JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2001, p. 3272–3278 Vol. 39, No. 9
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.9.3272–3278.2001

Evaluation of PCR-Restriction Profile Analysis and IS*2404* Restriction Fragment Length Polymorphism and Amplified Fragment Length Polymorphism Fingerprinting for Identification and Typing of *Mycobacterium ulcerans* and *M. marinum*

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Received 11 December 2000/Returned for modification 28 March 2001/Accepted 29 May 2001

***Mycobacterium ulcerans* and *M. marinum* are emerging necrotizing mycobacterial pathogens that reside in common reservoirs of infection and exhibit striking pathophysiological similarities. Furthermore, the interspecific taxonomic relationship between the two species is not clear as a result of the very high phylogenetic relatedness (i.e., >99.8% 16S rRNA sequence similarity), in contrast to only 25 to 47% DNA relatedness. To help understand the genotypic affiliation between these two closely related species, we performed a comparative analysis including PCR restriction profile analysis (PRPA), IS*2404* restriction fragment length polymorphism (RFLP), and amplified fragment length polymorphism (AFLP) on a set of *M. ulcerans* ($n = 29$) and *M. marinum* ($n = 28$) strains recovered from different geographic origins. PRPA was based on a triple restriction of the 3′ end region of 16S rRNA, which differentiated *M. ulcerans* into three types; however, the technique could not distinguish *M. marinum* from *M. ulcerans* isolates originating from South America and Southeast Asia. RFLP based on IS*2404* produced six *M. ulcerans* types related to six geographic regions and did not produce any band with *M. marinum*, confirming the previous findings of Chemlal et al. (K. Chemlal, K. DeRidder, P. A. Fonteyne, W. M. Meyers, J. Swings, and F. Portaels, Am. J. Trop. Med. Hyg. 64:270–273, 2001). AFLP analysis resulted in profiles which grouped *M. ulcerans* and *M. marinum* into two separate clusters. The numerical analysis also revealed subgroups among the *M. marinum* and *M. ulcerans* isolates. In conclusion, PRPA appears to provide a rapid method for differentiating the African *M. ulcerans* type from other geographical types but is unsuitable for interspecific differentiation of *M. marinum* and *M. ulcerans*. In comparison, whole- genome techniques such as IS *2404*-RFLP and AFLP appear to be far more useful in discriminating between *M. marinum* and *M. ulcerans*, and may thus be promising molecular tools for the differential diagnosis of infections caused by these two species.**

Mycobacterium ulcerans and *M. marinum* are slow-growing mycobacterial species with optimal growth temperatures of 30 to 33°C. These organisms are emerging as clinically significant pathogens associated with skin infections (5, 9). *M. ulcerans* infection, or Buruli ulcer (BU), was first described in Bairnsdale, Australia, in 1948 (17) and was subsequently found in numerous, mostly tropical countries in Africa, the Americas, Southeast Asia, and the central Pacific. Recent reports describe increases in the incidence of BU in Benin (13), Australia (6, 8, 12, 34), and Côte d'Ivoire (18). *M. ulcerans* causes chronic necrotizing ulcers in the skin of humans (22) and other mammals (22, 23). The epidemiology of BU is poorly understood, but most foci are associated with slow-flowing or stagnant water; however, the natural reservoir of *M. ulcerans* remains unknown. *M. marinum*, first described in Sweden (1), gives rise to infections in temperate climates and is the cause of fish tank and swimming pool granulomas (16).

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M. ulcerans is often difficult to isolate from clinical specimens and usually requires 6 to 8 weeks to produce visible growth in primary culture (23, 24). Definitive identification of *M. ulcerans* is thus time-consuming; however, it can be recognized by classic molecular and microbiologic methods (20, 24). *M. marinum*, once cultured, is readily identified by using conventional mycobacterial characterization methods. It grows relatively quickly (1 to 2 weeks) and is easily recognized as a result of its photochromogenicity (20). While infections due to *M. marinum* can usually be treated with antimycobacterial drugs, very few cases of BU lesions respond favorably to antimicrobial therapy (2), making wide surgical excision and skin grafting the treatment of choice.

In the last decade, various DNA-based techniques have been used to classify mycobacteria (15, 25, 26, 30). All such studies have demonstrated a high taxonomic affiliation between *M. ulcerans* and *M. marinum*. Other attempts have targeted the 3′ end of 16S rRNA gene and found four subtypes of *M. ulcerans* related to their geographic origin, except for one isolate from Suriname, which exhibited the same sequence as *M. marinum* (20). The use of IS*2404* resriction fragment length polymorphism (RFLP) analysis (2) led to the classification of *M. ulcer-*

TABLE 1. Source and origin of the mycobacterial strains used in this study

Species	Strain	Source	Received from (other strain designation)[a]	Geographical origin
M. ulcerans (n = 29)	ITM7922	Human	V.V., IPT141090018	French Guiana
	ITM842	Human	V.K. 701357	Suriname
	ITM 8756	Human	ATCC 33728	Japan
	ITM 5114	Human	P.L.	Mexico
	ITM 94-1330	Human	L.S., 143150	Australia
	ITM 94-1325	Human	L.S., 187859	Australia
	ITM 5122	Human	F.P.	Democratic Republic of Congo
	ITM 94-662	Human	F.P.	Ivory Coast
	ITM 94-339	Human	F.P.	Australia
	ITM 94-1327	Human	F.P.	Australia
	ITM 94-1329	Human	F.P.	Australia
	ITM 94-886	Human	F.P.	Benin
	ITM 97-111	Human	F.P.	Benin
	ITM 97-104	Human	F.P.	Benin
	ITM 9146	Human	F.P.	Benin
	ITM 94-815	Human	F.P.	Ivory Coast
	ITM 97-684	Human	F.P.	Benin
	ITM 97-490	Human	F.P.	Benin
	ITM 96-658	Human	F.P.	Angola
	ITM 97-680	Human	F.P.	Angola
	ITM 95-1112	Human	F.P.	Australia
	ITM 9114	Human	F.P.	Benin
	ITM 9550	Human	D.D., 17679	Australia
	ITM 9540	Human	D.D., 11098	Australia
	ITM 9537	Human	D.D., 11878	Papua New Guinea
	ITM 94-1324	Human	L.S., 176862	Australia
	ITM 8849	Human	D.D., 8471/69	Australia
	ITM 5147	Human	ATCC 19423^T	Australia
	ITM 94-1326	Human	L.S., 93160339	Australia
M. marinum (n = 28)	ITM 94-996	Fish	K.H.	South Africa
	US H35392/93	Human	P.S.	United States
	US M6	Fish	P.S.	United States
	ITM 7732	Fish	ATCC 927^T	United States
	ITM 94-979	Fish	K.H.	South Africa
	IPP 99000876	Human	V.V.	France
	IPP 99/890	Human	V.V.	France
	IPP 2000449	Human	V.V.	France
	IPP 99000843	Huamn	V.V.	France
	IPP 2000355	Human	V.V.	France
	US LS	Fish	P.S.	United States
	IPP 031038	Human	V.V.	France
	TON F106/91	Human	T.T.	Norway
	IPP 99/363	Human	V.V.	France
	ITM 8022	Human	F.P.	Belgium
	ITM 94-56	Human	F.P.	Belgium
	ITM 97-1321	Axololt.	F.P.	Belgium
	IPP 99000821	Human	V.V.	France
	ITM 98-852	Human	F.P.	Italy
	ITM 00-533	Human	F.P.	Belgium
	ITM 99-822	Human	F.P.	Belgium
	ITM 99-2570	Human	F.P.	Belgium
	ITM 99-3021	Human	F.P.	Belgium
	TON T25/84	Water	T.T.	Norway
	IPP CCUG533	Human	V.V.	France
	ITM 1717	Armadillo	F.P.	United States
	ITM 1726	Armadillo	F.P.	United States
	ITM 97-1320	Axololt.	F.P.	Belgium

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ans into six groups, including the isolate from Suriname as *M. ulcerans* type VI. Unfortunately, because only a few *M. marinum* isolates were included in the last two studies (2, 20), no reliable conclusions could drawn made on an interspecific relationship between *M. ulcerans* and *M. marinum*.

In the present study, three DNA-based methods were evaluated for the purpose of the identification and typing of *M. ulcerans* and *M. marinum* to define the taxonomic and phylogenetic relationship of these two species. PCR restriction profile analysis (PRPA) was used for the first time for studies of *M. ulcerans* and *M. marinum*. This approach is comparable to the PCR restriction enzyme analysis method described by

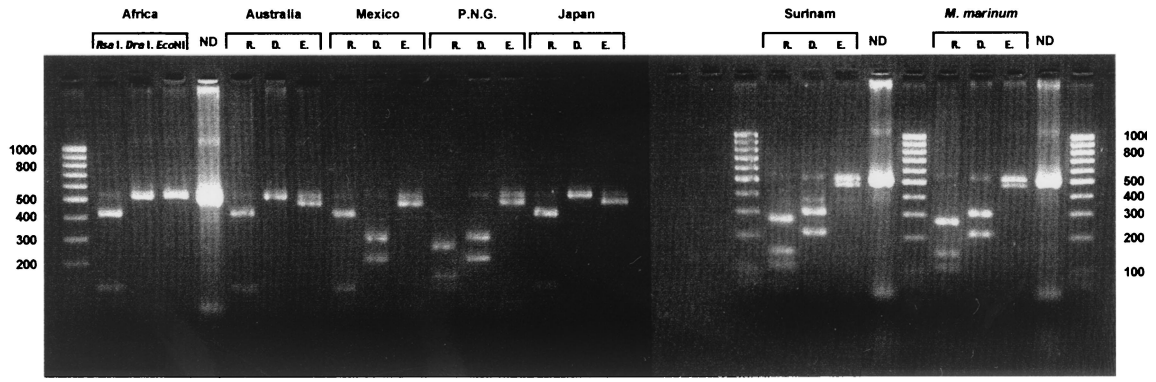

FIG. 1. Examples of PCR restriction profiles obtained from a representative set of strains using three restriction enzymes, *Rsa*I, *Dra*I, and *Eco*NI. The first and last lanes show the 100-bp ladder. ND, no digested PCR product; R, D, and E, *Rsa*I, *Dra*I, and *Eco*NI, respectively, P.N.G., Papua New Guinea.

Telenti et al. (32). PRPA differs from the latter technique in both the targeted region for PCR (i.e., the 3′ end of the 16S rRNA gene) and the use of three restriction enzymes (*Rsa*I, *Dra*I, and *Eco*NI). As a follow-up to our previous study (2) we applied IS*2404* RFLP to a comparable number of *M. ulcerans* and *M. marinum* strains to determine the phylogenetic relationship between these two species. Finally, in view of the ability of amplified fragment length polymorphism (AFLP) analysis to discriminate continental types of *M. ulcerans* (10), we have evaluated the usefulness of this technique in differentiating *M. ulcerans* from *M. marinum*.

MATERIALS AND METHODS

Strains used. All 57 isolates included in this study are part of the Institute of Tropical Medicine collection and were assigned to the species *M. ulcerans* and *M. marinum* by conventional biochemical methods (36). Fresh subcultures were made on tubes of Löwenstein- Jensen medium. The collection comprised type and reference strains originally obtained from clinical sources. Some strains were kindly provided by V. Vincent (Institut Pasteur de Paris, Paris, France), P. Lavalle (Centro Dermatologico Pascua, Mexico, Mexico), W. R. Faber and P. Van Keulen (Academic Medical Center, Amsterdam, The Netherlands), T. Tønjum (Institute of Microbiology, Oslo, Norway), P. L. Small (National Institutes of Health, Hamilton, Mont.), and H. F. A. K. Huchzermeyer (Veterinary Research Institute, Onderstepoort, South Africa).

PCR restriction profile analysis. The lysates from all isolates were obtained by resuspending a loopful of bacterial cells in 100 μl of TE (10 mM Tris, 1 mM EDTA [pH 8]) containing 1% (vol/vol) Triton X-100 and heating at 100°C for 15 min. Then 10 μl of lysate was added to 50 μl of PCR mixture containing 50 pmol each of primers P11 (5′-AGGAATTCTGGGTTTGACATGCACAGGA-3′) and P61 (5′-AAGGAGGTGATCCAGCCGCA-3′), 1 U of AmpliTaq DNA polymerase (Roche Molecular Systems), 200 μM each deoxyribonucleoside triphosphate, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 10 mM Tris-HCl (pH 8.4) and overlaid with mineral oil. Primers P11 and P61 target a 525-bp fragment of the 3′ end of the 16S rRNA gene of the genus *Mycobacterium*. Cycling was performed as follows: denaturation at 94°C for 5 min; amplification for 30 cycles at 94°C for 45 s, 56°C for 45 s, and 72°C for 45 s and a final extension at 72°C for 7 min. Subsequently, 7 μl of amplified DNA was electrophoresed through a 2% agarose gel, and bands were detected by ethidium bromide staining and UV transillumination. Restriction analysis of the amplification product was carried out for 2 h at 37°C in 20 μl of incubation buffer containing 15 U of restriction enzyme (*Rsa*I, *Dra*I, and *Eco*NI [Sigma]) and 8 μl of PCR product. Restriction fragment patterns were analyzed by gel electrophoresis of the restriction mixture at 50 V for 1.5 h in 3% small-fragment agarose gel (Eurogentec).

Southern blotting and preparation of the IS*2404* probe. The IS*2404* probe was prepared by chemical labeling of a PCR product as described by van Embden et al. for the preparation of the IS*6110* probe (35). The primers used were PGP3 and PGP4 as described previously (2).

For Southern blot analysis, *M. ulcerans* genomic DNA was digested with the appropriate restriction enzyme (*Pvu*II) and separated overnight by electrophoresis through a 0.8% agarose gel (35). DNA was transferred to the Hybond N^+ nylon membrane (Amersham Corp.) for 1 h in 0.4 M NaOH using a vacuum blotter system (Appligene-oncor). Hybridizations were performed at 42°C with high-stringency posthybridization washes (35). DNA was detected with the ECL direct system as specified by the manufacturer (Amersham Life Science).

AFLP analysis. The DNA was isolated and purified as described previously (35). All protocols relating to the preparation of DNA templates for AFLP analysis were performed essentially as described previously (11). Oligonucleotide sequences, amplification procedures, electrophoresis conditions, and data capture and analysis have been described elsewhere (10).

RESULTS

A collection of 29 *M. ulcerans* and 28 *M. marinum* isolates was used in this study (Table 1). These isolates originated from

TABLE 2. Fragment sizes obtained by triple restriction of the 16S rRNA PCR product of *M. ulcerans* and *M. marinum*

Strain[a]	*Rsa*I		*Dra*I		*Eco*NI	
	Length of fragments observed (bp)	No. of restriction sites	Length of fragments observed (bp)	No. of restriction sites	Length of fragments observed (bp)	No. of restriction sites
Africa (type I)[a]	419, 120	1	525	0	525	0
Australia (type II)[a]	419, 120	1	525	0	500	1
Mexico (type III)[a]	419, 120	1	300, 220	1	500	1
Papua New Guinea	272, 147, 120	2	300, 220	1	500	1
Japan[a] (type IV)[a]	419, 120	1	525	0	500	1
Suriname	272, 147, 120	2	300, 220	1	500	1
M. marinum	272, 147, 120	2	300, 220	1	500	1

[a] Classification reported by Portaels et al. (20).

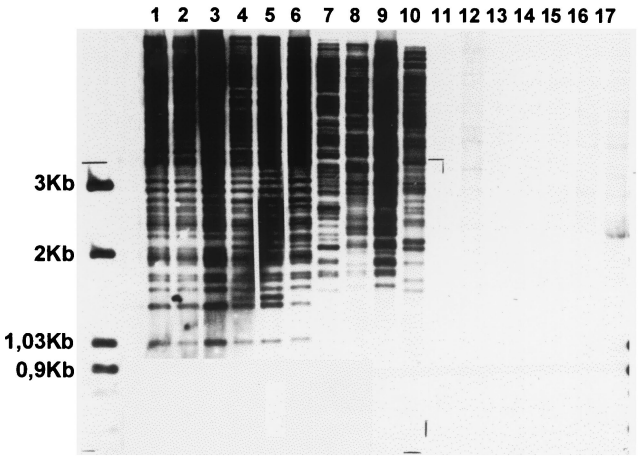

FIG. 2. A representative Southern blot obtained with 10 *M. ulcerans* (lanes 1 to 10) and 7 *M. marinum* (lanes 11 to 17) strains from different geographic origins. Lanes: 1 to 3, African; 4, reference strain ATCC 19423; 5, Australian; 6, Southeast Asian; 7, Asian; 8 and 9, South America; 10, Mexican; 11 and 12, United States; 13, reference strain ATCC 927; 14 and 15, Belgian; lanes 16 and 17, South African. The molecular size (in kilobases) is shown on the left.

a variety of sources and represent both temporal and geographic diversity. All the isolates were of human origin except for *M. marinum*, for which nine strains were of animal origin and one was from water (Table 1)

PCR restriction profile analysis. In Fig. 1, the various profiles derived from the three restrictions of the 525-bp fragment 16S rRNA amplicons are shown for *M. ulcerans* and *M. marinum* strains originating from different geographical regions. Table 2 lists the observed sizes of the fragments from the digested amplicons which are compatible with the predicted sizes obtained by GeneBank sequence analysis of the 3′-end 16S RNA gene. All the African *M. ulcerans* isolates tested yielded the same profile with *Rsa*I (data not shown), and a highly similar banding pattern was also produced by the Australian, Mexican, and Japanese strains. On the other hand, the Papua New Guinean and Surinamese strains of *M. ulcerans* and all the *M. marinum* isolates exhibited the same pattern with *Rsa*I. No *Dra*I restriction sites were found with *M. ulcerans* strains from Africa, Australia, or Japan. All the *M. marinum* strains and the *M. ulcerans* strains from Mexico, Papua New Guinea, and Suriname generated two bands at 300 and 220 bp. With *Eco*NI there was incomplete digestion with all the isolates except for the African *M. ulcerans* strains. By combining the three restriction profiles (Fig. 1), we found that all the *M. ulcerans* strains tested in this study are categorized into three types (African, Australian, and Mexican), except for the Papuan New Guinean and Surinamese isolates, which exhibited the same profiles as all the *M. marinum* isolates evaluated. PRPA applied to more than 50 African strains of *M. ulcerans* resulted in the same profile. Furthermore, 18 relatively closely related mycobacterial species, subjected to the same technique and with the same set of restriction enzymes, produced patterns that differed from the four profiles shown in Fig. 1 (K. Chemlal, unpublished data).

IS*2404* RFLP profiles. Representative patterns obtained with chromosomal DNA of *M. ulcerans* and *M. marinum* probed with IS*2404* are shown in Fig. 2. From the strains shown in Table 1, only representatives of *M. ulcerans* produced an IS*2404* RFLP band pattern (lanes 1 to 10) whereas no profile was obtained with seven selected *M. marinum* strains (lanes 11 to 17). Within the IS*2404* RFLP fingerprints of *M. ulcerans*, the 3-kb zone was polymorphic and allowed further subtyping of the *M. ulcerans* isolates into six groups (Fig. 2 legend).

AFLP analysis. AFLP patterns were obtained by using the primer combination A02 plus T02 (10). Typically, the AFLP patterns generated comprised 30 to 50 bands (data not shown). Following numerical analysis using the Pearson product-moment correlation coefficient, the 57 strains included in this study were grouped in two AFLP clusters at a delineation level of 60% (Fig. 3). These two clusters uniformly corresponded to the phenotypic species identifications of the strains, i.e., *M. ulcerans* and *M. marinum*. Within each of these clusters, a number of intraspecific subdivisions could be observed. Compared to the IS*2404* RFLP and PRPA results, there was no correlation with geographic origins.

DISCUSSION

The identification of mycobacterial species constitutes a critical step in patient management because the results obtained influence the choice of appropriate treatment. Classical procedures to establish the species of mycobacteria based on conventional biochemical tests can take several weeks and may generate inaccurate diagnoses. For *M. ulcerans*, there are only a few phenotypic characteristics, making additional molecular tests essential for conclusive identification. PCR-based methods offer several advantages including speed, sensitivity, and specificity (3, 4, 15, 21, 30). In the present investigation, a combination of PCR amplification of a 525-bp 16S rRNA fragment and a triple-restriction analysis (PRPA) was used to differentiate *M. ulcerans* from the closely related species *M. marinum*. The results of PRPA on a set of geographically diverse *M. ulcerans* isolates showed three different PRPA profiles (Table 2): subtype 1, representing the African strains; subtype 2, representing the Australian and Asian strains; and subtype 3, representing the Mexican strain. The *M. ulcerans* isolates from South America (strains ITM842 and ITM7922) gave the same profile as *M. marinum*, showing that PRPA is not suitable for a clear-cut differentiation between these two species. This result is in accord with previous findings that the 3′-end 16S rRNA sequence of the Surinamese *M. ulcerans* and *M. marinum* strains are identical (20). All the African and Australian *M. ulcerans* strains as well as all the *M. marinum* strains included in this study yielded highly similar PRPA patterns with the three restriction enzymes employed. This finding suggests that the discriminatory power of PRPA to differentiate strains within certain geographical regions is limited. However, PRPA proved to be a rapid method for the identification of *M. ulcerans* subtypes I and II compared to the laborious procedures involved in sequencing.

The pattern of conserved and variable domains within the 16S rRNA molecule offers the unique advantage of a single amplification reaction for identification of virtually all *Mycobacterium* spp. (14, 26, 29, 37). Unfortunately, the number of polymorphic sites in the 16S rDNA in the genus *Mycobacterium* is rather low since some species have the same sequence

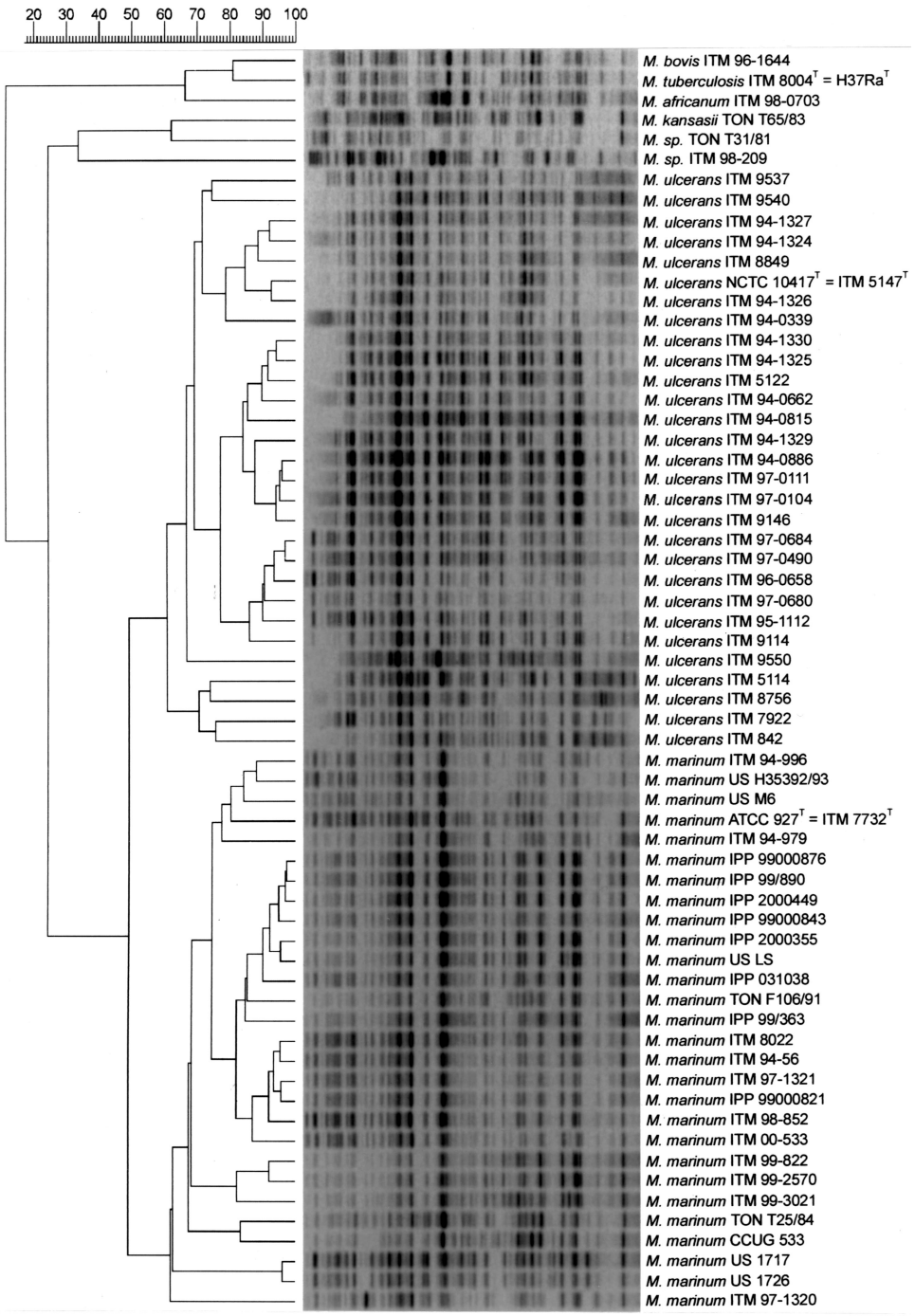

FIG. 3. Numerical analysis of normalized AFLP band patterns generated from *M. ulcerans* ($n = 29$) and *M. marinum* ($n = 28$) using primer combination A02 and T02. In addition, six outlying strains representing other mycobacterial taxa were included: *M. tuberculosis* (ITM 8004^T), *M. bovis* (ITM 96-1644), *M. africanum* (ITM 98-0703), *M. kansaii* (TON T65/83), and two *Mycobacterium* strains (TON T31/81 and ITM 98-209). The dendrogram was constructed using the unweighted paired-group using arithmetic averages with correlation levels expressed as percentages of the Pearson product-moment correlation coefficient. The clusters representing *M. ulcerans* and *M. marinum* were defined at a delineation level of 60%.

(*M. kansasii* and *M. gastri*) or possess a very high degree of sequence similarity (99.9%) (*M. malmoense* and *M. szulgai*) (26). Molecular distinction between *M. ulcerans* and *M. marinum* based on 16S rRNA is very difficult due to the existence of identical signature regions and only two single-nucleotide differences at the 3′ end of the gene (20). As shown in the present study (Fig. 1; Table 2), the high degree of conservation of the mycobacterial 16S rRNA gene may explain why PRPA of the 16S rRNA genes of *M. ulcerans* and *M. marinum* is not useful for discriminating between these two species. Other molecular methods have tried to circumvent this limitation in species discrimination (27), including sequence analysis of a

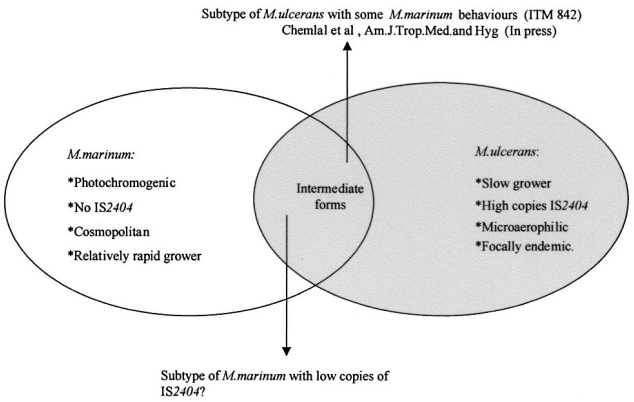

FIG. 4. Hypothetical presentation showing some differential characteristics of *M. marinum* and *M. ulcerans* and the postulated position of putative transitory forms between these two taxa.

360-bp gene fragment characteristic for GyrA lacking an intein and the 16-kDa HSP, an α-crystalline homologue (I. C. Shamputa, unpublished data). However, none of these methods so far permits an unequivocal differentiation between *M. ulcerans* and *M. marinum*.

To address the shortcomings of the PRPA method for identifying *M. ulcerans* and *M. marinum*, the current investigation was extended by an evaluation of two other molecular methods, namely, IS*2404* RFLP and AFLP. The IS*2404* RFLP technique was recently used in our laboratory (2) and was able to distinguish six groups in *M. ulcerans*. In the present study, the same results were obtained by analyzing the polymorphic region (<3 kb) of all the *M. ulcerans* profiles (Fig. 2). None of the *M. marinum* strains included in this study provided a band with the IS*2404*-specific probe (Fig. 2), confirming that this insertion sequence is specific to *M. ulcerans*. The presence of numerous copies of the IS*2404* insertion sequence in *M. ulcerans* (30) and its absence in *M. marinum* suggests that the highly related genomes of these two species may have been subjected to an evolutionary rearrangement by acquiring or losing insertion sequences. A recent genetic analysis of *M. ulcerans* and *M. marinum*, including multilocus sequencing and macrorestriction fragment polymorphism analysis, strongly supports this hypothesis (31). Because the IS*2404* RFLP method is not helpful at the subspecific level for the identification of *M. marinum*, an alternative DNA fingerprinting technique that encompasses the entire genome is essential. The PCR-based AFLP technique is such a whole-genome coverage technique and has already been successfully applied as a reproducible and reliable taxonomic tool for the differentiation of *M. tuberculosis, M. bovis*, and *M. ulcerans* (10). In the present study, AFLP was evaluated for its ability to discriminate among strains of *M. ulcerans* and *M. marinum* at the interspecific level. Using the primer combination A02 plus T02, both having one C extension at their 3′ ends (10), visual inspection as well as clustering analysis using the Pearson product-moment correlation coefficient (Fig. 3) revealed that *M. ulcerans* can be clearly separated from *M. marinum* by AFLP. In sharp contrast to their very high 16S rRNA sequence homology (>99.8%), DNA-DNA hybridization results have shown that *M. ulcerans* and *M. marinum* exhibit only 25 to 47% DNA homology (33). Since AFLP clustering is known to support classification based on DNA hybridization groups in a wide range of bacterial genera (28), it is not surprising that *M. ulcerans* and *M. marinum* represent two distinct AFLP groups. Furthermore, numerical analysis of normalized AFLP band patterns also revealed two or more subclusters in each of the two species-specific AFLP clusters (Fig. 3). Within *M. ulcerans*, these subgroupings did not correlate with the geographical origin of the strains as was observed with PRPA (Fig. 1). However, as previously demonstrated, the use of primer combination A02 and T01 in conjunction with a band-based similarity coefficient for numerical analysis differentiated African from Australian *M. ulcerans* types (10). Also, in the AFLP cluster encompassing *M. marinum*, there was no clear relationship between subgroupings and the source or origin of strains. Therefore, we recommend that the use of multiple AFLP primer combinations and pulsed-field gel electrophoresis be further explored for epidemiological studies on *M. marinum*.

In conclusion, the present study demonstrates the limitations of the 16S rRNA-based PRPA technique to differentiate *M. ulcerans* from *M. marinum* and the usefulness of the DNA fingerprinting techniques utilizing IS*2404* RFLP and AFLP to distinguish between these two species. Collectively, the striking phylogenetic closeness reported by Tønjum et al. (33) and the IS*2404* RFLP results presented in this study further support the recent findings of Stinear and et al. (31) in which a comparative genetic analysis revealed recent divergence of *M. ulcerans* from *M. marinum*. In our opinion, this hypothesis can be further supported by the following two observations: (i) the IS*2404* element is present in high copy number in *M. ulcerans* collected from different geographic sources (30) but absent in the closely related species *M. marinum*; and (ii) similar to the occurrence of IS*6110* in *M. tuberculosis* (7), the microaerophilic growth conditions required for *M. ulcerans* (19) may play a role in the stimulation of transposition of IS*2404* into the genome of these species. The key to confirming the hypothesized recent divergence of *M. ulcerans* from *M. marinum* would be finding a missing link between the two, e.g., an *M. marinum* strain with a low IS*2404* copy number (Fig. 4), indicating an evolving characteristic within the taxon.

ACKNOWLEDGMENTS

We thank D. Dawson, P. Lavalle, P. H. J. van Keulen, J. L. Stanford, P. L. C. Small, T. Tønjum, and F. A. K. Huchzermeyer for providing *M. ulcerans* and *M. marinum* isolates. We also thank J. C. Palomino and S. R. Pattyn for assistance and advice.

This work was generously supported by the Damien Foundation (Brussels) and the Belgian Agency for Development (Project: Buruli ulcer in Benin). It was also partially supported by The Fund for Scientific Research, Flanders (Belgium) (F.W.O.-Vlaanderen) (contract G.0368.98).

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