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Microsatellite analysis of *Toxoplasma gondii* shows considerable polymorphism structured into two main clonal groups

Daniel Ajzenberg[a], Anne-Laure Bañuls[b], Michel Tibayrenc[b], Marie Laure Dardé[a,*]

[a]*Laboratoire de Parasitologie–Mycologie, EA 3174, Faculté de Médecine, 2 Rue du Dr Marcland, 87031 Limoges, France*
[b]*Génétique des Maladies Infectieuses, UMR CNRS-IRD 9926, Centre IRD, BP 5045, 34032 Montpellier, France*

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Abstract

Previous studies on *Toxoplasma gondii* population structure, based essentially on multilocus restriction fragment length polymorphism analysis or on multilocus enzyme electrophoresis, indicated that *T. gondii* comprises three clonal lineages. These studies showed a weak polymorphism of the markers (2–4 alleles by locus). In this study, we used eight microsatellite markers to type 84 independent isolates from humans and animals. Two microsatellite markers were present in the introns of two genes, one coding for beta-tubulin and the other for myosin A, and six were found in expressed sequence tags. With 3–16 alleles detected, these markers can be considered as the most discriminating multilocus single-copy markers available for typing *T. gondii* isolates. This high discriminatory power of microsatellites made it possible to detect mixed infections and epidemiologically related isolates. Evolutionary genetic analyses of diversity show that the *T. gondii* population structure consists of only two clonal lineages that can be equated to discrete typing units, but there is some evidence of occasional genetic exchange that could explain why one of these discrete typing units is less clearly individualised than the other.

Keywords: *Toxoplasma gondii*; Multilocus genotyping; Microsatellites; Clonality; Polymerase chain reaction; Discrete typing unit

1. Introduction

Toxoplasma gondii is an obligate intracellular parasite infecting all warm-blooded animals with a world-wide distribution. It causes a large range of clinical manifestations in humans (Bossi et al., 1998; Dardé et al., 1998). Besides, there are marked biological differences among stocks concerning their pathogenicity to mice: most of the stocks are avirulent in mice producing asymptomatic chronic infections, while few which are highly virulent in mice stocks produce acute toxoplasmosis killing all mice with less than 10 tachyzoites.

Using mAb, it is possible to classify stocks in two or three groups demonstrating antigenic diversity in *T. gondii* (Bohne et al., 1993; Parmley et al., 1994; Meisel et al., 1996; Jensen et al., 1998). Population genetic analysis of published isoenzyme data led Tibayrenc et al. (1991) to propose that *T. gondii* exhibits a basically clonal population structure, similar to many other parasitic protozoa. Similar analyses, based on restriction fragment length polymorphism (RFLP) of six independent single-copy loci, amplified by PCR, indicated that *T. gondii* consists of only three clonal lineages designated types I, II and III, which occur in both animals and humans (Howe and Sibley, 1995). However, the question of the actual nature of these lineages remains entirely open: do they really correspond to clonal groups or, rather, to cryptic biological species within which sexual recombination occurs (Tibayrenc, 1993)?

Isoenzyme analysis using six different enzyme systems allowed the identification of 12 zymodemes among a population of 86 stocks (Dardé et al., 1992; Dardé, 1996; and unpublished data). Eight zymodemes comprise only one stock each, and four zymodemes (Z1, Z2, Z3 and Z4) cluster the majority of the stocks. Twenty-three strains were analysed by both multilocus enzyme electrophoresis (MLEE) and PCR-RFLP analysis of six single-copy genes: Z1 strains were equated to PCR-RFLP type I, Z2 and Z4 to type II and Z3 to type III (Dardé et al., 1996; Howe and Sibley, 1995). With these two kinds of markers (isoenzymes and single-copy genes), the allelic diversity is low (2–3 alleles/locus). These markers lack resolution for

* Corresponding author. Laboratoire de Parasitologie–Mycologie, CHU Dupuytren, 2 Avenue Martin Luther King, 87042 Limoges, France. Tel.: +33-555056160; fax: +33-555056177.

E-mail address: darde@unilim.fr (M.L. Dardé).

Abbreviations: DTU, discrete typing unit; EST, expressed sequence tags; MLEE, multilocus enzyme electrophoresis; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism

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phylogenetic and epidemiological studies, and for searching possible associations between genotypes and clinical forms of the disease.

Microsatellites represent another class of genetic markers. They are short tandem repeats of 2–6 nucleotides. Markers generated from these repeats are known to be highly polymorphic because of length variation of these repeats, and consequently, they exhibit multiple alleles, which makes them very informative for genetic studies. Polymorphism can be evaluated by PCR, which requires only a small amount of DNA, and allele sizing can be achieved with fluorescent primers and an automatic sequencer which assures reliability of the results.

We used multilocus genotyping with eight microsatellite markers on 84 stocks of *T. gondii* to design a molecular tool that has a higher resolution than isoenzymes for molecular epidemiology studies and to reconsider the problem of population structure in *T. gondii*.

2. Materials and methods

2.1. Source of Toxoplasma gondii stocks

We analysed 84 independent *Toxoplasma* stocks, previously typed by isoenzyme analysis (Table 1). Thirty-five of these stocks were analysed by SAG2 PCR-RFLP by other authors (Howe and Sibley, 1995; Honoré et al., 2000). Most of them originated from Europe (65) and North America (10). Six were from South America (French Guiana, Uruguay and Argentina). The geographic origin was not known for three. Sixty-one stocks were collected from human infections (38 congenital toxoplasmoses, 20 acquired toxoplasmoses and three without clinical history). The 23 animal stocks were isolated from different hosts (six sheep, seven pigs, three cats, one cow, one rabbit, one monkey, one chicken and one guinea pig). One stock (PMR) was without any information.

2.2. Selection of microsatellites in the Toxoplasma gondii database

A BLASTN search for dinucleotide repeats (Altschul et al., 1990) was performed at the *T. gondii* database maintained in the *Toxoplasma* Genome Web (http://www.ebi.ac.uk/parasites/toxo/toxpage.html). This made it possible to select 133 *T. gondii* sequences containing microsatellites: 83 contained $(TA)_n$, eight contained $(TG)_n$, and 42 contained $(CT)_n$. No sequence with $(GC)_n$ was found. Most of these sequences were from partial cDNA sequences called expressed sequence tags (ESTs). Currently, there are over 10,000 ESTs for *T. gondii* derived from RH tachyzoite and ME49 bradyzoite cDNA libraries (Ajioka et al., 1998; Manger et al., 1998). From this selection of microsatellite markers in ESTs, many sequences were excluded because of insufficient flanking sequences, or because the flanking sequences were unsuitable for primer design, usually due to a low G + C content. The second criterion of selection for ESTs was the number of dinucleotide repeats in the sequence published in GenBank, considering that those sequences that have more repeats generated more polymorphisms (Weber, 1990). We selected only ESTs with at least 10 dinucleotide repeats. Finally, eight microsatellite markers were selected (Table 2); six were located in ESTs of unknown function, identified by their GenBank accession numbers, and two were in the introns of two genes: *TUB2* coding for beta-tubulin, already used by Costa et al. (1997), and *TgM-A* coding for myosin A.

2.3. DNA extraction

Toxoplasma gondii stocks were stored in liquid nitrogen as tachyzoites cultivated in mouse peritoneal exudates or culture cells, or as bradyzoites in cysts of mouse infected brains. Some *T. gondii* stocks were obtained as tachyzoites cultivated in mouse peritoneal exudate, purified from host cells, and then stored as frozen pellets at −80°C. In all cases, DNA was extracted with the QIA amp DNA Mini Kit (Qiagen) and stored at +4°C.

2.4. Primers

For each microsatellite, primers were designed to amplify sequences with a size of less than 200 nucleotides and containing the microsatellite (Table 2). Primers used to amplify $(TG)_n$ repeats in the beta-tubulin gene have already been described (Costa et al., 1997). The forward primers were 5′ end labelled with fluorescein (6-FAM or HEX) to allow sizing of PCR products with an automatic sequencer. Primers were synthesised by Gibco BRL (Life Technologies) or Applied Biosystems.

2.5. DNA amplification

The amplification reaction mixture consisted of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dATP, dCTP and dGTP (Roche Diagnostics), 0.4 mM dUTP (Roche Diagnostics), 4 pmol of each primer, 5% (vol./vol.) dimethyl sulfoxide, 0.6 U *Taq* DNA polymerase (Amersham Pharmacia Biotech), 0.2 U uracyl DNA glycosylase (Roche Diagnostics) and 2.4 μl DNA in a 20 μl reaction volume. Amplifications were carried out in a Genecycler thermalcycler (Biorad), with 2 min at 50°C for uracyl DNA glycosylase action, 3 min at 94°C for initial denaturation, 35 cycles of denaturation at 94°C, annealing at 52°C for the *TgM-A* gene, 54°C for the *TUB2* gene, and for ESTs *AA519150*, *W35487*, *N61191*, *N82375* and *N83021*, and at 55°C for EST *N60608*, and extension at 72°C for 30 s each. The final cycle was followed by an additional 10 min at 72°C to complete partial polymerisation.

2.6. Electrophoresis of PCR products

PCR products were first separated by electrophoresis in 2% agarose gel to confirm DNA amplification. Then, PCR

Table 1
Characteristics of the 84 stocks of *Toxoplasma gondii*[a,b]

Stock	Host	Clinical findings	Geographic origin	Year	Z	*SAG2* type	Microsatellite group
ATIH	H	AIDS	Uruguay	–	Z1	ND	1
BK	H	CT (death)	Holland	1948	Z1	1	1
CT-1	A	Asymptomatic	USA	1989	Z1	1	1
ENT	H	CT	France (Strasbourg)	1985	Z1	1	1
FAJI	H	CT	France (Paris)	1991	Z1	1	1
GIL	H	CT	France (Nantes)	1988	Z1	1	1
MARTIN	H	Unknown	England	–	Z1	ND	1
MOR	H	CT	France (Nantes)	1988	Z1	1	1
P	H	CT	France (Toulouse)	1984	Z1	1	1
PIL	H	CT	France (Paris)	1994	Z1	ND	1
PMR	–	Unknown	Unknown	–	Z1	ND	1
RH	H	Encephalitis	USA	1939	Z1	1	1
FOU	H	DT (transplant)	France (Brest)	1992	Z1	1	1
DPHT	H	DT (transplant)	France (Paris)	1993	Z1	1	1
DUCH	H	Lymphadenopathy	Belgium	1971	Z1	ND	1
GPHT	H	CT	France (Paris)	1987	Z1	ND	1
WIK	H	Unknown	Unknown	–	Z1	ND	1
MAS	H	CT	France (Nice)	1991	Z5	1	2
P 80	A	Asymptomatic	USA (Iowa)	1991	Z11	ND	2
SSI 119	A	Asymptomatic	Denmark	1968	Z9	ND	2
P 89	A	Asymptomatic	USA (Iowa)	1991	Z10	3	2
CH1	A	Oocysts in faeces	France (Hte vienne)	1986	Z2	ND	2
C56	A	Asymptomatic	USA	1961	Z3	3	2
CEP	A	Oocysts in faeces	USA	1977	Z3	3	2
COR	H	DT (lymphoma)	France (Corbeil)	1992	Z3	3	2
LEG-NJA	H	AIDS	France (Nancy)	1995	Z3	3	2
LGE97-3	H	CT	France (Limoges)	1997	Z3	ND	2
M7741	A	Asymptomatic	USA	1958	Z3	3	2
NED	H	CT	France (Limoges)	1989	Z3	3	2
OPA	A	Asymptomatic	Uruguay	1993	Z3	ND	2
RUB	H	DT	French Guiana	1992	Z6	1	2
TONT	H	CT	France (Lyon)	1992	Z8	1	2
CAST	A	CT	Uruguay	1993	Z7	ND	2
VAND	H	DT	French Guiana	1997	Z12	1	2
GANGI	H	CT	Belgium	1984	Z2	ND	2
76K	A	Encephalitis	France	1963	Z2	ND	2
AUD	H	CT	France (Grenoble)	1988	Z2	ND	2
BEV	A	Asymptomatic	England	1959	Z2	2	2
BOU	H	AIDS	France (Limoges)	1985	Z2	2	2
CAL	H	CT	France (Limoges)	1989	Z2	ND	2
CH2	A	Oocysts in faeces	France (Hte vienne)	1987	Z2	ND	2
CHAM	H	CT (death)	France (Limoges)	1983	Z2	2	2
CHAT	H	CT	France (Lyon)	1988	Z2	ND	2
CRO	H	CT	France (Limoges)	1993	Z2	ND	2
DAM	H	DT (aplasia)	France (Amiens)	1993	Z4	2	2
DEGR	H	Meningitis	Belgium	1996	Z2	ND	2
FAR	H	CT	France (Limoges)	1991	Z2	ND	2
FOUA	H	CT	France (Lyon)	1988	Z2	ND	2
JONES	H	Lymphadenopathy	England	1986	Z2	ND	2
LGE 95-1	H	CT	France (Limoges)	1995	Z2	ND	2
ME49	A	Asymptomatic	USA	1965	Z2	2	2
MEZ	H	CT	France (Nice)	1999	–	ND	2
NTE	H	AIDS	Germany	1990	Z2	2	2
P100	A	Asymptomatic	USA (Iowa)	1991	Z2	ND	2
P101	A	Asymptomatic	USA (Iowa)	1991	Z2	ND	2
PIG3	A	Asymptomatic	Argentina	1993	Z2	ND	2
PON	H	CT	France (Limoges)	1986	Z2	ND	2
PRE	H	CT	France (Limoges)	1993	Z2	ND	2
PRU	H	CT	France (Limoges)	1964	Z2	2	2
PSP19	H	CT	France (Amiens)	1997	Z2	ND	2
PSP2	H	CT	France (Paris)	1994	Z2	ND	2
REN	H	CT	France (Limoges)	1989	Z2	ND	2

Table 1 (*continued*)

Stock	Host	Clinical findings	Geographic origin	Year	Z	*SAG2* type	Microsatellite group
ROD	H	CT	France (Limoges)	1988	Z2	ND	2
S1	A	CT	France (Aveyron)	1977	Z2	ND	2
S1K	A	CT	France (Aveyron)	1977	Z2	ND	2
S2K	A	CT	France (Hte vienne)	1979	Z2	ND	2
S3	A	CT	France (Hte vienne)	1980	Z2	ND	2
S3K	A	CT	France (Hte vienne)	1980	Z2	ND	2
SUR	H	AIDS	France (Nice)	1993	Z2	2	2
SZY	H	CT	France (Limoges)	1988	Z2	ND	2
BRE 97-1	H	DT (transplant)	France (Brest)	1997	Z4	2	2
C	H	CT	France (Paris)	1981	Z4	2	2
CHAMON	H	CT	France (Grenoble)	1988	Z4	2	2
DAS	H	CT	France (Limoges)	1992	Z4	ND	2
DEG	H	CT	France (Limoges)	1987	Z4	2	2
ELG	H	AIDS	France (Nice)	1990	Z4	2	2
GRE98-1	H	CT	France (Grenoble)	1998	Z4	ND	2
LGE 96-1	H	CT	France (Limoges)	1996	Z4	ND	2
LGE 94-2	H	CT	France (Limoges)	1994	Z4	ND	2
MAN-NJA	H	AIDS	France (Nancy)	1995	Z4	2	2
MILIS	H	Lymphadenopathy	Belgium	1962	Z4	ND	2
PSP12	H	CT	France (Paris)	1994	Z4	ND	2
SQM	A	DT	England	1990	Z4	ND	2
TG96	H	Unknown	Unknown	–	Z4	2	2

[a] *SAG2* types (PCR-RFLP) were described by Howe and Sibley (1995) and Honoré et al. (2000).
[b] Z, zymodeme; H, human; A, animal; CT, congenital toxoplasmosis; DT, disseminated toxoplasmosis.

products were diluted at 1/10, 1/5, or 1/2, or undiluted depending of the intensity of the band in agarose gels. One microlitre of each PCR product was mixed with 0.5 μl of the red dye labelled GeneScan™ size standard ROX 350 (Applied Biosystems) and 24.5 μl of deionised formamide. This mixture was then denatured and run on a polyacrylamide gel POP4 (Applied Biosystems) in a 47 cm/50 μm capillary for genetic analysis. Signals were read with an automatic sequencer (Abiprism 310 collection 1.0, mixed with 0.5 μl of the red dye labelled GeneScan™ size standard ROX 350 (Applied Biosystems, Courtabœuf, France)) and the data were stored and analysed with GeneScan™ analysis software (version 2.1, Applied Biosystems).

2.7. Diversity indices

A set of standard indices was evaluated in order to have an overall idea of the genetic and genotypic variability of this sample.

2.7.1. Genotypic diversity

The genotypic diversity is the number of multilocus genotypes on the total number of studied stocks (Eq. (1)):

$$G = g/n \tag{1}$$

where n is the number of individuals examined and g is the number of different multilocus genotypes.

2.7.2. Mean genetic diversity

According to Selander and Levin (1980), the mean genetic diversity can be evaluated by the following equation based on the frequency of electromorphs by locus (Eq. (2)):

$$H = \sum h/n \text{ with } h = 1 - \sum q_i^2 \tag{2}$$

where n is the number of locus, h is the genetic diversity for each locus and q_i^2 is the relative frequency of the ith allele for the considered locus. This formula is mathematically identical to that of the theoretical heterozygoty.

2.7.3. Discriminatory power: Simpson's index of diversity

By using Simpson's index of diversity (D), the ability of microsatellite polymorphism to discriminate between stocks was assessed and compared with that of the MLEE data. This index is based on the probability that two unrelated strain samples from the population under study will be attributed to different typing groups. It is calculated with the following equation (Eq. (3)):

$$D = 1 - \frac{1}{N(n-1)} \sum_{j=1}^{s} n_j\left(n_j - 1\right) \tag{3}$$

where N is the number of stocks in the sample population, s is the total number of different genotypes, and n_j is the number of strains belonging to the jth type. Simpson's index of diversity ranges from 0.0 to 1.0, where 1.0 indicates that with the typing method used, all stocks have a different genotype, and conversely, 0.0 indicates that all stocks are genetically identical.

Table 2
Microsatellite markers, PCR primers and allelic polymorphism

Marker	Definition	Repeat sequence[a]	PCR primers 5′–3′	Number of alleles	Size range of alleles (bp)
TUB 2	Beta-tubulin gene of TgRH tachyzoite	$(TG)_8$	1 **HEX**-CCAAGTTCTTCCGTCATTTC 2 CCTCATTGTAGAACACATTGAT	3	122–126
TgM-A	Myosin A gene of TgRH tachyzoite	$(TG)_9$	1 **6-FAM**-CATGTCCCTGTCGGTTTCTC 2 CGTAAATGCGGATGGAAACT	4	115–121
W35487[b]	TgESTzy77d12.r1 TgRH tachyzoite cDNA	$(CT)_{10}$	1 **6-FAM**-TGCTGCGGTCTTTTCTCTTC 2 AACATGCCGTTCCCTTCC	3	95–101
N60608[b]	TgESTzy20b09.r1 TgRH tachyzoite cDNA	$(TA)_{13}$	1 **6-FAM**-GAATCGTCGAGGTGCTATCC 2 AACGGTTGACCTGTGGCGAGT	7	131–145
N82375[b]	TgESTzy52d03.r1 TgRH tachyzoite cDNA	$(TA)_{13}$	1 **6-FAM**-TGCGTGCTTGTCAGAGTTC 2 GCGTCCTTGACATGCACAT	10	107–131
N83021[b]	TgESTzy58c09.r1 TgRH tachyzoite cDNA	$(TA)_{11}$	1 **6-FAM**-ACAACGACACCGCTATCTC 2 CTCTCTATACACAGACCGATTGG	10	125–161
N61191[b]	TgESTzy27f04.r1 TgRH tachyzoite cDNA	$(TA)_{11}$	1 **6-FAM**-CCGTATCACCAGATCATGTT 2 CTCTCACCTGATGTTGATGTAA	15	120–160
AA519150[b]	TgESTzz34e04.r1 TgME49 bradyzoite cDNA	$(TA)_{13}$	1 **HEX**-GTTGTCTATGCTGTCGTGCG 2 CACCCATAAACGGTTACTGGTC	16	134–170

[a] Number of dinucleotide repeats as published in GenBank for the corresponding strain.
[b] GenBank accession number.

2.8. Clustering and phylogenetic analysis

In order to establish the phylogenetic relationships among stocks, we applied a method based on genetic distances (Avise, 1994). Jaccard's distance (D_{ij}) was used according to the following formula (Jaccard, 1908; Eq. (4)):

$$D_{ij} = 1 - [a/(a + b + c)] \quad (4)$$

where:

a = number of bands that are common to the i and j stocks;
b = number of bands present in the i stock and absent in the j stock;
c = number of bands present in the j stock and absent in the i stock.

The neighbour-joining method (Saitou and Nei, 1987) was used to cluster the stocks from the distance matrix. The distances and dendrogram were computed using the Genetics Toolbox software elaborated in our laboratory (S. Noël and F. Chevenet), the PHYLIP software (Felsenstein, 1993, 3.5c ed. Seattle, WA. Department of Genetics, University of Washington) and analysed by the Treedyn software (Chevenet et al., 2000).

The robustness of the phylogenies inferred was tested by evaluating the agreement between MLEE and microsatellite genetic distances (concordance principle; Avise, 1994). Correlation between genetic distances for any pairs of stocks was evaluated with a non-parametric Mantel test (Mantel, 1967; Tibayrenc, 1995). Briefly, this test relies on a Monte Carlo simulation with 10^4 iterations, which randomly permutes the different cells of each distance matrix. Contrary to the classical correlation test, this randomisation procedure does not need any assumption about the number of degrees of freedom. In this case, the test was performed on the whole set of stocks. Tree methods were completed with a correspondence analysis carried out on the eight microsatellite data using the Genetix™ software.

2.9. Population genetics analysis

To evaluate the possible impact of genetic exchange on the population under survey, the population structure was explored by a set of complementary statistical tests. All of them take as the null hypothesis a situation of free genetic exchange (panmixia). The tests all explore the presence or absence of genetic recombination among loci and are based on the analysis of linkage disequilibrium, or non-random association of genotypes observed at different loci. They have been described by Tibayrenc et al. (1990), while the possible biases brought by geographical distance and natural selection have been exposed by Tibayrenc et al. (1991). In order to decrease the biases due to geographic separation, we have worked not only on the whole sample, but also on the sample from Limoges, France, only (see Table 1).

3. Results

3.1. GeneScan™ software analysis

PCR products consisted of a single peak of fluorescence after analysis by GeneScan™ software. Some additional peaks, shorter or longer by 2–4 bp, due to strand slippage of *Taq* polymerase on microsatellite sequence, were sometimes present, but their fluorescence intensity was always much less important than the main peak. This main peak

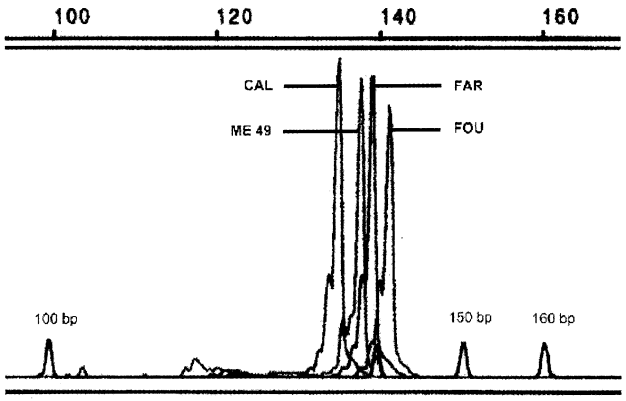

Fig. 1. Data analysed by GeneScan™ software after electrophoresis on an automated sequencer of PCR amplified DNA containing MS of expressed sequence tag *N60608* for CAL (135 bp), ME49 (137 bp), FAR (139 bp), and FOU (141 bp) stocks.

corresponding to the length of the PCR product was assigned to an allele. The resolution of electrophoresis in the automated sequencer is so high that we can easily separate peaks distant from 2 bp corresponding to a dinucleotide repeat. For example, in Fig. 1, we can separate four stocks having different alleles corresponding to 133, 135, 137 and 139 bp after amplification by PCR of the *N60608* microsatellite marker.

For the S3K stock, we observed two well-separated peaks with *N60608*, *N82375*, *N83021*, *N61191* and *AA519150* microsatellite markers. Since *T. gondii* is haploid, this means that S3K is a mixture of two stocks, one corresponding to S3 (Table 3).

3.2. Genetic diversity

The various statistical indices of genetic diversity were applied to only 83 stocks out of 84, since the S3K stock showed a mixture of two different genotypes (see above). Using the eight polymorphic microsatellite loci, 72 different multilocus genotypes were identified. The different results obtained with microsatellite markers on one hand and with MLEE data published in Dardé et al. (1992) on the other hand were compared in order to evaluate the resolution level of microsatellite markers.

All genetic diversity indices showed higher figures for the microsatellites than for MLEE. The results were 0.87 and 0.15, 0.66 and 0.34, 0.997 and 0.797, for genotypic diversity, mean genetic diversity and Simpson's index, respectively. Three MS markers showed a low allelic polymorphism: three alleles for EST *W35487* and *TUB2*, and four for *TgM-A*. The other microsatellite markers showed a higher allelic polymorphism, in particular ESTs *N61191* and *AA519150* with 15 and 16 alleles, respectively (Table 2). Association of the eight markers can differentiate 72 different multilocus genotypes in our population of 83 stocks, demonstrating a high discriminatory power.

3.3. Clustering analysis and phylogenetic classification

With the three less polymorphic microsatellites, we were able to classify most of the stocks (70) into three main groups corresponding to those previously described by isoenzyme or PCR-RFLP analysis. As shown in Table 4, 12 stocks characterised by an allelic association of 126 bp for *TUB2*, 101 bp for *W35487* and 121 bp for *TgM-A* corresponded to zymodeme 1, and 47 stocks characterised by an allelic association of 124 (*TUB2*), 95 (*W35487*), and 119 (*TgM-A*) corresponded to zymodemes 2 and 4, whereas 11 stocks characterised by an allelic association of 124 (*TUB2*), 95 (*W35487*) and 117 (*TgM-A*) corresponded to zymodeme 3 and to two atypical zymodemes (Z6 and Z8). However, 14 stocks could not be assigned to any of these three genetic groups. In some cases, their atypical genotype has already been noted by MLEE analysis (Z5, Z7, Z9, Z10, Z11 and Z12; Dardé, 1996). Besides, the microsatellite analysis distinguished a subgroup of five stocks (DPHT, FOU, DUCH, GPHT and WIK) among the stocks classified by MLEE as zymodeme 1.

When the eight microsatellite markers are used rather than only the three less variable ones, a graphic representation of correspondence analysis clearly shows the separation of our *Toxoplasma* population into two groups: group 1 stocks are differentiated from other stocks belonging all together to group 2 (Fig. 2). Stocks classified as zymodeme 3 or as atypical zymodemes (Z5–Z12) fall into group 2, even if, inside this group, they are the nearest to group 1. Besides, one can clearly distinguish in group 1, corresponding to zymodeme 1, two subgroups with five stocks differing from the other stocks by the allele 117 of *TgM-A* typically associated with stocks of group 2.

When tree analysis is considered, all stocks pertaining to zymodeme 1 fell into the same subdivision on the neighbour-joining tree built from microsatellite distances (Fig. 3). This cluster, group 1, was supported by a specific microsatellite marker (microsatellite of EST *W35487*, allele 101) and a specific MLEE marker (ASAT, type 1). The rest of the stocks fell into another cluster, group 2 (Fig. 3). However, this group was supported by no microsatellite marker and only by one specific MLEE marker (ASAT, type 2). These

Table 3
S3 and S3K genotypes obtained with the eight microsatellite markers

Stock	*TUB 2*	*W35487*	*TgM-A*	*N60608*	*N82375*	*N83021*	*N61191*	*AA519150*
S3	124	95	119	135	121	131	134	136
S3K	124	95	119	135/139	121/109	131/135	134/144	136/144

Table 4
Allelic combination of the three less polymorphic microsatellite markers, their repartition in the 84 stocks under study and their correspondence with zymodemes

Zymodeme	Microsatellite group	*W35487*	*TUB 2*	*TgM-A*	Number of stocks
Z1	1	101	126	121	12
Z1	1	101	126	117	5
Z2 and Z4	2	95	124	119	47
Z2	2	97	124	119	2
Z2	2	95	124	121	1
Z3	2	95	124	117	9
Z6 and Z8	2	95	124	117	2
Z5, Z9, Z10 and Z11	2	95	126	117	4
Z7	2	95	122	119	1
Z12	2	95	126	115	1

two subdivisions shown by tree analysis are identical to the correspondence analysis groupings. Stocks classified as zymodeme 3 are only a subgroup of group 2. The correlation between MLEE and microsatellite genetic distances, evaluated by the Mantel test (Mantel, 1967), was highly significant ($P < 10^{-4}$ with $r = 0.57$). This shows a strong statistical agreement between the phylogenies obtained from the two kinds of genetic markers.

3.4. Population structure analysis of *Toxoplasma gondii*

Linkage disequilibrium tests were applied to various subsets of the total sample in order to check some working hypotheses and to lower the bias due to geographic separation (see Section 4). The analysis of all the tests performed gave highly significant results (we obtained $P < 10^{-4}$ for most of them).

4. Discussion

4.1. Discriminatory power of microsatellites

Genetic typing methods of *T. gondii* strains have been extensively perfected in recent years. From a technical point of view, many tools usable for genetic studies on single-copy loci have been used: RFLP (Sibley and Boothroyd, 1992), PCR-RFLP (Sibley and Boothroyd, 1992; Parmley et al., 1994; Asai et al., 1995; Howe and Sibley, 1995; Howe et al., 1997; Binas and Johnson, 1998; Mondragon et al., 1998; Owen and Trees, 1999), sequencing (Luton et al., 1995; Rinder et al., 1995; Meisel et al., 1996; Windeck and Gross, 1996; Fazaeli et al., 2000; Lehmann et al., 2000), random amplified polymorphic DNA PCR (RAPD; Guo et al., 1997), and isoenzyme analysis (Dardé et al., 1992; Dardé, 1996; and unpublished data). Most of these studies were performed on a small sampling of stocks and described the use of only one locus, mainly the *SAG2* locus, for genetic typing. These limitations forbid any valuable conclusions about the population structure of *T. gondii*. Only two studies considered a larger population of stocks with several independent genetic markers: six enzymatic systems on 86 stocks (Dardé et al., 1992; Dardé, 1996; and unpublished data) and six independent single-copy loci on 106 stocks (Howe and Sibley, 1995). However, the polymorphism detected with these markers remained low (2–4 alleles by locus). A better resolution can be obtained by the use of multiple-copy loci, such as the BS probe (Sibley and Boothroyd, 1992; Howe and Sibley, 1994; Messina et al., 1996), TGR1E probe (Cristina et al., 1991), or mobile genetic elements (Terry et al., 2001), however, they only concern one genetic marker.

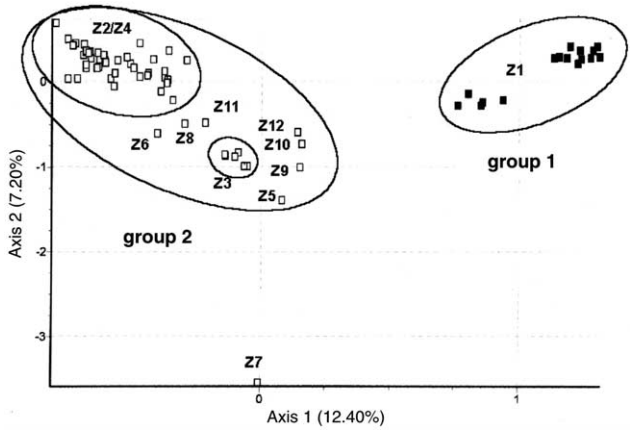

Fig. 2. Graphic representation of correspondence analysis carried out on the eight microsatellite data using Genetix™ software for 83 *Toxoplasma gondii* stocks and correspondence of microsatellite groups with zymodemes.

The aim of this study was to characterise *T. gondii* stocks with easily usable, highly polymorphic multilocus markers. Among the technical tools used in the study of DNA polymorphism, microsatellites are considered as the most informative, because allelic polymorphism is considerable and is caused by a genetic process that is totally different from those of other genetic markers previously described. Hypervariability is explained by the accumulation of length mutations by intra-allelic polymerase slippage on microsatellite sequence during replication.

Microsatellite markers have been used for typing several protozoan parasites, such as *Trypanosoma cruzi* (Oliveira et al., 1998), *Trypanosoma brucei* (Hide and Tilley, 2001),

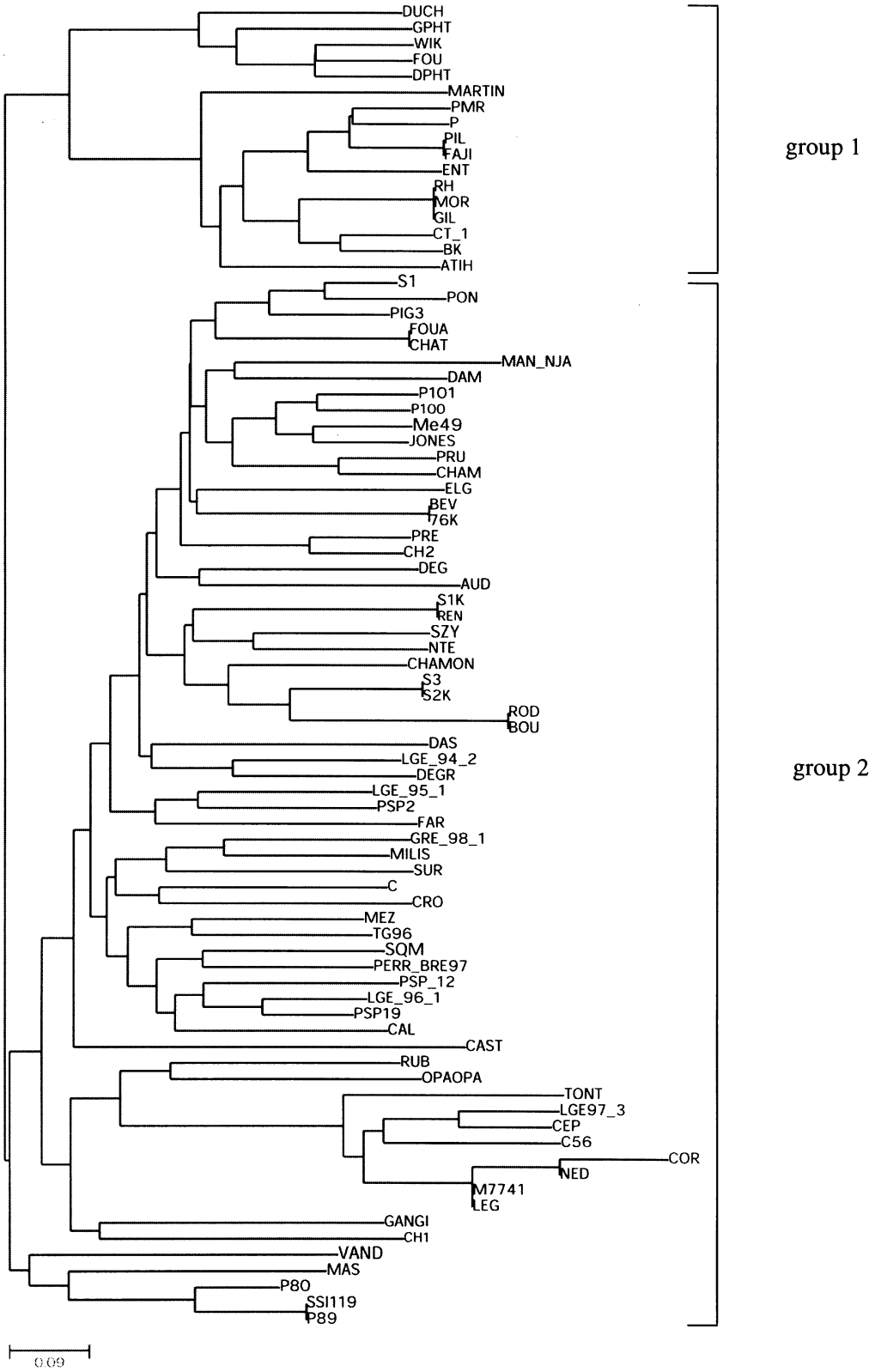

Fig. 3. Neighbour-joining tree derived from Jaccard's genetic distances calculated on the basis of the microsatellite data of the 83 stocks of *Toxoplasma gondii*.

Leishmania spp. (Rossi et al., 1994), *Plasmodium falciparum* (Su et al., 1998; Ferdig and Su, 2000) and *Cryptosporidium parvum* (Caccio et al., 2000, 2001; Feng et al., 2000). *Toxoplasma gondii* stocks have been typed with microsatellite markers only by Costa et al. (1997) using the polymorphism of the dinucleotide $(TG)_n$ repeat in the intron of the beta-tubulin gene. However, only two alleles of seven and eight (TG) repeats were found with this marker. In the present study, multilocus genetic typing was performed with eight microsatellite markers selected in GenBank (see Section 2).

The different genetic diversity indices confirm the high level of resolution of microsatellite markers by comparison with MLEE markers, but also with the PCR-RFLP data obtained by Howe and Sibley (1995). In our study, 3–16 alleles were found on a population of 83 stocks, whereas only 2–4 alleles were found with six single-copy loci by PCR-RFLP (1995) or with six enzymatic systems by isoenzyme analysis (Dardé et al., 1992; Dardé, 1996; and unpublished data). When the total number of multilocus genotypes is considered, the results are 15 out of 106 stocks, 12 out of 86 stocks, and 72 out of 83 stocks for the studies by Howe and Sibley (1995), Dardé (1996; and unpublished data), and the present study, respectively. This makes the association of these eight microsatellite markers one of the most discriminating tools used for genetic studies on *T. gondii* stocks.

4.2. Phylogenetic analysis

Both correspondence analysis and tree methods showed two genetic groups, termed group 1 and group 2. The first one (corresponding to zymodeme 1) is genetically well individualised and can be characterised by a specific microsatellite marker and a specific MLEE marker. It does not strictly correspond to type I as defined only by *SAG2* PCR-RFLP: among 14 stocks of our population exhibiting a type I with *SAG2* PCR-RFLP (Honoré et al., 2000; Howe and Sibley, 1995), four belonged to group 2. This demonstrates that multilocus analysis should be preferred for typing *Toxoplasma*. The robustness of group I is corroborated by the good correspondence between microsatellite typing and MLEE. Agreement between two kinds of genetic markers is a strong presumption that the phylogenetic subdivision uncovered is robust (Tibayrenc, 1998), according to the concordance principle (Avise, 1994). According to these criteria, group I can be equated to a 'discrete typing unit' (DTU): a set of multilocus genotypes that are genetically more related to each other than to any other ones and that can be characterised by one or more common genetic markers or 'tags' (Tibayrenc, 1998). The situation is slightly less clear for group 2, since this subdivision cannot be characterised by any microsatellite marker. However, it is a discrete subdivision, that can be characterised by a MLEE specific marker (ASAT type 2). For these reasons, it too can be equated to a DTU.

These results showing two distinct genetic groups within the species *T. gondii* are in agreement with the data by Lehmann et al. (2000), dealing with seven different gene sequences (five housekeeping genes and two antigen-coding genes). Our analysis suggests that group 1 is subdivided into two lesser subgroups: a subgroup of five stocks is characterised by allele 117 of the *TgM-A* MS marker, an allele that is typically observed only in group 2. However, these subgroups are not supported by specific microsatellite markers. The presence of two populations in type I has been also described by Fazaeli et al. (2000). It could be inferred that this subgroup shows a recombination event between group 1 and group 2. It is worth noting that all the stocks of group 1, besides their genetic individualisation, present other characteristics, both biological (high virulence for mice) and epidemiological (very rarely isolated in the field; Dardé, 1996; Johnson, 1997). Genetic diversity inside group 2 is more important and the behaviour in mice of group 2 isolates is more heterogeneous, a few of them being virulent in mice. The virulent stocks belonging to group 2 are those which were typed by other methods as atypical zymodemes (Z5, Z6, Z7, Z8, Z12) and as type I with *SAG2* PCR-RFLP. With microsatellite typing, they showed unique alleles (allele 115 of *TgM-A* marker for VAND (Z12), allele 122 of *TgM-A* marker for CAST (Z7)) or atypical recombination of alleles which could be the witness of recombination events.

Howe and Sibley (1995) showed that the vast majority of stocks were grouped into three types that were designated I, II and III. These three groups were not uncovered in the present study with eight microsatellite markers. Stocks belonging to type III or zymodeme 3 are a subgroup of group 2. They could be distinguished from other group 2 stocks by allele 117 of *TgM-A*, but as already noted, this allele is also detected in a subgroup of group 1 and in some stocks belonging to atypical zymodemes. These stocks could also be the results of genetic transfer between the two main groups.

4.3. Genetic recombination and population structure

Tibayrenc et al. (1991), by analysing published isoenzyme data (Dardé et al., 1988, 1992), proposed that *T. gondii* has a clonal population structure. This working hypothesis has been supported by Sibley and Boothroyd (1992), who proposed that *T. gondii* is subdivided into two major clonal lineages, of which one is virulent in mice. Later, Howe and Sibley (1995) postulated the existence of three lineages instead of two. Tibayrenc (1993) proposed to perform linkage disequilibrium analyses, not only on the whole *T. gondii* species, but also within each lineage, in order to decide whether they correspond to cryptic species or to clonal lineages (Maynard Smith et al., 1993). The question conditions the expected long-term stability of multilocus genotypes: if they are the result of clonal propagation, they are stable enough to be used as epidemiological markers; if they represent only individual

variants within a cryptic species, they are ephemeral and improper for epidemiological tracking (Tibayrenc, 1993). A difficulty in such an approach is that, by doing that, one undergoes a considerable loss of information with an important risk of a statistical type II error (Tibayrenc, 1993). The present work aimed to reconsider this debate with the help of a broad sample of strains and a highly discriminating marker, which lowers the risk of a statistical type II error increased by the use of poorly discriminating markers. Linkage disequilibrium tests were performed on the whole sample, on geographical subdivisions of the sample to avoid the bias due to geographical distance (Wahlund effect; Tibayrenc et al., 1991), and on each of the two genetic groups individualised here to check whether they correspond to clonal lineages or to cryptic species. Most of the tests were highly significant, with $P < 10^{-4}$. Interestingly, the tests remained positive when counting only once all repeated genotypes, a procedure recommended by Maynard Smith et al. (1993) to show that linkage disequilibrium is due to clonal evolution rather than to 'epidemic clonality' (occasional bouts of clonal propagation in a basically sexual species). These results make it possible to conclude: (i), that linkage disequilibrium in *T. gondii* is not due to a Wahlund effect; (ii), that the two main lineages identified in the present study are the result of clonal evolution rather than cryptic biological speciation or epidemic clonality. This result does not rule out the possibility of occasional bouts of sexuality. As a matter of fact, we have recorded here some indications of horizontal gene transfer (see the presence of allele 117 of the *TgM-A* marker in group 1), and Howe and Sibley (1995) have suggested the possibility of recombinant genotypes in *T. gondii*. Moreover, the fact that group 2 is poorly individualised could be explained by the existence of a certain rate of horizontal gene transfer, the main effect of which is to cloud phylogenies. Still, the fact remains that our data do suggest that long-term clonal propagation plays a major role in structuring *T. gondii* populations. It is interesting to note that identical genotypes are found in different countries (European and American countries) and in different hosts (humans and sheep). This result is epidemiologically relevant, since such unchanged genotypes are obviously the product of a common clonal descent, if one considers the high resolution of microsatellites. This result remains valid if we combine microsatellite and MLEE data, which even increases the resolution of this genetic typing.

4.4. Microsatellite markers applications: detection of mixed infections and epidemiological studies

The high discriminatory power of the eight microsatellite markers appears to be very useful in epidemiological studies of *T. gondii*, for detection of mixed infections or for parasite identification in laboratory cultures. Since *T. gondii* is haploid, only one peak is expected for a given locus corresponding to one allele. More than one peak will be detected if mixed infections with different alleles are present in the sample. A combination of eight single-copy microsatellite markers will provide enough information to distinguish stocks. This contrasts with typing with single-copy loci (not enough discrimination between strains) or even multicopy loci which cannot distinguish mixed infection in samples (Ferdig and Su, 2000). With the eight microsatellite markers, we were able to detect a natural mixed infection with S3K stock. S3K stock was collected from an epidemic abortion of sheep in France in 1980. When isolated, it was avirulent in mice. However, after several i.p. inoculations of cysts to mice, some mice developed ascites with few tachyzoites. This acute line (called S3) was then maintained as tachyzoites by i.p. inoculations of tachyzoites twice a week, whereas the chronic line (S3K) was maintained by i.p. inoculation of cysts every 6 months. Typing with eight microsatellite markers showed that S3K is a mixture of two group 2 stocks, one corresponding to S3 (Table 3). The acute line, S3, mixed at the origin with the chronic line contained in S3K has been selected by repeated inoculations to mice. They could not be differentiated with MLEE analysis.

Besides its interest in phylogenetic study, multilocus microsatellite analysis, which detects an extensive polymorphism in *T. gondii*, provides us with a powerful tool for strain typing and epidemiological tracking. Identical genotypes with the eight microsatellite markers were found in epidemiologically related stocks. That was the case for two group 1 stocks (MOR and GIL) isolated from the liver of two human foetuses at a 1 week interval in the same town and for two other stocks belonging to group 2 (CHAT and FOUA) and isolated at a 2 week interval from aborted foetuses in another town. The availability of this new tool will make it possible to reconsider the long-open question of the possible role of the parasite's genetic diversity on the different clinical forms of human toxoplasmosis.

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