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Genetic diversity and origins of invasive black rats (*Rattus rattus*) in Benin, West Africa

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Abstract. Black rats (*Rattus rattus*) are native to the Indian subcontinent but have now colonized most continents and islands following human movements and international trade. They are involved in the circulation and transmission to humans of many zoonotic agents as well as in massive damage to food stocks and native biodiversity in the regions they have settled. This study investigates the genetic diversity and possible origins of black rats from Benin, West Africa. We sequenced the complete mitochondrial cytochrome *b* gene in 90 individuals from nine localities in Benin. These sequences were subsequently compared to 390 other cytochrome *b* haplotypes from individuals from various European, Asian, American and African localities. Nucleotide polymorphism analysis, haplotype network and maximum likelihood phylogenetic tree reconstructions showed low mitochondrial diversity in black rats from Benin. Our results also suggest at least two distinct introduction events: one introduction probably occurred during the spice trade (15th-17th century) through the Indies Road connecting Europe to Asia. Other introduction events could have occurred more recently following the intensification of globalized trade from the eighteenth century, and onwards.

Key words: phylogeography, invasive species, cytochrome *b*, haplotype, polymorphism, Africa

Introduction

Though ancient, exchanges of people and goods throughout the world have greatly intensified in

recent decades as a result of extensive demographic increase and globalization. Globalized trade and human movements have led to animal, plant and microbe species introductions that are now

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considered the second most important cause of biodiversity loss (Briggs 2013). Such translocations of living organisms are also responsible for important threats to crop production and stored agricultural products (Swanepoel et al. 2017) as well as public and animal health (Kilpatrick & Randolph 2012). Altogether, surveillance, damage and management of invasive species cost 121 billion USD each year to the United States of America, representing 1% of GDP (Pimentel et al. 2008). This cost may explain the growing interest in biological invasion processes and control (Briggs 2013).

The black rat (*Rattus rattus*, Linnaeus 1758) is one of the most widely distributed invasive vertebrate species in the world (Drake & Hunt 2009). It belongs to a species complex originating in south-east Asia where the species displays its highest genetic diversity (Baig et al. 2018). Within this complex, six lineages have been described (Aplin et al. 2011); only lineage I, which corresponds to what is commonly referred to as the “ship rat”, has colonized the world following human migration and major trade routes (Audouin-Rouzeau & Vigne 1994, Drake & Hunt 2009, Varnham 2010). The phylogeny and phylogeography of black rats have

been the subject of several studies (e.g. Matisso-Smith & Robins 2004, Pagès et al. 2010, Tollenaere et al. 2010, Aplin et al. 2011, Colangelo et al. 2015, Baig et al. 2018). However, an understanding of its history in Africa is limited (see Kaleme et al. 2011, Konečný et al. 2013, Berthier et al. 2016). Archaeological data demonstrate that it arrived on South African coasts during the 6th century through the Arab trade that already connected the eastern and southern parts of the continent with the Middle East and Asia across the Indian Ocean (Prendergast et al. 2017). West Africa was probably colonized later from Europe (Konečný et al. 2013). For example, black rats seem to have colonized Senegal following the development of trade between West Africa and Europe during the 15th century (Rosevear 1969). Once settled along the coast, black rats then dispersed inland following road and fluvial routes (e.g. Kaleme et al. 2011, Konečný et al. 2013, Berthier et al. 2016), a process that is still ongoing (Konečný et al. 2013, Berthier et al. 2016). Considering their significance for the epidemiology of many zoonotic diseases (Goncalves da Cruz 2007), damage to foodstuffs (Meerburg et al. 2009) and impacts on native biodiversity (Morand et al. 2015), a good

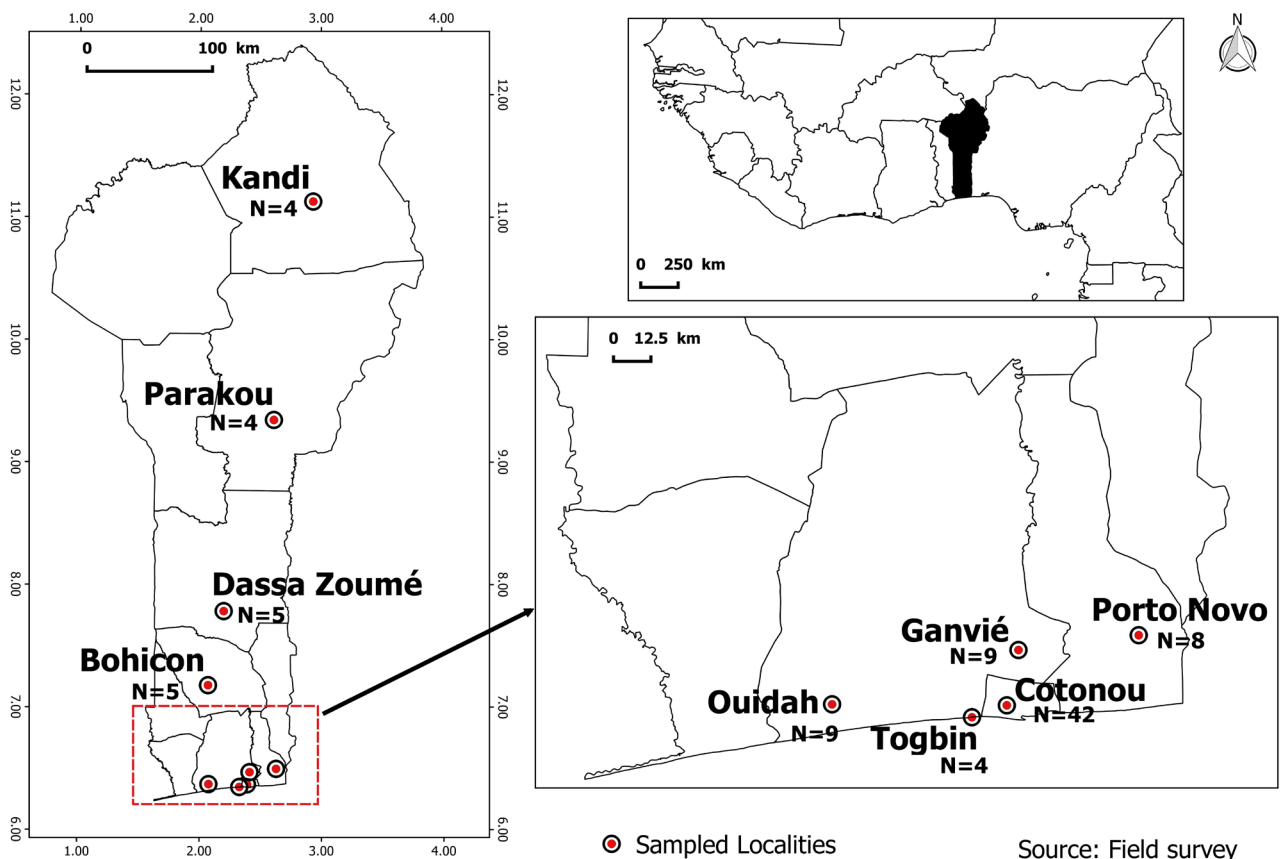


Fig. 1. Geographical location of the sampled localities in Benin.



understanding of their ecology and evolution is a prerequisite for the design of appropriate control strategies.

Benin is a West African country that has always been open to the world through its narrow but easily navigable coastline. From 17th to 19th century, Beninese coasts were heavily involved in the slave trade organized by the French, Portuguese and English (Cornevin 1970, Lovejoy 2017). While goods and people were initially disembarked from ships using canoes, a proper seaport was created in Cotonou, the economic capital of Benin, to allow for direct discharge of goods from the end of the 19th century (Janin 1964). Initially created as a 300 m long wharf in 1899 the International Sea port of Cotonou is now considered a major bulwark of the national economy. Traffic increased from 40,000 tons in 1914 to 272,000 tons in 1962 (Janin 1964). Following the modernisation of the Autonomous Sea port of Cotonou, trade with the rest of the world was estimated at > 6 million tons in the first half of 2019 (<https://www.lanationbenin.info/index.php/economie-2/144-economie/20004>). It also acts as the main import and export site for countries in the Sahelian hinterland (i.e. Mali, Niger and Burkina Faso) with which road connections from Cotonou are important. Since 1949, imports have outstripped exports (Brasseur-Marion 1953, Cockburn et al. 2010). For a long time, maritime trade mainly concerned capital goods (e.g. machinery, cement, vehicles) and foodstuffs (rice, sugar, flour), and was largely with France (75%), Italy and the Netherlands (Janin 1964). Thus, the increase in trade between Benin and the rest of the world, both within and outside Africa, is a major risk factor for the introduction of invasive species into Benin and its hinterland neighbouring countries (Hima et al. 2020).

To date, no genetic diversity and phylogeographic data on black rats are available for Benin while the species is thought to be present in all localities (De Visser et al. 2001, Hima et al. 2020) and abundant in most households in the south of the country (Houémènou et al. 2019, Hima et al. 2020), posing a risk of pathogen transmission to humans (Houémènou 2013, Houémènou et al. 2019, Dobigny et al. 2018, 2019). Therefore, the objective of this study was to fill this gap in our knowledge and to document the history of Benin's colonization by the black rat through the study of its mitochondrial genetic diversity.

Material and Methods

Black rat sampling

Small mammal trapping (rodents and shrews) was carried out in Benin from 2015-2018 (see Hima et al. 2020 for details). Each captured individual received a unique identification number associated with precise GPS coordinates. Captured animals were autopsied and a wide range of tissues were systematically collected and stored in 96° ethanol for subsequent genetic and epidemiological analyses. Among them, 90 *Rattus rattus* specimens from nine localities in Benin (Fig. 1) were used for the present study.

DNA extraction, amplification and cytochrome *b* gene sequencing

Total genomic DNA of each individual was extracted using the Biobasic Extraction Kit (EZ-10 Spin Column Genomic DNA Minipreps Kit, Animal). A 1,140 bp fragment of the mitochondrial region encoding for cytochrome *b* was then amplified by Polymerase Chain Reaction (PCR) using primers L14723 (5'-ACC AAT GAC ATG AAAAAT CAT CGT T-3') and H15915 (5'-TCT CCA TTT CTG GTT TAC AAG AC-3') (Tollenaere et al. 2010, Aplin et al. 2011, Colangelo et al. 2015). The amplification conditions consisted of an initial denaturation phase at 94 °C for 3 min, followed by 40 cycles each of denaturation at 94 °C for 45 s, hybridization at 52 °C for 60 s and elongation at 72 °C for 90 s; then a final elongation phase ran at 72 °C for 10 min. The quality and size of the PCR products were then verified by electrophoresis on 1% agarose and ethidium bromide-supplemented gel. The resulting PCR products containing an amplicon of the expected size (i.e. 1,140 bp) which were purified and sent for sequencing in both directions (@EuroFins MWG, South Korea). Cytochrome *b* sequences from Benin were deposited in GenBank with accession numbers MT294311-MT294400.

Diversity and phylogeographic analyses

The sequences obtained from the 90 Beninese black rats were verified in MEGA X software (Kumar et al. 2018), and then concatenated manually, allowing us to produce 1,127 bp long fragments of the mitochondrial cytochrome *b* gene. Total number of mutations (M), number of haplotypes (N), average number of nucleotide differences (k) (Tajima 1983), nucleotide diversity (π) and haplotypic diversity (Hd), (Nei 1987) were investigated under

Table 1. Number of cytochrome *b* sequences for each country used to generate Fig. 4 with references. Haplotype diversity ($Hd \pm SD$) and nucleotide diversity ($\pi \pm SD$) are also shown for each geographic region.

Geographic regions	Countries	Number of sequences	Haplotypes codes	$Hd \pm SD$	$\pi \pm SD$	Reference of sequences
East Africa	Ethiopia	17	A15, A24, A57, A59	0.746 ± 0.053	0.00534 ± 0.00045	Tollenaere et al. 2010, Tattard (unpublished)
	Kenya	1	A66			Tattard (unpublished)
	Tanzania	11	A55			Tollenaere et al. 2010, Lack et al. 2012, Tattard (unpublished)
North Africa	Egypt	2	A1	0.533 ± 0.180	0.00119 ± 0.00045	Lack et al. 2012
	Tunisia	1	A1			Colangelo et al. 2015
Europe	Italy	7	A1, A20, A21, A72			Colangelo et al. 2015
South Africa	Gabon	2	A1	0.723 ± 0.049	0.00488 ± 0.00042	Tattard (unpublished)
	Malawi	2	A66			Tattard (unpublished)
	Mozambique	27	A16, A14, A28,			Tollenaere et al. 2010, Tattard (unpublished)
	South Africa	23	A1, A6, A43, A44, A46, A56			Bastos et al. 2011, Tollenaere et al. 2010, Lack et al. 2012, Tattard (unpublished)
	Zambia	11	A14,			Tattard (unpublished)
	Zimbabwe	1	A14,			Tattard (unpublished)
West Africa	Benin	90	A1, A2, A3, A4, A5, A6, A7, A8	0.29 ± 0.056	0.00051 ± 0.00014	This study
	Niger	16	A1, A6, A30, A32			Tattard (unpublished)
	Nigeria	6	A1, A29, A30			Tattard (unpublished)
	Senegal	2	A1			Aplin et al. 2011, Tollenaere et al. 2010
North America	USA	137	A1, A6, A23, A26, A27, A32, A33, A34, A35, A36, A40, A41, A42, A45, A47, A49, A56, A63	0.749 ± 0.00062	0.00195 ± 0.00029	Aplin et al. 2011, Lack et al. 2012

Table 1. Continued

Geographic regions	Countries	Number of sequences	Haplotypes codes	Hd ± SD	π ± SD	Reference of sequences
Central America	Costa-Rica	7	A31	0.797 ± 0.00082	0.00211 ± 0.00022	Lack et al. 2012
	Honduras	1	A34			Lack et al. 2012
	West Indies	4	A1, A48			Lack et al. 2012
	Mexico	3	A1			Lack et al. 2012
South America	Nicaragua	11	A25, A34,			Lack et al. 2012
	Argentina	2	A6, A22			Lack et al. 2012
Australia	Indonesia	1	A69,	0.524 ± 0.209	0.00265 ± 0.00160	Lack et al. 2012, Bastos et al. 2011
	New Caledonia	4	A1, A6			Tatard (unpublished)
	New Zealand	2	A1			Aplin et al. 2011, Lack et al. 2012, Bastos et al. 2011, Robins et al. 2007
	India	11	A50, A52, A53, A54, A60, A61	0.964 ± 0.021	0.00577 ± 0.00052	Aplin et al. 2011, Tollenaere et al. 2010, Lack et al. 2012, Pages et al. 2010, Baig et al. 2018
Asia	Japan	3	A1, A6, A23,			Aplin et al. 2011, Lack et al. 2012, Bastos et al. 2011
	Oman	8	A18, A19, A51, A58, A62			Tollenaere et al. 2010, Lack et al. 2012
Indian Ocean islands	Yemen	2	A70, A71			Tollenaere et al. 2010
	Grand-Comore	7	A61, A65, A66, A68	0.863 ± 0.029	0.00594 ± 0.00043	Tollenaere et al. 2010
	Scattered islands	8	A14, A37, A38,			Russell et al. 2011
	Reunion island	5	A1			Tollenaere et al. 2010, Tatard (unpublished)
	Madagascar	20	A1, A10, A13, A14, A17, A67			Aplin et al. 2011, Tollenaere et al. 2010, Lack et al. 2012
Mayotte	10	A9, A11, A12, A14,			Tollenaere et al. 2010, Tatard (unpublished)	
Seychelles		15	A39, A64			Tatard (unpublished)

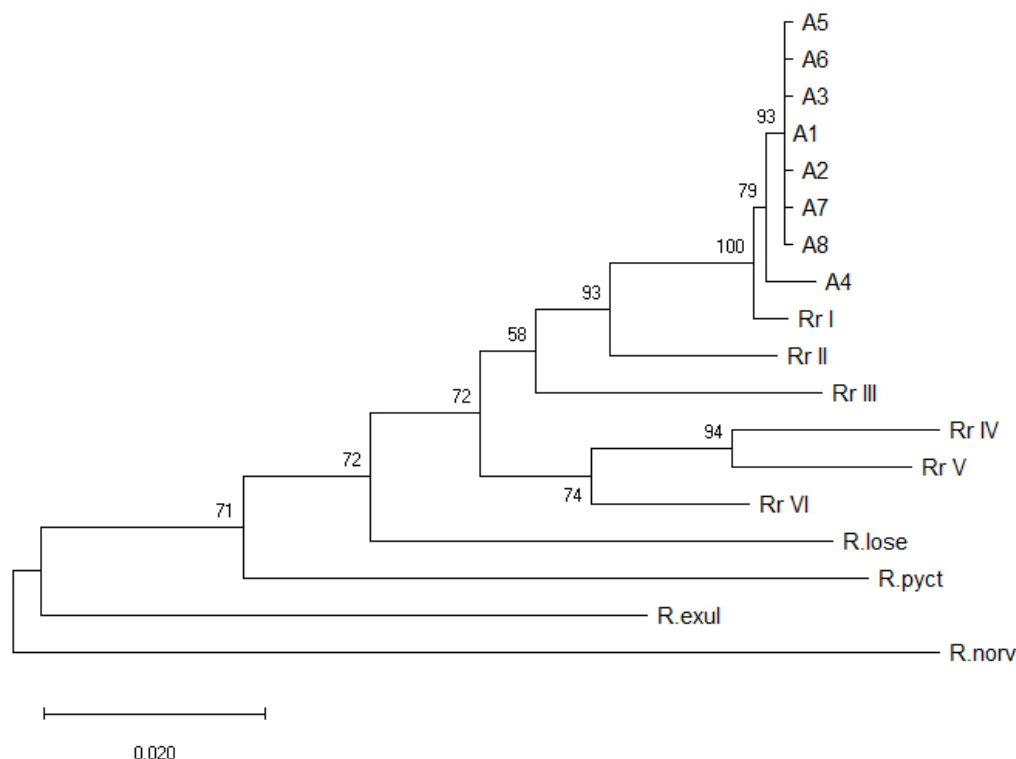


Fig. 2. Phylogenetic tree of Beninese *R. rattus* cytochrome *b* haplotypes constructed by the maximum likelihood method (GTR + G model) with the eight Beninese cytochrome *b* haplotypes, six sequences representing the different *R. rattus* lineages (Rr I to VI) described in Aplin et al. (2011) and *Rattus losea*, *Rattus pyctoris*, *Rattus exulans* and *Rattus norvegicus* used as outgroups. Bootstrap values are indicated above each node. The tree is drawn to scale, branch length is proportional to the number of nucleotide substitutions. A: Haplotype; Rr: *Rattus rattus*, R. lose: *Rattus losea*, R. pyct: *Rattus pyctoris*, R. exul: *Rattus exulans* and R. norv: *Rattus norvegicus*.

DNA_{sp} V5.10 (Librado & Rozas 2009). Neutrality tests of Tajima (Tajima 1989) and Fu and Li (Fu & Li 1993) were conducted to investigate the mutation-drift equilibrium hypothesis which can thus detect selection signatures or deviation from the demographic equilibrium of the population, such as a demographic bottleneck or a population expansion. Significance was tested using 1,000 coalescence simulations under the null hypothesis of an equilibrium. The significance threshold was set *a priori* at 0.05.

Of the 90 Beninese black rats, only eight distinct haplotypes were recovered (see below). These eight Beninese haplotypes were aligned with one representative of each *Rattus rattus* lineage (namely lineages I to VI) as defined by Aplin et al. (2011). In order to identify the phylogeographic lineage of individuals, this dataset was used to reconstruct a phylogenetic tree using the Maximum Likelihood method (Guindon & Gascuel 2003). The evolution model that best matches our data was chosen using AIC (Posada & Buckley 2004), as implemented on the ATCG PhyML platform (Guindon et al. 2010). We used the BioNJ tree as the starting tree, and performed 1,000 bootstrap iterations. The

conspecific rat species *Rattus losea*, *Rattus pyctoris*, *Rattus exulans* and *Rattus norvegicus* were used as outgroups (Pagès et al. 2010).

A local Median-Joining Network for the 90 1,127 bp-long sequences from Benin was reconstructed under Network v.5.0 (Network © Copyright Fluxus Technology Ltd 1999-2018) (Brandelt et al. 2009) in order to explore the genetic affinities within Benin. Secondly, in order to determine the possible geographic origins of invasive black rats of Benin, the 90 Beninese sequences were aligned with 390 other sequences from lineage I sensu

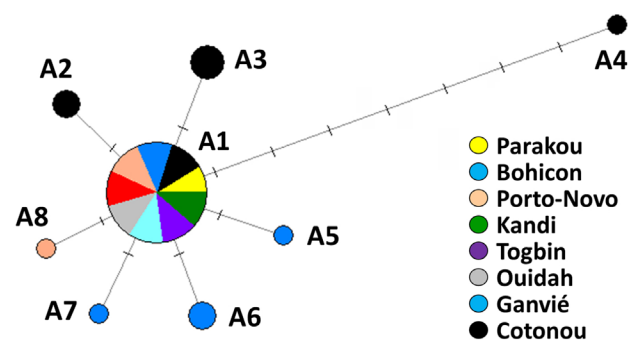


Fig. 3. Median-joining network of Benin *Rattus rattus* haplotypes. A: haplotype.

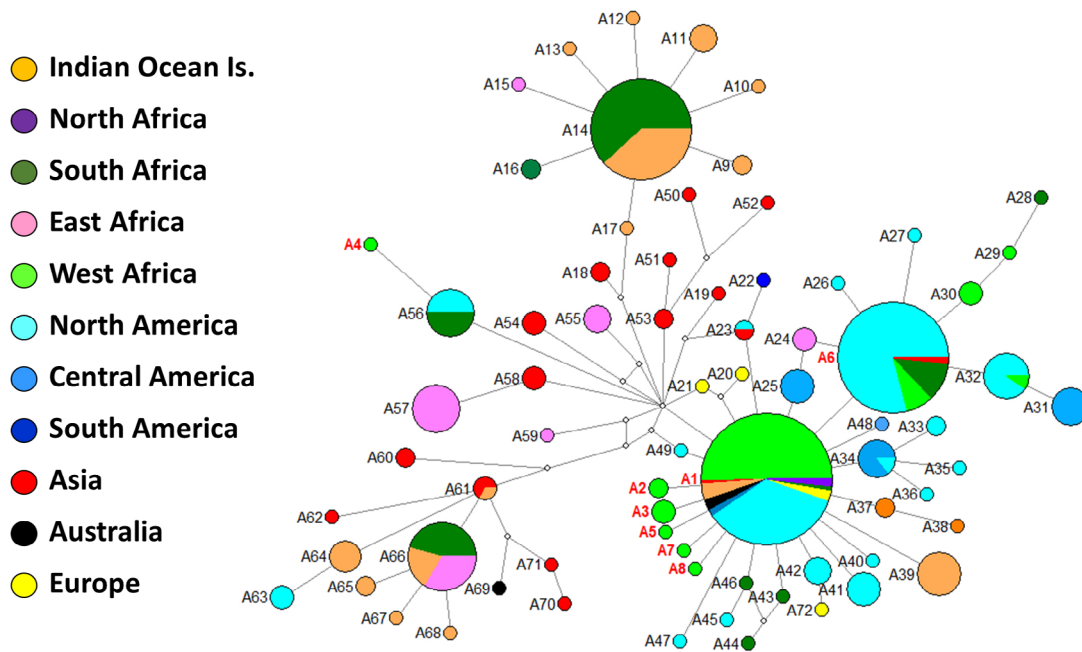


Fig. 4. Global Median-joining network of *R. rattus* lineage I sensu Aplin et al. (2011). This network was built with 480 cytochrome *b* sequences of 969 bp. The eight haplotypes from Benin are in red text. Circles size is proportional to the number of individuals who share a given haplotype. Branches length is not proportional to the number of mutations.

Aplin et al. (2011). These sequences correspond to the cytochrome *b* sequences of black rats described in the literature with at least 969 bp that are available in the GenBank database (Robins et al. 2007, Pagès et al. 2010, Tollenaere et al. 2010, Aplin et al. 2011, Bastos et al. 2011, Lack et al. 2013, Colangelo et al. 2015, Baig et al. 2018, Tatard unpublished; Table 1). Due to the presence of shorter sequences

in the previously published studies, the 90 Beninese sequences (1,127 pb) were truncated and reduced to the 969 bp shared with the 390 non-Beninese available sequences, thus leading to a final matrix of 480 sequences. Using this dataset, a global Median-Joining Network was reconstructed in order to explore genetic affinities between black rats from Benin and those from elsewhere in the world.

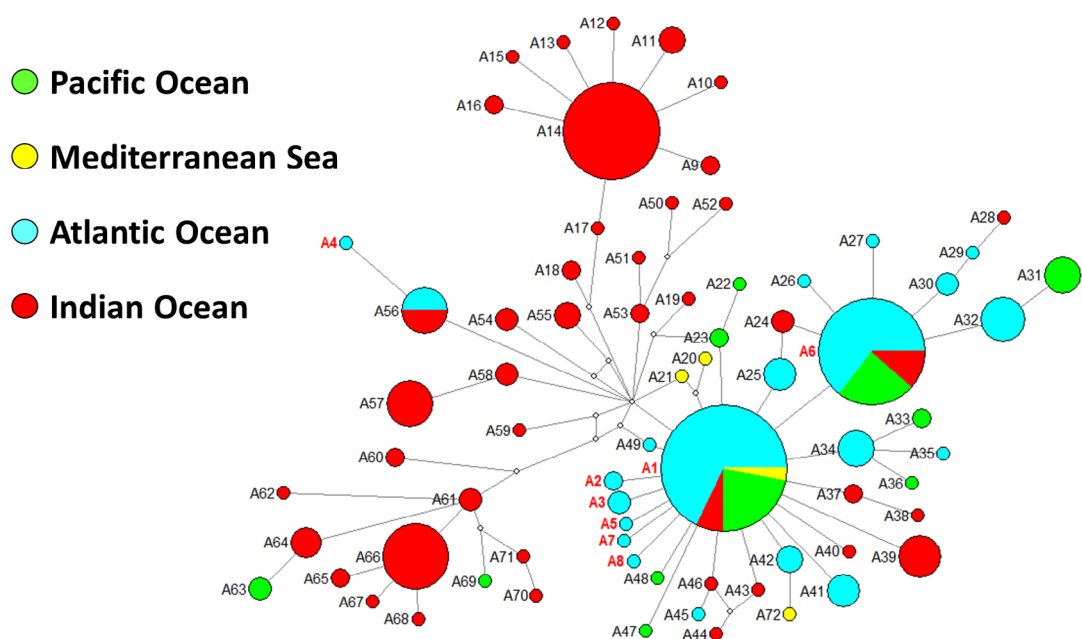


Fig. 5. Global Median-joining network of *R. rattus* and haplotype distribution according to ocean and maritime fronts.



Results and Discussion

Black rats from Benin all belong to lineage I sensu Aplin et al. (2011). Although this result was anticipated, our phylogenetic tree (Fig. 2) unambiguously shows that all eight Beninese haplotypes (see below) belong to the *Rattus rattus* lineage I sensu Aplin et al. (2011), the most cosmopolitan and invasive lineage in the world (Aplin et al. 2011). Their grouping within Lineage I was supported by a maximum bootstrap value (100%). As a consequence, our dataset can be investigated as a whole and confidently compared to other black rats from lineage I.

Genetic diversity of black rats in Benin

Of all 90 black rats from Benin, we identified 13 polymorphic sites defining eight different haplotypes. Haplotypic diversity ($H_d = 0.2295 \pm 0.059$), nucleotide diversity ($\pi = 0.00033 \pm 0.00014$) and average number of nucleotide differences ($k = 0.37528$) all suggest quite low genetic diversity. A low genetic diversity is also observed in most localities recently colonized by black rats around the world, such as the USA (Lack et al. 2013), the western Mediterranean Basin (Colangelo et al. 2015) and south-west of Niger (Berthier et al. 2016). Accordingly, the low genetic diversity across Benin suggests a relatively recent colonization originating from a limited number of individuals. However, alternative explanations such as sampling bias or selective pressure on certain mitochondrial types could also explain the observed pattern. Nevertheless, our individuals came from localities that in some cases are more than 500 km apart (see Fig. 1) and display markedly different environmental characteristics. Therefore, it is reasonable to conclude that the low diversity of *R. rattus* in Benin is explained by historical factors, namely a recent introduction and local evolution from a few individuals only. Fu & Li (1993) neutrality tests ($F^* = -6.806$ and $D^* = -2.33616$) were all negative and significant ($P < 0.05$), which usually characterizes a population expansion pattern, and which is observed in most other areas where *Rattus rattus* has invaded (Tollenaere et al. 2010, Lack et al. 2013, Colangelo et al. 2015) and proliferated rapidly after its introduction.

The local Median-Joining Network shows a clear star-like genetic structure (Fig. 3) with a central major haplotype (A1) that was found in all the sampled localities throughout Benin. This pattern also strongly suggests local evolution with a rapid

demographic expansion from an ancestral pool of individuals that was characterized by a single ancestral haplotype (namely A1). This ancestral haplotype is still found in all the Beninese sampled localities, thus supporting its wide, relatively recent dispersal. Six other haplotypes differ from the ancestral A1 haplotype by one single mutational step (Fig. 3); most likely representing recent derivative variants from A1. In contrast, one specific haplotype retrieved from a single specimen trapped in Cotonou, A4, was seven mutational steps away from A1. Considering the relatively low mutation rate of cytochrome *b* in mammals in general, and rodents in particular (Nabholz et al. 2008), the presence of this rather rare and genetically distant haplotype A4 strongly suggests that some black rats in Benin may have a different origin (temporal and/or spatial) than A1 and its derivatives. Taken together, our data indicate that at least two independent introductions of black rats have occurred. Unfortunately, the low variability of cytochrome *b* in Benin black rats does not provide sufficient resolution to enable us to further explore the history of this species within the country.

Putative origin(s) of black rats from Benin

The global Median-Joining Network (i.e. 90 sequences from Benin and 390 sequences from the literature, Table 1) enabled us to explore the genetic affinities between black rats from Benin and those from elsewhere in the world. In total, 72 different haplotypes were obtained. The most common haplotype A1, first described by Hingston et al. (2005), is widespread on all continents: it occurs in West Africa, North America, Europe, Asia and Australia (Table 1). This haplotype is also the most common haplotype in Benin, which suggests a successful introduction event and subsequent dispersal. Given its cosmopolitan distribution today, the possible geographical origins of this haplotype in Benin are diverse and impossible to trace. However, it is highly probable that its introduction all over the world is associated with a recent and massive dispersal associated with maritime trade that has intensified from the 18th century onwards. Therefore, the arrival of this haplotype in West Africa, particularly in Benin, may have occurred within the last three centuries. It could be related to the later stages of the slave trade (which originated in the 17th century but increased considerably in the 18th and 19th centuries when it gradually gave way to the so-called “illegitimate” trade; Lovejoy 2017). It could also



have been introduced more recently as a result of the further expansion of maritime trade during the 20th century.

The haplotype A6 was identified in Benin and also in Niger, South Africa, Japan and North America, thus showing a wide geographic distribution. It differs from the A1 haplotype by a single nucleotide mutational step, and is thus closely related. As a consequence, it is highly plausible that A6 was also introduced into Benin during the same period as A1. Unfortunately, once again, the genetic variability provided by the cytochrome *b* gene does not allow us to be more precise. Kaleme et al. (2011) suggested that black rats have been introduced into the DRC by two routes: the first through Kinshasa seaport where specimens of *R. rattus* show affinities with European haplotypes, and the second from the east where specimens of *R. rattus* show genetic similarities with Arab and South Asian haplotypes and may suggest introductions through the Arab trade route along the eastern coast of Africa. Similarly, a study of black rats in Senegal showed that the end of the 17th century, particularly when the Saint-Louis seaport was founded, is the likely initial period of black rat introduction into this region of West Africa, probably from European countries (Konečný et al. 2013). The A1 and A6 haplotypes also match these hypotheses for Benin.

Haplotype A4 differs from haplotypes A1 and A6 by seven and eight mutational steps, respectively (Fig. 3). It diverges from its closest relative, haplotype A56 (shared only by specimens from South Africa and North America) by a single mutation, thus pointing towards a putative link between some North American, South African and West African black rats. It is also genetically closer to a panel of haplotypes that are found in the Indian Ocean region and towards the Indian subcontinent. The latter region is known as the area of origin of *R. rattus* (Aplin et al. 2011, Baig et al. 2018) and, consequently, the genetic diversity of lineage I in this region is much greater (Figs. 4, 5 and Table 1). Thus, it is tempting to hypothesize that the introduction of haplotype A4 into Benin could date back to a slightly earlier period when the maritime trade intimately linked Africa and Asia through the so-called Indian Spice Route (Howe 1994). In support of such Asian/African translocations, Tollenaere et al. (2010) and Prendergast et al. (2017) have also shown that the

first arrivals of black rats in Africa were made from Asia to South African coasts following the Arab trade route. Genetic diversity indices (Table 1) also appear to confirm this pattern: they decrease from Asia to the Indian ocean area, East, South and finally West Africa. The sharing of haplotype A56 with North American individuals could then be explained by two different hypotheses: either (i) the introduction into Benin took place when the Indian Route was operating (i.e. between the 16th and 17th centuries) with the triangular slave trade involving Europe, Africa and America, or (ii) the same haplotype was independently introduced into North America at a later date.

The representation of haplotype distribution in accordance with oceanic and maritime fronts (Fig. 5) fits the hypothesis for at least two waves of colonization of Benin by black rats. According to this hypothesis, maritime exchanges with the Indian Ocean and Indian subcontinent would have contributed to the first introduction event (now represented by haplotype A4) some four to five centuries ago, while the other Beninese haplotypes (A6, A1 and their derivatives) would have been introduced more recently following increased maritime trade over the three last centuries. Additional analyses are required to test this hypothesis and, if confirmed, to explain why the older introduction event was not followed by a massive demographic expansion in Benin, since A4 was only represented by a single individual within our sample.

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