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1 **Diversity of *Toxoplasma gondii* strains shaped by commensal communities of small**  
2 **mammals**

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26

27 **Abstract**

28 Commensal rodent species are key reservoirs for *Toxoplasma gondii* in the domestic  
29 environment. In rodents, different *T. gondii* strains show variable patterns of virulence  
30 according to host species. *Toxoplasma gondii* strains causing non-lethal chronic infections in  
31 local hosts will be more likely to persist in a given environment, but few studies have  
32 addressed the possible role of these interactions in shaping the *T. gondii* population structure.  
33 In addition, the absence of validated techniques for upstream detection of *T. gondii* chronic  
34 infection in wild rodents hinders exploration of this issue under natural conditions. In this  
35 study, we took advantage of an extensive survey of commensal small mammals in three  
36 coastal localities of Senegal, with a species assemblage constituted of both native African  
37 species and invasive species. We tested 828 individuals for *T. gondii* chronic infection using  
38 the modified agglutination test (MAT) for antibody detection in serum samples and a  
39 quantitative PCR assay for detection of *T. gondii* DNA in brain samples. The infecting *T.*  
40 *gondii* strains were genotyped whenever possible by the analysis of 15 microsatellite markers.  
41 We found (i) a very poor concordance between molecular detection and serology in the  
42 invasive house mouse, (ii) significantly different levels of prevalence by species and (iii) the  
43 autochthonous *T. gondii* Africa 1 lineage strains, which are lethal for laboratory mice, only in  
44 the native African species of commensal small mammals. Overall, this study highlights the  
45 need to reconsider the use of MAT serology in natural populations of house mice and  
46 provides the first known data about *T. gondii* genetic diversity in invasive and native species  
47 of small mammals from Africa. In light of these results, we discuss the role of invasive and  
48 native species, with their variable adaptations to different *T. gondii* strains, in shaping the  
49 spatial structure of *T. gondii* genetic diversity in Africa.

50 *Keywords:* *Toxoplasma gondii*, *Hammondia*, *Neospora caninum*, Genetic diversity, Rodents,  
51 West Africa

52

## 53 1. Introduction

54 Toxoplasmosis is a ubiquitous parasitic zoonosis, caused by the obligate intracellular  
55 protozoan parasite *Toxoplasma gondii*. Felids are the definitive hosts, while all other warm-  
56 blooded animals are intermediate hosts for this parasite. Birds and mammals, including  
57 humans, develop dormant tissue cysts after ingestion of oocysts shed in the environment by  
58 cats in the form of contaminated feces. Another source of infection for human and other meat-  
59 consuming species is raw or undercooked meat from animals harboring infective tissue cysts.  
60 In the domestic environment, rodents are believed to be the most important intermediate hosts  
61 in the *T. gondii* cycle (Dubey et al., 1995b; Hejlíček et al., 1997) as they are usually the main  
62 prey species of domestic cats (Langham, 1990; Molsher et al., 1999; Turner and Bateson,  
63 2013). However, while certain rodent species demonstrate resistance to a number of more or  
64 less virulent strains and develop lifelong chronic infection with *T. gondii* (Lilue et al., 2013;  
65 Dubey et al., 2016), other rodent species show a high susceptibility to most *T. gondii* strains  
66 and rapidly die from acute toxoplasmosis (Fujii et al., 1983; Jokelainen and Nylund, 2012).  
67 Hence, the patterns of genetic resistance specific to the local rodent species will determine *T.*  
68 *gondii* strain transmission in a given environment, and this mechanism might strongly shape  
69 the different *T. gondii* population structures observed around the world (Khan et al., 2009;  
70 Lilue et al., 2013). Strains that are able to persistently infect local rodent species according to  
71 their respective resistance patterns may represent, at least partially, those that are involved in  
72 local transmission to cats, contamination of the environment and infection of humans (Lilue et  
73 al., 2013). In Africa, which was the focus of our study, human infection mainly occurs from  
74 local sources, often due to contact with contaminated soil (Duong et al., 1992; Adou-Bryn et  
75 al., 2004; Uneke et al., 2007; Abu et al., 2015). Therefore, assessing *T. gondii* circulation  
76 among rodents and characterizing the strains causing chronic infections in these intermediate  
77 hosts could be of importance in order to determine which species are the local reservoirs of

78 potentially pathogenic strains. Screening of rodents for *T. gondii* is useful to estimate the  
79 prevalence of *T. gondii* infection, but also to identify the chronically infected individuals  
80 before performing bioassays and isolating the infecting strains or to directly genotype those.  
81 Serological screening has been widely used in rodents, mainly within the framework of  
82 prevalence studies. However, serological techniques have never been validated by a ‘gold  
83 standard’ technique, which brings into question their reliability in identifying infected  
84 individuals (summarized by Afonso et al., 2007; Dabritz et al., 2008; Mercier et al., 2013).  
85 The most commonly used serological test is the modified agglutination test (MAT). However,  
86 this test does not give reliable results for all species (Aroussi et al., 2015).

87         In the present study, we took advantage of an extensive survey of commensal small  
88 mammals in three coastal urban settlements of Senegal. Species assemblages of small  
89 mammals in those regions were composed of native African species (the giant pouch rat,  
90 *Cricetomys gambianus* and the shrew, *Crocidura olivieri*) and of invasive species (the house  
91 mouse, *Mus musculus domesticus* and the black rat, *Rattus rattus*) that were introduced during  
92 colonial times. Given their distinct origins, invasive and native species have probably been  
93 exposed to different *T. gondii* strains during their evolutionary history. In addition, the  
94 probable differences in the history of establishment of the domestic cat based on region, in  
95 addition to the geographical variability in climate (which influences the viability of oocysts in  
96 the environment), have probably exposed these intermediate hosts to varying levels of  
97 environmental contamination by *T. gondii*. These putative differences in the evolutionary  
98 history of invasive and African native small mammals may have led to distinct host-parasite  
99 co-adaptations. Hence, we hypothesize that invasive and native species would exhibit  
100 different patterns of immune responses to *T. gondii* infection associated with different innate  
101 susceptibilities to various *T. gondii* strains.

102           The sampling conducted during trapping sessions allowed the collection of sera and  
103 brain samples from four species of small mammals sampled in these three regions. As a first  
104 step, we compared antibody detection using MAT serology with the detection of *T. gondii*  
105 DNA in brain samples using a quantitative PCR (qPCR) assay for each small mammal  
106 species. In addition, we considered the differences in *T. gondii* prevalence levels between  
107 each species based on the bioecological traits that characterize them. Finally, we genotyped  
108 strains from infected individuals. Identifying different strains in invasive and native African  
109 species of small mammals would support the hypothesis that different host species might be  
110 reservoirs for distinct *T. gondii* strains. The expected results of this study would be that rodent  
111 invasions are key events in shaping the *T. gondii* population structure in a given area.

112

## 113   **2. Materials and methods**

114

### 115   *2.1. Small mammal sampling*

116           Fieldwork was carried out under the framework agreements established between the  
117 Institut de Recherche pour le Développement (France), and the Republic of Senegal, as well  
118 as with the Senegalese Head Office of Waters and Forests, and the Ministry of Health and  
119 Social Action, Senegal. Handling procedures were performed under our laboratory agreement  
120 in relation to experiments on wild animals (no. D-34-169-1), and follow the official guidelines  
121 of the American Society of Mammalogists (Sikes and Gannon, 2011). Trapping campaigns  
122 within districts were systematically performed with prior explicit agreement from relevant  
123 local authorities. Small mammals were sampled by live trapping in 12 districts of the city of  
124 Dakar, Senegal, in the international port of Dakar and on Goree Island, Senegal, in four  
125 districts in Joal-Fadiouth, Senegal, and two districts in Rufisque, Senegal, according to a  
126 standardized protocol described by Dalecky et al. (2015). The primary aim of the sampling

127 was to describe small mammal communities in these urban areas and the population genetic  
128 structure of the dominant species. In each district, we conducted one live trapping session of  
129 two to five consecutive days between 2016 and 2017, on a median surface area of 0.04 km<sup>2</sup>  
130 (min: 0.01, max: 1.51). In general, two traps (one wire mesh trap and one Sherman trap) were  
131 set per room or courtyard in buildings corresponding to dwelling houses, boutiques,  
132 workshops, offices or warehouses, and whose locations were precisely recorded with a GPS  
133 device. Small mammals were brought back to our laboratory and euthanized by cervical  
134 dislocation before autopsy. For this study, blood samples were collected from the heart or  
135 from a blood clot within the thoracic cavity, and then centrifuged to separate the serum. The  
136 brain of each rodent was also collected and weighted. In total 828 small mammals were  
137 included in the present screening for *T. gondii* infection within the three study zones: 671 *M.*  
138 *m. domesticus*, 78 *R. rattus*, 47 *C. gambianus*, and 32 *C. olivieri* (shrews) (Fig. 1).

139

## 140 2.2. Seroprevalence, molecular prevalence and comparison of MAT serology with qPCR

141

### 142 2.2.1. Serological examination

143 Sera were transferred to microtubes and stored at -20 °C until used for serological  
144 analyses. Sera of rodents and shrews were screened for *T. gondii*-specific IgG antibodies  
145 (Dubey and Desmonts, 1987) following a slightly modified previously published protocol  
146 (Bolais et al., 2017) using four serial dilutions (1:20, 1:40, 1:100 and 1:800) of the serum. The  
147 antigen consisting of formalin-fixed RH strain *T. gondii* tachyzoites was provided by the  
148 Laboratory of Parasitology, Centre Hospitalier Universitaire de Reims, Reims, France.

149

### 150 2.2.2. *Toxoplasma gondii* DNA detection

151 After being rinsed in physiological serum (NaCl 0.9%), brains were placed in 1 ml of  
152 physiological serum, and extruded through a 5 cm 23 gauge needle several times to yield a  
153 liquid tissue homogenate. Given that the qPCR method used is able to detect *T. gondii* DNA  
154 extracted from a single cyst, homogenization was performed to optimize *T. gondii* DNA  
155 detection in the case of a non-random tissue cyst distribution in the brain. In highly resistant  
156 *T. gondii* hosts such as rats, the numbers of cysts in the brains of infected individuals is  
157 generally in the order of tens to thousands (Dubey, 1996; Freyre et al., 2001, 2003). In our  
158 case, DNA was extracted from 200 µl of liquid brain homogenate following the protocol  
159 designed for biological fluid processing using a commercial kit (Qiagen QIAamp DNA Mini  
160 Kit, Courtaboeuf, France), as recommended by the manufacturer. This proportion represents  
161 15% to 20% of the total brain volume for most of the samples and makes it likely to detect *T.*  
162 *gondii* DNA in brains with very low tissue cyst numbers. We calculated that there was a  
163 probability of 90% that at least one cyst should occur in the 200 µl of liquid homogenate if the  
164 whole brain contained 15 cysts or more (data not shown). The extraction products were tested  
165 by a qPCR assay as described by Ajzenberg et al. (2016) on a thermocycler Rotor-Gene 6000  
166 (Corbett Life Science, Sydney, Australia), targeting the 529 bp repeat region (REP529,  
167 GenBank accession no. **AF146527**) of *T. gondii* DNA (Homan et al., 2000).

168 In brief, each PCR contained 5 µl of extracted DNA, mixed with 15 µl of a PCR mix  
169 with 1X LightCycler FastStart DNA Master Hybridization Probes kit (Roche diagnostics,  
170 Mannheim, Germany), 0.5 U of UDG (Roche Diagnostics, Mannheim, Germany), 5 mmol/L  
171 of MgCl<sub>2</sub>, 0.5 µmol/L of each primer, 0.1 µmol/L of TaqMan probe (Eurofins, Ebersberg,  
172 Germany) which is labeled with a fluorescent dye (6-carboxyfluorescein, 6-FAM) at 5' end  
173 and a dark quencher (Black Hole Quencher, BHQ1) at the 3' end. The cycling protocol was as  
174 follows: initial decontamination by UDG at 50 °C for 2 min and denaturation at 95 °C for 10  
175 min, followed by 50 cycles at 95 °C for 20 s and 60 °C for 40 s. The results obtained were



176 expressed in cycle threshold ( $C_t$ ) values. Each sample was run in duplicate and extraction  
177 products that were positive in at least one test were considered positive.

178

### 179 2.2.3. Assessment of the specificity of MAT and qPCR results

180 To assess whether positive results obtained by MAT serology could have been caused  
181 by infections with the *T. gondii*-related species *Hammondia hammondi*, *Hammondia heydorni*  
182 or *Neospora caninum*, all seropositive individuals were tested by three different PCR assays  
183 on brain DNA extracts using primers specifically targeting each of those three organisms.  
184 Also, the occurrence of possible cross-reactions between the primers targeting the  
185 *Toxoplasma* 529 bp repeat region and *H. hammondi* or *H. heydorni* was verified by  
186 performing two different PCR assays on each positive brain sample for *T. gondii* using the  
187 primers specifically targeting *H. hammondi* and *H. heydorni*.

188 For the detection of *H. hammondi*, the primer pair Hham34F/Hham3R was used  
189 (Schaes et al., 2008). Briefly, PCR was carried out in a 25  $\mu$ l reaction mixture consisting of 1  
190 U/25  $\mu$ l of Dynazyme II F-501L DNA polymerase (Finzyme, Espoo, Finland), 250  $\mu$ M of  
191 each of the dNTPs (Amersham Biosciences, Piscataway, USA), 0.5  $\mu$ M of each primer and 2  
192  $\mu$ l of DNA. The thermal cycling protocol followed included an initial denaturation at 94°C for  
193 5 min, followed by 35 cycles of 60°C for 1 min, 72°C for 1 min and 94°C for 1 min. The PCR  
194 ended with incubation at 60°C for 1 min and a final extension at 72°C for 10 min. The  
195 amplification products were resolved in 1.5% agarose gels using a 100 bp DNA ladder  
196 (Invitrogen, Carlsbad, USA) as the reference.

197 For the detection of *H. heydorni*, the primer pair JS4/JS5 (Slapeta et al., 2002) was  
198 used. In brief, a final reaction volume of 25  $\mu$ l was applied, composed of 1 U/25  $\mu$ l of  
199 Dynazyme II F-501L DNA polymerase (Finzyme, Espoo, Finland), 250  $\mu$ M of each of the  
200 dNTPs (Amersham Biosciences, Piscataway, USA), 0.5  $\mu$ M of each primer and 2  $\mu$ l of DNA.

201 Cycling conditions were: 5 min at 94.8 °C (initial denaturation); 1 min at 56 °C (including a  
202 0.5 °C decrement per cycle after the first), 1 min at 72 °C, and 1 min at 94 °C (10 cycles); 1  
203 min at 51 °C, 1 min at 72 °C, and 1 min at 94 °C (40 cycles); and 5 min at 72 °C (final  
204 elongation). The amplicons were analysed in 1.5% agarose gels using a 100 bp DNA ladder  
205 (Invitrogen, Carlsbad, USA) as the reference.

206 For the detection of *N. caninum*, DNA was analysed by a previously published qPCR  
207 targeting the Nc-5 gene by using the primer pair NeoF/NeoR and the probe Neo-probe as  
208 described previously (Constantin et al., 2011; Legnani et al., 2016). Briefly, qPCRs were  
209 performed on a CFX96 instrument (Bio-Rad Laboratories, Cary, USA) in a final volume of 20  
210 µl, using a commercial master mix (iQ supermix, Bio-Rad Laboratories GmbH, Munich,  
211 Germany). qPCR primers (800 nM) and a probe (200 nM) were employed as reported  
212 (Legnani et al., 2016). The cycling conditions were 95 °C for 5 min, followed by 46  
213 amplification cycles of 95 °C for 10 s and 58 °C for 30 s as described. After each cycle the  
214 light emission by the fluorophore was measured. qPCR results were analysed using CFX  
215 manager software Version 1.6 (Bio-Rad Laboratories).

216

#### 217 2.2.4. Prevalence of *T. gondii* infection

218 *Toxoplasma gondii* infection prevalence levels were defined on the basis of molecular  
219 detection of *T. gondii* using qPCR and on the results of MAT serology (positive cut-off at  
220 1:20 dilution titer). The occurrence of a species effect on the prevalence levels was tested by  
221 Fisher's exact test, adopting a 95% confidence interval (CI).

222

#### 223 2.2.5. Level of agreement between tests

224 For each species of rodent or shrew, a Kappa coefficient calculation was performed  
225 online (<http://graphpad.com/quickcalcs/kappa2/>) and used to estimate agreement between the

226 two tests for the detection of *T. gondii* infection. The Kappa coefficient values (k) were  
227 classified according to the benchmark scale (Landis and Koch, 1977). The extent of  
228 agreement was qualified as very poor ( $0 < k < 0.20$ ), poor ( $0.20 < k < 0.40$ ), moderate ( $0.40 <$   
229  $k < 0.60$ ), good ( $0.60 < k < 0.80$ ), and almost perfect ( $0.80 < k < 1$ ) depending on the  
230 magnitude of Kappa. A Kappa value below 0 was qualified as being a disagreement.

231

### 232 2.3. Genotyping of *T. gondii* strains and neighbor-joining clustering

233 To characterize the infecting *T. gondii* strains in the different species of rodents and  
234 shrews, *T. gondii*-positive DNA samples with a  $C_t$  value  $\leq 32$  by qPCR were genotyped using  
235 the polymorphism of 15 microsatellite markers located on 11 different chromosomes in a  
236 multiplex PCR assay described elsewhere (Ajzenberg et al., 2010).

237 Reference strains representing the 16 *T. gondii* haplogroups (HGs) described to date  
238 (Su et al., 2012; Lorenzi et al., 2016) were used for comparison with the new small mammal  
239 isolates: GT1 (HG1), ME49 (HG2), VEG (HG3), MAS (HG4), RUB (HG5), FOU (HG6),  
240 CAST (HG7), TgCtBr5 (HG8), P89 (HG9), VAND (HG10), COUG (HG11), ARI (HG12),  
241 TgCtPRC04 (HG13), TgA105004 (HG14), TgCtCo5 (HG15) and CASTELLS (HG16). In  
242 addition, a number of field isolates from Algeria (Yekkour et al., 2017), Ethiopia  
243 (Gebremedhin et al., 2014), and Gabon (Mercier et al., 2010) were included for comparison  
244 with other African strains.

245 To evaluate the extent of genetic distance among Senegalese strains from our sample  
246 and evaluate their positions towards the reference strains mentioned above, an unrooted  
247 Neighbor-joining tree was reconstructed from microsatellite data with Populations 1.2.32  
248 (<http://bioinformatics.org/populations/>) based on Cavalli-Sforza and Edwards chord distance  
249 estimator (Cavalli-Sforza and Edwards, 1967) and generated with MEGA 6.05  
250 (<http://www.megasoftware.net/history.php>).

251

### 252 3. Results

253

#### 254 3.1. Different *T. gondii* seroprevalence and molecular prevalence levels according to small 255 mammal host species

256 We detected no DNA from *H. hammondi*, *H. heydorni* or *N. caninum* in the brains of  
257 any seropositive individuals by MAT ( $n=61$ ). In brains positive for *T. gondii* by qPCR  
258 ( $n=110$ ), only two gave positive PCR results for *H. heydorni* (Supplementary Table S1;  
259 available at Mendeley Data via <http://dx.doi.org/10.17632/m4dcd7f8h5.1>).

260 Estimates of *T. gondii* prevalence varied from 2.6% to 37.5% by MAT serology and  
261 from 3.8% to 27.7% by qPCR, depending on the species of small mammals (Table 1).  
262 Seroprevalence and molecular prevalence levels were both significantly different between  
263 species ( $P<0.001$  and  $P<0.01$ , respectively).

264

#### 265 3.2. Different levels of agreement between MAT and qPCR in detecting *T. gondii* infection 266 according to small mammal host species

267 For *M. m. domesticus*, the larger sample size ( $n=671$ ) allowed a robust comparison of  
268 the results with both techniques (Table 1). From the 113 individuals positive by at least one of  
269 the two techniques, 24 individuals were MAT-positive and qPCR-negative, 81 were MAT-  
270 negative and qPCR-positive and only eight were positive with both techniques. Accordingly,  
271 MAT and qPCR results demonstrated very poor agreement with a Kappa of 0.067 (95% CI: -  
272 0.017; 0.150).

273 For *R. rattus*, a limited sample size ( $n=78$ ) and a low prevalence did not allow a robust  
274 estimation of the concordance between MAT and qPCR results. However, a noteworthy  
275 observation is that the only two rats with MAT titers  $\geq 20$  were negative using qPCR and the

276 only three qPCR-positive individuals had MAT titers < 20. This observation was confirmed  
277 by the disagreement between the results of the two techniques shown by a kappa coefficient  
278 calculation of -0.032 (95% CI: -0.062; -0.002).

279 For *C. gambianus*, the Kappa coefficient value showed a good agreement between  
280 both tests (0.695 (95% CI: 0.471; 0.920)), although it lacked accuracy as its confidence  
281 interval ranged from moderate to almost perfect agreement categories.

282 For *C. olivieri*, the Kappa coefficient value (0.472 (95% CI: 0.178; 0.765)) lacked  
283 accuracy and was considered non-informative due to the limited size of the available sample.  
284 All the PCR-positive individuals from this species ( $n=5$ ) were seropositive. However, seven  
285 individuals positive by MAT that were PCR-negative were also observed.

286

### 287 3.3. Identification of the *T. gondii* lineage Africa 1 only in native African small mammal host 288 species

289 Most of the small mammals detected as infected by qPCR displayed a burden of *T.*  
290 *gondii* in their brains that was too low to allow genotyping (Supplementary Fig. S1). Out of  
291 the 110 brain samples which were qPCR-positive for *T. gondii*, genotyping with 15  
292 microsatellite markers was attempted for only 16 DNA samples (19.4%) which had a  $C_t$  value  
293  $\leq 32$ . In total, 11 DNA samples were successfully amplified for all 15 microsatellites, one  
294 DNA sample was successfully amplified for 11 microsatellites and four DNA samples were  
295 not amplified at all (Supplementary Table S2). The proportion of the successfully genotyped  
296 samples among the qPCR positive samples varied between the different species. Among the  
297 12 obtained genotypes, none was from the three qPCR-positive *R. rattus*, six genotypes were  
298 from the 89 qPCR-positive *M. m. domesticus* (6.7%), one genotype was from the five qPCR-  
299 positive *C. olivieri* (20%) and five genotypes were from the 13 qPCR-positive *C. gambianus*  
300 (38.5%). The neighbor-joining tree (Fig. 2) showed that 11 out of 12 genotypes could be

301 clustered in three main groups: a group clustering type II strains (6/12), a group clustering  
302 Type III strains (3/12), and a group clustering *Africa I* strains (2/12). In addition, one atypical  
303 genotype clustered with the genotype of an African strain, TgCkGh01, from Ghana. The six  
304 successfully genotyped strains infecting *M. m. domesticus* clustered only with type II (4/6)  
305 and type III (2/6) strains whereas the five genotyped strains infecting *C. gambianus* clustered  
306 with the three main groups and with the Ghanaian TgCkGh01 strain. The unique strain  
307 characterized in *C. olivieri* clustered with Africa 1 strains. No mixed strain infection was  
308 found.

309         Among those 12 genotyped individuals, 10 were seropositive and showed high  
310 antibody titers (seropositive at 1:100 and 1:800 dilutions titers). The only two strains from  
311 seronegative individuals were found in *M. m. domesticus*.

312

### 313 3.4. Data accessibility

314         Supplementary Table S1 is available at Mendeley Data via  
315 <http://dx.doi.org/10.17632/m4dcd7f8h5.1>.

316

317

## 318 4. Discussion

319         Testing the hypothesis of different adaptation patterns for *T. gondii* strains according  
320 to small mammal host species under natural conditions implies dealing with several obstacles.  
321 The levels of prevalence which are often low in this category of intermediate hosts (Gotteland  
322 et al., 2014) and the absence of validated techniques for detection of chronic *T. gondii* carriers  
323 among natural populations of rodents make it difficult to isolate the parasite or its DNA in  
324 sufficient amounts for genotyping purposes. Here, we show that the agreement level between  
325 MAT serology and qPCR results varies substantially according to species. We found a poor

326 agreement between MAT serology and qPCR results in *M. m. domesticus*, with a high  
327 proportion of PCR-positive individuals not detected by MAT serology. The same result was  
328 found in *R. rattus*, although a larger sampling is required to draw a robust conclusion. In  
329 contrast, the good agreement between the two tests in *C. gambianus* suggests that MAT  
330 serology can be useful for the detection of *T. gondii* infection in this species, but needs  
331 confirmation using a larger sample. For *C. olivieri*, the limited number of tested individuals  
332 did not allow a clear conclusion to be drawn about the concordance between the two tests in  
333 relation to this species.

334         In *M. m. domesticus*, the large proportion of qPCR-positive individuals found negative  
335 by MAT serology indicates that MAT serology is not a reliable test to detect chronic infection  
336 among natural populations of this species. This is not consistent with results obtained in  
337 conventional laboratory strains of mice (Dubey et al., 1995a; Owen and Trees, 1998), and  
338 emphasizes the need to validate MAT serological tests in natural rodent populations. Our  
339 results, based on extensive sampling, confirm previous observations of true infection in wild  
340 mice found to be negative by MAT serology (Dubey et al., 1995b; Owen and Trees, 1998;  
341 Araújo et al., 2010; Gotteland et al., 2014). In the present study, to further confirm that PCR-  
342 positive individuals were infected by *T. gondii*, we took into account the possible weak cross-  
343 reactivity of the primers targeting the *Toxoplasma* 529 bp repeat region with *Hammondia*  
344 DNA as was reported in a previous study (Schaes et al., 2008). Only two of the *T. gondii*  
345 qPCR-positive individuals (n=110) were found to be positive for *H. heydorni*. We could not  
346 conclude whether only *H. heydorni* DNA was occurring in brain extracts of these two  
347 individuals or whether both *T. gondii* and *H. heydorni* DNA were present, but this result  
348 confirms the good specificity of the qPCR in detecting *T. gondii* DNA in our sample. High  
349 proportions of chronically infected individuals with undetectable levels of anti-*T. gondii*  
350 antibodies cannot be solely explained by cases of recent infections in which humoral

351 responses may not have developed yet. A number of studies have noticed that the titer of  
352 antibodies in sera of infected hosts may more or less strongly correlate with the parasitic  
353 burden in tissues (Opsteegh et al., 2010; Singh et al., 2010). Most of the infected individuals  
354 exhibited very low concentrations of *T. gondii* DNA in their brains, which may have led to  
355 low antibody titers in sera that were undetectable by MAT serology. Also, Beverley (1959)  
356 demonstrated that congenitally infected outbred mice did not develop levels of antibodies that  
357 are detectable by serology, an observation later confirmed by Jacobs (1966;  
358 <https://doi.org/10.1016/B978-1-4832-2913-3.50154-4>). Furthermore, experimental co-  
359 infections of laboratory mice by *T. gondii* and other parasitic microorganisms showed the  
360 occurrence of complex interactions between the different host immune pathways for the  
361 control of these unrelated parasitic infections (Welter et al., 2006; Khan et al., 2008). Unlike  
362 laboratory rodents, natural populations of rodents are reservoirs of important parasitic  
363 populations (Brouat et al., 2007; Diagne et al., 2017). The occurrence of complex interactions  
364 between the host immune response pathways for dealing with this diversity of parasitic  
365 species could be reasonably expected. Notably, *T. gondii* was shown to inhibit the antigen-  
366 specific Th2 immune responses against a number of parasitic species (Santiago et al., 1999;  
367 Liesenfeld et al., 2004; Miller et al., 2009; Ahmed et al., 2017) Although no study has shown  
368 the reverse, inhibitions of immune responses related to coinfections that may be common in  
369 natural populations of rodents could work against serological detection.

370 In addition, variable proportions of seropositive individuals that were negative using  
371 qPCR were noted in our sample for all four studied species. Serological cross-reactions  
372 between the *T. gondii* antigen used in MAT serology and antibodies against the cyst-forming  
373 coccidians *H. hammondi*, *H. heydorni* and *N. caninum* (summarized by Gondim et al., 2017)  
374 are unlikely, because PCR assays for the detection of these organisms in the brain extracts of  
375 seropositive individuals yielded negative results in all individuals. However, *Hammondia* spp.



376 exhibit lower tropism for the brain compared with the muscles (Frenkel and Dubey, 1975) and  
377 checking for *Hammondia* DNA in muscles may have led to slightly different conclusions.  
378 Unfortunately, no muscle tissues could be tested in this study. Another explanation could be  
379 the occurrence of unspecific agglutination reactions, which is a recurring problem in  
380 agglutination tests (Dubey et al., 1985; Weinberg and Storch, 1985; Becker et al., 2007;  
381 Schares et al., 2018). This phenomenon may be caused by the high protein concentrations that  
382 could be found in some samples (Le Potier et al., 1998; Villena et al., 2012). It is noteworthy  
383 that the proportion of MAT-positive/qPCR-negative individuals in our sample was lower in  
384 the higher categories of dilution titers, which could be attributed to the decrease in protein  
385 concentrations by increasing the dilution of tested serum samples. Finally, the occurrence of  
386 truly infected individuals that go undetected by PCR cannot be fully excluded. If an infected  
387 seropositive animal has a low tissue burden and an inhomogeneous cyst distribution in its  
388 tissues, the tissue sample collected for the DNA extraction could be free of *T. gondii* cysts  
389 (Opsteegh et al., 2011; Aroussi et al., 2015). In most species, neural and muscular tissues  
390 usually harbor the highest burden of tissue cysts, with the brain being the preferred site of *T.*  
391 *gondii* in mice (Dubey, 2009). This latter statement needs to be confirmed in other species of  
392 rodents. Dubey et al. (2016) have summarized the results from studies investigating the  
393 distribution of tissue cysts in the brains of various laboratory lineages of rats and mice. The  
394 majority of the studies reported a non-random distribution of tissue cysts across the different  
395 brain areas, although it was not obvious. Even though precautions were taken to optimize  
396 detection (see section 2.2.2.), we cannot fully exclude the occurrence of a negative qPCR  
397 result in infected brains in which the cyst count was extremely low. To our knowledge, such  
398 low numbers of cysts (< 10 cysts per brain) have never been reported in brains of rodents.  
399 This issue could be of key importance in the *T. gondii* life cycle as a single viable bradyzoite  
400 is enough for cat infection (Dubey, 2006). Altogether, this discussion has emphasized the

401 clear limitations of MAT serology in detecting chronic *T. gondii* infection compared with  
402 qPCR in natural populations of *M. m. domesticus*, but defining the latter as a ‘gold standard’  
403 test for the detection of *T. gondii* in chronically infected rodents requires further study.

404 We found statistically significant differences in prevalence levels between the four  
405 species studied here, by the calculation of both seroprevalence and molecular prevalence.  
406 Given the important limits of MAT serology that we highlighted above, we only considered  
407 the results of molecular prevalence in the interpretation of our results. The differences in  
408 molecular prevalence according to small mammal host species reported in this study were in  
409 accordance with the biology and the life habits of each species. *Cricetomys gambianus* had  
410 the highest prevalence level (27.7%) compared with the three other species. In captivity, this  
411 species has the longest lifespan (more than 4 years and up to 8 years in the closely related  
412 *Cricetomys ansorgei* from southern Africa (Goodman and Monadjem, 2017)), compared with  
413 the three other species (from 12-18 months in the wild to 4 years in captivity for *Crocidura*  
414 spp. (Nowak, 1999); approximately 1 year under natural conditions for *M. m. domesticus* and  
415 *R. rattus* (Berry and Bronson, 1992; Pocock Michael et al., 2004; Wilson, 2009)). *Cricetomys*  
416 *gambianus* should also have the widest home range (*C. ansorgei* has been shown to have a  
417 home range of several hectares (Skinner and Smithers, 1990)). These bioecological traits may  
418 multiply the likelihood of contact with oocysts in time and space. Ecological characteristics  
419 may also be invoked to explain differences in prevalence levels between *C. olivieri* (15.6%),  
420 *M. m. domesticus* (13.3%) or *R. rattus* (3.8%). For instance, *Crocidura* spp. could have a  
421 higher likelihood of contact with oocysts in soil due to frequent digging and consumption of  
422 paratenic hosts of *T. gondii* such as earthworms (Clausnitzer et al., 2003; Churchfield et al.,  
423 2004). Hence, higher levels of *T. gondii* prevalence have been found by Afonso et al. (2007)  
424 in fossorial species compared with other rodent species. Conversely, the arboreal species *R.*  
425 *rattus* that often builds nests in rooves of houses could have limited contact with the soil.

426 Relying on prevalence patterns for these species characteristics suggests an hypothesis of an  
427 orally acquired infection for these species and an environmental source of infection. This  
428 interpretation, which has been used in a number of previous studies (Afonso et al., 2007;  
429 Reperant et al., 2009), should however be taken with caution because the main mode of  
430 transmission of *T. gondii* in natural populations of small mammals is still unresolved (Dubey,  
431 2009). High levels of prevalence have been reported in *M. m. domesticus* (Murphy et al.,  
432 2008) and *Apodemus sylvaticus* (Thomasson et al., 2011) in areas relatively free of cats. These  
433 previous studies suggest a perpetuation of the *T. gondii* life cycle through only congenital  
434 transmission between the successive generations of rodents, although the use of nested PCR  
435 in these studies is methodologically questionable due to possible PCR cross-contamination  
436 issues.

437         The outcome of *T. gondii* infection according to the virulence of the infecting strain  
438 and host resistance may also shape the pattern of infection prevalence in rodents. Indeed,  
439 higher levels of prevalence are expected in host species that survive and develop chronic  
440 infection regardless of the infecting *T. gondii* strains, compared with host species developing  
441 fatal toxoplasmosis when infected with certain *T. gondii* strains. In our sample, genotypic  
442 diversity of *T. gondii* strains identified in rodents and shrews consisted mainly of clonal  
443 lineages of type II (6/12), type III — and type III-like — (3/12) and Africa 1 (2/12) lineages.  
444 This diversity is consistent with the intertropical position of Senegal in West Africa, type II  
445 strains being predominant in North Africa and the Africa 1 strain in the tropical countries of  
446 West Africa (e.g., Guinea, Ghana and Côte D'Ivoire) (Galal et al., 2017). Due to the low  
447 parasite burden in most samples, the limited number of successfully genotyped *T. gondii*  
448 strains did not allow a robust comparison of strain diversity according to the host species. The  
449 five genotyped strains infecting *C. gambianus* showed a substantial diversity for such a small  
450 sample size as they were represented in four different groups in the neighbor-joining tree. The

451 identification of the Africa 1 lineage in the two native African species, *C. gambianus* and *C.*  
452 *olivieri*, shows that these species are resistant to *T. gondii* strains with this genotype. Africa 1  
453 and type I strains are closely related (Mercier et al., 2010) and known to be lethal in all the  
454 conventional strains of laboratory mice (Khan et al., 2009; Mercier et al., 2010; Behnke et al.,  
455 2011). These strains harbor the type I alleles at the *ROP5* and *ROP18* genes, both known as  
456 major virulence determinants in laboratory mice (Shwab et al., 2016). The resistance of native  
457 host species to most of the African *T. gondii* strains including Africa 1 can be expected as  
458 they have probably shared the same environment since ancient times and had co-adapted in a  
459 pattern allowing both the parasite's transmission and host survival.

460 In the invasive species *M. m. domesticus*, only type II and type III strains were  
461 identified. Unlike laboratory mice that often develop high parasitic burdens following  
462 infection by *T. gondii* — and this is why they are commonly used in strain bioassays (Dubey,  
463 2009) — most wild mice in our sample had very low parasitic burdens in their brains which  
464 could be used for genotyping. Due to the small number of successfully genotyped strains, we  
465 could not determine whether the absence of Africa 1 lineages in this species should be  
466 attributed to the limits of our sampling or to virulence of the *T. gondii* strains with this  
467 genotype in *M. m. domesticus* from Senegal, as is the case for most laboratory mouse strains.  
468 Historical and genetic evidence point to a European origin for the *M. m. domesticus*  
469 populations in Senegal (Dalecky et al., 2015; Lippens et al., 2017) that were introduced in  
470 port cities through ships of European explorers and settlers. In Europe type II, followed by  
471 type III, which are not pathogenic for laboratory mice, are by far the predominant lineages.  
472 We speculate that mice therefore may not be adapted to the more virulent *T. gondii* strains  
473 found in Africa, a hypothesis previously proposed by Jensen et al. (2015). This may partly  
474 explain the higher prevalence of *T. gondii* infection in native species over invasive species, as  
475 the latter (at least *M. m. domesticus*) would die after the infection with certain African strains.

476 The genes involved in *T. gondii* resistance in mice (the immunity regulated GTPases (IRGs))  
477 show a high degree of polymorphism among natural mouse populations (Lilue et al., 2013).  
478 This diversification of the alleles involved in *T. gondii* resistance may be driven by the  
479 virulence of strains specific to each region of the world. For example, the southeastern Asian  
480 house mouse (*Mus musculus castaneus*) shows resistance to type I strains (Lilue et al., 2013)  
481 in southeastern Asia, where type I and other genetically related strains are not uncommon  
482 (Chaichan et al., 2017). This subspecies of mouse has been shown to inhibit the parasite-  
483 derived kinase complex ROP5/ROP18, a putative adaptive trait to survive infection by type I  
484 strains (Lilue et al., 2013).

485 In conclusion, the results presented here contribute to our understanding of the  
486 complex interactions that may occur between *T. gondii* and commensal small mammals under  
487 natural conditions. Our results support the hypothesis of variable adaptations of commensal  
488 small mammal species to the different strains of the parasite and provide insight into the  
489 putative mechanisms shaping the spatial structure of *T. gondii* genetic diversity. Our findings  
490 also highlight the important discrepancies that could occur between laboratory mice and  
491 natural populations of mice regarding humoral responses to *T. gondii* infection and parasite  
492 burdens in tissues of infected mice, which pose new challenges in detecting and  
493 characterizing the parasite in one of its most important reservoir species.

494

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- 756

757

758 **Figure legends**

759

760 **Fig. 1.** Sampling regions and distribution of rodents and shrews collected in this study. The  
761 three studied regions of Senegal are highlighted on the maps. The pie charts indicate the  
762 numbers of rodents and shrews from each species that have been included in the present study  
763 (for details refer to Supplementary Fig. S1).

764

765 **Fig. 2.** Neighbor-joining tree of genotypes inferred from Cavalli-Sforza distances calculated  
766 for the data of 15 microsatellite markers for the 12 strains described in this study and a set of  
767 reference strains. Circles next to the identifiers of genotypes indicate individuals belonging to  
768 *Cricetomys gambianus* species, squares indicate *Mus musculus domesticus* and triangles  
769 indicate *Crocidura olivieri*. The lineage clustering genotypes from this study are indicated  
770 where available. Reference strains and their respective haplogroups (HG) are indicated in bold  
771 letters when available (for details refer to Supplementary Table S2).

772

773 **Supplementary Fig. S1.** Flow chart indicating the number of *Mus musculus domesticus*,  
774 *Rattus rattus*, *Cricetomys gambianus* and *Crocidura olivieri* collected during the field  
775 sampling in Dakar, Rufisque and Joal-Fadiouth (Senegal), submitted to PCR assays for  
776 detection of *Toxoplasma gondii*, *Hammondia heydorni* and *Hammondia hammondi* DNA, and  
777 with *T. gondii* DNA successfully genotyped with microsatellite markers.

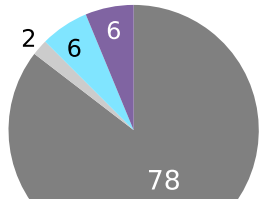
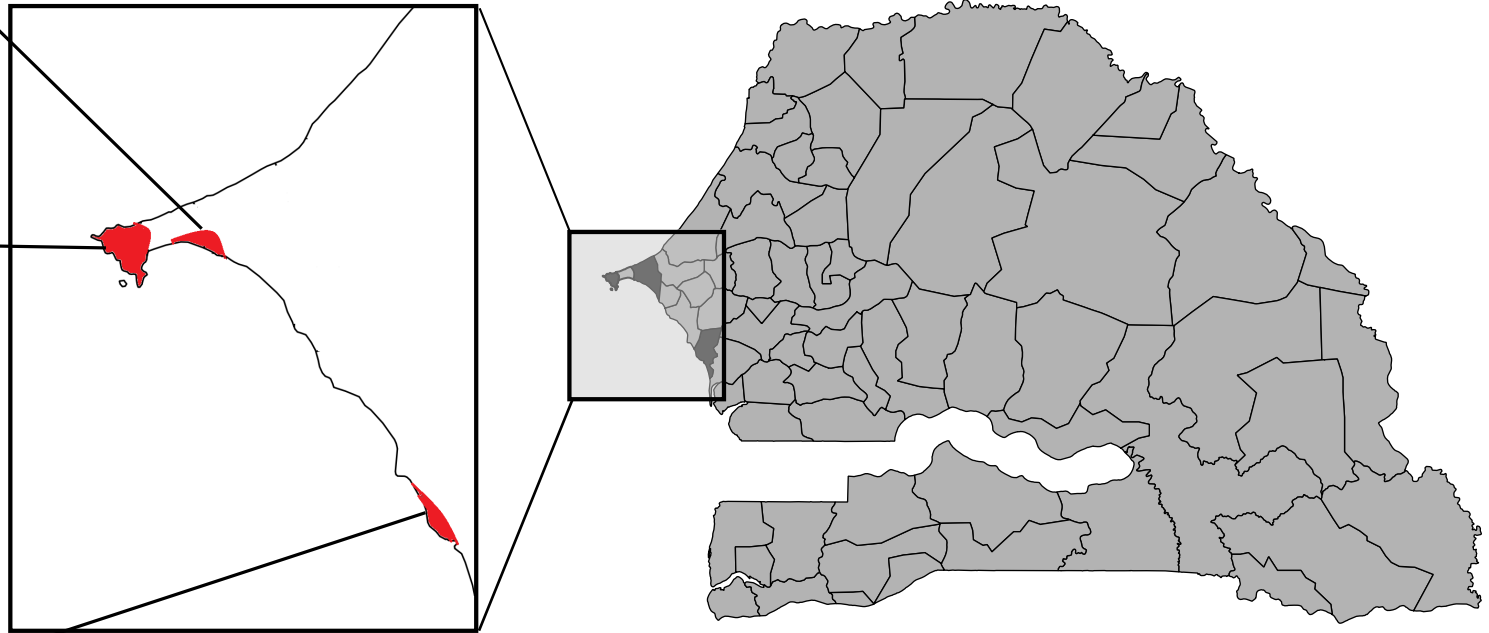
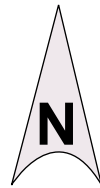
778

779 **Supplementary Table S1.** Results of the serological screening using the modified  
780 agglutination test (MAT) and of PCR assays for detection of *Toxoplasma gondii*, *Hammondia*  
781 *heydorni*, *Hammondia hammondi* and *Neospora caninum* DNA from small mammals of

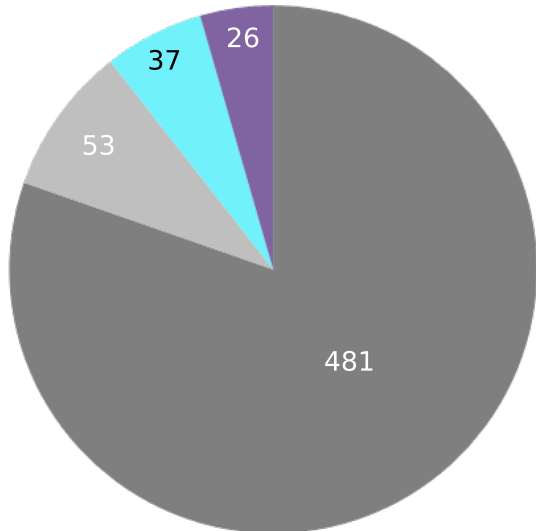
782 Senegal. The Table is available at Mendeley Data via  
783 <http://dx.doi.org/10.17632/m4dcd7f8h5.1>.

784

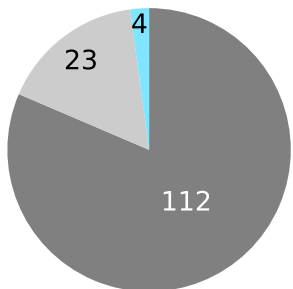
785 **Supplementary Table S2.** Genotyping results obtained by the analysis of 15 microsatellite  
786 markers for the 16 genotyped strains from this study (in bold letters), for 25 strains previously  
787 collected in Africa and for 17 reference strains representing the *Toxoplasma gondii* global  
788 diversity (including three strains from Africa).



Rufisque



Dakar



Joal-Fadiouth

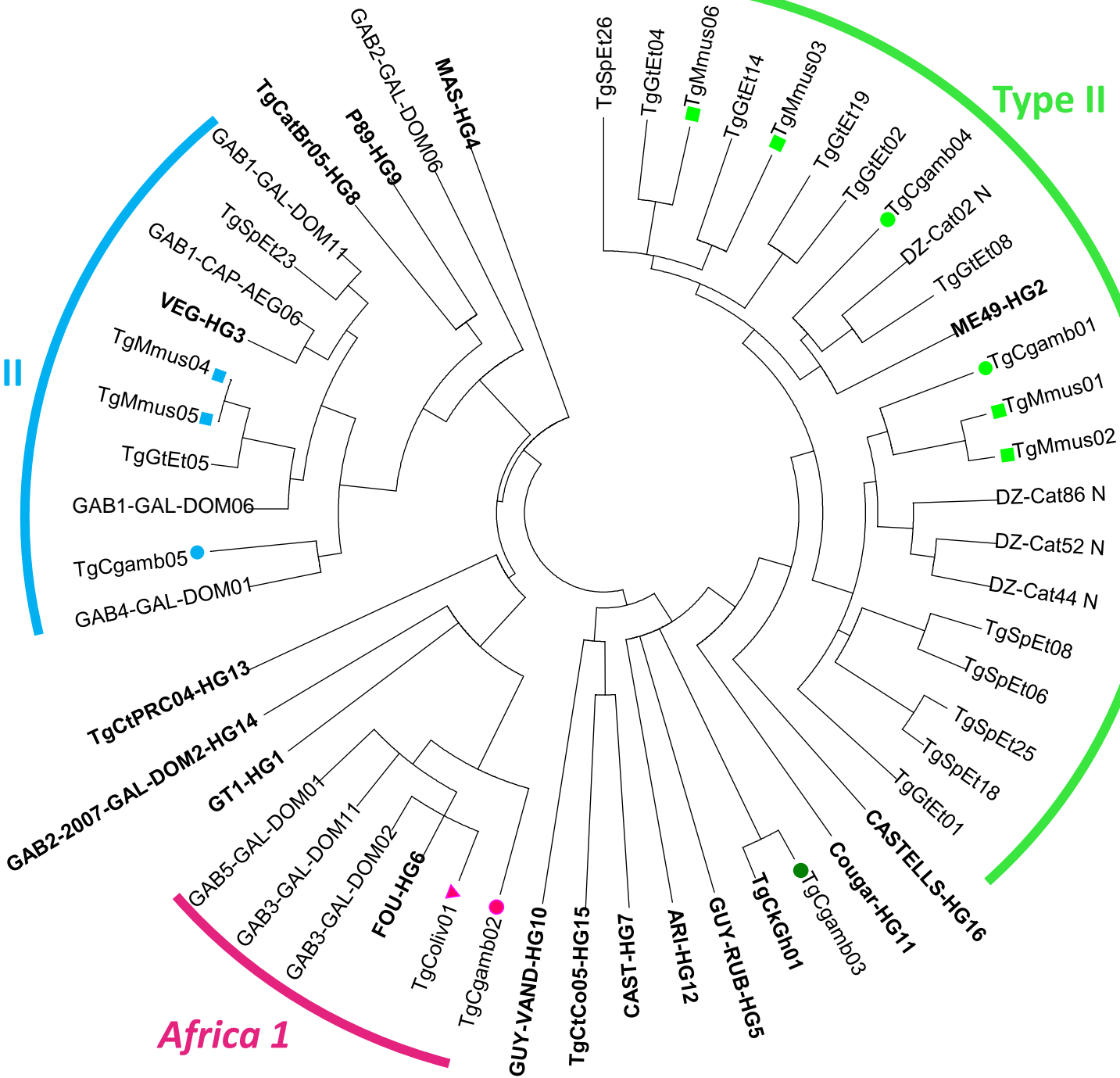
■ *Mus musculus domestiscus*

■ *Rattus rattus*

■ *Cricetomys gambianus*

■ *Crocidura olivieri*

Type III



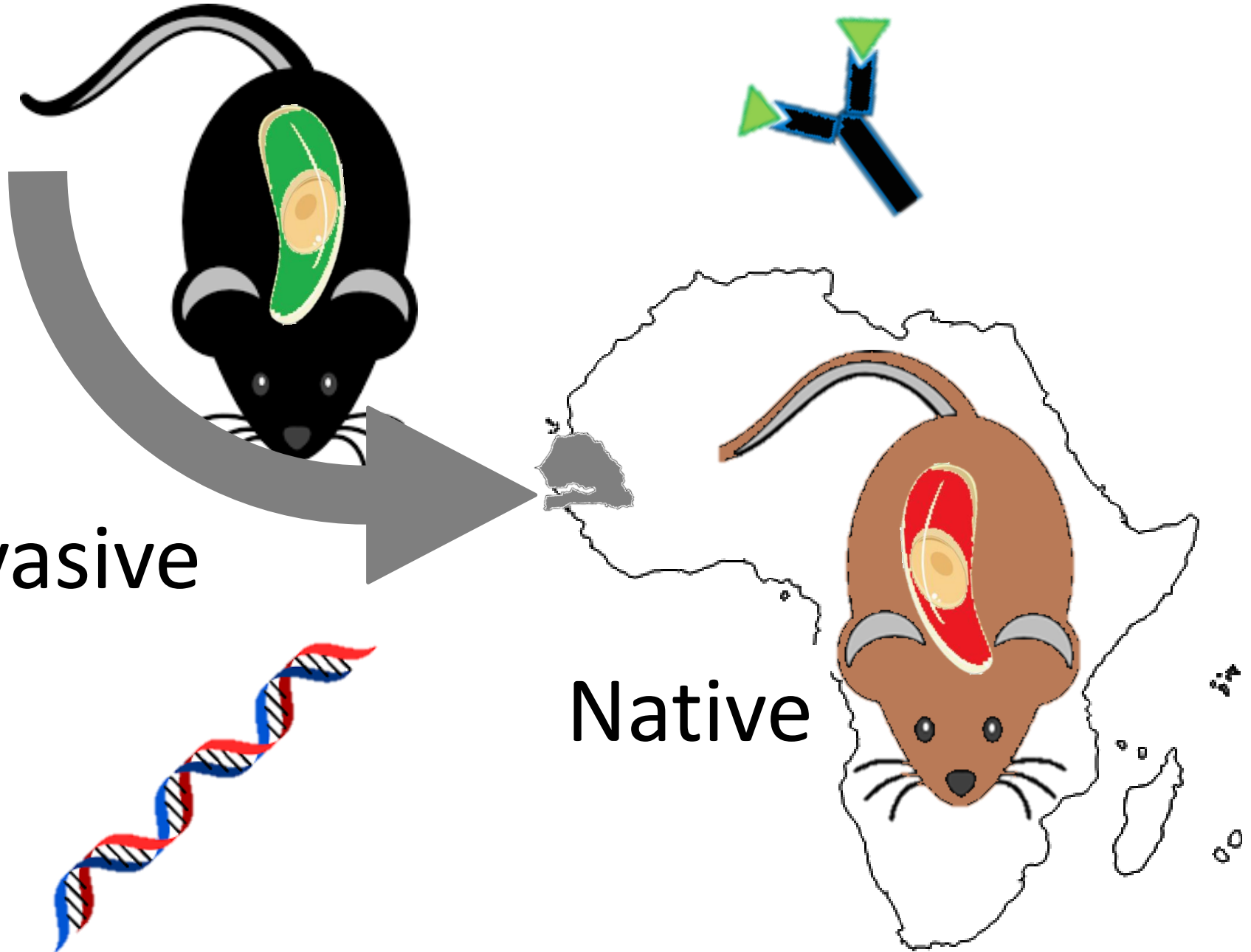


**Table 1.** Prevalence of *Toxoplasma gondii* infection in the sampled regions of Senegal and comparison of the results obtained by serology using the modified agglutination test (MAT) and by quantitative PCR on brain samples for each species.

Species	<i>Mus musculus</i>			
	<i>domesticus</i>	<i>Rattus rattus</i>	<i>Cricetomys gambianus</i>	<i>Crocidura olivieri</i>
<b>Sample size</b>	671	78	47	32
<b>Seroprevalence MAT % (CI at 95%)</b>	4.8 (3.2; 6.4)	2.6 (-0.9; 6.1)	31.9 (18.6; 45.2)	37.5 (20.7; 54.3)
<b>Molecular prevalence PCR % (CI at 95%)</b>	13.3 (10.7; 15.9)	3.8 (-0.4; 8)	27.7 (14.9; 40.5)	15.6 (3.0; 28.2)
<b>Kappa coefficient (CI at 95%)</b>	0.067 (-0.017; 0.150)	-0.032 (-0.062; -0.002)	0.695 (0.471; 0.920)	0.472 (0.178; 0.765)
<b>Kappa agreement</b>	very low agreement	disagreement	good agreement	moderate agreement

CI, confidence interval

# *Toxoplasma gondii* in small mammals



Invasive

Native