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Haematozoa of the Great Blue Turacos, *Corythaeola cristata* (Vieillot, 1816) (Aves: Musophagiformes: Musophagidae) imported to Singapore Jurong Bird Park with description and molecular characterisation of *Haemoproteus* (*Parahaemoproteus*) *minchini* new species (Apicomplexa: Haemosporidia: Haemoproteidae)

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Abstract. Investigation of blood parasites from eleven *Corythaeola cristata* (Vieillot, 1816) imported from Tanzania to the Jurong Bird Park, Singapore reveals a high prevalence of Haematozoa (81.8%) and the presence of a polyparasitism by microfilaria, *Plasmodium* and a distinctive new species of haemoproteid: *Haemoproteus* (*Parahaemoproteus*) *minchini* new species. The present study provides a description of the parasite based on the morphology and morphometric measurements of its blood stages that highlights a sexual dimorphism as well as its larger size when compared to the other haemoproteid reported from the Musophagiformes (i.e, *Haemoproteus montezi* Travassos Santos Dias, 1953). The study also provides a molecular characterisation of *Haemoproteus* (*Parahaemoproteus*) *minchini* new species by four different gene fragments (cytochrome *b*, cytochrome c oxidase 1, caseinolytic protease C and elongation factor Tu) spread across the organellar genomes of the parasite. Phylogenetic analyses were performed for each gene to assess the genetic relationship of this new parasite species with the other available sequences of identified avian haemoproteids, and it is referable to the subgenus *Parahaemoproteus*. This study also proposes for the first time a sequence of the elongation factor Tu gene for a haemoproteid parasite. The geographic origin and the possible pathogeny and pathology of *Haemoproteus* (*Parahaemoproteus*) *minchini* new species are discussed, as is the importance of using these data in conjunction with morphological and the molecular methods to study avian Haemosporidia.

Key words. *Haemoproteus* (*Parahaemoproteus*) *minchini* new species, *Corythaeola cristata*, morphology, morphometric measurement, molecular biology, Jurong Bird Park Singapore.

INTRODUCTION

The Great Blue Turaco *Corythaeola cristata* (Vieillot, 1816) is the largest species of the order Musophagiformes. The turacos are a distinctive group of sub-Saharan arboreal, frugivorous and folivorous birds that inhabits the dense broad-leaved evergreen forest. Wild *C. cristata* are often hunted for their feathers coloured by unique copper based pigments (Turner, 1997). Due to its general shape, large size, long tail, blue and yellow plumage, stout beak, and prominent head crest, this stunning bird is often kept and raised in captivity for a long time (Delacour, 1917; Tocidlowski, 2015). Despite being common in aviaries and throughout its wide geographical range in the wild (BirdLife International, 2016), little is known about the blood parasites of *C. cristata*.

A literature review related to this topic only returns: *i)* five reports of parasites non-identified at the species level, namely: *Haemoproteus* sp. (Minchin, 1910; Hamerton, 1931; Schwetz, 1931), *Leucocytozoon* sp. (Schwetz, 1931, 1935), *Trypanosoma* sp. (Minchin, 1910; Schwetz, 1933) and *Microfilaria* sp. (Minchin, 1910; Schwetz 1931, 1935); *ii)* two descriptions of new parasites: *Microfilaria schizorhinos* Ringenbach, 1914 and *Trypanosoma cristatae* Schwetz, 1931, both species were described briefly without detail and for the last considered as nomen dubium (Valkiūnas et al., 2011); and *iii)* two catalogue entries (Bennett et al., 1982, 1992) listing some of the references cited above. This bird species and the other Musophagiformes are hardy species in aviaries and only one death related to *Haemopoteus* sp. infection in captivity has been reported (Hamerton, 1931), while other deaths were not related to blood parasite infections (Hamerton, 1931; Tocidlowski, 2015).

In late September 2013, nine among 11 Great Blue Turacos imported from Tanzania died at the Jurong Bird Park in Singapore. The putative cause of death in these cases were

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investigated and we here report our findings of the blood parasites and of an undescribed *Haemoproteus* species.

MATERIAL AND METHODS

Biological material. Eleven Great-blue Turacos *Corythaeola cristata* (Vieillot, 1816) imported from Tanzania to the Jurong Bird Park, Singapore by an authorised company at the end of September 2013. After shipment, direct transfer from the airport and physical check-up, all the birds were placed in individual cages for a thirty-day quarantine. To adhere to the quarantine protocol, the birds were sampled by cloaca swabs for avian influenza, Newcastle disease and *Salmonella* isolation.

With a view to breed this species, the birds underwent laparoscopy to confirm their gender and gonadal status. Blood samples were collected during the laparoscopy procedure. Each bird was sedated with isoflurane, placed in a lateral recumbence and 0.5 to 1.0 ml of blood was collected from the right jugular vein. The sample was divided into two, one EDTA and the other in heparine for complete blood counts and chemistry panel while one drop of blood was placed on microscopic glass slide directly from syringe for smearing. Two thin blood smears for microscopy were prepared immediately for each bird using standard procedures (Campbell 1994). After the haematological test procedures, the leftover EDTA blood samples were kept at 4°C until DNA extraction. All samples were collected and processed between 21 and 24 October 2013.

All procedures performed in this study involving animals were in accordance with the ethical standards or practices of the institution.

Morphological analysis. Thin blood smears for microscopy were fixed with absolute methanol and stained 1 hour by 10% Giemsa stain (Merck) in pH=7.4 phosphate buffer. Then the smears were protected by a cover slip mounted with Eukitt® mounting medium. Blood smears were entirely screened, with an Olympus BX61 microscope, at low magnification (×100 to ×200) for the detection of large Haematozoa and at high magnification (×1,000) for a minimum of 10,000 red blood cells (RBCs) for the detection of small parasites. Hapanthotype and parahapanthotype slides were screened entirely at low magnification to check the possible concomitant infection by another haemosporidian parasite. Parasitemia was estimated on 10,000 RBCs. Microphotographs and morphometric measurements were taken with a Nikon Eclipse 80i microscope equipped with a Nikon DS Ri1 camera and the Nikon NIS Elements D Imaging Software. Statistical comparisons were performed with R Software (R Core Team 2014). Sample displaying obvious mixed infection by several haemosporidian parasites were not retained for molecular tests.

Nucleic acid extraction. DNA was extracted from 20 µL of EDTA whole blood ten times diluted into sterile PBS using the QIAamp DNA Mini Kit from Qiagen® following manufacturers' recommendations. DNA was eluted in 100µL of elution buffer (EB) and frozen at -30°C until usage.

DNA amplification. Presence of haemosporidian parasites were tested by polymerase chain reaction (PCR) assay of a DNA fragments of a mitochondrial genome: the cytochrome b gene (cytb), using the protocol proposed by Perkins & Schall (2002). Briefly, this assay is a nested-PCR (nt-PCR) designed to amplify the almost complete cytb gene using two consecutive steps that are performed with two sets of pan-haemopsoridian oligonucleotide primers: DW2/DW4 and DW1/DW6 respectively, and where the products of the first reaction are used as template for the second reaction. For positive samples, genus specific nt-PCR reactions following the protocols of Waldenström et al. (2004) and Hellgren et al. (2004), and using the products of the reaction DW2/DW4 as template were run to discriminate *Haemoproteus*, *Plasmodium* and *Leucocytozoon* and to check for any possible mixed infection not detected by morphology.

Presence of submicroscopic *Plasmodium spp.* infection was further assessed by a separate PCR assay targeting the nuclear 18S ssrRNA gene as described by Leclerc et al. (2014). All PCR reactions were run on GeneAmp® PCR System 9700 from Applied Biosystems® and PCR products were visualised after electrophoresis on 1.5% agarose gel. The PCR products from all the assays were purified using the QIAquick® PCR Purification Kit (Qiagen®) following manufacturers' recommendations, eluted in 30µL of EB buffer and frozen at -30°C until usage.

Sequencing. The purified PCR products were prepared for sequencing in both direction using the BigDye® Terminator v3.1 cycle sequencing Kit (Applied Biosystems®) and the respective oligonucleotide primers, following manufacturers' recommendations. The products from the BigDye® reactions were purified using the BigDye® XTerminator™ Purification Kit (Applied Biosystems®) following manufacturers' recommendations before to be sequenced on a 3500xl Genetic Analyzer (Applied Biosystems®).

Sequences analysis. Alignment and cross-checking of the sequences were performed with CLC Main Workbench 7.7 software from CLC bio (Qiagen®). In case the electropherogram revealed a mixed infection the samples were not selected for phylogenetic studies. Comparison of our new sequences with published one deposited in GenBank was done by Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

Additional molecular targets. On the sample confirmed to harbour a pure *Haemoproteus* infection, three other conserved genes used for phylogenetic and evolution studies were tentatively amplified, namely: the cytochrome c oxydase 1 gene (cox1) also located into the mitochondrial genome; the elongation factor Tu gene (tufA) and the caseinolytic protease C gene (clpC) both from the apicloplast genome. Segment of the cox1 gene, a gene selected for the Barcode of Life project, was obtained following the protocol developed

Table 1. Haematozoa recorded from the blood of the Great Blue Turacos from the Jurong Bird Park.

Bird no.	Blood film no.	Haematozoa			
		P	***H***	***L***	**Other**
H2909	JM06	+	–	–	M
H2910	JM07	–	+	–	M
H2911	JM08	+	–	–	M
H2912	JM09	–	–	–	M
H2913	JM10	–	–	–	–
H2914	JM11	+	+	–	M
H2915	JM12	+	+	–	M
H2905	JM13	+	–	–	M
H2906	JM14	–	–	–	–
H2907	JM15	+	–	–	M
H2908	JM16	+	+	–	M
Prevalence (%)		63.6	36.4	0.0	81.8

P: *Plasmodium*, *H*: *Haemoproteus*, *L*: *Leucocytozoon* and M for microfilaria.

by Duval et al. (2009) while fragments of tufA and clpC genes were amplified as proposed by Perkins et al. (2007). All amplifications, purifications and sequencing steps were performed as described for the cytb gene.

Phylogenetic analysis. Multiple alignments of the sequences with published sequences from GenBank and MalAvi databases were created using Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm (Edgar 2004) Molecular phylogeny was performed by Maximum Likelihood (ML) method with GTR+Γ+I model using PhyML 3.0 software (Guindon et al., 2010). Statistical robustness of the tree was evaluated by non-parametric bootstrapping (1000 replicates).

RESULTS

Total prevalence of birds found positive for Hematozoa was 81.8% (9/11) with detection of protozoan parasites from the genus *Plasmodium* and *Haemoproteus* and also detection of microfilaria. Mixed infections by parasites belonging to these different groups were frequent 72.8% (8/11). Details about the infection of each bird are provided in Table 1.

Morphological observation of the *Plasmodium* parasites clearly highlighted several species belonging to distinct subgenera. Polyparasitism by more than one *Plasmodium* species was noted and as all the stages were not seen for each morpho-species, it was not possible to identify them.

Morphological features and measurements of the microfilaria were recorded and appear compatible with the ones provided for *Microfilaria schizorhinos* by Ringenbach (1914). However similarly to this author, without any adult worms, it is impossible to confidently assign these larvae to any taxon. Investigations by molecular methods could potentially be helpful to solve this issue and will be attempted in a separate study.

Detailed investigation of the *Haemoproteus* by combined morphology, morphometric measurements and molecular methods is reported below.

TAXONOMY

Order Haemosporidia Danilewsky, 1885

Family Haemoproteidae Doflein, 1916

Genus *Haemoproteus* Kruse, 1890

Haemoproteus (*Parahaemoproteus*) *minchini* new species
(Fig. 1)

Material examined. Hapanthotype: one thin blood film numbered JBP7-1, made from the blood of *Corythaeola cristata* number H2910, location Jurong Bird Park, Singapore, coll. Dr. C. Okumura, 22 October 2013; deposited into the Parasitology collection of the Muséum National d'Histoire Naturelle, Paris under the accession number 617YY-PXX50, parasitemia is approximately: 0.5%.

Parahapanthotype: a second thin blood film numbered JBP7-2, made from the blood of *Corythaeola cristata* number H2910, other information as for the hapantotype. Paratypes: thin blood films numbered JBP11, JBP12, JBP16 from *Corythaeola cristata* H2914, H2915, H2909, other data as for the hapanthotye are deposited into the Parasitology collection of the Muséum National d'Histoire Naturelle, Paris under the accession numbers: 222BF-PXX108, 221BF-PXX109 and 218BF-PXX106 respectively.

Type host. *Corythaeola cristata* (Vieillot, 1816) – Great Blue Turaco, Musophagiformes, Musophagidae.

Site of infection. Mature erythrocytes; tissue stages unknown.

Locality. Tanzania.

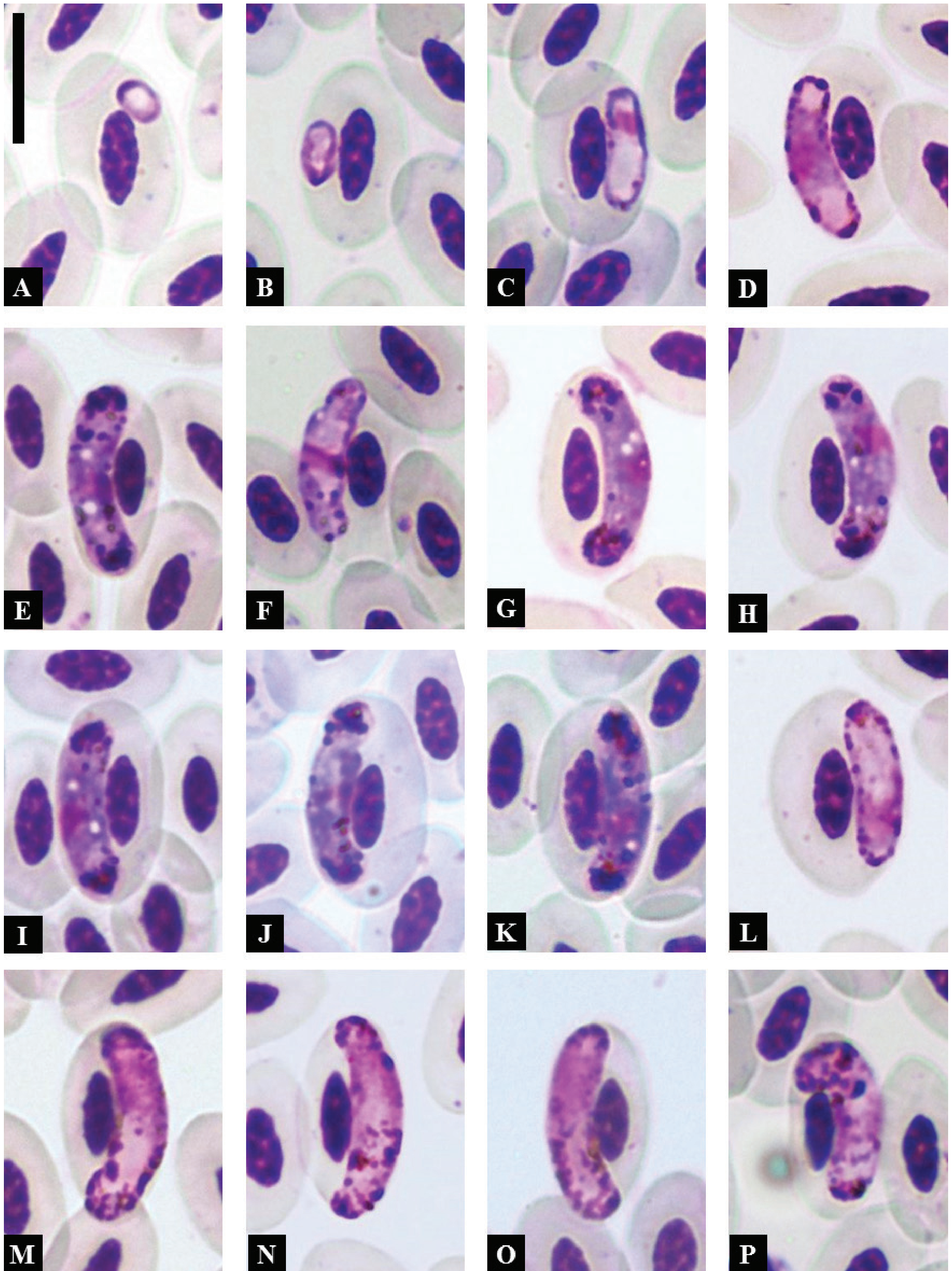

Fig. 1. Microphotographs of *Haemoproteus* (*Parahaemoproteus*) *minchini* new species from the blood of the Great Blue Turaco (*Corythaeola cristata*). A, ring; B–C, young gametocytes; E–K, macrogametocytes; D, L, early microgametocyte; M–P, microgametocytes. Giemsa stained thin blood film from hapantotype material. Scale bar = 10 µm.

Distribution and additional hosts. Tanzania and Uganda where a similar parasite has been recorded by Minchin (1910) from the blood of the type host. No other record neither DNA sequences were found identical.

DNA sequences. Fragment of the cytb (1,258bp) and cox1 (1,336bp) genes from the mitochondrial genome and fragment of the clpC (647bp) and tufA (814bp) genes from the apicoplast genome; all isolated from the blood of the type specimen *C. cristata* H2910; respective GenBank accession numbers are [KU160476] to [KU160479]; sequences will also be deposited into MalAvi database.

Vector. Unknown.

Etymology. The species name is given in honour of Edward Alfred Minchin, Professor of Protozoology in the University of London who first reported this parasite from the blood of *C. cristata*.

Description. Rings and young gametocytes (Fig. 1A–C): Develop in mature red blood cell (RBC), are round or oval in shape, with a peripheral dark nucleus and a large white vacuole. They are usually found in sub-apical or lateral position in the RBC and are rarely in contact with the nucleus or the membrane of the RBC. Growing they elongate in lateral position parallel to the nucleus of the RBC and extend up to a similar size. Their nucleus stains a bright fuchsia colour, adopts a subapical and transversal position, the vacuole becomes wider and the first thin granules of volutin start to appear peripherally.

Macrogametocytes (Fig. 1E–K): When young they continue to elongate, present an even thickness, and are almost straight or only very slightly curved. Their cytoplasm is loose and stains a pale blue, their nucleus becomes submedian and transversal; the first thin grains of dark brown pigment appear; the vacuole recesses while more granules of volutin accumulate at the apexes and around the vacuole. Thereafter the macrogametocytes continue to elongate, their maximum expand is from one pole of the RBC to the other but the majority remains shorter, lying onto the membrane of the RBC and separated or just loosely touching the nucleus of the RBC; they take a slightly more curved shape but never encircled the nucleus of the RBC. Their cytoplasm becomes denser, thinly granular and stains a deeper blue, with few small, clear and round vacuoles; the dark brown pigment grains coarse and more abundant with yellow reflects can be found anywhere but tend to scatter near the nucleus and the ends; the nucleus no longer transversal recesses in a dense, deeper fuchsia mass of variable size often attached to the membrane of the parasite; the amount of volutin continues to increase creating clumps of dark violet aggregates filing the polar regions. All along the growth the margin of the parasite remains even and the extremity rounded, sometime a subapical throttling is observed, accentuating the accumulation of volutin granules and the darkness of the extremities of the parasites. Only the largest fully grown macrogametocytes touch the nucleus of the RBC and induced a slight lateral displacement of it.

Microgametocytes (Fig. 1D, L–P): General development and configuration are similar to the macrogametocytes with the usual sexual variations. However, they display a slightly bigger size at all stages; a larger nucleus made of a very loose and diffused chromatin, only differentiated from the cytoplasm by its deeper pink colour; less condensed volutin granules accumulating onto the membrane of the polar regions of the parasite and usually not filling them entirely. Due to their larger size, they tend to hypertrophy more the RBC, to touch more often the nucleus of the RBC and to displace it more laterally. Parasites that curved above one of the nucleus pole are seldom seen.

Remarks. Minchin (1910) gave the following brief description of a *Haemopoteus* sp., he observed into the blood of a *C. cristata* from Uganda: "The Halteridia are abundant and of a distinct type, of even thickness, very slightly curved, with coarse pigment-grains, and with red-staining grains at the two ends of the body in addition to a more diffuse red patch which apparently represents the nucleus and is usually situated near the middle of the body". Along with this short description Minchin (1910) published six coloured drawings that illustrated the above mentioned characters of his parasite and also clearly depicted the yellowish reflect of the pigment-grains, the even margin of the extremities, the condensed nucleus of the macrogametocyte, the presence of a space between the macrogametocyte and the nucleus of the RBC and its absence for the microgametocyte. When compared with our parasite, we noticed that all the morphological features described or illustrated by Minchin are encompassed into our description. Therefore, it seems very likely that we are looking at the same parasite that Minchin a century ago and we have therefore decided to dedicate this parasite to him.

Differential diagnosis. One other haemoproteid species has been described from the Musophagiformes: *Haemoproteus montezi* Travassos Santos Dias, 1953 from the blood of the Purple-crested Turaco, *Tauraco porphyreolophus* (Vigors, 1831) in Mozambique. This parasite has been reported from several species of Turacos (Bennett & Herman, 1976; Bennett et al., 1982; Bennett & Pierce, 1990; Bennett et al., 1992; Valkiūnas, 2005; Vrána et al., 2005), and also from some Nectariniidae (Bennett & Herman, 1976) but never from the Great Blue Turaco. Travassos Santos Dias (1953), in the original description based on blood samples recovered from two sacrificed birds noted the presence of round forms that displace the nucleus of the RBC to the periphery and the presence of halteridian forms that did not displace it. He might have dealt with a mixed infection or with parasites that have undergone typical post-mortem changes as suggested by Bennett & Pierce (1990). Focusing onto the halteridian forms described by Travassos Santos Dias (1953) they are readily distinctive of *H. minchini* new species by several characters: the more or less narrowed extensions at the extremities in both sex; the margin sometime tortuous; the presence in the macrogametocytes of one or two large refractile vacuoles located into the polar region; the dark chatain almost black, mid-size grains of pigment scattered throughout the cytoplasm but that tend to accumulate at the extremities; and their lack of volutin granules.

Bennett & Pierce (1990) redescribed *H. montezi* based on material they deposited into the IRCAH and designated as: *i)* neohapantotype: blood film No. 39042a obtained from *Tauraco hartlaubi* (Fischer & Reichenow, 1884) collected by C.M. Herman, Kabete, Kenya in March 1939 and *ii)* several paraneohapantotypes: blood film No. 39042b (same as above); blood film No. 36767 obtained from *Tauraco livingstoni* (Gray, 1864) collected by M. Lips, near Lubumbashi, Zaire in February 1956. On their material, these authors noted an amoeboid margin of the young stages; noticed as Travassos Santos Dias (1953) the slightly amoeboid polar margin of the gametocytes; highlighted the parasite tendency to displace the nucleus of the RBC longitudinally toward one extremity of the RBC and to loop around the other pole of the nucleus giving it an asymmetrical aspect; mentioned that the yellow-brown pigment was small, difficult to see and scattered throughout the cytoplasm while the volutin granules were rarely seen but when present located in the poles. All these morphological features made their parasite distinct from *H. minchini* new species.

Valkiūnas (2005) gave also a description of *H. montezi* from the IRCAH material. He highlighted the same morphological features that Bennett & Pierce (1990); presented their morphometric measurements; insisted on the asymmetrical aspect of the parasite, the narrowed extremities and the "cleft" left between the young and growing stages and the nucleus of the RBC; but to their contrary mentioned that the outline of parasites is even and the volutin granules usually present and gathered at the ends of the parasite.

Minchin (1910) also observed a *Haemoproteus* sp. from the blood of the Ross's Turaco *Musophaga rossae* Gould, 1852 from Uganda. The description and illustrations he provided led him to readily differentiate this parasite from the one he observed in *C. cristata* and actually seem to be more corresponding to the description of *H. montezi*.

Morphometric measurements. Bennett & Pierce (1990) provided measurements of *H. montezi* that are recalled for reference along with those of *H. minchini* new species in Table 2. As the only results and not the measurement data of *H. montezi* are known, it was not possible to perform a statistical comparisons between the morphometric measurement of *H. minchini* new species and *H. montezi*, however some difference can be noticed with caution: *i)* the macrogametocytes and the microgametocytes of *H. minchini* new species appear respectively slightly larger in both length and width than those of *H. montezi* (Table 2); *ii)* the infected RBC seem more enlarged in both length and width by the gametocytes of *H. minchini* new species than by the gametocytes of *H. montezi* (Table 2); *iii)* the gametocytes of *H. minchini* new species tend to displace more laterally the nucleus of the infected RBC (NDR reduced) than the gametocytes of *H. montezi* (Table 2); *iv)* the gametocytes of *H. minchini* new species harbour less pigment granules than the gametocytes of *H. montezi* (Table 2). All these comparisons apparently confirm again the differences between these two parasites.

Morphometric measurements of the gametocytes of *H. minchini* new species, were assessed by Shapiro-Wilk test (Shapiro & Wilk, 1965) and did not follow a normal distribution. Therefore, they were analysed by the Mann-Whitney *U* test (Mann & Whitney, 1947) ($\alpha=0.05$) and highlight a sexual dimorphism. Mature microgametocytes are significantly larger in both length ($p=0.00126$) and width ($p=0.01072$) than the macrogametocytes. The sexual dimorphism is also noted on the impact onto the infected RBC. Gametocytes of *H. minchini* new species significantly enlarge the infected RBCs in both length and width ($p<0.00001$ and $p=0.00006$ respectively) for RBCs infected by macrogametocytes and ($p<0.00001$ and $p<0.00001$ respectively) for RBCs infected microgametocytes. The enlargement induced by the microgametocytes is significantly bigger in both directions than the one induced by the macrogametocytes ($p<0.00001$ and $p<0.00001$ respectively). The sexual dimorphism is not observed on the impact onto nucleus of the infected RBC. Gametocytes of *H. minchini* new species do not significantly modify the length and width of the nucleus of the infected RBCs ($p=0.48803$ and $p=0.08226$ respectively) for RBCs infected by macrogametocytes and ($p=0.17879$ and $p=0.06178$ respectively) for RBCs infected microgametocytes. No significant difference between the microgametocytes and the macrogametocytes are observed on their impact onto the nucleus of the infected RBCs neither in its length and width ($p=0.17619$ and $p=0.08226$ respectively) nor in its displacement ($p=0.05821$).

Molecular data and phylogenetic analysis. The PCRs for haemosporidian parasite detection are congruent with the microscopy and confirm the 3 negative birds, the 2 mixed *Plasmodium* species infected birds and the single pure *Haemoproteus* infection. *Haemoproteus minchini* new species is further characterised at the genetic level by four sequences of conserved genes namely cytb (GenBank: KU160476), cox1 (GenBank: KU160477), clpC (GenBank: KU160478) and tufA (GenBank: KU160479) obtained from the type specimen, no cloning and sequencing were attempted from the mixed infected samples. Alignment and BLAST comparison of the sequences for cytb, cox1 and clpC confirm that *H. minchini* new species belongs to the Haemoproteidae and it is distinct from all other deposited sequences. Regarding tufA, it is the first time that a sequence of this gene is obtained from a *Haemoproteus* parasite.

Phylogenetic analysis of cytb gene: It was performed with our new sequence [KU160476] and a selection of 79 identified morpho-species sequences recorded into the Avian haemosporidian uniform database: MalAvi (sequence [KU160476] was trimmed according to the standard fragment size of the MalAvi database to a fragment of 479bp) (Fig. 2). It shows that *H. minchini* new species is a distinct from all other sequences of identified Haemosporidia parasites deposited into the MalAvi database. Analysis of this short fragment shows that *H. minchini* new species belongs to the *Parahaemoproteus* subgenus and falls within a small clade forms by *Haemoproteus balmorali* Pierce, 1984; *Haemoproteus attenuatus* Valkiūnas, 1989; *Haemoproteus*

Table 2. Morphometric measurements of *Haemoproteus* (*Parahaemoproteus*) *minchini* new species and *Haemoproteus montezi* Travassos Santos Dias, 1953.

Features	***Haemoproteus minchini*** **new species**			***Haemoproteus montezi****		
	n	**X̄**	**SD**	**n**	**X̄**	**SD**
Uninfected RBC:	40			35		
L		13.62	0.64		13.4	0.8
W		7.54	0.44		7.4	0.6
NL		6.19	0.58		6	0.8
NW		2.27	0.27		2.2	0.3
Macrogametocyte RBC:	40			35		
L		14.51	0.89		13.5	1.2
W		8.09	0.59		7.6	0.6
NL		6.20	0.49		6	0.6
NW		2.34	0.27		2.3	0.3
NDR		0.70	0.14		0.8	0.2
Macrogametocyte:	40			35		
L		14.77	1.14		14.2	1.5
W		3.64	0.60		3.4	0.6
NL		3.25	0.59		3.2	0.8
NW		2.16	0.51		2.1	0.5
NPG		12.03	1.60		16.3	1.5
Microgametocyte RBC:	30			10		
L		15.55	0.76		14	1
W		8.71	0.51		8.1	0.7
NL		6.32	0.54		6.1	0.5
NW		2.43	0.42		2.3	0.3
NDR		0.65	0.15		0.8	0.2
Microgametocyte:	30			10		
L		15.64	0.84		14.2	1.1
W		3.97	0.51		3.5	0.5
NL		6.36	0.73		6.3	1
NW		3.49	0.40		3.3	0.5
NPG		11.03	2.29		16.5	2

n: sample size, *X*: mean, *SD*: standard deviation. L: length, W: width, NL: nucleus length, NW: nucleus width, NDR: nuclear displacement ratio (calculated according to Bennett & Campbell, 1972), NPG: number of pigment granules. * Measurements of *Haemoproteus montezi* are from Bennett & Pierce, 1990. All sizes are given in µm.

gavrilovi Valkiūnas & Iezhova, 1990; *Haemoproteus paranucleophilus* Iezhova, Dodge, Sehgal, Smith & Valkiūnas, 2011; and *Haemoproteus ptilotis* Clark, Adlard & Clegg, 2015; with divergences ranging from 2.8% to 5.2% (Fig. 2). A similar analysis conducted with all the sequences deposited into Genebank and MalAvi (data not shown) highlights a more closely related sequence: H_AFR59 [KM056456] that diverges by only 1% on this short fragment. Interestingly this sequence derivate from an unidentified *Parahaemoproteus* species isolated from another Musophagiformes host, the *Tauraco corythaix schalowi* (Reichenow, 1891) screened during a blood parasites survey in Malawi (Lutz et al., 2015).

Phylogenetic analysis of cox1 gene: It was performed with our new sequence [KU160477] and 28 other sequences of identified avian Haemosporidia downloaded from Genbank (fragment size of 918 bp) (Fig. 3). This analysis confirms that *H. minchini* new species belongs to the *Parahaemoproteus* subgenus. It also shows that *H. minchini* new species is distinct from all the other sequences included into the analysis and forms a sister group to two others clades containing parasites of the Passeriformes: *Haemoproteus fringillae* (Labbé, 1894), *Haemoproteus magnus* Valkiūnas & Iezhova, 1992, *Haemoproteus lanii* Mello, 1936, *Haemoproteus belopolskyi* Valkiūnas, 1989 and *Haemoproteus parabelopolskyi* Valkiūnas, Križanauskienė, Iezhova, Hellgren & Bensch, 2007, and *Haemoproteus tartakovskyi* Valkiūnas, 1986, *Haemoproteus passeris* Kruse, 1890 and *Haemoproteus vireonis* Bennett, Caines & Woodworth-Lynas, 1987 respectively with divergences ranging from 5.1% to 9.3% (Fig. 3).

Phylogenetic analysis of clpC gene: It was performed with our new sequence [KU160478] and 31 other sequences of identified avian Haemosporidia downloaded from Genbank

Fig. 2. Molecular phylogeny based on the mitochondrial cytb gene illustrating the position of *Haemoproteus* (*Parahaemoproteus*) *minchini* new species within the avian haemosporidian parasites. The analysis was performed by Maximum Likelihood (ML) method with GTR+Γ+I model on 79 identified morpho-species sequences (49 *Haemoproteus*, 21 *Plasmodium* and 9 *Leucocytozoon* used as out-group to root the tree) recorded into the MalAvi database (fragment size 479bp) and our sequence of *H. minchini* new species [KU160476]. Statistical branch support (>60%) is provided by bootstrap values (1,000 replicates) on the branches. MalAvi accession numbers are in square bracket and GenBank accession numbers are in vertical bars.

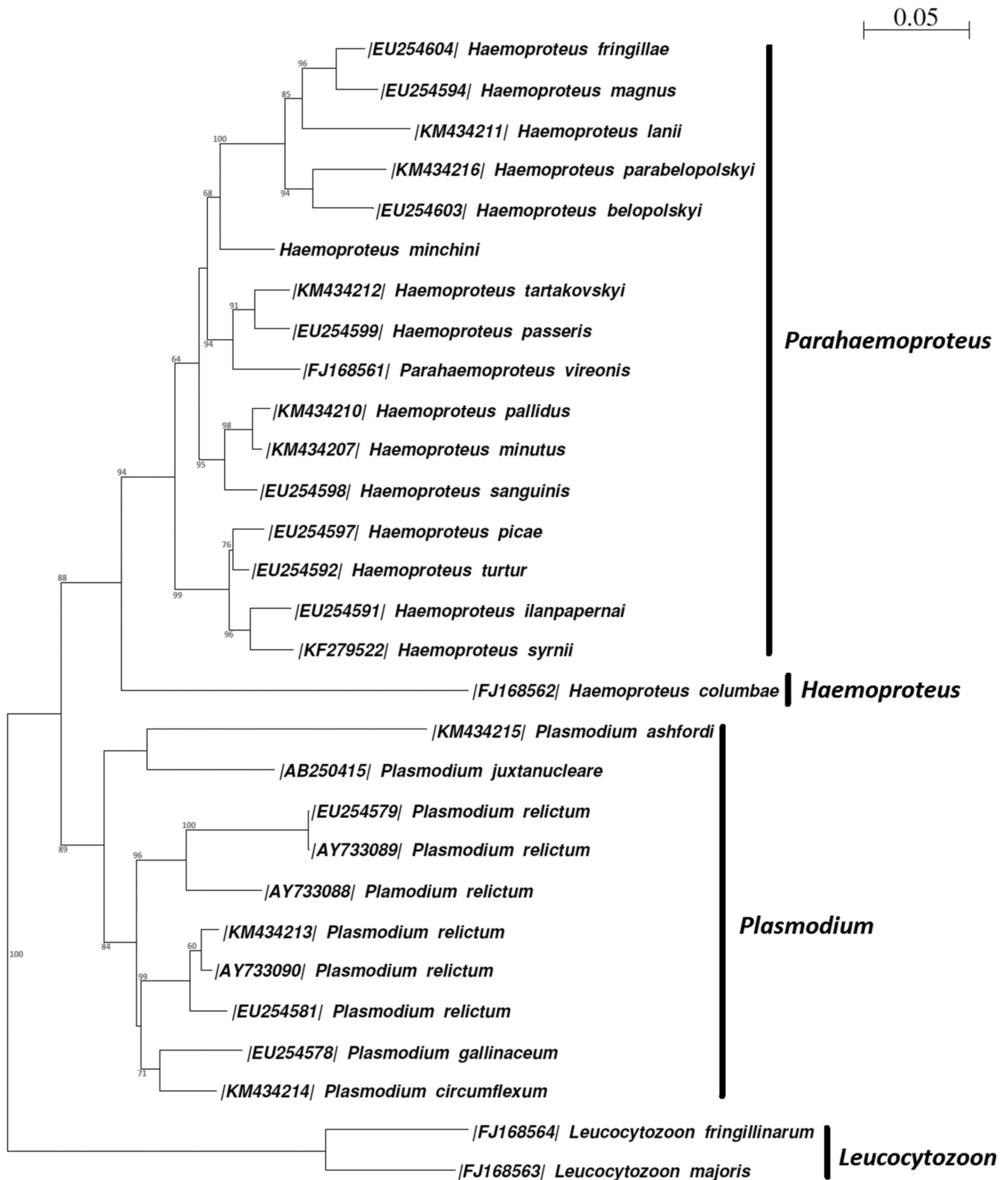

Fig. 3. Molecular phylogeny based on the mitochondrial cox1 gene illustrating the position of *Haemoproteus minchini* new species within the avian haemosporidian parasites. The analysis was performed by Maximum Likelihood method (ML) with GTR+Γ+I model on 28 sequences from identified species (16 *Haemoproteus*, 10 *Plasmodium* and 2 *Leucocytozoon* used as out-group to root the tree) downloaded from GenBank (fragment size 918bp) and our sequence of *H. minchini* new species [KU160477]. Statistical branch support (>60%) is provided by bootstrap values (1,000 replicates) on the branches. GenBank accession numbers are in vertical bars.

(fragment size of 505bp) (Fig. 4). Analysis of this gene from the apicoplast confirms also that *H. minchini* new species belongs to the *Parahaemoproteus* subgenus. Similarly to the results obtained from the cox1 gene, it appears related to some parasites of the Passeriformes and is placed at the base of clade containing *H. lanii*, *H. belopolskyi*, *H. magnus* and *H. fringillae* with divergence ranging from 8.9% to 13.2% (Fig 4).

Phylogenetic analysis of tufA gene: It was performed with our new sequence [KU160479] and 19 other sequences of identified Haemosporidia downloaded from Genbank (fragment size of 814 bp) (Fig. 5). It is the first time that a sequence of tufA gene is obtained from a haemoproteid parasite. Phylogenetic analysis on this gene confirms that *H. minchini* new species is not related to the parasites of the genus *Plasmodium* and forms a sister group to all the *Plasmodium* species from Birds, Rodents and Primates, included in to the analysis, that cluster within a single clade. The genetic divergence is ranging from 12.7% to 15.5% and equal to 14.3% with the *Plasmodium* species and *Leucocytozoon caulleryi* Mathis & Leger, 1909, respectively, clearly separating *H. minchini* new species from both *Plasmodium* and *Leucocytozoon* genera (Fig. 5).

DISCUSSION

Subgenus of *Haemoproteus minchini* new species: Results of the genetic studies obtained for cytb, cox1 and clpC genes are congruent and clearly highlight that *Haemoproteus minchini* new species belongs to the clade formed by the species of haemoproteids transmitted by *Culicoides* vectors. Based on these results and despite an unknown arthropod vector, *H. minchini* new species is classified into the *Parahaemoproteus* subgenus. This taxonomic rank could be assessed biologically by studying the exoerythrocytic schizogony and the sporogony in a competent vector that would also highlight the inputs of the genetic methods into the taxonomy of the avian Haemoproteidae.

Geographic origin of *Haemoproteus* (*Parahaemoproteus*) *minchini* new species: Based on the geographical origin of the Great Blue Turacos, their non-migrant status and their keeping in vector-proof quarantine condition in Jurong Bird Park before the release into the open-air aviaries, it is very unlikely that the infection was acquired in Singapore from other birds in the park or from autochthonous birds. Therefore, the transmission of *H. minchini* new species is very likely to have occurred in the Afrotropical region. This idea seems to be confirmed by the follow up over two years of the two non-infected birds (H2906 and H2913) that have never displayed any parasite in their blood neither sign of infection.

Polyparasitism: It is the rule in natural infection with concurrent polyinfection by several species from the same genus and also frequently by several genera (Landau et al., 1989; Chavatte et al., 2007, 2009). It has been reported that usually the different species occupy distinct spatial and temporal niches into the host (Cambie et al., 1990). Our Great Blue Turacos that are wild caught birds did not escape this rule with 72.8% of polyinfection by different parasitic agents namely *Haemoproteus*, *Plasmodium* and microfilaria and with detection of several *Plasmodium* species belonging to distinct subgenus. The diversity of parasites recorded during their screening in quarantine might have been exacerbated by the inherent stress related to their captures, handling, captivity and geographical relocation as noticed for the *Plasmodium* of the Magpies (Chavatte et al., 2007).

Possible pathogeny and pathology related to *H. minchini* new species: In wild populations it is difficult to evaluate the impact of haemosporidian infections on their host due to the natural polyparasitism with several Hematozoa and the difficulty to recover sick or dying individuals and carcasses that are rapidly scavenged by predators (Bennett et al., 1993; Atkinson et al., 2008). Some dramatic cases are well documented e.g. the transmission of *Plasmodium relictum* (Grassi & Feletti, 1891) in the endemic naïve Hawaiian bird populations following the anthropogenic introduction of a competent vector, that had a high detrimental effect and led to host species extinctions (Warner, 1968; van Riper et al., 1986). So far, no similar extreme consequence has been associated with the haemoproteids parasites that are usually considered non-pathogenic (Sibley & Werner, 1984). However several reports identify *Haemoproteus* infection as the cause of pathology and death in wild birds (Kučera et al., 1982; Atkinson & Forrester, 1987). Certain studies also highlight a negative correlation of *Haemoproteus* infection on the host especially with the reproductive success, body condition, immunity status and survival (Allander & Bennett, 1995; Nordling et al., 1998; Ots & Hõrak, 1998; Hõrak et al., 2001; Sol et al., 2003). Some of these correlative studies were assessed with a more experimental approaches based on medication experiment and confirm that the birds infected with *Haemoproteus* had significant reductions in fledging success, higher nestling mortality rate and reduction in survival when compared with other infected birds that were treated with antimalarial medications (Merino et al., 2000; de la Puente et al., 2010). Although, it remains difficult to evaluate the real impact of haemoproteids infections due to the biases induced by the stress of capture, handling and sampling.

In captive breeding conditions of domestic birds, *Haemoproteus* have been reported to induce a wide range of infection from asymptomatic to severe clinical diseases and death (Rae, 1995). Several species of haemoproteids and their related infection are well documented e.g. *Haemoproteus columbae* Kruse, 1890 in doves and pigeons (Earlé et al., 1993), *Haemoproteus meleagridis* Levine, 1961 in the turkey (Atkinson et al., 1986, 1988), *Haemoproteus nettionis* (Johnston & Cleland, 1909) in anatids (Julian & Galt, 1980), *Haemoproteus lophortyx* Earl, 1929 in bobwhite quails (Cardona et al., 2002; Pacheco et al., 2011b) or also *Haemoproteus* sp. in parrots (Olias et al., 2011).

In zoological collections, stress, high density and promiscuity, increase the risk of pathogen transfer from species that would have never get an opportunity to be in contact naturally but

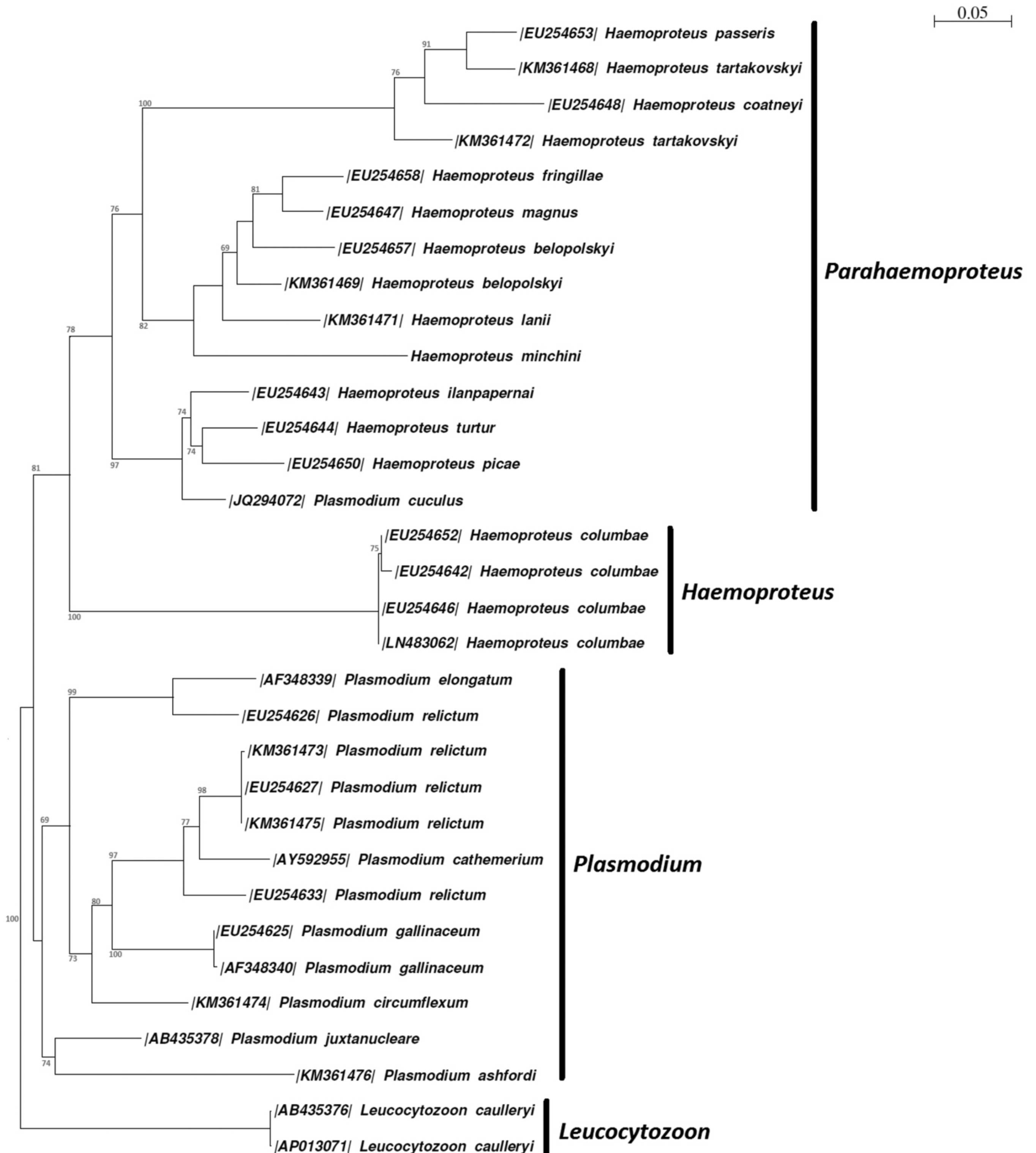

Fig. 4. Molecular phylogeny based on the apicoplast clpC gene illustrating the position of *Haemoproteus* (*Parahaemoproteus*) *minchini* new species within the avian haemosporidian parasites. The analysis was performed by Maximum Likelihood (ML) method with GTR+Γ+I model on 31 sequences from identified species (16 *Haemoproteus*, 13 *Plasmodium* and 2 *Leucocytozoon* used as out-group to root the tree) downloaded from GenBank (fragment size 505bp) and our sequence of *H. minchini* new species [KU160478]. Statistical branch support (>60%) is provided by bootstrap values (1,000 replicates) on the branches. GenBank accession numbers are in vertical bars.

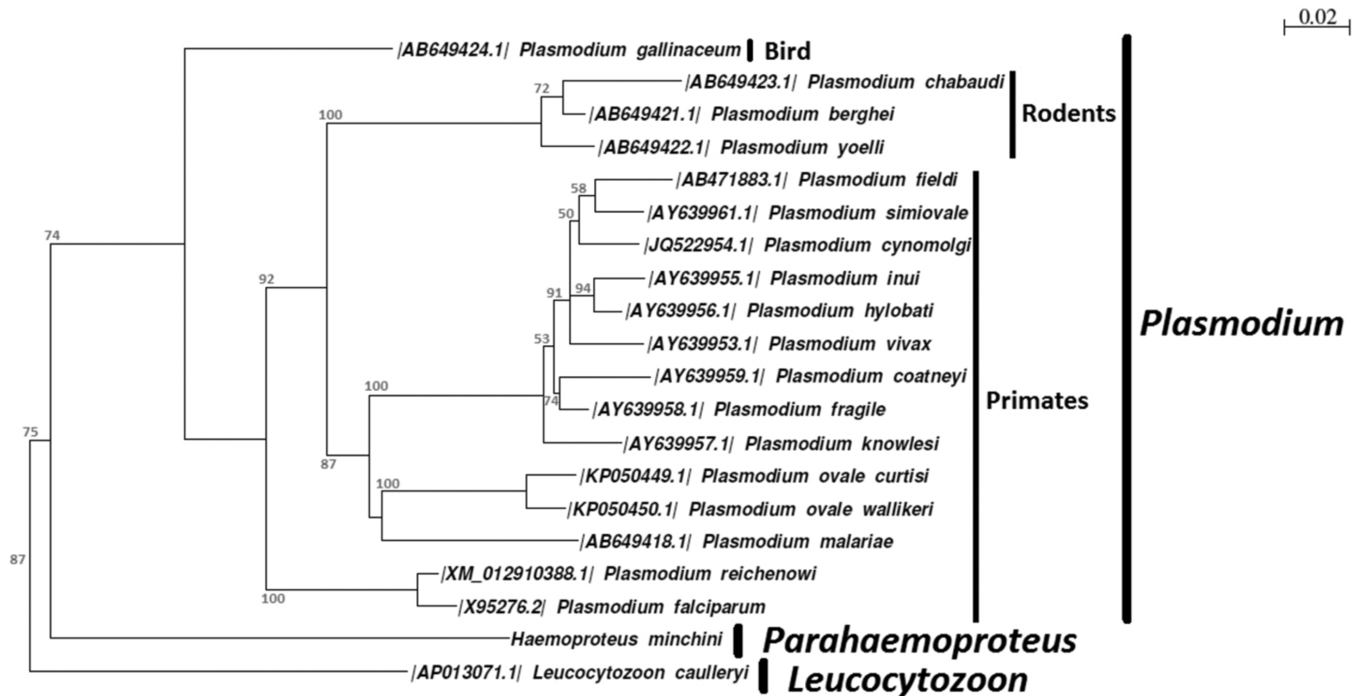

Fig. 5. Molecular phylogeny based on the apicoplast tufA gene illustrating the position of *Haemoproteus* (*Parahaemoproteus*) *minchini* new species within the haemosporidian parasites. The analysis was performed by Maximum Likelihood (ML) method with GTR+Γ+I model on 19 sequences from identified species (18 *Plasmodium* from Bird, Rodents and Primates and 1 *Leucocytozoon* used as out-group to root the tree) downloaded from GenBank (fragment size 814 bp) and our sequence of *H. minchini* new species [KU160479]. Statistical branch support (>50%) is provided by bootstrap values (1,000 replicates) on the branches. GenBank accession numbers are in vertical bars.

also from autochthonous animals to those in the collection. A typical and well-known example related to haemosporidian parasites is the infection of penguins by *Plasmodium* species from local wild birds that often lead to dramatic outbreaks with high mortality rate in absence of chemoprophylaxis (Fix et al., 1988; Cranfield et al., 1990; Bueno et al., 2010; Leclerc et al., 2014). Similarly *Haemoproteus* has been incriminated as causative agent of pathologies and deaths into different bird species in several zoos (Ferrell et al., 2007; Donovan et al., 2008).

The multiple Haemoprotozoan infections detected among the Great Blue Turacos imported into the Jurong Bird Park might possibly be the main and/or an aggravating cause of their high mortality rate 81.8% (9/11). Detailed histo-pathological studies are ongoing to investigate more precisely the causes of the death but some typical macroscopic anatomo-pathological symptoms of haemosporidian infection such as hepatomegaly, haemorrhages and anaemic organs were directly recorded during the necropsy.

cytb gene: along with the generalisation of the use of the molecular methods such as PCRs for the detection of haemosporidian infections among bird populations, the cytochrome b gene has been plebiscited and is also widely used to study genetic variations, phylogeny and evolution history of these parasites (Bensch et al., 2000; Perkins & Schall 2002; Waldenström et al., 2004). PCRs methods are many folds more sensitive that microscopy (Jarvi et al., 2002; Durrant et al., 2006) and especially in the context of low or very low infection (Jarvi et al., 2002). Assessing this question Valkiūnas et al. (2008b) conclude that both methods have similar sensitivities and both underestimate the overall prevalence of the infection. In the present study a good agreement was found between the detections of haemosporidian parasites by microscopy and molecular methods as well as for their generic identification. At the species level, it was not possible to assess confidently the diversity of *Plasmodium* parasites by morphology since not all stages were not observed and no attempt was performed using molecular tools to assess this point. Sometime PCRs methods detect the presence of more taxa than the microscopy (Bensch et al., 2004) but sometime they also detect only one taxon, that is not always the predominant one in mixed infection (Valkiūnas et al., 2006) or detect the DNA of sporozoites from parasites that are not developing in the investigated host (Valkiūnas et al., 2009). Despite these caveats, many studies still only focused on molecular methods and neglect microscopy, leading to a high number of cytb sequences deposited in GenBank that are just linked to a genus or misidentified when linked to a species as pointed by Valkiūnas et al., (2008a). Once used in phylogeny and evolution studies these incorrectly identified sequences lead to erroneous conclusion or incomprehensible results and need to be corrected (Karadjian et al., 2014). In order to limit this risk, the MalAvi Data Base was settled in 2009 (Bensch et al., 2009). It is a very valuable initiative, although the length of the fragment is quite short (479bp) and it now also includes many of unidentified sequences e.g. H_AFR59 [KM056456] the closest related to *Haemoproteus* (*Parahaemoproteus*) *minchini* new species isolated from another Musophagiforme species in Africa (Lutz et al., 2015). It would have been extremely useful to have morphological data and also a wider fragment of the cytb or information from other genes to allow

a reliable comparison of this parasite with *H. minchini* new species and *H. montezi*.

tufA gene: it is a gene encoding the elongation factor Tu which is a conserved prokaryotic elongation factor implied into translation mechanism for the protein synthesis by the ribosome. It is the first time that a sequence of tufA gene is obtained for a *Haemoproteus* parasite while it is common for the *Plasmodium*. Sequence [KU160479] will therefore be a useful reference for any subsequent studies that would include more genes targets to achieve the molecular characterisation of haemoproteids and their relationship with the other Haemosporidia.

Multiple genes approach: it was adopted in this work and based on four genes (cytb, cox1, clpC and tufA) from the organellar genomes of the parasite because different genes have different phylogenetic value and provide distinct evolution history and relationship between related organisms. In haemosporidian parasites results obtained on a single gene or even an entire organellar genome have been proved to be radically divergent: e.g. *Plasmodium ovale* that was reported related to the *Plasmodium* of Lemurs by the phylogeny built on the mitochondrial genome (Pacheco et al., 2011a) and related to the *Plasmodium* of Rodents by the phylogeny built on the apicoplast genome (Arisue et al., 2012). Additional recent phylogenetic information provided from a nuclear gene seems to support the first hypothesis (Chavatte et al., 2015) and also highlight that is desirable to adopt an approach based on multiple genes across the different genomes to increase the likelihood of deriving a true phylogenetic relationship between the various species and not just the evolution story of the gene studied (Perkins et al., 2007; Martinsen et al., 2008). In this view, it would also be advisable whenever possible to generate supermatrix by concatenation of the different genes sequenced (Martinsen et al., 2008) or alternatively to consider whole genome sequencing approach (Bensch et al., 2016).

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