

## The rural–urban effect on spatial genetic structure of type II *Toxoplasma gondii* strains involved in human congenital toxoplasmosis, France, 2002–2009



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### ABSTRACT

Congenital toxoplasmosis involves *Toxoplasma gondii* type II strains in 95% of cases in France. We used spatial principal component analysis (sPCA) and 15 microsatellite markers to investigate the spatial genetic structure of type II strains involved in 240 cases of congenital toxoplasmosis in France from 2002 through 2009. Mailing addresses of patients were geo-referenced a posteriori in decimal degrees and categorized into urban or rural areas of residence. No spatial genetic structure was found for type II strains that infected mothers who were living in urban areas, but a global spatial genetic structure was found for those that infected mothers who were living in a rural environment. Our results suggest that sources of infection by *T. gondii* are different in rural and urban areas in France, and advocate for targeted messages in the prevention of toxoplasmosis according to the type of residence of susceptible people.

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### 1. Introduction

*Toxoplasma gondii* is considered to be one of the most successful parasites because this protozoan is virtually able to invade any nucleated cell of any warm-blooded animal at all latitudes. This parasite is

efficiently transmitted by ingestion of oocysts shed by felids in the environment or cysts present in tissues of mammals and birds. Toxoplasmosis is considered a benign disease except in fetuses infected during early pregnancy and in severely immunocompromised patients because they are at risk of severe brain and eye damage, and in tropical South America where the prevalence of ocular disease is high and where life-threatening cases of pulmonary toxoplasmosis may occur in otherwise healthy people (Carne et al., 2009; de-la-Torre et al., 2013). It is estimated that 25% of the human population is currently chronically infected by *T. gondii*. In fact, seroprevalence in the general population can tremendously vary with geography and also with time. Less than 10% of people born in the USA have antibodies against *T. gondii* whereas in certain tropical areas of South America and Africa, the prevalence is as high as 80% (Krueger et al., 2014). Big variations of prevalence can also be observed at a country scale. For example in France, the median prevalence among women of childbearing age was 43.8% in 2003 but the prevalence varied substantially by French region, being lowest in North-eastern France (29.5%) and highest in the greater Paris area (52.7%) (Berger et al., 2009). Seroprevalence varies also with time as shown in France with a decrease in prevalence from 83% in 1965 to 37% in 2010 (Nogareda et al., 2014). These data indicate that the epidemiology of

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human toxoplasmosis is complex and involves many different aspects including intensity of *T. gondii* circulation in the environment, geoclimatic variables such as temperatures, precipitations or altitude, and human factors such as personal hygiene, cultural and food habits, socio-economic status, level of education and residence in a rural or urban area.

The population structure of this cosmopolitan parasite is more complex than initially thought and has distinct geographic patterns. The hotspot of *T. gondii* genetic diversity seems to be tropical South America, especially in the Amazonian forest, where a combination of a large gene pool with frequent genetic exchanges have generated a wide variety of so-called atypical genotypes (Ajzenberg et al., 2004). Elsewhere, and especially in Europe, *T. gondii* displays a striking clonal population structure. Based on genotyping data obtained from domestic and wild animals in France, it is estimated that 99% of *T. gondii* strains that circulate in the French environment belong to type II (Aubert et al., 2010; Halos et al., 2010). Type III strains are much less frequent in animals in France than in animals from other areas such as North America or some regions of Africa (Shwab et al., 2014). To date, type I and atypical strains have never been isolated from any animal species in France. As a consequence, isolating a non-type II strain in a patient in France raises the possibility that this patient had been infected with an imported strain during a journey abroad or because he had eaten imported food, as already shown in some reports (Ajzenberg et al., 2009; Pomares et al., 2011).

The aim of this study was to better understand the epidemiology of human toxoplasmosis in rural versus urban areas by investigating the spatial genetic structure of *T. gondii* strains in both environments at a country scale in France. If people who live in rural areas are predominantly infected by local food produced in their region of residence, then a significant spatial genetic structure of *T. gondii* strains should be observed in rural areas. Conversely, if we consider that people living in urban areas are more likely to get *T. gondii* infection from food purchased from supermarkets, no spatial genetic structure should be observed in urban areas because goods distributed in supermarkets come from many different suppliers all over the French territory. To test this hypothesis, we used strains isolated in France from congenital cases because these strains are predominant in the collection of human strains at the French national reference center of toxoplasmosis and because women are infected during a short period of time during pregnancy, which minimizes the likelihood of *T. gondii* infection outside their residency area.

## 2. Material and methods

### 2.1. *T. gondii* strains

From 1992 to 2009, the French national reference center of toxoplasmosis genotyped 325 *T. gondii* strains responsible for congenital toxoplasmosis in 16 French teaching hospitals. These strains had been isolated in mice after inoculation of amniotic fluids, placenta, or blood cord samples for evidencing congenital toxoplasmosis in children or fetuses from mothers infected by *T. gondii* during pregnancy in this period. All these strains were genotyped with 15 microsatellite markers as previously described (Ajzenberg et al., 2010). Of these 325 strains, 305 (94%) were type II, 10 (3%) were type III, and 10 (3%) could not be classified into one of the three major clonal types and therefore considered as atypical.

For the analysis, we restricted the sample to the strains isolated during a shorter period of time, from 2002 through 2009, in order to minimize temporal effect on genetic variability. We also excluded the type III and atypical strains in order to minimize the genetic variability due to imported strains from non-European countries. In total, 240 type II strains isolated from 240 cases of congenital toxoplasmosis in France from 2002 through 2009 were included in the analysis (supplementary material).

### 2.2. Geo-referencing *T. gondii* strains in France

The 240 *T. gondii* strains were geographically associated to the place of residence of the corresponding mothers in whom these strains had been collected. The mailing addresses (number, street and commune names) of the 240 patients were geo-referenced *a posteriori* in decimal degrees using the web portal of the French National Institute of Geography (IGN, <http://www.geoportail.fr/>). The spatial distribution in France of the 240 strains is displayed in Fig. 1. Some gaps can be identified in this map. The big gap in the center of the country is mainly due to the fact that this area (called Massif Central) is the least densely populated area in France. Other gaps in certain densely populated areas are explained by the poor availability of strains from these areas at the French national reference center of toxoplasmosis.

### 2.3. Classification of *T. gondii* strains into rural and urban

In order to investigate the relative degree of spatial genetic structure of *T. gondii* strains isolated from women living in a rural environment versus those living in an urban environment, the geographic coordinates of the 240 strains were classified as rural or urban based on two different classifications.

#### 2.3.1. TUU classification

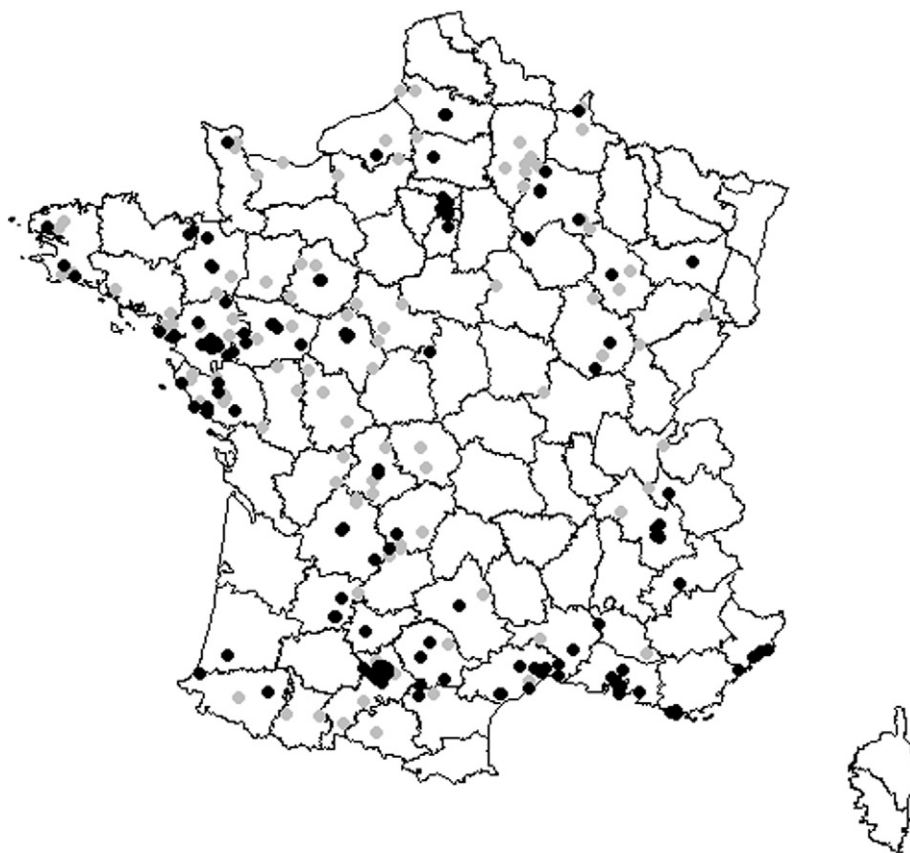
A detailed classification of the 36,569 French communes within rural versus urban socio-economic environments was delivered in 1999 by the French National Institute for Statistics and Economic Studies (INSEE, available at <http://www.insee.fr/en/default.asp>) and updated in 2009. This classification is based on urban unit sizes ("Taille d'Unité Urbaine" or TUU in French) and includes several criteria such as population size, socio-economic activity within the commune, or frequency of travel between place of residence and work. Based on the TUU classification, the rural communes are therefore those that are not urban. The TUU classification allowed us to classify  $n = 85$  *T. gondii* strains as rural and  $n = 155$  *T. gondii* strains as urban (supplementary material).

#### 2.3.2. GE classification

The TUU classification can be completed by another one that classifies urban communes as those that are not rural. We used the Google Earth (GE) application (<http://earth.google.com/>) to display a satellite windows (10 km × 10 km) centered on the mailing addresses of the 240 patients. When no commune of more than 5000 inhabitants was found at less than five kilometers of a given mailing address, the *T. gondii* strain isolated from this given patient was classified as rural. A strain was thus classified as urban when the previous condition was not met. Overall, it allowed us to classify  $n = 98$  *T. gondii* strains as rural and  $n = 142$  *T. gondii* strains as urban (Fig. 1 and supplementary material). Matching between the TUU and GE classifications was very good since 79 out of the 85 rural *T. gondii* strains that were defined with the TUU classification were also rural with the GE classification (observed Cohen's unweighted Kappa coefficient = 0.78).

### 2.4. Genetic and genotypic diversity within rural and urban populations

Genetic polymorphism was measured by allelic richness (A) per locus and sample and by Nei's unbiased genetic diversity within sub-samples (Hs) (Nei and Chesser, 1983). Allelic richness was corrected for unequal sample size using the rarefaction method (El Mousadik and Petit, 1996). The A and Hs values were calculated with the FSTAT software version 2.9.4 (Goudet, 1995). Genotypic diversity was calculated from the number of multilocus genotypes on the total number of strains for both rural and urban populations. Linkage disequilibrium (LD) between pairs of loci was assessed with a randomization test performed in FSTAT. The statistics used was the log likelihood ratio G summed over all subpopulations. Because this procedure was repeated on all pairs of polymorphic loci, we applied the sequential Bonferroni



**Fig. 1.** Spatial distribution of 240 type II strains isolated from congenital toxoplasmosis cases from 2002 through 2009 in France. The black symbols are for the urban strains and the grey symbols are for the rural strains according to the GE classification (see [Material and methods](#)).

correction (Holm, 1979) to the p values. LD was calculated for both the urban and the rural populations. To test for genetic isolation by geographical distance (IBD) within both rural and urban populations, Mantel's tests (Mantel, 1967) ( $n = 9999$  permutations) were performed for matrix correspondence to compare pairwise geographical (km) and genetic distances based on the Cavalli-Sforza and Edwards chord-distance estimator  $D_c$  (Cavalli-Sforza and Edwards, 1967) among *T. gondii* strains using R 2.10.1 software (R Development Core Team, 2009).  $D_c$  was computed using Populations 1.2.30 (1999, Olivier Langella, CNRS UPR9034, [http://bioinformatics.org/\\_tryphon/populations/](http://bioinformatics.org/_tryphon/populations/)).

### 2.5. Spatial genetic structure of *T. gondii* strains

We used spatial principal component analysis (sPCA), implemented in the package *adegenet* for the software R (R development Core team, 2013), to investigate the spatial genetic structure of *T. gondii* strains isolated from congenital cases in France (Jombart, 2008; Jombart et al., 2008). The sPCA seeks principal components that optimize the product of the variance and Moran's Index (Cliff and Ord, 1981; Moran, 1948), ensuring that both the genetic diversity and the spatial patterns are taken into account. The sPCA is particularly appropriate to our study because the spatial distribution of *T. gondii* strains is continuous in our sampling. The sPCA identifies global structures that correspond to a positive spatial auto-correlation between genotypes, indicating the occurrence of patches or clines in the spatial distribution of genetic diversity (Jombart et al., 2008). As in other multivariate analyses, such global structures are typically detected graphically by an abrupt decrease in positive eigenvalues (Jombart et al., 2009). The assessment of the strength of the inferred global structure is facilitated by a non-parametric randomized Monte-Carlo test (Jombart et al., 2008). The sPCA and non-parametric randomized Monte-Carlo tests were

performed for each type of *T. gondii* strain (rural vs. urban) using successively the GE and the TUU rural/urban classification.

The sPCA needs a matrix of proximities between *T. gondii* strains to be incorporated explicitly in the analysis for the calculation of the Moran's Index. As seen in Fig. 1, *T. gondii* rural strains are more evenly distributed than the more aggregated urban *T. gondii* strains. This difference in spatial distribution of rural versus urban strains implied to use different proximity matrices, for both urban and rural strains, to ensure that our results were not influenced by the proximity matrix type. First, we used a binary connection matrix  $M$  (e.g., with value equal to 1 if two urban *T. gondii* strains were neighboring or equal to 0 if they were not). Neighborhood was defined by building a connection network (also called neighboring graph) following Delaunay's triangulation (Upton and Fingleton, 1985). The Delaunay's graphs were modified by removing unrealistic connections crossing the sea (e.g., from northwest to southwest of France). Secondly, we used the matrix of the inverse of Euclidean distances between each pair of urban *T. gondii* strains. Such a proximity matrix implies that the strength of the spatial auto-correlation between two *T. gondii* strains is linearly related to the distance separating these two *T. gondii* strains. This distance-based neighborhood is appropriate to aggregated or irregular distributions (Gabriel and Sokal, 1969) such as the one observed in urban *T. gondii* strains whereas Delaunay's triangulation would lead to unrealistic connection in that case. All p values  $< 0.05$  were considered significant.

## 3. Results

### 3.1. Genetic diversity

Allelic richness ( $A$ ) and genetic diversity ( $H_s$ ) values per locus and population were reported in Table 1. Among the 15 microsatellite markers, only 7 (*M48*, *M102*, *N60*, *N82*, *AA*, *N61*, and *N83*) showed a

**Table 1**  
Genetic diversity (Hs) and allelic richness (A) per locus and rural/urban classification.

Locus <sup>a</sup>	Genetic diversity (Hs)				Allelic richness (A)			
	TUU classification		GE classification		TUU classification		GE classification	
	Rural	Urban	Rural	Urban	Rural	Urban	Rural	Urban
TUB2	0	0	0	0	1.00	1.00	1.00	1.00
W35	0.02	0.05	0.02	0.06	2.00	2.00	2.00	2.00
TgM-A	0	0	0	0	1.00	1.00	1.00	1.00
B18	0	0	0	0	1.00	1.00	1.00	1.00
B17	0	0	0	0	1.00	1.00	1.00	1.00
M33	0	0	0	0	1.00	1.00	1.00	1.00
MIV.1	0	0	0	0	1.00	1.00	1.00	1.00
MXL1	0	0	0	0	1.00	1.00	1.00	1.00
M48	0.85	0.80	0.83	0.81	16	16.48	15.00	17.69
M102	0.68	0.64	0.66	0.65	7.00	6.75	6.00	7.79
N60	0.66	0.62	0.63	0.64	7.00	7.55	6.00	7.71
N82	0.70	0.69	0.75	0.65	11.00	14.26	12.00	13.61
AA	0.91	0.91	0.91	0.91	17.00	17.29	16.00	17.78
N61	0.92	0.91	0.92	0.91	16.00	15.54	16.00	15.71
N83	0.71	0.70	0.71	0.69	6.00	4.80	6.00	4.91
Mean	0.78	0.75	0.77	0.77	11.43	11.81	11.00	12.17

<sup>a</sup> Among the 15 microsatellite markers, only 7 (M48, M102, N60, N82, AA, N61, and N83) are highly polymorphic and are called fingerprinting markers. The mean calculations were based on the 7 fingerprinting markers data only.

high polymorphism (Table 1). These 7 markers are called fingerprinting markers (Ajzenberg et al., 2010) and are able to detect slight genetic differences among closely related strains such as type II strains. When we combined the results from the seven fingerprinting markers based on the GE classification, the mean A in the rural population was significantly lower than for the urban population (11.00 vs. 12.17; unilateral Wilcoxon signed rank test,  $p = 0.04$ ). Based on the TUU classification, the mean A in the rural population was also lower than for the urban population but without reaching statistical significance (11.43 vs. 11.81; unilateral Wilcoxon signed rank test,  $p = 0.27$ ). On the contrary, Hs tended to be higher in rural strains than in urban strains (Wilcoxon signed rank test,  $p = 0.04$  and  $p = 0.15$  for the TUU and GE classification, respectively).

Genotypic diversity was nearly equal in both populations:  $94/98 = 0.959$  in rural strains versus  $136/142 = 0.958$  in urban strains for the GE classification, and  $98.80\%$  in rural strains versus  $94.80\%$  in urban strains for the TUU classification. After sequential Bonferroni correction, few pairs of polymorphic loci were in significant linkage disequilibrium with a maximum of four out of the 28 pairs for the urban population by using the GE rural/urban classification. Finally, IBD was not detected in any of the population whatever the classification used (data not shown).

### 3.2. Spatial genetic structure of *T. gondii* strains

No spatial genetic structure was found by the sPCA for the urban strains whatever the rural/urban classification and the proximity matrix

**Table 2**  
P-values for the randomization tests performed to assess the significance of the global spatial genetic structure of urban and rural strains in each sample depending on the rural/urban classification and the proximity matrix used.

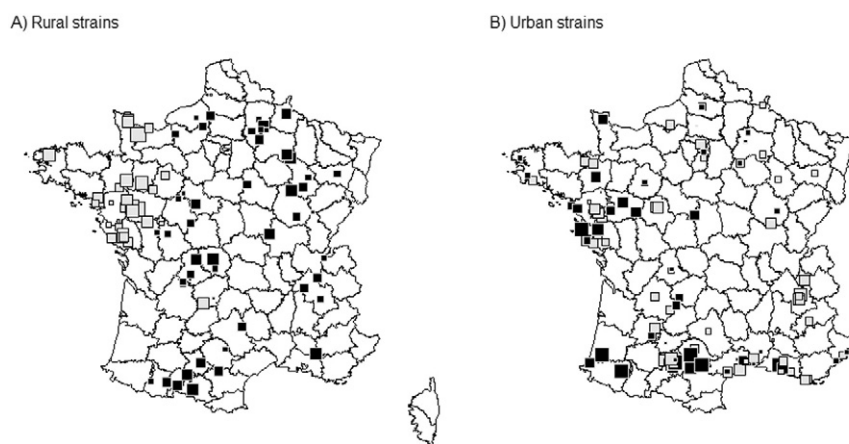
Strains	Urban/rural classification	Proximity matrix	P-value for global test
Urban	GE (n = 142)	Delaunay	0.48
		Distance-based	0.78
	TUU (n = 155)	Delaunay	0.65
		Distance-based	0.89
Rural	GE (n = 98)	Delaunay	0.13
		Distance-based	0.05
	TUU (n = 85)	Delaunay	0.03
		Distance-based	0.22

used. All p values for global test on urban strains were non-significant (Table 2). On the contrary and in agreement with our prediction, an overall spatial genetic structure was found in rural strains since p values for global test reached statistical significance in two out of the four tests (Table 2). That significance level was reached for both the GE and TUU classification by using distance-based and Delaunay proximity matrix, respectively. This result suggests that the global spatial structure for rural strains is not an artefact of a particular classification or proximity matrix. The spatial structure of rural strains according to TUU classification and the Delaunay proximity matrix is displayed in Fig. 2A. Of note, this graphical representation of rural strains matched the one displayed for the GE rural/urban classification with the distance-based proximity matrix (result not shown). This global structure separated mainly the northwestern part of France from the rest of the country. Grey squares corresponding to negative sPCA scores for strains were mainly observed in three administrative regions of the Northwestern France (Bretagne, Pays de la Loire, and Normandie) whereas black squares corresponding to positive sPCA scores were in the rest of the country for most of them. For comparison and despite no spatial genetic structure was found in urban strains, Fig. 2B displayed a graphical representation of the sPCA scores obtained for the urban strains with the GE rural/urban classification and the Delaunay proximity matrix, i.e. the combination of classification and proximity matrix that led to the lowest p-value for urban strains (Table 2).

## 4. Discussion

Based on bivariate or multivariate analysis with calculation of the odds ratios, most epidemiological studies try to identify the variables associated with an increased risk of toxoplasmosis by interviewing a target population (mostly pregnant women) with standardized questionnaires in a given geographic area (Cook et al., 2000; Jones et al., 2009). The results are interesting and often useful for making preventive strategies and public health policy decisions, but have serious limitations due to the subjective answers given in interviews. Because people may be unaware of their exposures or may have difficulty recalling specific exposure that occurred, it is considered that interviews with questionnaires are not able to explain roughly 50% of the risk for *T. gondii* infections in these studies (Cook et al., 2000; Jones et al., 2009). When a more objective test is applied, unexpected results may arise. For example, a new blood test detecting a specific antibody to an 11-kDa oocyst protein showed that oocyst-induced infections were largely underestimated in the USA and that about 50% of women infected with oocysts did not identify significant risk factors for oocysts infection (Boyer et al., 2011). It is therefore important to address the epidemiological issues of toxoplasmosis by using different approaches than the classical interviews with questionnaires. Our work is original because it aimed to understand the epidemiology of *T. gondii* infection in rural versus urban areas by using spatial principal component analysis (sPCA). Restriction fragment length polymorphism markers or intron sequencing are unable to detect enough polymorphism among closely-related strains such as type II strains, but this is possible with highly polymorphic microsatellite markers such as those used in this study (Ajzenberg et al., 2010). Among the 240 type II strains genotyped with 15 microsatellite markers in this study, 222 different multilocus genotypes were identified with 211 unique genotypes and only 11 genotypes common to at least 2 strains. Based on these data, the Simpson's Index of Diversity  $1 - D$  (Ajzenberg et al., 2002) is 0.999 which is almost maximal and shows the very high discriminatory power of the microsatellite markers used in this study.

There was no spatial genetic structure for type II strains that infected pregnant women who are living in urban areas in France. This means that people living in an urbanized area may be infected by local strains but also by strains from different geographical regions. These conditions are met in supermarkets and restaurants where food has multiple origins from all over the French territory and also from foreign



**Fig. 2.** A) Graphical display of the global spatial genetic structure found in rural *T. gondii* type II strains from 2002 through 2009 in France with the sPCA by using the TUU classification and the Delaunay's proximity matrix. B) Graphical display of the lack of global spatial genetic structure found in urban *T. gondii* type II strains from 2002 through 2009 in France with the sPCA by using the GE classification and the Delaunay's proximity matrix. sPCA scores for georeferenced strains: black squares correspond to positive sPCA scores and grey squares to negative sPCA scores. The size of any square is proportional to the absolute value of the sPCA scores (i.e., strains with large black squares and strains with large grey squares are the most differentiated ones).

countries. Food imports from extra-European countries, especially from South-America, increase the risk of being infected with atypical strains that are considered more pathogenic than type II strains (Pomares et al., 2011). As a corollary, we might expect higher genetic diversity in urban strains than in rural ones. This was partially observed as the allelic richness (A) tended to be higher in urban than in rural strains but genetic diversity (Hs) tended to be higher in rural than in urban strains and genotypic diversity was nearly equal in both populations.

Conversely, our results showed a spatial genetic structure only for type II strains that infected pregnant women who are living in rural areas in France. This global structure clearly separated the strains associated to rural environments in the northwestern part of France from those associated to rural areas in the rest of the country. Because we restricted the analysis to a short period of time from 2002 through 2009, the genetic structure observed in rural areas was not due to a temporal effect but was indeed spatial. Some limitations include the fact that pregnant women who were living in rural areas may have been infected with food purchased in supermarkets from urban areas. However, this effect was not strong enough to alter the spatial genetic structure observed in rural areas, and suggests that rural strains are strongly structured. In agreement with our hypothesis, people who live in rural areas are more likely to be infected with local strains that are circulating in a limited perimeter around their residence.

If the observed spatial genetic structure in rural strains can be plausibly explained, the processes leading to such a separation between Northwestern France and the rest of the country are not obvious. Northwestern rural strains are genetically homogenous and different from those of the rest of the country. One might question whether this current separation could be a legacy of an historical structure that tend to disappear due to higher admixture with strains from diverse origins or is an ongoing process of spatial genetic structuring due to the effects of anthropization on the spatial distribution of *T. gondii* strains. One way to better understand the current involved process would be to develop a landscape genetic approach (Manel et al., 2003) in order to identify the main landscape elements facilitating or decreasing gene flow between *T. gondii* populations. More isolates are needed to assess the spatial structure of rural and urban *T. gondii* strains at smaller scales in France, for example in different administrative regions. Genotyping strains from animals in rural areas should also be encouraged to test whether the strains detected in infected people are similar to the ones found in the local fauna. Finally, future works have to be done to link the spatial genetic structure of *T. gondii* strains to the spatial distribution of well-known risk factors in the epidemiology of toxoplasmosis.

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There was no funding source and the authors have no conflict of interest related to this article.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.meegid.2015.08.025>.

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