dispersal of

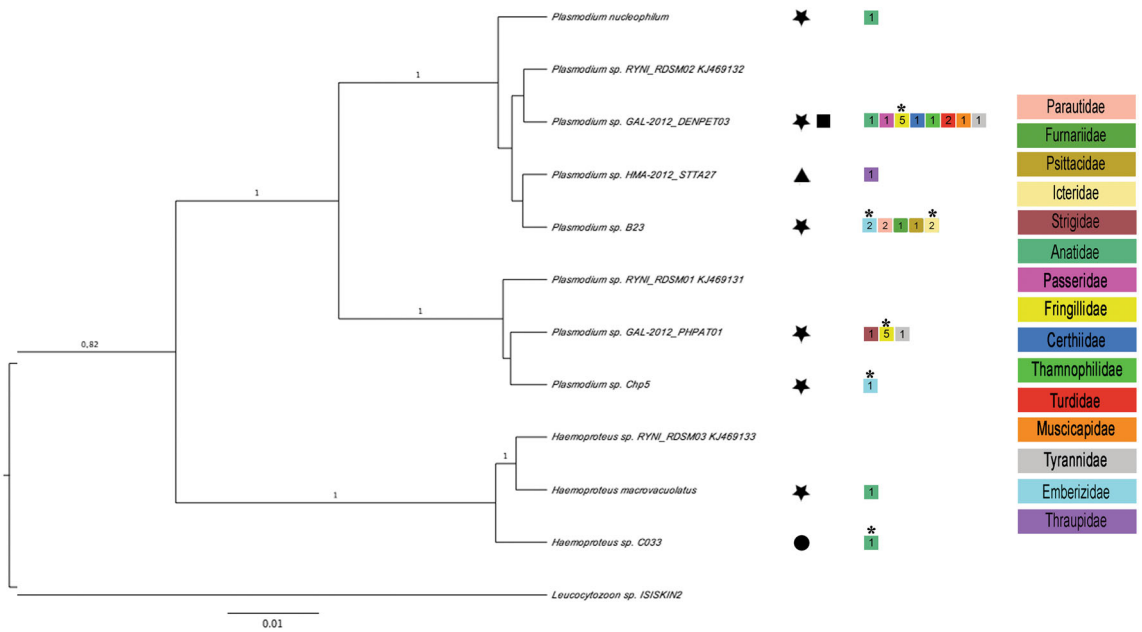

Fig. 3 Phylogram of *Plasmodium* spp. and *Haemoproteus* spp. from *R. niger* inferred using Bayesian analysis. Support values indicate Bayesian posterior probabilities (only values above 50 % are shown). *Symbols* represent the continents where the lineages were described, according to GenBank and MalAvi: Asia, *black circle*; South America, *black star*; Central America, *black diamond*; North America, *black square*. *Squares* to the right of lineage names indicate host species in which a particular lineage was recovered and are color coded to indicate the family to which the host species belongs. *Numbers within squares* indicate the number of host species infected. *Asterisks* indicate that the hemosporidian parasite was observed in migratory bird species

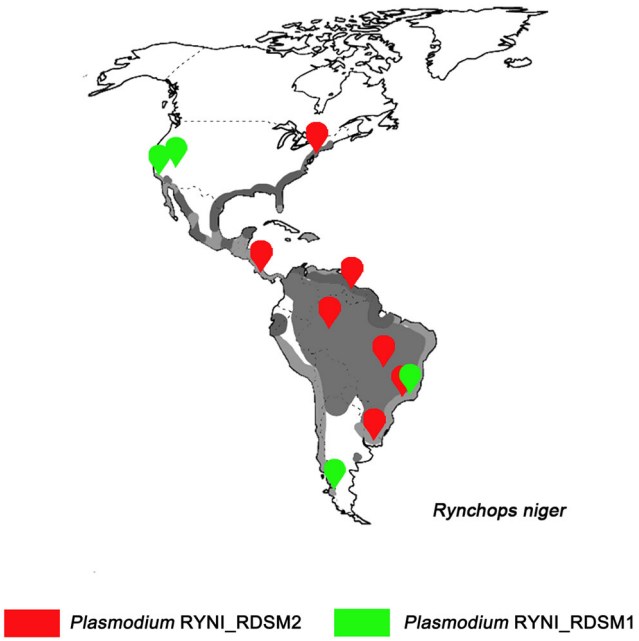

Fig. 4 Map depicting overlapping distribution of the two *Plasmodium* lineages recovered from *R. niger*. The *balloons* represent regions where the *Plasmodium* lineages related in this study were observed earlier

new *Plasmodium* lineages in the Galapagos Archipelago (Levin et al. 2013). The fact that *Plasmodium* lineages recovered from *R. niger* seem be the same as those found in bobolinks allows us to discuss the importance of this migratory songbird in the expansion of a common *Plasmodium* lineage in different parts of the Americas, including the Brazilian Amazon Basin. We could not demonstrate that this *Plasmodium* lineage (*P.* sp. RYNI isolate RDSM2) naturally occurs in young *R. niger* in the Amazon region due to the low parasitemia observed and poor quality of the sequences recovered. However, we observed infections by this same lineage in other bird species from different biomes in Brazil across different years, which suggests that the lineage is well established and is transmitted regularly in Brazil. Indeed, this lineage has been described in Brazil in 11 bird species across eight distinct bird families in Southeast Brazil, including biomes such as Cerrado, Atlantic rainforest, and seasonally dry forest (Lacorte et al. 2013), and also in a synanthropic bird species (Marzal et al. 2011). This lineage received distinct denominations as can be verified in two different databases, GenBank and MalAvi, including lineage HMA-2012 proposed by Archer et al. (2011). Recently, this *Plasmodium* lineage was characterized at morphological and molecular levels and identified as *P. nucleophilum* (Chagas et al. 2013). Further studies would be valuable for precise identification of this widespread *Plasmodium* lineage.

Another hemoparasite found in this study was the *Plasmodium* sp. RYNI isolate RDSM1. This lineage also was described in three families of the order Passeriformes, including two bird species from southwestern and northeastern USA (Pagenknopp et al. 2008; Levin et al. 2013) and three bird species from Brazil (Lacorte et al. 2013). This lineage was registered in a passerine species in Chile and is denominated ChP5 (Merino et al. 2008). This *Plasmodium* lineage has been observed only in the American continent. As discussed earlier, characterization of the *Plasmodium* species was not possible due to the low parasitemia observed in blood smears. However, it is important to emphasize that the two *Plasmodium* lineages found infecting *R. niger* are generalist lineages since they have been found in taxonomically distinct birds in heterogeneous habitats. Host generalist *Plasmodium* infecting migratory birds can spread to different hosts, causing the risk of extinction of susceptible wild birds.

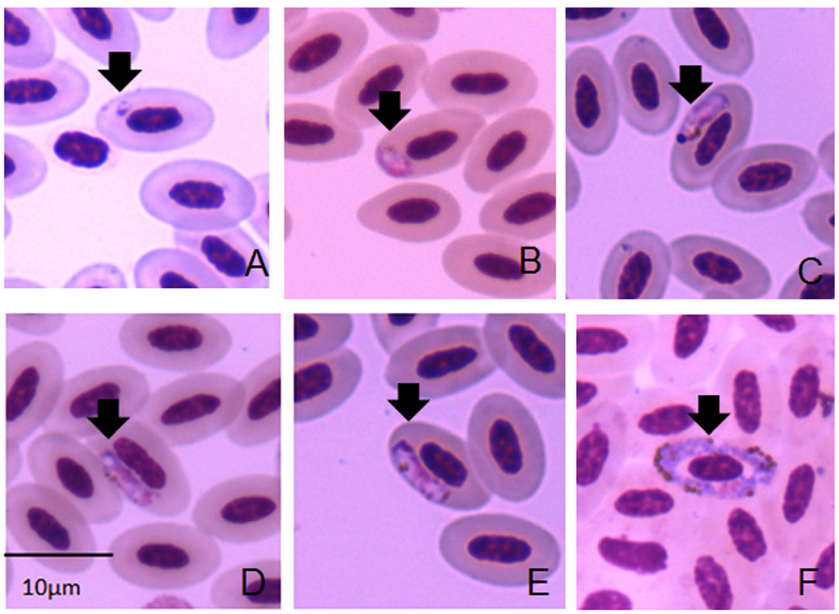

Fig. 5 Different maturation stages of gametocytes of *Haemoproteus* sp. in erythrocytes: immature (**a**, **b**), intermediary (**c**, **d**), and mature (**e**, **f**)

The single lineage of *Haemoproteus* sp. (*H.* sp. RYNI isolate RDSM3) is the hemosporidian species most abundant among *R. niger* adults and matched to *Haemoproteus macrovacuolatus*, which was described recently in another waterfowl, the black-bellied whistling duck, *Dendrocygna autumnalis*, from Colombia (Matta et al. 2014). Colombia is located on the migration route of *R. niger*. Thus, this waterbird may be transporting the parasite to this region or it may become infected in the Colombian region. This *Haemoproteus* species was genetically distinct from *Haemoproteus* C033 with a pairwise genetic divergence of approximately 1 %. *Haemoproteus* has been considered a relatively host-specific taxon, being restricted to a few bird species belonging to the same family (Bennett et al. 1994; Savage and Greiner 2004; Peirce and Adlard 2005; Hellgren et al. 2009). *Haemoproteus* parasites show a degree of host specificity that may be related to specific dynamics of vertebrate host and vector populations independent of regional differentiation (Bethany et al. 2014). Further studies aiming to determine the occurrence of *H.* sp. RYNI isolate RDSM3, a possible specialist hemoparasite lineage, in other parts of the American continent may be useful to establish its potential use as a geographical marker for migratory Black Skimmers in Neotropical environments.

The parasite–host dynamics in migratory bird populations may be subject to pressures contrasting those faced by populations of sedentary birds. Since the migratory birds can be infected in breeding, wintering, and migratory sites, they can harbor a greater genetic diversity of parasite lineages than nonmigrant species (Smith et al. 2004; Jenkins et al. 2012). Thus, there is a greater likelihood of migratory birds carrying parasite lineages that can establish in specific regions in accordance with existing hosts and vectors during local migration.

The Amazon region has a high diversity of wild birds along and also has many different mosquito species (Barbosa et al. 2008), making it an environment favorable to successful infections by hemosporidians. The presence of migratory wild birds in this region can lead to the expansion of blood parasite lineages to areas of high ecological importance. Our results highlight the potential role of migratory birds in the prevalence, distribution, and diversity of avian malarial parasites and show that these birds can act as effective dispersers of such microorganisms that could threaten the conservation of wild bird species.

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Ethics Committee in Animal Experimentation—CETEA/UFMG—Protocol 254/2011 approved in December 7, 2011 and Ethics Committee of the Instituto de Desenvolvimento Sustentavel Mamiraua—IDSM—Protocol CEP IDSM 005/2012, approved in October, 2012).

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