

Genetic Diversity and Population Structure of *Mycobacterium marinum*: New Insights into Host and Environmental Specificities

Vincent Broutin,^{a,b,c} Anne-Laure Bañuls,^c Alexandra Aubry,^d Nicolas Keck,^e Marc Choisy,^c Jean-François Bernardet,^f Christian Michel,^f Jean-Christophe Raymond,^g Cédric Libert,^h Antoine Barnaud,ⁱ Pieter Stragier,^j Françoise Portals,^j Dominique Terru,^b Claudine Belon,^b Olivier Dereure,^{a,k} Cristina Gutierrez,^l Maria-Laura Boschioli,^m Philippe Van De Perre,^{a,b} Emmanuelle Cambau,^d and Sylvain Godreuil^{a,b}

INSERM U1058 Infection by HIV and by agents with mucocutaneous tropism: from pathogenesis to prevention, Montpellier, France^a; Centre Hospitalier Régional Universitaire de Montpellier, Hôpital Arnaud de Villeneuve, Département de Bactériologie-Virologie, Montpellier, France^b; MIVEGEC, UMR IRD 224-CNRS 5290-Universités Montpellier 1 et 2, Centre IRD, Montpellier, France^c; Centre National de Référence des Mycobactéries et de la Résistance aux Antituberculeux, Paris, France^d; Laboratoire Départemental Vétérinaire de l'Hérault, Montpellier, France^e; Unité de virologie et immunologie moléculaires, Institut National de la Recherche Agronomique, Jouy-en-Josas, France^f; Comité National des Pêches Maritimes et des Elevages Marins, Paris, France^g; Parc zoologique Darwin, Montpellier, France^h; Le Gouessant Aquaculture, Lamballe, Franceⁱ; Mycobacteriology Unit, Institute of Tropical Medicine, Antwerp, Belgium^j; Department of Dermatology, Hôpital Saint-Eloi, University of Montpellier^k; FIND, Geneva, Switzerland^l; and ANSES, Laboratoire de Santé Animale de Maisons-Alfort, Unité des Zoonoses Bactériennes, Maisons-Alfort, France^m

Mycobacterium marinum causes a systemic tuberculosis-like disease in fish and skin infections in humans that can spread to deeper structures, resulting in tenosynovitis, arthritis, and osteomyelitis. However, little information is available concerning (i) the intraspecific genetic diversity of *M. marinum* isolated from humans and animals; (ii) *M. marinum* genotype circulation in the different ecosystems, and (iii) the link between *M. marinum* genetic diversity and hosts (humans and fish). Here, we conducted a genetic study on 89 *M. marinum* isolates from humans ($n = 68$) and fish ($n = 21$) by using mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) typing. The results show that the *M. marinum* population is genetically structured not only according to the host but also according to the ecosystem as well as to tissue tropism in humans. This suggests the existence of different genetic pools in the function of the biological and ecological compartments. Moreover, the presence of only certain *M. marinum* genotypes in humans suggests a different zoonotic potential of the *M. marinum* genotypes. Considering that the infection is linked to aquarium activity, a significant genetic difference was also detected when the human tissue tropism of *M. marinum* was taken into consideration, with a higher genetic polymorphism in strains isolated from patients with cutaneous forms than from individuals with deeper-structure infection. It appears that only few genotypes can produce deeper infections in humans, suggesting that the immune system might play a filtering role.

Mycobacterium marinum is a slow-growing and ubiquitous waterborne mycobacterial species with optimal growth temperatures between 25 and 35°C (11, 20, 33). *M. marinum* infection occurs in a variety of hosts, such as fish and amphibians, and occasionally in humans who have been exposed to contaminated fish and water. Human infections are generally limited to cutaneous lesions, referred to as “swimming pool granuloma” and “fish tank granuloma,” according to where the infection was contracted (4, 11, 12, 19–21, 33); however, in some cases, the infection can spread to deeper structures, resulting in tenosynovitis, arthritis, and osteomyelitis (2, 5, 6, 10, 14, 15, 18).

M. marinum is a known fish pathogen causing a chronic granulomatous disease that bears many similarities to mammalian mycobacterioses, including tuberculosis. The rapid development of fish farming and of the ornamental fish industry has similarly led to a worldwide increase in the number of reports of *M. marinum* infections in fish, with two major consequences: (i) a substantial financial loss for the two sectors concerned and (ii) an increased risk of contamination for people who handle fish (4, 8, 9, 12, 16, 19, 22, 37).

The mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) genotyping method for *M. marinum* (1, 25, 26, 30, 39) appears to be a powerful tool with which to study the genetic polymorphism of this bacterium. However, little information is available concerning (i) the overall and intraspecific (from humans to animals) genetic diversity of *M.*

marinum and (ii) the impact of the ecosystem (offshore aquaculture versus aquarium environments) on *M. marinum* genotype circulation and human transmission.

The main goal of this study was to assess *M. marinum* genetic diversity in relation to ecosystems and hosts in order to better define the epidemiology of this mycobacterium and improve our understanding of human infection.

MATERIALS AND METHODS

Patients and fish. The origins and other information concerning the bacteria are presentation in Tables 1 and 2. Sixty-three *M. marinum* isolates from humans were from the collection of the National Reference Center for Mycobacteria, Hôpital Pitié-Salpêtrière, Paris, France, and had been collected during a national survey in France from January 1996 to December 1998 (2). Five other samples were provided by the Mycobacteria Reference Laboratory, Pasteur Institute (Paris, France). These 68 *M. marinum* isolates were from 38 men and 30 women with a median age of 46 years (range, 4 to 77 years). Cutaneous exposure to fish tank water was

Received 14 May 2012 Returned for modification 8 June 2012

Accepted 28 August 2012

Published ahead of print 5 September 2012

Address correspondence to Sylvain Godreuil, s-godreuil@chu-montpellier.fr.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JCM.01274-12

TABLE 1 Sources of *M. marinum* isolates from patients and the clinical presentation

Sample no.	Geographical origin	Source ^a	Source of infection ^b	Clinical presentation ^c	Year of isolation
1	France	NRCM	AFT	ADSI; synovitis	1996
2	France	NRCM	OSC; pond water	ADSI; arthritis and tenosynovitis	1996
3	France	NRCM	AFT	ESL	1996
4	France	NRCM	INA	INA	1995
5	France	NRCM	INA	INA	1995
6	France	NRCM	OSC; fish spine	ADSI; tenosynovitis	1996
7	France	NRCM	AFT	ESL	1996
8	France	NRCM	AFT	ADSI; synovitis	1996
9	France	NRCM	OSC; fish spine	ADSI; tenosynovitis	1996
10	France	NRCM	AFT	ESL	1997
11	France	NRCM	INA	INA	1995
12	France	NRCM	AFT	ESL	1997
13	France	NRCM	AFT	ESL	1996
14	France	NRCM	AFT	ESL	1998
15	France	NRCM	INA	INA	1995
16	France	NRCM	INA	INA	1995
17	France	NRCM	AFT	ESL	1994
18	France	NRCM	AFT	ADSI; arthritis	1996
19	France	NRCM	INA	INA	1996
20	France	NRCM	AFT	ADSI; tenosynovitis	1997
21	France	NRCM	AFT	ESL	1997
22	France	NRCM	AFT	ESL	1997
23	France	NRCM	AFT	ESL	1997
24	France	NRCM	AFT	ESL	1996
25	France	NRCM	AFT	ESL	1997
26	France	NRCM	AFT	ESL	1997
27	France	NRCM	AFT	ESL	1997
28	France	NRCM	OSC; fish spine	ADSI; tenosynovitis	1996
29	France	NRCM	AFT	ADSI; synovitis, arthritis	1996
30	France	NRCM	AFT	ESL	1996
31	France	NRCM	INA	ADSI; synovitis, arthritis	1996
32	France	NRCM	AFT	ESL	1997
33	France	NRCM	AFT	ADSI; synovitis	1996
34	France	NRCM	AFT	ESL	1997
35	France	NRCM	AFT	ESL	1997
36	France	NRCM	OSC; pond water	ADSI; osteoarthritis	1996
37	France	NRCM	INA	ADSI; tenosynovitis	1997
38	France	NRCM	INA	ESL	1996
39	France	NRCM	INA	ESL	1997
40	France	NRCM	AFT	ESL	1997
41	France	NRCM	AFT	ESL	1996
42	France	NRCM	AFT	ESL	1997
43	France	NRCM	AFT	ESL	1998
44	France	NRCM	OSC; fish spine	ADSI; tenosynovitis	1998
45	France	NRCM	OSC; fish spine	ADSI; synovitis	1997
46	France	NRCM	AFT	ESL	1998
47	France	NRCM	AFT	ESL	1997
48	France	NRCM	AFT	ESL	1998
49	France	NRCM	OSC; swimming pool	ESL	1998
50	France	NRCM	INA	INA	1998
51	France	NRCM	INA	INA	1998
52	France	NRCM	AFT	ESL	1997
53	France	NRCM	AFT	ESL	1998
54	France	NRCM	AFT	ESL	1997
55	France	NRCM	AFT	ESL	1996
56	France	NRCM	AFT	ESL	1997
57	France	NRCM	AFT	ESL	1997
58	France	NRCM	INA	INA	INA
59	France	NRCM	AFT	ESL	1998
60	France	NRCM	AFT	ESL	1998

(Continued on following page)

TABLE 1 (Continued)

Sample no.	Geographical origin	Source ^a	Source of infection ^b	Clinical presentation ^c	Year of isolation
61	France	NRCM	INA	INA	INA
62	France	NRCM	AFT	ADSI; arthritis	1997
63	France	NRCM	NA	INA	INA
64 (IP310)	France	MRPI	AFT	INA	2000
65 (IP355)	France	MRPI	AFT	INA	2000
66 (IP821)	France	MRPI	AFT	INA	1999
67 (IP843)	France	MRPI	AFT	INA	1999
68 (IP876)	France	MRPI	AFT	INA	1999

^a NRCM, National Reference Center for Mycobacteria, Paris, France; MRPI, Mycobacteria Reference Laboratory at the Pasteur Institute, Paris, France.

^b AFT, aquarium fish tank; OSC, other source of contamination; INA, information not available.

^c ESL, exclusively skin lesions; ADSI, associated deeper-structure infection; INA, information not available.

reported by 45 patients and to pond water by 2 patients; injury from or contact with a fish spine was reported by 5 individuals (Table 1). Swimming pool contamination was reported in one case. The source of infection was unknown for 15 patients. The clinical presentation was documented for 52 patients (among whom 36 had only skin lesions and 16 also had deep-structure infection) and not available for 16 (Table 1).

Eighteen fish isolates of *M. marinum* were obtained from the Hérault Departmental Veterinary Laboratory (Montpellier, France) and from the French network of veterinary laboratories, and three were from the collection of the Institute of Tropical Medicine in Antwerp (Belgium) (Table 2). The fish species, environment (aquarium, 8 isolates; offshore aquaculture, 13 isolates), geographic origin, and year of isolation are documented in Table 2.

Mycobacterium culture and species identification. Mycobacteria were cultured on Lowenstein-Jensen (LJ) slants. All 89 cultures (human and fish isolates) were positive by Ziehl-Neelsen staining. Based on conventional biochemical methods and the commercial multiplex line-probe assay Geno-Type Mycobacterium AS/CM (Hain Lifescience GmbH, Nehren, Germany), all 89 isolates were assigned to the species *M. marinum* (17, 23).

DNA preparation and MIRU-VNTR typing. DNA was extracted as described previously (32). Three or four mycobacteria colonies were resuspended in TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and digested

with 1 mg/ml lysozyme. After treatment with 0.1 mg/ml proteinase K and 1% sodium dodecyl sulfate, suspensions were incubated with 0.6 M NaCl and 0.27 M *N*-acetyl-*N,N,N*-trimethyl ammonium bromide. DNA was extracted with chloroform-isoamyl alcohol and precipitated with isopropanol. Alternatively, DNA was obtained by resuspending bacteria in 100 to 200 μ l of TE followed by heat inactivation at 100°C for 10 min and centrifugation (10,000 \times *g* at 4°C for 20 min) to remove cellular debris.

MIRU loci 2, 4, 5, 7, 9, and 20 and VNTR loci 1, 4, 6, 8, 9, 14, 15, 18, and 19 were individually amplified and analyzed as previously described (1, 25). PCRs were performed in 30- μ l mixtures containing 1.0 U HotStar *Taq* polymerase (Qiagen, Hilden, Germany), 3.0 μ l of 10 \times PCR buffer, 6.0 μ l Q solution, 1.5 mM MgCl₂, a 200 μ M concentration of each deoxynucleoside triphosphate, a 0.6 pM concentration of each primer, and 5 μ l DNA sample (50 ng/ μ l). All PCRs were preceded by 15 min denaturation at 95°C and consisted of 40 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. An aliquot (3 μ l) of each PCR product was electrophoretically separated through 3% small-fragment agarose gels (Eurogentec, Seraing, Belgium) in 0.5 \times TAE (20 mM Tris-acetate, 0.5 mM EDTA [final concentration]) buffer at 100 V. Gels were then stained with ethidium bromide, and the amplicon size was estimated by compar-

TABLE 2 *M. marinum* isolates from fish

Sample no.	Geographical origin	Source ^a	Fish species	Environmental origin	Year of isolation
69	France	INRA	Fighting fish (<i>Betta splendens</i>)	Aquarium (ornamental fish)	1990
70	France	INRA	Pearl gourami (<i>Trichogaster leerii</i>)	Aquarium (ornamental fish)	1990
71	France	INRA	Medaka or Japanese killfish (<i>Oryzias latipes</i>)	Aquarium (experimental fish facilities)	1998
72	North Africa	HDVL	European sea bass (<i>Dicentrarchus labrax</i>)	Offshore aquaculture (Mediterranean Sea)	2005
73	North Africa	HDVL	European sea bass (<i>Dicentrarchus labrax</i>)	Offshore aquaculture (Mediterranean Sea)	2005
74	North Africa	HDVL	European sea bass (<i>Dicentrarchus labrax</i>)	Offshore aquaculture (Mediterranean Sea)	2005
75	North Africa	HDVL	European sea bass (<i>Dicentrarchus labrax</i>)	Offshore aquaculture (Mediterranean Sea)	2005
76	North Africa	HDVL	European sea bass (<i>Dicentrarchus labrax</i>)	Offshore aquaculture (Mediterranean Sea)	2007
77	North Africa	HDVL	European sea bass (<i>Dicentrarchus labrax</i>)	Offshore aquaculture (Mediterranean Sea)	2007
78	North Africa	HDVL	European sea bass (<i>Dicentrarchus labrax</i>)	Offshore aquaculture (Mediterranean Sea)	2007
79	North Africa	HDVL	European sea bass (<i>Dicentrarchus labrax</i>)	Offshore aquaculture (Mediterranean Sea)	2007
80	North Africa	HDVL	European sea bass (<i>Dicentrarchus labrax</i>)	Offshore aquaculture (Mediterranean Sea)	2007
81	North Africa	HDVL	European sea bass (<i>Dicentrarchus labrax</i>)	Offshore aquaculture (Mediterranean Sea)	2007
82	North Africa	HDVL	European sea bass (<i>Dicentrarchus labrax</i>)	Offshore aquaculture (Mediterranean Sea)	2007
83	France	HDVL	Four-eyed fish (<i>Anableps anableps</i>)	Aquarium (ornamental fish)	2007
84	France	HDVL	Four-eyed fish (<i>Anableps anableps</i>)	Aquarium (ornamental fish)	2007
85	Réunion Island	HDVL	Red drum (<i>Sciaenops ocellatus</i>)	Offshore aquaculture (Indian ocean)	2006
86	Réunion Island	HDVL	Red drum (<i>Sciaenops ocellatus</i>)	Offshore aquaculture (Indian ocean)	2008
87	Portugal	ITM-01-935	Turbot (<i>Scophthalmus maximus</i>)	Aquaculture	2001
88	South Africa	ITM-94-979	Four-eyed fish (<i>Anableps anableps</i>)	Aquarium	1994
89	South Africa	ITM-94-996	Four-eyed fish (<i>Anableps anableps</i>)	Aquarium	1994

^a INRA, Institut National de la Recherche Agronomique, HDVL, Hérault Departmental Veterinary Laboratory, Montpellier, France; ITM, Institute of Tropical Medicine, Antwerp, Belgium.

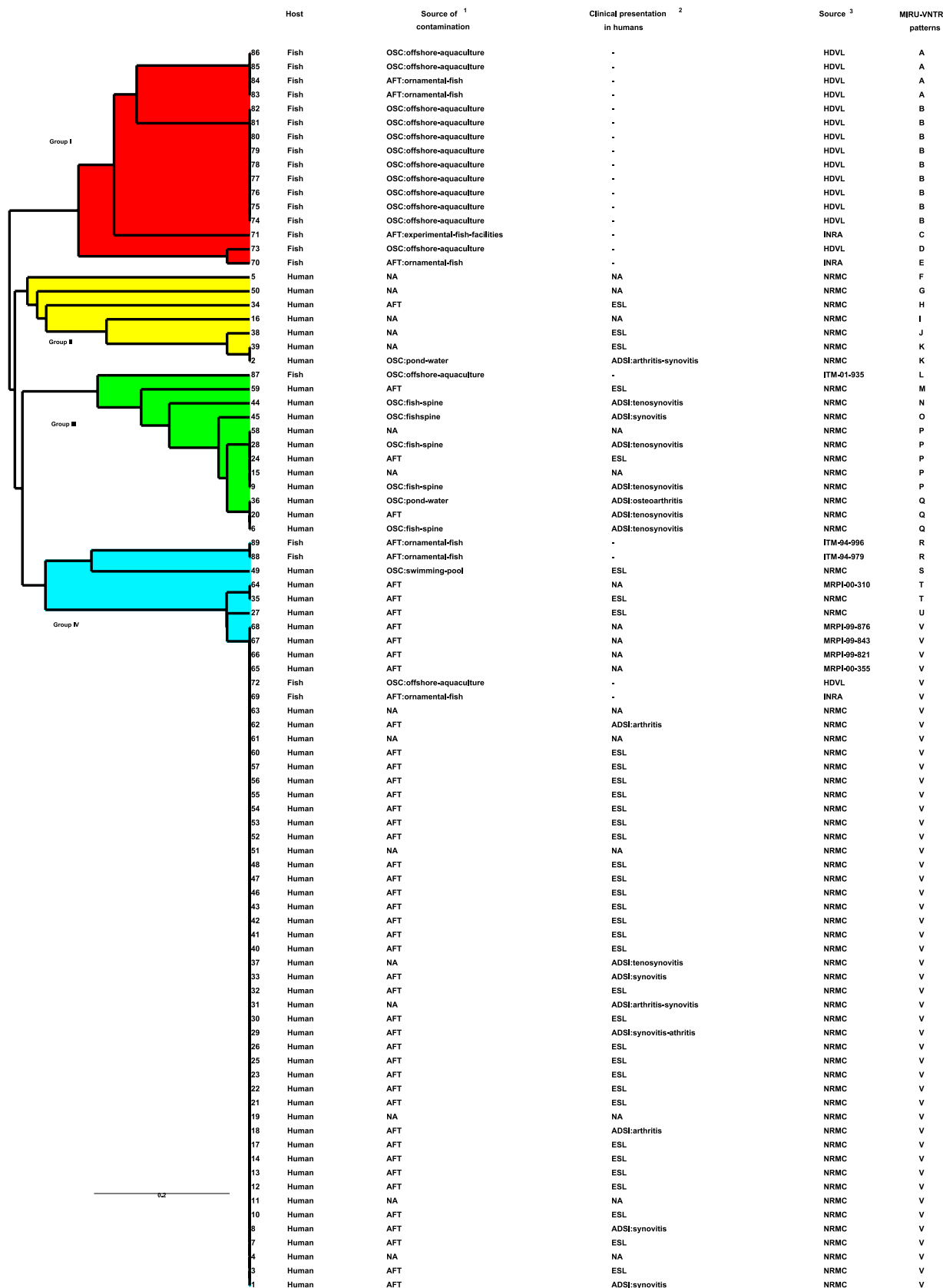


FIG 1 UPGMA tree based on the MIRU-VNTR (15 loci) data for the 89 samples under study. The relationships between patterns were assessed using the UPGMA dendrogram. 1, aquarium fish tank (AFT) or other source of contamination (OSC); 2, exclusively skin lesions (ESL) or associated deeper-structure infection (ADSI); 3, National Reference Center for Mycobacteria, Paris, France (NRMC); Mycobacteria Reference Laboratory at the Pasteur Institute, Paris, France (MRPI); Hérault Departmental Veterinary Laboratory, Montpellier, France (HDVL); Institute of Tropical Medicine, Antwerp, Belgium (ITM); Institut National de la Recherche Agronomique (INRA).

TABLE 3 Diversity indices calculated using the MIRU-VNTR data for the different *M. marinum* groups under study

<i>M. marinum</i> group	No. of isolates	Genotypic diversity	Mean genetic diversity (<i>h</i>)
1: total <i>M. marinum</i> sample (human + fish)	89	0.25	0.44
2: isolates from human hosts	68	0.22	0.31
3: isolates from fish hosts	21	0.38	0.47
4: isolates from infected patients exposed to fish tank water	45	0.15	0.12
5: isolates from infected patients exposed to other sources of contamination	8	0.18	0.45
6: isolates from infected aquarium fish	8	0.75	0.67
7: isolates from infected offshore aquaculture fish	13	0.30	0.26
8: isolates from patients and fish exposed to fish tank water	53	0.22	0.40
9: isolates from patients exposed to other sources of contamination and from offshore aquaculture fish	21	0.48	0.31
10: isolates from patients with exclusively skin lesions	36	0.25	0.20
11: isolates from patients with deeper-structure infection	16	0.38	0.41
12: isolates from patients with exclusively skin lesions exposed to fish tank water	33	0.18	0.19
13: isolates from patients with exclusively skin lesions exposed to other sources of contamination	1	1	Not applicable
14: isolates from patients with skin lesions associated with deep structure infection exposed to fish tank water	7	0.28	0.12
15: isolates from patients with skin lesions associated with deep structure infection exposed to other sources of contamination	7	0.71	0.28

ison with 50- and 100-bp step ladders (Promega, Leiden, The Netherlands). Amplicon size and amplicon sequencing (when the size was not described) were used to estimate the number of repeats at each locus as described by Ablordey et al. and Stragier et al. (1, 25).

Genetic diversity and population structure analyses. To study the genetic variability, several diversity indices, including the genotypic diversity and the mean genetic diversity (*h*), were calculated. The population structure was explored by estimating the value of F_{st} (index of genetic differentiation between samples), which ranges between 0 (no differentiation) and 1 (all samples fixed for a different allele). These parameters were calculated using F-STAT, version 2.9.3 (13).

Phylogenetic analysis. The genetic relationships among isolates were inferred from the MIRU-VNTR data using the UPGMA (unweighted pair group method with arithmetic average) clustering method. PAUP 4.0 (27) was used for tree elaboration and Treedyn (7) for tree visualization and annotation.

Statistical analysis. Statistical analyses were performed using the StatView software, version 4.5 (SAS Institute Inc., Cary, NC). Associations between variables were assessed using Student's *t* test. *P* values of <0.05 were considered statistically significant.

TABLE 4 Comparison of mean genetic diversity (*h*) among groups of *M. marinum* isolates

Population X/population Y	<i>P</i> ^a
Isolates from fish hosts (group 3)/isolates from human hosts (group 2)	1.5×10^{-4}
Isolates from patients exposed to other sources of contamination (group 5)/isolates from patients exposed to fish tank water (group 4)	1.2×10^{-5}
Isolates from infected offshore aquaculture fish (group 7)/isolates from infected aquarium fish (group 6)	1.2×10^{-6}
Isolates from patients with exclusively skin lesions exposed to fish tank water (group 12)/isolates from patients with skin lesions associated with deep structure infection exposed to fish tank water (group 14)	9×10^{-3}

^a *P* values of <0.05 were considered statistically significant (Student's *t* test).

We investigated how the population structure of the strains, as quantified by *F* statistics (35), is influenced by (i) host species, (ii) year of sampling, (iii) type of contact, and (iv) clinical symptoms. Since clinical symptom is defined only for human hosts and contacts are not the same for the two hosts, we adopted a hierarchical approach, nesting contact and clinical symptoms within host species to explore their effects on the variance. An additional complexity arises from the fact that there is a substantial colinearity between year of sampling and host species (human samples having been collected earlier than fish ones). To deal with possible confounding effects arising from this issue, we compared the cases (i) where year of sampling is nested within host species (thus correcting the effect of year of sampling by the effect of host species) and (ii) where host species is nested within year of sampling (thus correcting the effect of host species by the effect of year of sampling). The calculations of these hierarchical *F* statistics were performed by the algorithms proposed by Yang (38) as implemented in the Hierfstat R package (36). F_{st} estimations (and their confidence intervals) are those of Weir and Cockerham (36).

RESULTS

MIRU-VNTR typing and cluster analysis. Twenty-two different MIRU-VNTR patterns (designated A to V) were detected among the 89 isolates that were distributed in 9 clusters comprising 75 isolates (84.3%) and 14 unique patterns (15.7%) (Fig. 1). The largest cluster included 48 samples (pattern V) and the smallest clusters ($n = 2$; patterns K, R, and S) comprised only two isolates each; the other four clusters included nine (pattern B), five (pattern P), four (pattern A), and three (pattern Q) isolates each. Nine MIRU-VNTR loci (MIRU loci 2 and 5 and VNTR loci 1, 6, 8, 9, 14, 18, and 19) showed a high diversity index ($h > 0.5$), and five (MIRU loci 1, 9, and 20 and VNTR loci 4 and 15) had a low diversity index ($h < 0.5$), while MIRU locus 7 was the least discriminating locus ($h < 0.1$).

The dendrogram (Fig. 1) was generated using the UPGMA (unweighted-pair group method using arithmetic mean) method and the MIRU-VNTR data. From the phylogenetic tree, we distinguished four groups (I, II, III, and IV). Human and fish *M.*

TABLE 5 Comparison of genetic differentiation (F_{st} index) in the *M. marinum* groups under study

Population X/population Y	F_{st}	P
Isolates from human hosts (group 2)/isolates from fish hosts (group 3)	0.42	<0.05
Isolates from patients exposed to fish tank water (group 4)/isolates from infected patients exposed to other sources of contamination (group 5)	0.60	<0.05
Isolates from infected aquarium fish (group 6)/isolates from infected offshore aquaculture fish (group 7)	0.25	<0.05
Isolates from patients exposed to fish tank water (group 4)/isolates from infected aquarium fish (group 6)	0.58	<0.05
Isolates from infected patients exposed to other sources of contamination (group 5)/isolates from infected offshore aquaculture fish (group 7)	0.49	<0.05
Isolates from patients and fish exposed to fish tank water (group 8)/isolates from patients exposed to other sources of contamination and from offshore aquaculture fish (group 9)	0.46	<0.05
Isolates from patients with exclusively skin lesions exposed to fish tank water (group 12)/isolates from patients with skin lesions associated with deep structure infection exposed to fish tank water (group 14)	0	>0.05
Isolates from patients with skin lesions associated with deep structure infection exposed to fish tank water (group 14)/isolates from patients with skin lesions associated with deep structure infection exposed to other sources of contamination (group 15)	0.6	<0.05

marinum strains were not fully separated in the tree. Nevertheless, cluster A was mostly represented by fish isolates from offshore aquacultures (patterns A to E) (Fig. 1). Cluster II, with the exception of two isolates, contained only human isolates with an unknown mode of contamination (patterns F to K), while group III, except for one fish isolate from an aquarium (ITM 01-935), was composed only of clinical isolates from patients who were not exposed to fish tank water (patterns L to Q). Finally, cluster IV, which included 60.7% of all *M. marinum* samples under study (54/89 isolates, of which only 4 were from fish; patterns R to V), was mostly composed of human isolates with the same MIRU-VNTR profile ($n = 46$, mainly from aquarists). Moreover, these clinical isolates originated from patients who had developed different clinical forms of the infection (simple skin disease or with deeper tissue lesions). No specific clusters or subclusters could be distinguished based on the different clinical presentation.

Genetic structure relative to the host, environment, and clinical forms in humans. Next, to thoroughly investigate the genetic structure of *M. marinum* in different environments and hosts, the set of 89 *M. marinum* isolates (group 1) was subdivided into several groups (Table 3) based on (i) the host [humans (group 2) or fish (group 3)], (ii) the source of contamination for humans [aquarium (group 4) or other sources of contamination (group 5)], (iii) the ecosystem of the infected fish [aquarium tanks (group 6) or offshore aquaculture (group 7)], (iv) the human and fish ecosystems together [aquarium (group 8) or other sources of contamination and offshore aquaculture (group 9)], (vii) the clinical presentation in humans [exclusively skin involvement (group 10) or skin lesions associated with deep structure infection (group 11)], and (viii) the clinical presentation in humans according to the source of contamination [exclusively skin involvement after exposure to fish tank water (group 12) or exposure to other

sources of contamination (group 13) or skin lesions associated with deep structure infection exposed to fish tank water (group 14) or exposed to other sources of contamination (group 15)].

An important polymorphism was found in the global *M. marinum* population (group 1, humans and fish) with a mean genetic diversity of 0.44 (Table 3). Comparison of genetic diversity in the different *M. marinum* groups revealed a greater genetic diversity in fish isolates than in human isolates (group 3 versus group 2, $P = 1.5 \times 10^{-4}$; group 6 versus group 4, $P = 6.7 \times 10^{-9}$) (Table 4). Genetic differentiation between human and fish isolates of *M. marinum* was high and significant (Table 5). These data suggest different pools of genotypes according to the host. Moreover, genetic differentiation was also significantly high when the *M. marinum* isolates were classified based on the ecosystem (Table 5) (group 4 versus group 5, $P < 0.05$; group 6 versus group 7, $P < 0.05$). Specifically, genetic diversity was significantly higher in clinical isolates from patients exposed to other sources of contamination than to fish tank water (group 5 versus group 4, $P = 1.2 \times 10^{-5}$) (Table 4) and conversely was higher in *M. marinum* samples from aquarium fish than from aquacultures (group 6 versus group 7, $P = 1.2 \times 10^{-6}$) (Table 4). The comparison of *M. marinum* genotypes of human isolates classified according to the clinical presentation and to the source of contamination showed that (i) among *M. marinum* isolates from infected patients exposed to fish tank water, genetic diversity (h) was higher in isolates from patients with exclusively skin forms than in isolates from skin lesions associated with deeper-structure infections (Table 4), and there was no significant genetic differentiation (F_{st}) between these two groups (Table 5); (ii) among *M. marinum* isolates from infected patients exposed to other sources of contamination, the majority of these isolates (7/8; 87.5%) are involved in skin lesions associated with deeper-structure infections with a high genetic diversity

TABLE 6 Effects of host species and year of sampling on the strain population structure^a

Measurement	Species	Year/species	Year	Species/year
F_{st}	0.4261 (0.3423–0.4841)	0.1454 (0.1187–0.1821)	0.3079 (0.2650–0.3546)	0.5372 (0.4489–0.6010)
Variance	3.8615	0.8044	2.1498	5.2889
Percentage	42.37	8.81	30.64	75.11

^a “Species” and “Year” show data for the effect considered alone. “Year/species” shows the effect of year nested within the effect of species (i.e., the effect of year corrected by the effect of species). “Species/year” shows the effect of species nested within the effect of year (i.e., the effect of species corrected by the effect of year). F_{st} is Weir and Cockerham’s estimate of F_{st} (36); 95% confidence intervals are in parentheses. “Variance” shows the variance components of each effect, and “percentage” shows the percentage of the variances that accounted for these factors.

(Table 3) in this group; (iii) among *M. marinum* isolates from patients with deeper-structure infection, the genetic differentiation (F_{st}) is high and significant between infected patients exposed to fish tank water and those exposed to other sources of contamination (Table 5).

Table 6 shows that the F_{st} estimates and the variance components of species effect are substantially and significantly higher than those of the year-of-sampling effect. Furthermore, considering the two effects at the same time shows that most of the year effect is actually due to the underlying confounding host species effect (compare the results for year and those for year/species in Table 6). Correcting species effect by year of sampling increases the magnitude of its effect (compare results for species with those for species/year). This shows that there is a strong host species effect that tends to be concealed by a colinear year-of-sampling effect.

DISCUSSION

M. marinum is the etiologic agent of fish tuberculosis and of a granulomatous disease observed mainly in aquarists and professional fish breeders (3, 4, 8, 9, 12, 16, 19, 20, 31, 34, 37). However, little information is available on the organization of *M. marinum* genetic diversity relative to the host, environment, and clinical forms in humans.

A challenge of our data set was the fact that samples from human and fish hosts were not collected during the same period. By adopting a nested analysis of the population structure, we managed to disentangle these two confounding effects, and the results clearly showed that collected strains are strongly genetically structured according to the host (human versus fish) species and much less according to the year of sampling. Our analysis by MIRU-VNTR typing of 89 fish and human isolates shows that, overall, the genetic polymorphisms in *M. marinum* isolates vary according to the host (human versus fish), and the genetic polymorphism value (genetic diversity and genotypic diversity) is higher for fish isolates. These results were expected, since fish are the natural host of *M. marinum* species, while humans are only accidental hosts and normally an epidemiological impasse (because patients are successfully treated and interhuman transmission has never been detected). Moreover, the strong genetic differentiation demonstrates that fish and human *M. marinum* populations are characterized by different gene pools and that a limited number of genotypes can infect humans. Our results suggest that only some *M. marinum* strains have zoonotic potential and/or that few *M. marinum* genotypes have a large host spectrum that includes humans as well, as previously proposed by Ucko and Colorni (28).

M. marinum genetic diversity varies also in function of the ecosystem (aquarium versus aquaculture). Considering only the fish samples, the significant genetic differentiation between *M. marinum* isolates from aquarium and farmed fish suggests that the circulating genotypes are influenced by the ecological niche. These results are in agreement with the findings of Sechi et al. and Ucko et al. (24, 28, 29), who reported that based on the molecular characterization of the 16S rRNA and *hsp65* genes, the distribution of *M. marinum* genotypes depends on the ecosystem (marine versus freshwater environments) and on the geographical origin of isolates. The two ecosystems studied here present specific features which might have a different influence on the gene pool and circulation of *M. marinum* strains: the aquarium is a “closed” environment but generally composed of a large number of different

fish species coming from various geographic areas that are normally poorly controlled from a bacteriologic point of view, while offshore aquaculture is an open but restricted environment with an overcrowding population but generally composed of only one fish species. It is obvious that these ecological and population characteristics may influence the genetic structure of *M. marinum* and the emergence of specific genotypes. In our study, the genetic diversity of *M. marinum* was significantly less important in the group of isolates from farmed fish than from aquarium fish, in agreement with their different levels of sanitary control and different levels of biodiversity in terms of fish species. Nevertheless, there may be a bias due to the relatively small number of fish isolates under study and due to the fact that the majority of *M. marinum* samples from aquaculture fish came from the same North African fish farm. A larger sample of strains of each population from different areas of the different same countries would provide a more accurate measure of genetic diversity according to the ecosystem. However, other factors, such as host species-bacterium interactions, may also play a role.

Concerning *M. marinum* from clinical isolates, our results suggest that the pool of genotypes varied according to the clinical form and to the source of contamination.

Indeed, among *M. marinum* isolates from patients exposed to fish tank water, the genetic diversity of isolates from patients with exclusively skin lesions was significantly higher than that of isolates from patients with skin and deeper-structure infection. These data are consistent with the higher frequency of the cutaneous forms of disease. However, the lack of significant genetic differentiation between these two groups of *M. marinum* clinical isolates suggests that all *M. marinum* strains that infect humans might potentially also infect deeper structures, independently of their genotype. The lower genetic diversity of the *M. marinum* isolates from patients with more serious infections could be explained by immune system activity eliminating an important part of the genotypic variants. Nevertheless, in the cases of deeper-structure infection, when we compared the isolates from aquarium and those from other sources of contaminations, we observed significant genetic differentiation, in agreement with the existence of different genetic pools as a function of ecosystems. It is worth noting that the majority of patients infected by other sources than aquarium environment presented deeper-structure infections. This could suggest strain-specific virulence or pathogenic properties within *M. marinum*, as the study of van der Sar et al. also seems to suggest (30).

In conclusion, our results show different patterns of genetic structuring in *M. marinum* isolates that were grouped based on their host, ecosystem, and tissue tropism in humans, suggesting different gene pools according to the biological or ecological compartment and different epidemiologic potential of the strains. It would be relevant to identify coding genes that might be involved in these different abilities in order to understand the mechanisms of transmission, virulence, and pathogenicity and the specific interactions between host and pathogen.

ACKNOWLEDGMENTS

We thank Isabelle Zorogniotti for excellent technical assistance. We thank François Renaud for helpful discussions. We thank Elisabetta Andermacher for assistance in preparing and editing the manuscript.

We are grateful to the IRD (Institut de Recherche pour le Développement), the CNRS (Centre National de la Recherche Scientifique), and the

Laboratoire de Bactériologie, Hôpital Arnaud de Villeneuve, Montpellier, France, for financial and technical support.

We have no conflict of interest.

REFERENCES

- Ablordey A, et al. 2005. Multilocus variable-number tandem repeat typing of *Mycobacterium ulcerans*. *J. Clin. Microbiol.* 43:1546–1551.
- Aubry A, Chosidow O, Caumes E, Robert J, Cambau E. 2002. Sixty-three cases of *Mycobacterium marinum* infection: clinical features, treatment, and antibiotic susceptibility of causative isolates. *Arch. Intern. Med.* 162:1746–1752.
- Borradori L, Baudraz-Rosselet F, Beer V, Monnier M, Frenk E. 1991. *Mycobacterium marinum* granuloma in a fish fancier. Apropos of a case with sporotrichoid lesions. *Schweiz. Med. Wochenschr.* 121:1340–1344.
- Bozzetta E, et al. 2010. *Mycobacterium marinum* infection in a hybrid striped bass farm in Italy. *J. Fish Dis.* 33:781–785.
- Caron J, et al. 2011. Aggressive cutaneous infection with *Mycobacterium marinum* in two patients receiving anti-tumor necrosis factor- α agents. *J. Am. Acad. Dermatol.* 65:1060–1062.
- Causero A, Screm C, Beltrame A, Mastidoro L. 2003. *Mycobacterium marinum*: a case of skin granuloma complicated by tenosynovitis of the extensors. *Chir. Organi. Mov.* 88:93–97.
- Chevenet F, Brun C, Banuls AL, Jacq B, Christen R. 2006. TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC Bioinformatics* 7:439.
- dos Santos NM, Ado Vale Sousa MJ, Silva MT. 2002. Mycobacterial infection in farmed turbot *Scophthalmus maximus*. *Dis. Aquat. Organ.* 52:87–91.
- Durborow RM. 1999. Health and safety concerns in fisheries and aquaculture. *Occup. Med.* 14:373–406.
- Eberst E, et al. 2012. Epidemiological, clinical, and therapeutic pattern of *Mycobacterium marinum* infection: a retrospective series of 35 cases from southern France. *J. Am. Acad. Dermatol.* 66:e15–e16.
- Falkinham JO, III. 1996. Epidemiology of infection by nontuberculous mycobacteria. *Clin. Microbiol. Rev.* 9:177–215.
- Finkelstein R, Oren I. 2011. Soft tissue infections caused by marine bacterial pathogens: epidemiology, diagnosis, and management. *Curr. Infect. Dis. Rep.* 13:470–477.
- Goudet J. 1995. Fstat (vers. 1.2): a computer program to calculate F-statistics. *J. Hered.* 86:485–486.
- Lahey T. 2003. Invasive *Mycobacterium marinum* infections. *Emerg. Infect. Dis.* 9:1496–1498.
- Lee EY, Rubin DA, Brown DM. 2004. Recurrent *Mycobacterium marinum* tenosynovitis of the wrist mimicking extraarticular synovial chondromatosis on MR images. *Skeletal Radiol.* 33:405–408.
- Lehane L, Rawlin GT. 2000. Topically acquired bacterial zoonoses from fish: a review. *Med. J. Aust.* 173:256–259.
- Levy-Frebault VV, Portaels F. 1992. Proposed minimal standards for the genus *Mycobacterium* and for description of new slowly growing *Mycobacterium* species. *Int. J. Syst. Bacteriol.* 42:315–323.
- Liao CH, et al. 2007. Skin and soft tissue infection caused by nontuberculous mycobacteria. *Int. J. Tuberc. Lung Dis.* 11:96–102.
- Passantino A, Macri D, Coluccio P, Foti F, Marino F. 2008. Importation of mycobacteriosis with ornamental fish: medico-legal implications. *Travel Med. Infect. Dis.* 6:240–244.
- Petrini B. 2006. *Mycobacterium marinum*: ubiquitous agent of waterborne granulomatous skin infections. *Eur. J. Clin. Microbiol. Infect. Dis.* 25:609–613.
- Prearo M, et al. 2004. Mycobacterioses: emerging pathologies in aquarium fish. *Vet. Res. Commun.* 28(Suppl 1):315–317.
- Rhodes MW, et al. 2003. *Mycobacterium shottsii* sp. nov., a slowly growing species isolated from Chesapeake Bay striped bass (*Morone saxatilis*). *Int. J. Syst. Evol. Microbiol.* 53:421–424.
- Richter E, Rusch-Gerdes S, Hillemann D. 2006. Evaluation of the Geno-Type *Mycobacterium* assay for identification of mycobacterial species from cultures. *J. Clin. Microbiol.* 44:1769–1775.
- Sechi LA, et al. 2002. Strain variation in Mediterranean and Red Sea *Mycobacterium marinum* isolates. *New Microbiol.* 25:351–356.
- Stragier P, Ablordey A, Meyers WM, Portaels F. 2005. Genotyping *Mycobacterium ulcerans* and *Mycobacterium marinum* by using mycobacterial interspersed repetitive units. *J. Bacteriol.* 187:1639–1647.
- Sun G, et al. 2011. Discriminatory potential of a novel set of Variable Number of Tandem Repeats for genotyping *Mycobacterium marinum*. *Vet. Microbiol.* 152:200–204.
- Swofford DL. 1998. posting date. PAUP. Sinauer Associates, Sunderland, MA.
- Ucko M, Colorni A. 2005. *Mycobacterium marinum* infections in fish and humans in Israel. *J. Clin. Microbiol.* 43:892–895.
- Ucko M, et al. 2002. Strain variation in *Mycobacterium marinum* fish isolates. *Appl. Environ. Microbiol.* 68:5281–5287.
- van der Sar AM, et al. 2004. *Mycobacterium marinum* strains can be divided into two distinct types based on genetic diversity and virulence. *Infect. Immun.* 72:6306–6312.
- Van Seymourtier P, Verellen K, De Jonge I. 2004. *Mycobacterium marinum* causing tenosynovitis. 'Fish tank finger.' *Acta Orthop. Belg.* 70:279–282.
- van Soelingen D, de Haas PE, Hermans PW, van Embden JD. 1994. DNA fingerprinting of *Mycobacterium tuberculosis*. *Methods Enzymol.* 235:196–205.
- Vincent V, Guttierrez MC. 2005. *Mycobacterium marinum*. *Antibiotiques* 7:133–137.
- Wayne LG, Sramek HA. 1992. Agents of newly recognized or infrequently encountered mycobacterial diseases. *Clin. Microbiol. Rev.* 5:1–25.
- Weir BS. 1996. Genetic data analysis II, 2nd ed. Sinauer, Sunderland, MA.
- Weir BS, Cockerham CC. 1985. Estimating F-statistics for the analysis of population structure. *Evolution* 38:1358–1370.
- Weir M, et al. 2012. Zoonotic bacteria, antimicrobial use and antimicrobial resistance in ornamental fish: a systematic review of the existing research and survey of aquaculture-allied professionals. *Epidemiol. Infect.* 140:192–206.
- Yang RC. 1998. Estimating hierarchical F-statistics. *Evolution* 52:950–956.
- Yip MJ, et al. 2007. Evolution of *Mycobacterium ulcerans* and other mycolactone-producing mycobacteria from a common *Mycobacterium marinum* progenitor. *J. Bacteriol.* 189:2021–2029.