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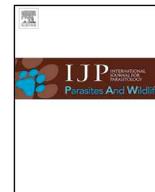
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Invited article

Rodent malaria in Gabon: Diversity and host range

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ABSTRACT

Malaria parasites infect a wide range of vertebrate hosts, such as reptiles, birds and mammals (i.e., primates, ungulates, bats, and rodents). Four *Plasmodium* species and their subspecies infect African Muridae. Since their discoveries in the 1940s, these rodent *Plasmodium* species have served as biological models to explore many aspects of the biology of malaria agents and their interactions with their hosts. Despite that, surprisingly, little is known about their ecology, natural history and evolution. Most field studies on these parasites, performed from the 1940s to the early 1980s, showed that all rodent *Plasmodium* species infect only one main host species, the thicket rat. In the present study, we re-explored the diversity of *Plasmodium* parasites infecting rodent species living in peridomestic habitats in Gabon, Central Africa. Using molecular approaches, we found that at least two *Plasmodium* species (*Plasmodium vinckei* and *Plasmodium yoelii*) circulated among five rodent species (including the invasive species *Mus musculus*). This suggests that the host range of these parasites might be larger than previously considered. Our results also showed that the diversity of these parasites could be higher than currently recognized, with the discovery of a new phylogenetic lineage that could represent a new species of rodent *Plasmodium*.

1. Introduction

Malaria is a mosquito-borne disease caused by protozoan parasites of the genus *Plasmodium* (Déchamps et al., 2010). This genus comprises many species that infect a large range of vertebrate groups including birds, reptiles and mammals (Boundenga et al., 2016, 2017; Yotoko and Elisei, 2006). In mammals, besides the parasites infecting humans, those infecting rodents and more specifically Muridae (*Plasmodium berghei*, *Plasmodium chabaudi*, *Plasmodium vinckei*, and *Plasmodium yoelii*) have been the most studied and have served, since their discoveries in the 1940s, as biological models to explore many aspects of the biology of malaria agents and their interactions with their hosts (vertebrate and arthropod vectors) (LaCrue et al., 2011; Landau and Chaubaud, 1994; Stephens et al., 2012; Vanderberg, 1991; Wargo et al., 2007). Due to their importance as model systems, rodent malaria agents were among the first eukaryotes (and among the first protozoans) to have their genome entirely sequenced (in 2002 for *P. yoelii yoelii* 17XNL, in 2005 for *P. chabaudi chabaudi* AS) (Carlton et al., 2002; Hall et al., 2005).

Despite the huge amount of knowledge acquired on the biology of *Plasmodium* species infecting rodents during the last 80 years, paradoxically little is known about their ecology, natural history, and evolution (Landau et al., 1970; Ramakrishnan et al., 2013; Vanderberg, 1991). Indeed, most field studies on these parasites were performed from the 1940s to the early 1980s (Landau and Chaubaud, 1994), and to our knowledge, no study has been carried out in their natural habitat since then.

Previous studies have shown that the distribution of rodent malaria agents is limited to the Congo basin: Cameroon, Central African Republic, Congo, Democratic Republic of the Congo, and Nigeria (Culleton, 2005; Killick-Kendrick, 1978; Landau and Chaubaud, 1994). It is thought that most of these agents (except *P. berghei*) infect one main host species, the thicket rat (*Grammomys poensis* – previously called *Thamomys rutilans*) that inhabits tropical lowland forests (Killick-Kendrick, 1978; Landau and Chaubaud, 1994). A recent study carried out in Uganda, Kenya, Zambia and Mozambique did not detect any infection in sylvatic rodents, confirming the absence or the rarity of these rodent *Plasmodium* species outside the Congo basin (Lutz et al., 2016).

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Fig. 1. Location of the provinces of Gabon where rodent samples were collected. The map shows the sites where rodents were captured (in red) and the site where some *Anopheles* infected with rodent malaria parasites were found in a previous study (in green) (Makanga et al., 2016). The number of individuals collected in each province is indicated between brackets. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

These parasites were first characterized and defined using morphological features, leading to the definition of four species (*P. yoelii*, *P. chabaudi*, *P. berghei* and *P. vinckei*) and several subspecies (e.g., *P. yoelii yoelii* and *P. yoelii nigeriensis*) (Landau and Chaubaud, 1994). Isoenzyme analysis first and later multiple gene sequencing of several isolates collected in the 1970s (Carter, 1978) and stored at the European Malaria Reagent Repository (www.malariaresearch.eu) largely confirmed this diversity, and suggested that most subspecies should be considered as species. This is particularly true for the *P. vinckei* subspecies because they show high genetic divergence and do not seem to recombine when present in sympatry (Carter, 1978; Perkins et al., 2007; Ramiro et al., 2012).

The only known vector of murine malaria parasites was, up to recently, *Anopheles durenii millescampsii*, the invertebrate host of *P. berghei* and *P. v. vinckei* in Katanga (Democratic Republic of the Congo) (Killick-

Kendrick, 1978; Stephens et al., 2012). However, a recent study in the forests of Gabon identified five other anopheles species infected by rodent *Plasmodium* parasites (*Anopheles moucheti*, *Anopheles nili*, *Anopheles gabonensis*, *Anopheles vinckei*, and *Anopheles marshalii*), suggesting that a far larger number of anopheles species could play the role of vector within their geographic range (Makanga et al., 2016; Rahola et al., 2014).

Recent studies on *Plasmodium* parasites of different groups of vertebrates demonstrated the utility of re-evaluating their diversity in the molecular era, by showing that their diversity has been, most of the time, historically underestimated (Boundenga et al., 2016; Prugnolle et al., 2010; Valkiūnas et al., 2008). For instance, the use of molecular tools has led to revising the diversity of the *Plasmodium* parasites infecting apes and ungulates (Boundenga et al., 2016; Liu et al., 2010; Perkins and Schaefer, 2016; Prugnolle et al., 2010).

Table 1 Description of the rodent species analysed in this study. The table shows the number of samples collected per species and the infection rate by *Plasmodium* agents.

Province	Sites	Year of samples collection												Total of samples				
		2009	2010	2011	2012	2013	2014	2015	2016	2015		2016						
		Blood (n/N)	Organ (n/N)	Blood (n/N)	Organ (n/N)	Blood (n/N)	Organ (n/N)	Blood (n/N)	Organ (n/N)	Blood (n/N)	Organ (n/N)	Blood (n/N)	Organ (n/N)	Blood (n/N)	Organ (n/N)	Blood (n/N)	Organ (n/N)	(n/N)
Haut-Ogooue	Franceville	-	16	-	-	-	-	1/10	-	-	-	3	10	2/8	9	3/71	-	3/71
	Djoumou	-	-	-	-	-	-	-	9	2/20	-	-	-	10	3	2/42	-	2/42
	Lekoni	-	5	-	1/17	-	-	2/19	-	-	-	-	-	-	-	-	-	3/41
Ogooue-Lolo	Koullamoutou	-	-	-	-	-	-	20	-	-	-	-	-	-	-	-	-	1/62
	Mimongo	-	-	15	-	-	-	-	1/14	10	9	9	10	-	1/30	-	-	1/58
Ogooue-Ivindo	Makokou	-	-	6	15	10	10	9	2/10	14	14	-	1/7	-	-	-	-	3/71
	Total of samples	-	21	30	32	10	10	48	24	36	21	21	47	18	42	18	42	13/345

In the present study, we examined the genetic diversity and the host range of malaria parasites infecting Muridae in Gabon (Central Africa) by analysing the mitochondrial cytochrome *b* gene (*CYTB*). We focused on rodent parasites because information regarding their distribution and the ecology in these hosts is currently non-existent in Gabon. The present study is the first molecular overview of protozoan blood parasites of the order Haemosporida recorded in rodents from the forested regions of Gabon.

2. Materials and methods

2.1. Study area and sample collection

Gabon (Fig. 1) is a Central African country located in the west part of the Congo basin. Most of its surface (85%) is covered by forest that is amongst the richest African forests in terms of diversity and endemism (Lee et al., 2006). Gabon has a humid and hot climate, typical of equatorial regions. There are four seasons: two dry (from June to September and from mid-December to mid-February) and two rainy (from mid-September to mid-December and from mid-February to mid-June).

The *Plasmodium* diversity circulating among wild rodents in Gabon was analysed by screening rodent samples collected by different teams of the Centre International de Recherches Médicales de Franceville (CIRMF) in four provinces (Fig. 1) as part of a research programme on rodent pathogens between 2009 and 2016 (See Table 1 and Table S1 for more details on sampling). Rodents were captured using Tomahawk and Sherman traps, as described in (Duplantier, 1989), in peridomestic habitats (up to 250m from the houses). Each individual was identified using morphological features described in previous studies (Duplantier et al., 1993). For this study, analyses were performed on 345 rodent samples that belonged to seven rodent species [*Lemniscomys striatus* (n = 50), *Lophuromys nudicaudus* (n = 40), *Mastomys natalensis* (n = 35), *Praomys* sp. (n = 30), *Grammomys poensis* (n = 10), *Rattus rattus* (n = 100), and *Mus musculus* (n = 80)]. For each animal, whole blood was preferentially used when available, otherwise a mix of liver/spleen was chosen (see Supplementary Table S1 for details on sampling). Biological samples (whole blood or liver/spleen) were collected and kept in liquid nitrogen until their arrival at the CIRMF where they were stored at -80 °C for molecular analyses.

2.2. Ethical approval

The study was performed outside protected areas in Gabon. Rodent trapping and sampling were conducted after authorization by the Wildlife and Hunting Department of the Gabonese Ministry of Water and Forestry (N°003/MEFE-PA/SG/DGEF/DCF and N°0021/MEFE-PA/SG/DGEF/DCF). Animal capture, handling, euthanasia and sample transfer across country borders were performed in accordance with the guidelines of the American Society of Mammalogists (<http://www.mammalsociety.org/committees/animal-care-and-use>) and in strict accordance with the recommendations of the Gabonese National Ethics Committee (Authorization N°PROT/0020/2013I/S G/CNE).

2.3. Molecular analyses

2.3.1. Molecular characterization of malaria parasites

For each sample, total DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Courtaboeuf, France) from approximately 200 µl of blood or 100 mg of liver/spleen according to the manufacturer's procedures. Total DNA was then used as template for the detection of malaria parasites according to a previously described protocols based on the amplification by nested PCR of a portion of the *Plasmodium* *CYTB* gene (Boundenga et al., 2017; Prugnolle et al., 2010). All amplified products (10 µl) were run on 1.5% agarose gels in Tris-acetate-EDTA buffer. Amplicons (700bp) were then sequenced by Eurofins MWG (France). Moreover, in all positive samples, the nature of the host species was confirmed by amplifying and sequencing a portion

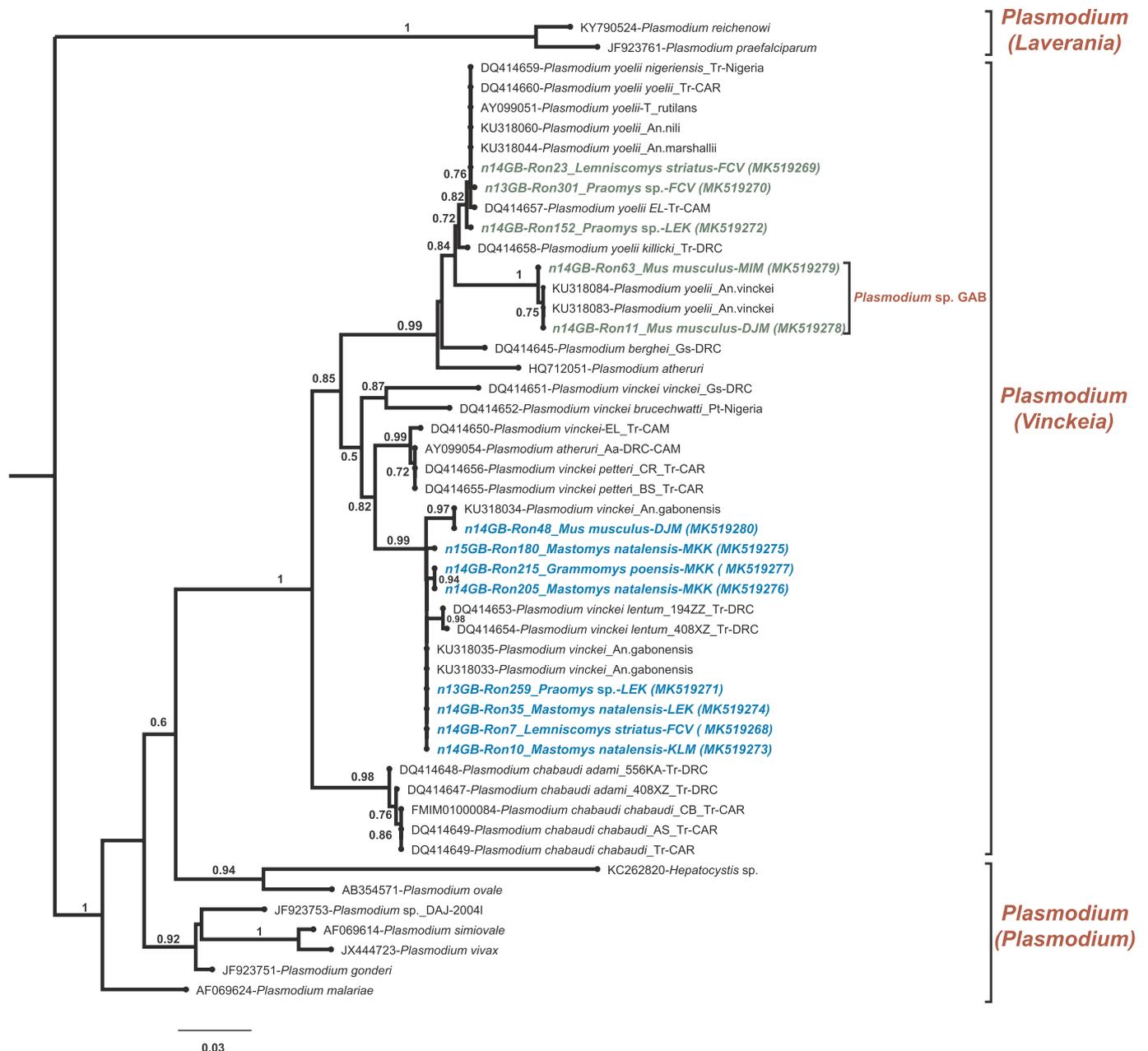


Fig. 2. Phylogenetic relationships between the *CYTB* sequences of *Plasmodium* parasites obtained in our study (in colour) and the sequences obtained from existing databases (in black). The tree was built using partial *CYTB* sequences (700 bp-long). The names of our isolates (for instance, n14GB-Ron48_Mus musculus-DJM) include: 1) the year and country of collection (n14GB: n14: 2014 and GB: Gabon); 2) the sample number (Ron48: Rodent number 48); 3) the rodent species and 4) the abbreviation of the sample site (FCV: Franceville; MIM: Mimongo, LEK: Lekoni, DJM: Djoumou; MKK: Makokou; KLM: Koulamoutou). The name of isolates clustering with *Plasmodium yoelii* is in green, and of isolates clustering with *Plasmodium vinckei* is in blue. CAM: Cameroon and CAR: Central African Republic. In our study we called *P. sp. GAB* the new *Plasmodium* lineage found in some Gabonese rodents. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of the cytochrome B gene, as previously described in (Steppan et al., 1999; Steppan and Schenk, 2017). All sequences identified in our study were deposited in GenBank under the following accession numbers: MK395253 to MK395265 (*Plasmodium* species), and MK519268 to MK519280 (host species). Due to the nature of the used biological material (frozen blood or organs), microscopic analyses could not be performed.

2.3.2. Phylogenetic tree analyses and estimation of divergence

The phylogenetic analyses were done after multiple alignments of the obtained partial *CYTB* sequences (700 nucleotides) and of the Genbank reference sequences using ClustalW (v 1.8.1 in BioEdit

v.7.0.9.0. Software) (Hall, 1999). It was previously shown that enough phylogenetic data can be obtained from *CYTB* sequences to study the phylogenetic relationships between malaria parasites and to recover major clades (Pacheco et al., 2011; Perkins, 2008). Maximum Likelihood (ML) methods were used for tree construction (Boundenga et al., 2017). Sequence evolution was modelled with General Time Reversible (GTR) + Gamma, as determined using ModelTest (Posada and Crandall, 1998). The highest-likelihood DNA tree and the corresponding bootstrap support values were obtained with PhyML (Guindon et al., 2010) (freely available at the ATGC bioinformatics facility: <http://www.atgc-montpellier.fr/>) using Nearest Neighbour Interchange (NNI) + Subtree Pruning Regrafting (SPR) branch swapping

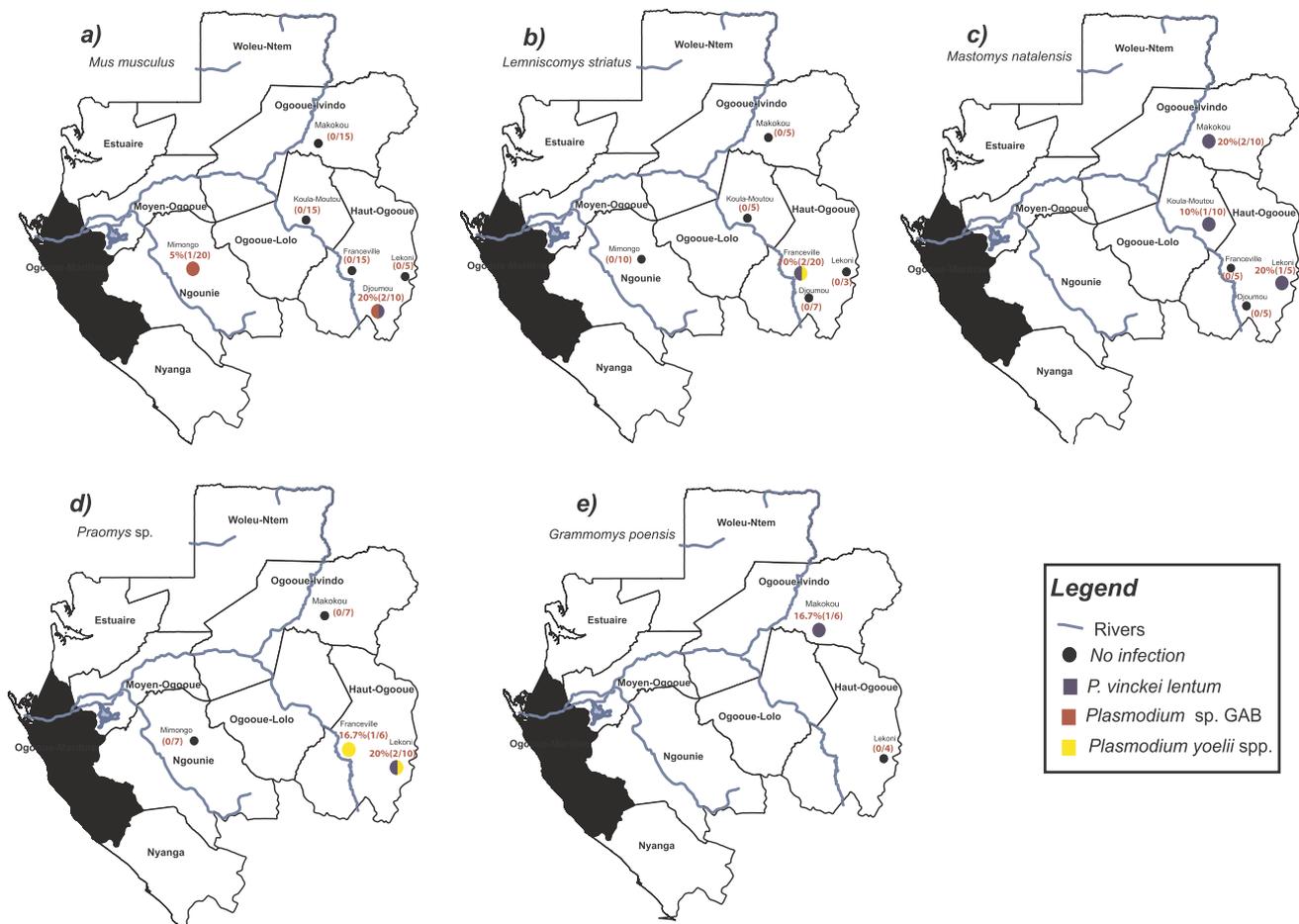


Fig. 3. Map of malaria parasite distribution for each host species and infection rate for each *Plasmodium* species or lineage (*Plasmodium yoelii* spp., *Plasmodium vinckei lentum*, and *Plasmodium* sp. GAB). a) for *Mus musculus*; b) for *Lemniscomys striatus*; c) for *Mastomys natalensis*; d) for *Praomys* sp. and e) for *Grammomys poensis*. *Plasmodium* sp. GAB (*P. sp. GAB*) corresponds to the new phylogenetic lineage of rodent *Plasmodium* described in our study.

and 100 bootstrap replicates. In our study, ML trees were rooted using *Plasmodium praefalciparum* and *Plasmodium reichenowi*. The pairwise genetic distances (*p*-distances) between species were estimated using MEGA 6 (Tamura et al., 2013).

3. Results

This study investigated the diversity of species belonging to the *Plasmodium* genus and infecting rodents in four regions of Gabon (Fig. 1). *Plasmodium* parasites were detected in five of the seven studied rodent species (for more details see Table 1). The global infection rate was 3.71% ($n/N = 13/345$) and varied among rodent species: 11.43% for *M. natalensis* ($n/N = 4/35$), 3.33% for *L. striatus* ($n/N = 2/50$), 10% for *Praomys* sp. ($n/N = 3/30$), 3.75% for *M. musculus* ($n/N = 3/80$), and 10% for *G. poensis* ($n/N = 1/10$). No infection was found in the captured *R. rattus* and *L. nudicaudus* samples (for more details, see Table 1).

Phylogenetic analyses revealed that the 13 parasite sequences could be grouped with those of two *Plasmodium* species (*P. vinckei* and *P. yoelii*) that had been previously identified in central African rodents (Fig. 2). Specifically, eight sequences clustered with the subspecies *P. vinckei lentum*. All the five positive rodent host species were found to be infected by this subspecies: *M. natalensis* ($n = 4$); *G. poensis* ($n = 1$), *L. striatus* ($n = 1$), *Praomys* sp. ($n = 1$), and *M. musculus* ($n = 1$) (Figs. 2 and 3). These eight sequences clustered with other sequences found in *An. gabonensis* (KU318034; KU318035 and KU318033) from Gabon (Makanga et al., 2016) and the sequences found among rodents (DQ414653 and DQ414654) of the Democratic Republic of the Congo

(Carter and Walliker, 1976) (Fig. 2). Moreover, *P. vinckei lentum* was found in different regions of Gabon during our study (see Fig. 3).

The other five *Plasmodium* samples were related to *P. yoelii*, but formed two distinct phylogenetic groups (Fig. 2). The first group contained three sequences obtained from *L. striatus* and *Praomys* sp. (n14GB-Ron23_ *L. striatus*; n14GB-Ron301_ *Praomys* sp. and n14GB-Ron152_ *Praomys* sp.), and clearly clustered with the clade including both *P. yoelii nigeriensis* and *P. yoelii yoelii* (Fig. 2). These subspecies were not genetically different based on the *CYTB* sequence portion used in our study, and therefore could not be distinguished. The second group contained two sequences isolated from *M. musculus*, in association with two sequences previously obtained from infected forest *Anopheles* of Gabon (KU318084 and KU318083) (Makanga et al., 2016). Although this clade (*Plasmodium* sp. GAB) is related to *P. yoelii*, it was genetically quite different from all subspecies identified so far. It was well supported (high bootstrap values), and the average divergence (*p*-distance) measured between the *P. yoelii* subspecies and our two sequences (n14GB-Ron11- *Mus musculus* and n14GB-Ron63- *Mus musculus*) was $d = 0.03$. This value was higher than the one observed between the different *P. yoelii* subspecies ($d = 0.01$) and similar to the divergence currently observed between different species of rodent *Plasmodium* (e.g., *P. berghei* – *P. yoelii*: $d = 0.03$). Like for *P. vinckei*, these infections were discovered in different regions of Gabon (Fig. 3).

4. Discussion

The present study is the first molecular overview of malaria parasites recorded in rodents from Gabon and the first on the natural history

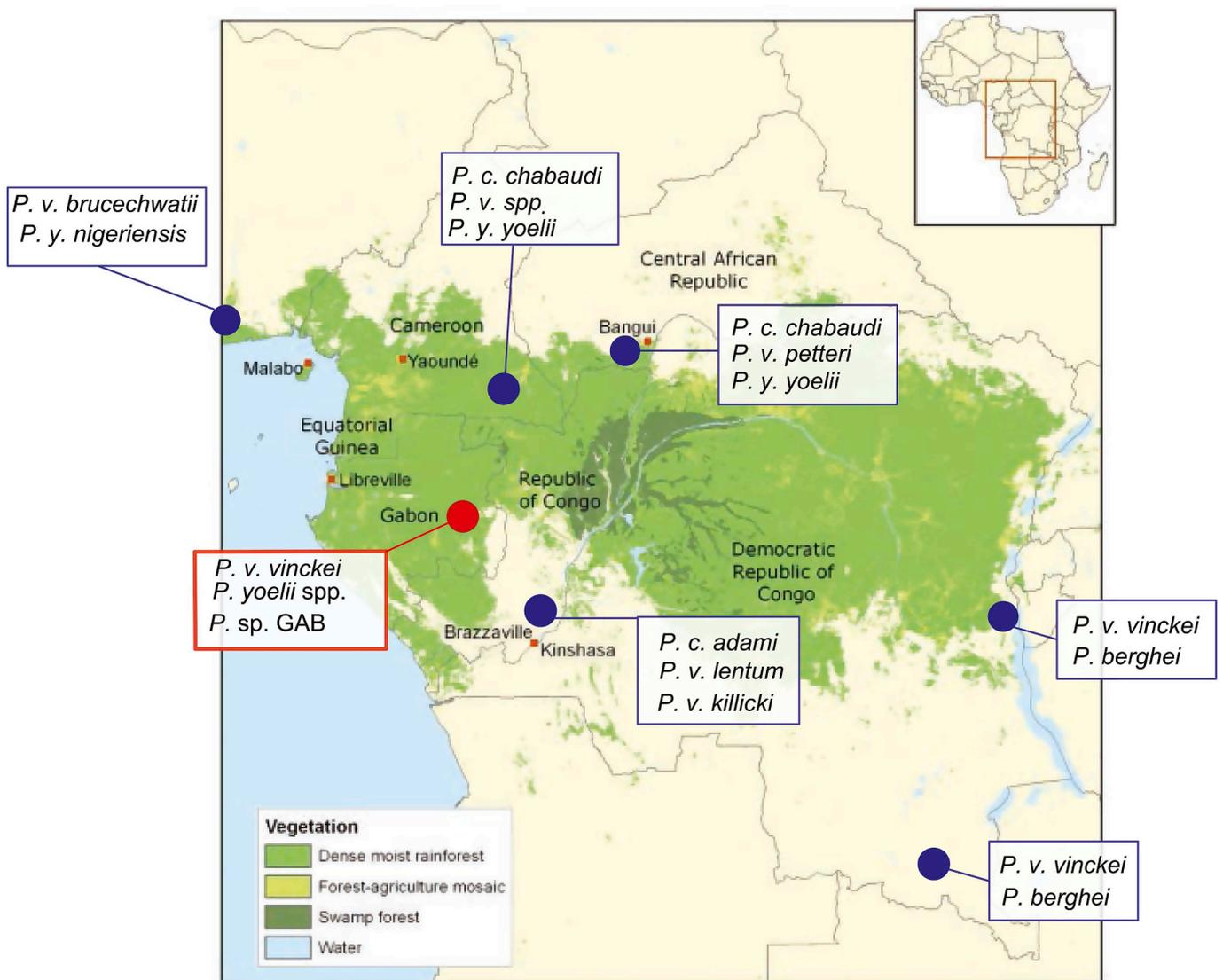


Fig. 4. Map showing the distribution of rodent malaria parasite species in Central Africa. This map is based on the data provided in Landau and Chabau (1994) (blue) and the data obtained in our study (red). *P. v.*: *Plasmodium vinckei*; *P. y.*: *Plasmodium yoelii*; *P. c.*: *Plasmodium chabaudi*; *P. v.*: *Plasmodium vinckei*; *P. berghei*: *Plasmodium berghei* and *P. sp. GAB*: *Plasmodium sp. GAB*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of rodent malaria agents since almost 40 years. The analyses were performed on a set of 345 samples (whole blood, liver, or spleen) obtained from seven rodent species (Muridae family), all captured in proximity or within villages or cities of Gabon. All parasites detected using molecular methods belonged to the *Plasmodium* genus and were identified in five of the seven rodent species captured (*L. striatus*, *Praomys* sp., *M. natalensis*, *G. poensis*, and *M. musculus*). Among the four *Plasmodium* species that are currently recognized to infect African Muridae (*P. chabaudi*, *P. berghei*, *P. vinckei*, and *P. yoelii*) (Bafort, 1971; Landau et al., 1970; Perkins et al., 2007; Stephens et al., 2012), two were identified in our study: *P. yoelii* and *P. vinckei* (with sequences that clustered specifically within the *P. vinckei lentum* subspecies). The discovery of these two parasite species in this region was not a surprise because they had been previously identified in neighbouring countries (Cameroon, Congo, Democratic Republic of the Congo, and the Central African Republic) (Landau and Chabaud, 1966; Landau and Chabaud, 1994; Perkins et al., 2007; Stephens et al., 2012) (Fig. 4). The circulation of these two *Plasmodium* species was also reported recently in Gabonese forests in a study on *Anopheles* vectors showing that approximately 11% of the infected mosquitoes captured carried rodent *Plasmodium* parasites of these two species (Makanga et al., 2016, 2017).

4.1. Host range

On the other hand, the diversity of rodent host species infected by these parasites was more a surprise. Indeed, murine malaria agents are considered to naturally infect one main host in the Congo basin, as reviewed by Killick-Kendrick (1978): “With the exception of one of the parasites of the Cameroon and *P. berghei* of Katanga, the principal and probably sole host of murine malaria parasites in Africa south of the Sahara in natura is the thicket rat, *T. rutilans*” (now known as *G. poensis*). Our study confirmed that *G. poensis* (*T. rutilans*) was infected, but it was not the only host or the host with the highest recorded prevalence (10%; n/N = 1/10, Table 1). Rodent malaria agents were detected with similar prevalence rates in two other native species of Gabon (11.42% in *M. natalensis* and 10% in *Praomys* sp.; Table 1) and with lower rates in another native species (*L. striatus*: 4%) and in one invasive species (*M. musculus*: 3.75%). Although these results need to be confirmed in other areas, they suggest that rodent malaria agents might be less specific than previously thought, and they are more in agreement with previous data on *Plasmodium* infectivity towards different murid hosts (Killick-Kendrick, 1978). Indeed, it has been experimentally shown that *P. yoelii* and *P. vinckei* can infect a wide range of murid rodents (Bafort, 1971;

Culleton, 2005; Fagbenro-Beyioku and Oyerinde, 1989). Our findings are also coherent with the fact that different *Anopheles* species can play the role of vector (Makanga et al., 2016). This diversity of vectors could favour the infection of different rodent species due to different trophic behaviours.

This difference compared with all previous studies could be explained by the fact that at that time, malaria infection diagnosis was only based on microscopy analysis (Garnham, 1966; Landau and Chaubaud, 1994). This might not have provided enough power (compared with molecular methods) to detect chronic infections or low parasitaemia infections in other species than *G. poensis*. Another explanation could also be that the previously studied ecosystems and consequently the communities of rodents were different from those of the present study. We think that a more systematic analysis of rodent communities from different habitats (peridomestic and wild) and their *Plasmodium* infections should be performed in the Congo basin.

Finally, the presence of *Plasmodium* species in *M. musculus* is an interesting observation. *M. musculus* is an invasive species that might have been introduced in Africa following the arrival of European settlers and has expanded across the continent (Terashima et al., 2006), capturing new local pathogens along the way. It would be interesting to understand how this parasite adapted to this new host species and to determine the physiological responses of this host species to these *Plasmodium* infections.

4.2. Parasite diversity

Our study also shows that the rodent *Plasmodium* species diversity has historically been underestimated. Indeed, against all expectations, two of our sequences (n14GB-Ron11_Mus musculus and n14GB-Ron63_Mus musculus), in addition to two sequences previously obtained from infected *Anopheles*, form a distinct and divergent clade, although close to *P. yoelii* (*P. sp.* GAB, Fig. 2). We think that this lineage may represent a new species of rodent *Plasmodium* on the basis of the divergence measured between this clade and *P. yoelii* (*p*-distance = 0.03), the closest known species. This observation needs to be confirmed using whole genome data (our analysis was based only on *CYTB* sequencing data) to ensure that recombination does not occur between this and other rodent *Plasmodium* clades (e.g., *P. yoelii*).

5. Conclusion

In conclusion, this study contributes to the knowledge and understanding of the diversity of *Plasmodium* species that circulate among rodents of central Africa, particularly in Gabon. Our results show that at least two *Plasmodium* species circulate in Gabon and infect several rodent species. Our molecular analysis reveals the existence of a potentially new species of *Plasmodium* (*Plasmodium sp.* GAB), closely related to *P. yoelii*. Additional information must be obtained to assign this new lineage to a new species. Better characterizing the diversity of naturally circulating rodent *Plasmodium* species is a prerequisite to understand their evolution and to determine the forces that drove lineage diversification (geographic isolation, host shift, co-speciation with host, vectors ...). Additional studies on wild and commensal rodents over the entire range are necessary to re-define this diversity in the molecular era.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijppaw.2019.07.010>.

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