JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2004, p. 461–466 Vol. 42, No. 1
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.1.461–466.2004

Genetic Diversity and Population Structure of *Mycobacterium tuberculosis* in Casablanca, a Moroccan City with High Incidence of Tuberculosis

Loubna Tazi,[1]* Jamila El Baghdadi,[2] Sarah Lesjean,[3] Camille Locht,[3] Philip Supply,[3] Michel Tibayrenc,[1] and Anne-Laure Bañuls[1]

Génétique des Maladies Infectieuses, UMR CNRS-IRD 9926, IRD, 34394 Montpellier Cedex 5,[1] and Laboratoire des Mécanismes Moléculaires de la Pathogenèse Bactérienne, INSERM U447, Institut Pasteur de Lille, F-59019 Lille Cedex,[3] France; and Service des Mycobactéries, Institut Pasteur du Maroc, Casablanca, Morocco[2]

Received 31 March 2003/Returned for modification 11 June 2003/Accepted 11 August 2003

Although lower-resource countries have by far the highest burden of tuberculosis, knowledge of *Mycobacterium tuberculosis* population structure and genetic diversity in these regions remains almost nonexistent. In this paper, 150 Moroccan *M. tuberculosis* isolates circulating in Casablanca were genotyped by random amplified polymorphic DNA analysis using 10 different primers and by mycobacterial interspersed repetitive units-variable number of tandem repeats typing at 12 loci. The population genetic tests revealed a basically clonal structure for this population, without excluding rare genetic exchanges. Genetic analysis also showed a notable genetic polymorphism for the species *M. tuberculosis*, a weak cluster individualization, and an unexpected genetic diversity for a population in such a high-incidence community. Phylogenetic analyses of this Moroccan sample also supported that these isolates are genetically heterogeneous.

Today, tuberculosis remains the leading cause of mortality due to a single infectious agent. The incidence is increasing worldwide, especially in developing countries. It has been estimated that one third of the world's population is infected, which represents a huge reservoir for the disease (7, 32).

The understanding of tuberculosis transmission dynamics has been greatly enhanced by the development of various DNA typing methods (5, 14, 20, 22, 36, 47, 48). New markers based on variable number of tandem repeats (VNTR) in eukaryotic-like minisatellites have been identified in 12 independent loci of the *Mycobacterium tuberculosis* genome. These elements, named mycobacterial interspersed repetitive units (MIRUs), have already been used for strain typing and for molecular epidemiology studies of tuberculosis (27, 40). Moreover, contrary to the standard restriction fragment length polymorphism based on IS*6110*, these markers reveal the variability of independent genetic loci and are therefore better usable for population genetics purposes, since the analysis of linkage disequilibrium requires that independent loci are identified.

Besides these specific markers for *M. tuberculosis* typing, the random amplified polymorphic DNA (RAPD) technique has been used for various kinds of microorganisms (2, 52), including *M. tuberculosis* isolates (16, 33). Moreover, since it samples the overall diversity of genomes and corresponds therefore to a generalist marker (43), this method allows the comparison of genetic diversity between different microorganisms.

In Morocco, the global incidence of all clinical forms of tuberculosis is very high, with nearly 104 new cases per 100,000 inhabitants yearly, representing about 30,000 new cases per year. Casablanca, the biggest city and the economic capital of Morocco, includes almost one-fifth of the total cases recorded in the country (National Anti-tuberculosis Fight Program, Department of Health, Rabat, Morocco). Despite the use of the directly observed treatment short-course strategy for controlling the disease since 1991, the incidence is still increasing (29). Several reports on the incidence and the distribution of tuberculosis in the different communities of the country have been published (1, 11, 23), but genetic studies are rare (9, 10, 24). In this work, RAPD and MIRU-VNTR were used to analyze the genetic diversity and the population structure of the *M. tuberculosis* isolates circulating in Casablanca.

The study included 150 *M. tuberculosis* sputum isolates, mostly collected in 1997 (49 isolates) and 1998 (60 isolates). They came from six different districts of Casablanca: "Hay Mohammadi Aïn Sebaa" (HMAS) (97 isolates), "Ben Msik Sidi Othmane" (22 isolates), "Casablanca Anfa" (16 isolates), "Fida Derb Sultan" (3 isolates), "Hay Hassani Aïn Chock" (6 isolates), and "Mohammedia" (2 isolates), except 1 isolate, which came from Benslimane, a city located about 50 km from Casablanca. For three isolates, the patients lived in Casablanca, but there is no information about the district and the year of diagnosis of tuberculosis. Most of the isolates were collected from patients living in the HMAS district, where the incidence of pulmonary tuberculosis is even higher than in the other districts. Nearly 450 new cases per year were recorded in this district, but not all the isolates were cultured and available for genetic analysis. All the isolates included in this study were cultured on Löwenstein-Jensen medium and identified as *M. tuberculosis* by conventional biochemical methods (15). The DNA was extracted by a standardized protocol described previously (49).

* Corresponding author. Present address: Department of Ecology and Evolutionary Biology, University of California, Irvine, 321 Steinhaus Hall, Irvine CA 92697-2525. Phone: (949) 824-8295. Fax: (949) 824-2474. E-mail: ltazi@uci.edu.

TABLE 1. Sequences of the 10 RAPD primers used in this study

Primer	Sequence
A2	5′-TGCCGAGCTG-3′
N9	5′-TGCCGGCTTG-3′
R8	5′-CCCGTTGCCT-3′
R13	5′-GGACGACAAG-3′
U10	5′-ACCTCGGCAC-3′
U13	5′-GGCTGGTTCC-3′
U16	5′-CTGCGCTGGA-3′
U17	5′-ACCTGGGGAG-3′
U19	5′-GTCAGTGCGG-3′
U20	5′-ACCTCGGCAC-3′

TABLE 2. Diversity indices obtained with RAPD and MIRU data

Sample (n^a)	Genotypic diversity (RAPD/MIRU)	Polymorphism rate (RAPD/MIRU)	Mean genetic diversity (H) (RAPD/MIRU)
Moroccan isolates (150)	0.91/0.63	1/0.8	0.48/0.38
HMAS (97)	0.89/0.66	0.9/0.8	0.42/0.37
Year 1997 (49)	0.96/0.73	0.9/0.8	0.43/0.35
Year 1998 (60)	0.95/0.78	1/0.7	0.44/0.38
HMAS—year 1997 (44)	0.95/0.73	0.9/0.7	0.4/0.35
HMAS—year 1998 (30)	0.97/0.9	0.9/0.7	0.36/0.38

[a] n, sample size.

Twenty-two additional stocks were kindly provided to us in the form of purified DNA and were added to the collection for comparison purposes and phylogenetic analyses, bringing the total samples analyzed up to 172. They include three Moroccan stocks of *Mycobacterium bovis* (J. Berrada, Institut Agronomique et Vétérinaire, Rabat, Morocco), 10 *M. tuberculosis* stocks from other countries, and 9 stocks from other species of the *M. tuberculosis* complex (K. Kremer and D. van Soolingen, RIVM, Bilthoven, The Netherlands). These 19 stocks had already been described and analyzed in previous studies (22, 40).

The RAPD technique was performed according to the method of Williams et al. (52). For each of the 10 primers used (Table 1), a negative control was added to check the specificity of the pattern obtained. The reproducibility of the patterns was tested for each primer and each stock. All bands obtained on RAPD gels were numbered and scored as presence or absence data.

MIRU typing with loci 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, and 40 was performed by using the method described previously (27, 41). These data are available at http://www.ibl.fr/mirus/mirus.html. Fifty stocks were also analyzed by a system combining multiplex PCR analysis on a fluorescence-based DNA analyzer with computerized automation of the genotyping. The protocol for the amplification and the computer-assisted typing was described previously (27, 40).

To obtain the genetic variability of the isolates circulating in Casablanca, a set of diversity indices including genotypic diversity, polymorphism rate (30), and mean genetic diversity (35) was evaluated.

The population structure was explored by a set of complementary statistical tests (*d1*, *d2*, *e*, *f*, and *g* tests) (45, 46). All of them take panmixia (random genetic exchange) as a null hypothesis. They all explore the presence or absence of genetic recombination among loci and are based on the analysis of linkage disequilibrium (nonrandom association of genotypes occurring at different loci) (45, 46). The *g* test estimates the correlation between independent genetic markers by a nonparametric Mantel test (25), which is based on Monte Carlo simulation with 10^4 iterations (44). A correlation between independent genetic markers is a particularly strong evidence of linkage disequilibrium (44, 46).

Phylogenetic relationships among the isolates were inferred from RAPD and MIRU data by using neighbor-joining analysis (34) based on Jaccard's distances matrix (19) and the Wagner analysis (12, 21) with bootstrapping (8) to test the robustness of the nodes. For all the analyses, a *Mycobacterium canettii* stock was used as an outgroup (22). The distance matrix and the phylogenetic trees were computed using the Genetics Toolbox and Treedyn softwares (6) designed in our laboratory and the PHYLIP software (J. Felsenstein, 3.5c ed., 1993; Department of Genetics, University of Washington, Seattle).

For the genetic diversity analysis and the study of the population structure of *M. tuberculosis* in Casablanca, only the 150 Moroccan isolates were considered. Different subgroups were defined in order to better understand the distribution of the diversity in the sample under study and to avoid biases due to geographical and/or temporal separation (Wahlund effect) (46). Due to weak sampling, the districts "Ben Msik Sidi Othmane" and "Casablanca Anfa" were not included in these analyses. The different genetic indices were calculated in each group for the RAPD and MIRU data (Table 2).

The values obtained with the RAPD data were almost always higher than those obtained with the MIRU data, which suggests that the RAPD technique allows detection of more mutational events than the MIRU technique. However, for both genetic markers, the diversity was notable and almost equivalent in all the subgroups under study (Table 2). The polymorphism rate showed that all the RAPD primers gave polymorphic patterns in all the groups, and for the MIRU-VNTR loci only 1 out of the 12 loci (locus 24) was totally monomorphic (data not shown).

The phylogenetic analysis showed that the Moroccan *M. tuberculosis* isolates were not separated from the other *M. tuberculosis* stocks, regardless of the analysis used (Wagner or neighbor-joining analysis based on MIRU or RAPD data). Figure 1 shows a neighbor-joining tree based on the MIRU-VNTR data, with the overall sample under study. All 22 reference stocks were interspersed among the Moroccan *M. tuberculosis* isolates, except for *Mycobacterium microti* P56, which fell apart from the whole group together with *M. canettii* P48, used as an outgroup. Two out of three Moroccan stocks of *M. bovis* were also mixed with the Moroccan *M. tuberculosis* isolates, except for K5. Moreover, except for *Mycobacterium africanum* P55, all the other reference stocks not belonging to the *M. tuberculosis* species were clustered apart from the Moroccan *M. tuberculosis* isolates. From the phylogenetic trees, we could distinguish different groups, but none was associated with the geographical origin of the patients. Moreover, the Wagner analysis with bootstrapping performed on the same data gave mainly low bootstrap values (data not shown).

The linkage disequilibrium, the degree of overrepresentation of multilocus genotypes, and the absence of recombinant

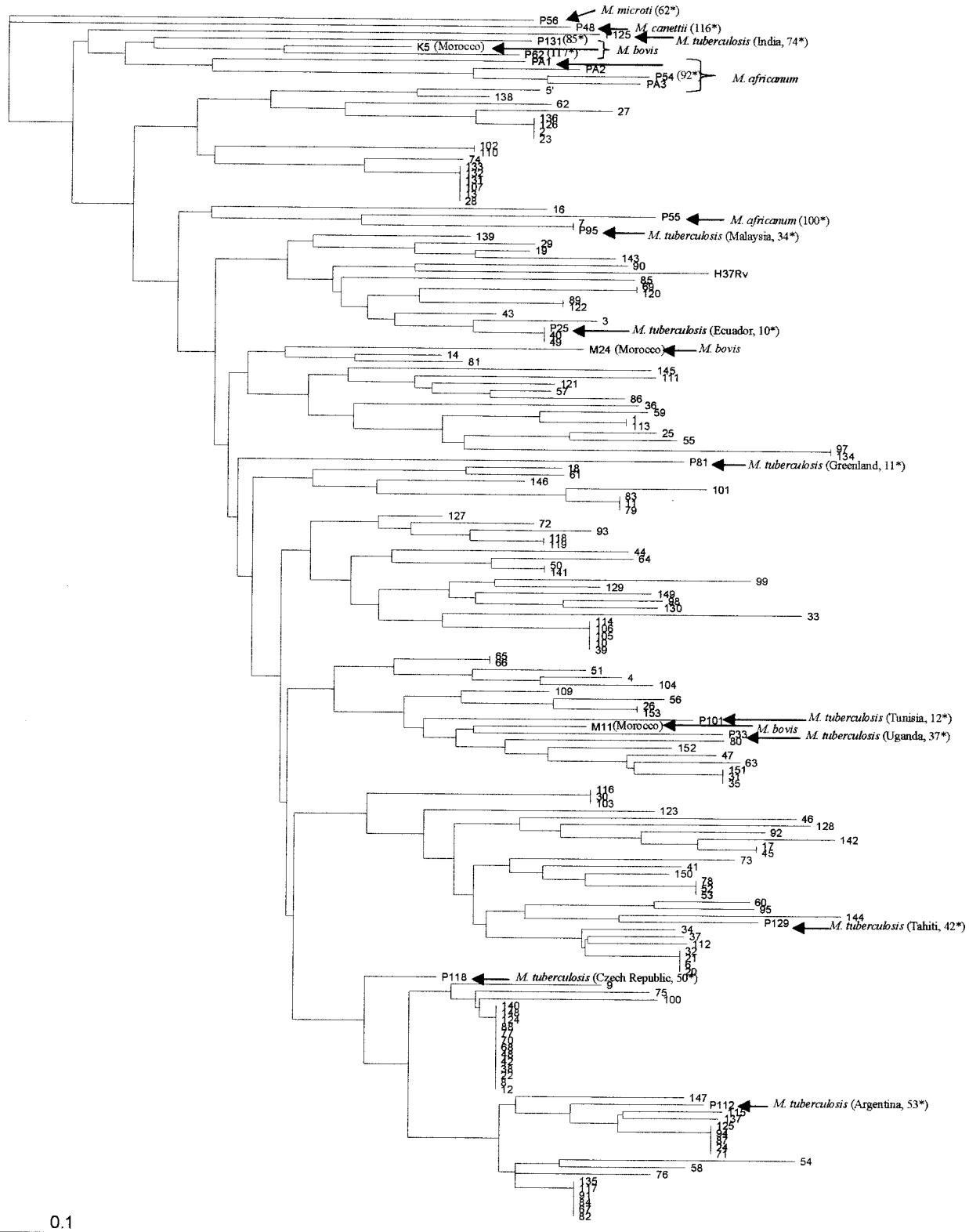

FIG. 1. Neighbor-joining tree based on MIRU-VNTR data with all the samples under study (172 stocks). An *M. canettii* stock (P48) is used as an outgroup. The reference samples with an asterisk have been analyzed in previous studies (22, 40). For the reference *M. tuberculosis* stocks, the geographic origin is indicated. The other stocks in the tree correspond to the Moroccan *M. tuberculosis* sample.

TABLE 3. Population genetics analysis for the Moroccan *M. tuberculosis* population with RAPD and MIRU data[a]

Sample (n^b)	Test *f* (RAPD/MIRU)	Test *d1* (RAPD/MIRU)	Test *d2* (RAPD/MIRU)	Test *e* (RAPD/MIRU)
Moroccan isolates (150)	$P \leq 10^{-4}/P \leq 10^{-4}$	$P \leq 10^{-4}/P \leq 10^{-4}$	$2.6 \times 10^{-2}/P \leq 10^{-4}$	$P \leq 10^{-4}/P \leq 10^{-4}$
HMAS (97)	$P \leq 10^{-4}/P \leq 10^{-4}$	$1.3 \times 10^{-3}/P \leq 10^{-4}$	$3.6 \times 10^{-1}/P \leq 10^{-4}$	$P \leq 10^{-4}/P \leq 10^{-4}$
Year 1997 (49)	$3.7 \times 10^{-3}/P \leq 10^{-4}$	$4.6 \times 10^{-2}/P \leq 10^{-4}$	$4.9 \times 10^{-1}/P \leq 10^{-4}$	$1.4 \times 10^{-1}/P \leq 10^{-4}$
Year 1998 (60)	$P \leq 10^{-4}/P \leq 10^{-4}$	$5.7 \times 10^{-3}/P \leq 10^{-4}$	$2.8 \times 10^{-2}/2.4 \times 10^{-3}$	$1.5 \times 10^{-2}/P \leq 10^{-4}$
HMAS—year 1997 (44)	$4.6 \times 10^{-3}/P \leq 10^{-4}$	$8.1 \times 10^{-2}/P \leq 10^{-4}$	$6.4 \times 10^{-1}/P \leq 10^{-4}$	$2.6 \times 10^{-1}/P \leq 10^{-4}$
HMAS—year 1998 (30)	$1.6 \times 10^{-1}/10^{-4}$	$1.3 \times 10^{-1}/P \leq 10^{-4}$	$6.2 \times 10^{-1}/1.5 \times 10^{-1}$	$6.2 \times 10^{-1}/3 \times 10^{-4}$

[a] The *P* value (statistical significance) is settled at 0.01 for the *f* test and at 0.05 for the other tests (*d1*, *d2*, *e*, and *g*) (45, 46).
[b] *n*, sample size.

genotypes were analyzed for all RAPD primers and MIRU-VNTR loci. To lower the potential occurrence of geographical and/or temporal bias within the population (Wahlund effect), the linkage disequilibrium tests were applied separately per district and per year of collection. The samples studied and the results obtained are listed in Table 3. The values obtained for the *f* tests were almost constantly highly significant regardless of the genetic marker, indicating significant linkage disequilibrium in all the samples. The Mantel tests (*g* tests) performed between the two sets of data were also significant for almost all the groups studied ($P \leq 0.05$), indicating also significant linkage disequilibrium in the population under study. This was further confirmed by the results obtained with *d1*, *d2*, and *e* tests, suggesting globally significant overrepresentation of certain multilocus genotypes (tests *d1* and *d2*) and the absence of recombinant genotypes (test *e*), two additional complementary criteria of clonality. In order to distinguish the occasional spread of ephemeral clonal genotypes in a basically sexual species, referred to as epidemic clonality by Maynard Smith et al. (26), from a predominant clonal evolution, the linkage disequilibrium tests were done by processing each genotype as a single individual. Again, globally significant results were obtained for these analyses ($P \leq 0.01$ for the *f* test; data not shown).

This work represents the first genetic study on tuberculosis transmission and *M. tuberculosis* population structure in Morocco. Among the 150 Moroccan isolates, 149 have been randomly selected in different districts of Casablanca. Therefore, this sample, although not exhaustive, provides a good prospective basis for population genetics analysis. With appropriate quality controls, RAPD is usable for any organism. MIRU-VNTR genotyping was used as a convenient reference, since it has recently shown its potential for molecular epidemiology and population structure studies of *M. tuberculosis* (27, 40, 42).

The results obtained with the two markers gave convergent indications of different manifestations of linkage disequilibrium, which is considered strong evidence for a basically clonal population structure (44–46). Moreover, the hypothesis of epidemic clonality proposed by Maynard Smith et al. (26) is not corroborated by the significant results obtained when each genotype was treated as a single individual in the linkage disequilibrium tests. These data and those obtained by Supply et al. (42) in a South African population with MIRU-VNTR and IS*6110*-restriction fragment length polymorphism analysis support the hypothesis that *M. tuberculosis* undergoes predominant clonal evolution, even in regions in which different strains may have ample opportunities for genetic exchange. However, the low bootstrap values recorded on this Moroccan sample lead us to not completely exclude the occurrence of occasional horizontal gene transfer, since such transfers tend to cloud the individualization of distinct phylogenetic lines.

A notable polymorphism was revealed with both markers in this population. This finding is consistent with results obtained on different pathogenic microorganisms with clonal evolution. On the basis of RAPD, regardless of the primer used, we could compare the level of polymorphism obtained in this *M. tuberculosis* population with those obtained for other bacterial species and for certain protozoan species with a well-known population structure (43, 45). As examples, we give below several values of mean genetic diversity obtained for microorganisms undergoing predominant clonal evolution: 0.14 for *Leishmania infantum* (18), 0.8 for *Trypanosoma cruzi* (3), 0.42 for *Saccharomyces cerevisiae* (S. Joly, unpublished data), 0.51 for *Candida albicans* (M. Darce, unpublished data), and 0.85 for *Escherichia coli* (M. Grandhomme, unpublished data), whereas the value obtained for *M. tuberculosis* in this study is around 0.4. Therefore, the genetic diversity of *M. tuberculosis* appears to be significant by comparison with other predominantly clonal species.

The notable genomic diversity observed in this study is in apparent contrast with data obtained by sequencing *M. tuberculosis* structural genes (28, 39), suggesting an extreme reduction of silent nucleotide substitutions in *M. tuberculosis* compared to *E. coli* and other bacterial species. However, the results reported here are supported by those obtained from a recent whole-genome comparison between different *M. tuberculosis* strains (13), indicating more extensive overall genetic polymorphism. These apparently divergent observations can be reconciled by the fact that (i) specific gene families may display distinct variabilities in the *M. tuberculosis* genome (13), and (ii) genetic variation is also driven by insertion/deletion events (4), by transposition (39), and by variations at numerous loci containing repetitive sequences, including MIRU-VNTR and other VNTRs (14, 36, 37, 41). Such multiple sources of polymorphism interspersed in a backbone of conserved structural genes are detectable by RAPD, which samples overall genome variability.

This relatively high genetic diversity was recorded in the Moroccan population, regardless of the subsample (per year or per district) considered, and for all diversity indices under study (Table 2). The results obtained were rather unexpected, since other studies have demonstrated lower levels of genetic diversity in high-incidence communities (17, 31, 50, 51). The MIRU-VNTR genotypic diversity in this sample (0.63) was higher than that observed in a sample from a Cape Town suburb area (0.46) (42), another setting with a very high inci-

dence of tuberculosis. From the phylogenetic analysis, no obvious dominant groups of very closely related strains were apparent in the Moroccan collection (Fig. 1), even when the HMAS district of Casablanca or the year of isolation was considered alone (data not shown). This is also in contrast with observations made in other regions with high incidence (42, 50).

In conclusion, the data obtained in this study and the results published on a South African population (42) suggest that predominant clonal evolution is not a local specificity but rather is the rule for *M. tuberculosis*. This hypothesis will have to be fully confirmed by studies of *M. tuberculosis* population structures in additional communities with different incidence levels, since microorganisms may develop different strategies according to the environment in which they evolve (e.g., *E. coli* 38).

The genetic diversity recorded in this study and previously published data (13, 28, 39) suggest that the genome of *M. tuberculosis* contains hypervariable regions and other less variable regions, suggesting that different selection pressures occur along the genome. This implies that the choice of the molecular marker is crucial with respect to the objectives of a given study.

We gratefully acknowledge Kristin Kremer, Dick van Soolingen (National Institute of Public Health and the Environment, The Netherlands), Jaouad Berrada, and Ouafae Bendadda (Institut Agronomique et Vétérinaire, Morocco) for providing the stocks used for comparison purposes. We thank also Nouzha Bencheikh (Department of Health, Morocco) for providing the data concerning tuberculosis distribution in Morocco and Pablo Bifani for critical reading of the manuscript.

REFERENCES

1. **Aghandous, R., M. Zouhdi, M. Zizi, M. Errami, and L. Idrissi.** 1999. *Mycobacterium* resistance to antimycobacterial reagent. Therapie **54:**623–625.
2. **Bazzicalupo, M., and R. Fani.** 1996. The use of RAPD for generating specific DNA probes for microorganisms. Methods Mol. Biol. **50:**155–175.
3. **Brisse, S., C. Barnabé, and M. Tibayrenc.** 2000. Identification of six *Trypanosoma cruzi* phylogenetic lineages by random amplified polymorphic DNA and multilocus enzyme electrophoresis. Int. J. Parasitol. **30:**35–44.
4. **Brosch, R., S. V. Gordon, M. Marmiesse, P. Brodin, C. Buchrieser, K. Eiglmeier, T. Garnier, C. Gutierrez, G. Hewinson, K. Kremer, L. M. Parsons, A. S. Pym, S. Samper, D. van Soolingen, and S. T. Cole.** 2002. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proc. Natl. Acad. Sci. USA **99:**3684–3689.
5. **Cave, M. D., K. D. Eisenach, P. F. McDermott, J. H. Bates, and J. T. Crawford.** 1991. IS*6110*: conservation of sequence in the *Mycobacterium tuberculosis* complex and its utilization in DNA fingerprinting. Mol. Cell. Probes **5:**73–80.
6. **Chevenet, F., A. L. Bañuls, and C. Barnabé.** 2000. TreeDyn: un éditeur interactif d'arbres phylogénétiques, p. 87–90. *In* G. Caraux, O. Gascuel, and M. F. Sagot (ed.), Actes des premières journées ouvertes biologie, informatique et mathématiques. ENSAM/LIRMM, Montpellier, France.
7. **Dye, C., S. Scheele, P. Dolin, V. Pathania, and M. C. Raviglione.** 1999. Consensus statement: global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. JAMA **282:**677–686.
8. **Efron, B.** 1979. Bootstrap methods: another look at the jackknife. Ann. Stat. **7:**1–26.
9. **El Baghdadi, J., R. Lazraq, A. Benani, M. Naciri, S. Ibrahimy, and A. Benslimane.** 1997. PCR detection of *Mycobacterium tuberculosis* lacking IS*6110*. Bull. Soc. Pathol. Exot. **90:**303–306.
10. **El Baghdadi, J., R. Lazraq, S. Ibrahimy, and A. Benslimane.** 1997. Molecular typing of Moroccan strains of *Mycobacterium tuberculosis*. Bull. Soc. Pathol. Exot. **90:**156–159.
11. **El Baghdadi, J., R. Lazraq, S. Ibrahimy, Z. Bouayad, R. Guinet, and A. Benslimane.** 1997. Survey of primary drug resistance of *Mycobacterium tuberculosis* in Casablanca, Morocco. Int. J. Tuberc. Lung Dis. **1:**309–313.
12. **Farris, J. S.** 1970. Methods for computing Wagner trees. Syst. Zool. **19:**83–92.
13. **Fleischmann, R. D., D. Alland, J. A. Eisen, L. Carpenter, O. White, J. Peterson, R. DeBoy, R. Dodson, M. Gwinn, D. Haft, E. Hickey, J. F. Kolonay, W. C. Nelson, L. A. Umayam, M. Ermolaeva, S. L. Salzberg, A. Delcher, T. Utterback, J. Weidman, H. Khouri, J. Gill, A. Mikula, W. Bishai, W. R. Jacobs, Jr., J. C. Venter, and C. M. Fraser.** 2002. Whole-genome comparison of *Mycobacterium tuberculosis* clinical and laboratory strains. J. Bacteriol. **184:**5479–5490.
14. **Frothingham, R., and W. A. Meeker-O'Connell.** 1998. Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. Microbiology **144:**1189–1196.
15. **Grosset, J., H. Boisvert, and C. Truffot-Pernot.** 1990. Mycobactéries, p. 965–1017. *In* L. Le Minor and M. Veron (ed.), Bactériologie médicale. Flammarion, Paris, France.
16. **Harn, H. J., K. L. Shen, L. I. Hon, K. W. Yu, G. C. Liu, K. C. Yueh, and J. H. Lee.** 1997. Evidence of transmission of *Mycobacterium tuberculosis* by random amplified polymorphic DNA (RAPD) fingerprinting in Taipei City, Taiwan. J. Clin. Pathol. **50:**505–508.
17. **Hermans, P. W., F. Messadi, H. Guebrexabher, D. van Soolingen, P. E. de Haas, H. Heersma, H. de Neeling, A. Ayoub, F. Portaels, D. Frommel, M. Zribi, and J. D. A. van Embden.** 1995. Analysis of the population structure of *Mycobacterium tuberculosis* in Ethiopia, Tunisia, and The Netherlands: usefulness of DNA typing for global tuberculosis epidemiology. J. Infect. Dis. **171:**1504–1513.
18. **Hide, M., A. L. Bañuls, and M. Tibayrenc.** 2001. Genetic heterogeneity and phylogenetic status of *Leishmania* (*Leishmania*) *infantum* zymodeme MON-1: epidemiological implications. Parasitology **12:**425–432.
19. **Jaccard, P.** 1908. Nouvelles recherches sur la distribution florale. Bull. Soc. Vaudoise Sci. Nat. **44:**223–270.
20. **Kamerbeek, J., L. Schouls, A. Kolk, M. van Agterveld, D. van Soolingen, S. Kuijper, A. Bunschoten, H. Molhuizen, R. Shaw, M. Goyal, and J. van Embden.** 1997. Simultaneous detection and strain differentiation of *M. tuberculosis* for diagnosis and epidemiology. J. Clin. Microbiol. **35:**907–914.
21. **Kluge, A. G., and J. S. Farris.** 1969. Quantitative phyletics and the evolution of anurans. Syst. Zool. **18:**1–32.
22. **Kremer, K., D. van Soolingen, R. Frothingham, W. H. Haas, P. W. Hermans, C. Martin, P. Palittapongarnpim, B. B. Plikaytis, L. W. Riley, M. A. Yakrus, J. M. Musser, and J. D. van Embden.** 1999. Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility. J. Clin. Microbiol. **37:**2607–2618.
23. **Laraqui, C. H., S. Ottmani, M. A. Hammou, N. Bencheikh, and J. Mahjour.** 2001. Study of tuberculosis in health care workers in the public sector of Morocco. Int. J. Tuberc. Lung Dis. **5:**939–945.
24. **Lazraq, R., J. El Baghdadi, J. L. Guesdon, and A. Benslimane.** 1999. Evaluation of IS*6110* as amplification target for direct tuberculosis diagnosis. Pathol. Biol. **47:**790–796.
25. **Mantel, N.** 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. **27:**209–220.
26. **Maynard Smith, J., N. H. Smith, M. O'Rourke, and B. G. Spratt.** 1993. How clonal are bacteria? Proc. Natl. Acad. Sci. USA **90:**4384–4388.
27. **Mazars, E., S. Lesjean, A. L. Bañuls, M. Gilbert, V. Vincent, B. Gicquel, M. Tibayrenc, C. Locht, and P. Supply.** 2001. High-resolution minisatellite-based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. Proc. Natl. Acad. Sci. USA **98:**1901–1906.
28. **Musser, J. M., A. Amin, and S. Ramaswamy.** 2000. Negligible genetic diversity of *Mycobacterium tuberculosis* host immune system protein targets: evidence of limited selective pressure. Genetics **155:**7–16.
29. **Ottmani, S., L. Laasri, P. Chaulet, P. Dolin, N. Bencheikh, A. Lahlou, and J. Mahjour.** 1998. La tuberculose et la lutte antituberculeuse au Maroc de 1980 à 1998. Bull. Epidemiol. **35:**2–11.
30. **Pasteur, N., G. Pasteur, F. Bonhomme, J. Catalan, and J. Britton-Davidian.** 1987. Manuel technique de génétique par electrophorèse des protéines, p. 232. Lavoisier, Paris, France.
31. **Pineda-Garcia, L., A. Ferrera, and S. E. Hoffner.** 1997. DNA fingerprinting of *Mycobacterium tuberculosis* strains from patients with pulmonary tuberculosis in Honduras. J. Clin. Microbiol. **35:**2393–2397.
32. **Raviglione, M. C., D. E. Snider, Jr., and A. Kochi.** 1995. Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic. JAMA **273:**220–226.
33. **Richner, S. M., J. Meiring, and R. Kirby.** 1997. A study of the genetic diversity of *Mycobacterium tuberculosis* isolated from patients in the eastern province of South Africa using random amplified polymorphic DNA profiling. Electrophoresis **18:**1570–1576.
34. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
35. **Selander, R. K., and B. R. Levin.** 1980. Genetic diversity and structure in *Escherichia coli* populations. Science **210:**545–547.
36. **Skuce, R. A., T. P. McCorry, J. F. McCarroll, S. M. Roring, A. N. Scott, D. Brittain, S. L. Hughes, R. G. Hewinson, and S. D. Neill.** 2002. Discrimination of *Mycobacterium tuberculosis* complex bacteria using novel VNTR-PCR targets. Microbiology **148:**519–528.
37. **Smittipat, N., and P. Palittapongarnpim.** 2000. Identification of possible loci

of variable number of tandem repeats in *Mycobacterium tuberculosis*. Tuber. Lung Dis. **80:**69–74.
38. **Souza, V., M. Rocha, A. Valera, and L. E. Eguiarte.** 1999. Genetic structure of natural populations of *Escherichia coli* in wild hosts on different continents. Appl. Environ. Microbiol. **65:**3373–3385.
39. **Sreevatsan, S., X. Pan, K. E. Stockbauer, N. D. Connell, B. N. Kreiswirth, T. S. Whittam, and J. M. Musser.** 1997. Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proc. Natl. Acad. Sci. USA **94:**9869–9874.
40. **Supply, P., S. Lesjean, E. Savine, K. Kremer, D. van Soolingen, and C. Locht.** 2001. Automated high-throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on mycobacterial interspersed repetitive units. J. Clin. Microbiol. **39:**3563–3571.
41. **Supply, P., E. Mazars, S. Lesjean, V. Vincent, B. Gicquel, and C. Locht.** 2000. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Mol. Microbiol. **36:**762–771.
42. **Supply, P., R. M. Warren, A. L. Bañuls, S. Lesjean, G. D. van Der Spuy, L. A. Lewis, M. Tibayrenc, P. D. van Helden, and C. Locht.** 2003. Linkage disequilibrium between minisatellite loci supports clonal evolution of *Mycobacterium tuberculosis* in a high tuberculosis incidence area. Mol. Microbiol. **47:**529–538.
43. **Tibayrenc, M.** 1995. Population genetics of parasitic protozoa and other microorganisms. Adv. Parasitol. **36:**47–115.
44. **Tibayrenc, M.** 1995. Population genetics and strain typing of microorganisms: how to detect departures from panmixia without individualizing alleles and loci. C. R. Acad. Sci. Ser. III **318:**135–139.
45. **Tibayrenc, M., F. Kjellberg, and F. J. Ayala.** 1990. A clonal theory of parasitic protozoa: the population structure of *Entamoeba*, *Giardia*, *Leishmania*, *Naegleria*, *Plasmodium*, *Trichomonas*, and *Trypanosoma* and its medical and taxonomical consequences. Proc. Natl. Acad. Sci. USA **87:**2414–2418.
46. **Tibayrenc, M., F. Kjellberg, J. Arnaud, B. Oury, S. F. Breniere, M. L. Darde, and F. J. Ayala.** 1991. Are eukaryotic microorganisms clonal or sexual? A population genetics vantage. Proc. Natl. Acad. Sci. USA **88:**5129–5133.
47. **van Embden, J. D. A., M. D. Cave, J. T. Crawford, J. W. Dale, K. D. Eisenach, B. Gicquel, P. Hermans, C. Martin, R. McAdam, T. M. Shinnick, and P. M. Small.** 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J. Clin. Microbiol. **31:**406–409.
48. **van Soolingen, D., P. E. de Haas, P. W. Hermans, P. M. Groenen, and J. D. van Embden.** 1993. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **31:**1987–1995.
49. **van Soolingen, D., P. E. de Haas, P. W. Hermans, and J. D. van Embden.** 1994. DNA fingerprinting of *Mycobacterium tuberculosis*. Methods Enzymol. **235:**196–205.
50. **van Soolingen, D., L. Qian, P. E. de Haas, J. T. Douglas, H. Traore, F. Portaels, H. Z. Qing, D. Enkhsaikan, P. Nymadawa, and J. D. van Embden.** 1995. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of east Asia. J. Clin. Microbiol. **33:**3234–3238.
51. **Warren, R., J. Hauman, N. Beyers, M. Richardson, H. S. Schaaf, P. Donald, and P. van Helden.** 1996. Unexpectedly high strain diversity of *Mycobacterium tuberculosis* in a high-incidence community. S. Afr. Med. J. **86:**45–49.
52. **Williams, J. G., A. R. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingey.** 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. **18:**6531–6535.