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rRNA gene RFLP as an identification tool for *Corynebacterium* species

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The value of rRNA gene RFLP analysis (ribotyping) as a tool for *Corynebacterium* and *Turicella* species identification was evaluated. Seventy-four strains representing 26 different species or subspecies were analysed by *Bst*EII, *Sma*I and *Sph*I ribotyping. Numerical analysis of the resulting rDNA banding patterns was performed by Dice coefficient correlation in order to establish a database for species identification. In general, most of the strains belonging to the same species clustered together. Interestingly, *Bst*EII clustering of many species followed known phylogenetic lineages. This was not evident with the more heterogeneous *Sma*I and *Sph*I patterns. The *Sma*I patterns contained a 1800 bp band in the digests of all species studied with the exception of *Corynebacterium urealyticum*. *Sph*I digestion resulted in the most heterogeneous patterns. The information provided by all three enzymes was considered essential for the reliable linking of strains of unknown identity with defined species in the database. It is concluded that ribotyping provides an useful tool for screening and characterization of potentially new *Corynebacterium* species.

Keywords: *Corynebacterium*, rRNA gene restriction patterns, ribotyping, identification, taxonomy

INTRODUCTION

Within the group of coryneform bacteria (i.e. aerobic, asporogenous, non-partially acid-fast, irregular, Gram-positive rods), the genus *Corynebacterium* contains the largest number of defined species (40 in March 1998). *Bergey's Manual of Systematic Bacteriology* (Collins & Cummins, 1986) lists only 16 valid *Corynebacterium* species; however, between 1987 and 1995, 12 new species were defined (Funke *et al*., 1997d), and between 1996 and March 1998, another 11 new species were defined. Most of the species described during 1986–1998 were isolated from human clinical samples. The identification of corynebacteria from clinical samples, together with the appearance of newly established species, is still causing confusion for laboratories not used to dealing with the characteristics of this diverse genus.

Since it is most likely that a plethora of further new *Corynebacterium* species will be described in the near future, it was considered appropriate to evaluate the application of rRNA gene restriction fragment patterns (ribotyping) as a means of identification of coryneforms. The concept of ribotyping was established in the 1980s (Grimont & Grimont, 1986) and it has been used mainly for tracing the possible clonality of epidemiologically associated isolates. However, it was originally described as a potential tool for taxonomy and it is now used more and more for species identification. Several studies have shown that ribotyping deals with a genetically constant feature, resulting in species-specific bands or typical banding patterns within a species. For Gram-positive microbes, it has been used for identification within the genera *Enterococcus* (Hall *et al*., 1992), *Streptococcus* (Rudney & Larson, 1993), *Lactobacillus* (Björkroth & Korkeala, 1996, 1997) and *Leuconostoc–Weissella* (Björkroth *et al*., 1998).

The systematic use of ribotyping within the genus *Corynebacterium* has never been studied before. This study set out to evaluate the potential of this technique for the identification of *Corynebacterium* species. Several restriction enzymes were evaluated in order to establish a revealing database aiding the identification of these bacterial species.

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METHODS

Strains. Seventy-one strains representing 25 different medically relevant *Corynebacterium* species or subspecies were used in the present study (Table 1). All strains originated from epidemiologically unrelated patients and belonged to the culture collection of the Department of Medical Microbiology, University of Zürich (DMMZ), established by G. Funke. *Turicella otitidis* was included because this genus is the closest phylogenetic neighbour of the genus *Corynebacterium* (Pascual *et al*., 1995; Ruimy *et al*., 1995) and the taxon is often confused with *Corynebacterium* species in the routine clinical laboratory (Funke *et al*., 1997d).

Isolation of DNA. Depending on the growth rate, strains were incubated for 18–48 h at 37 °C on Columbia agar base (Gibco-BRL) supplemented with sheep blood (7 %). For the lipophilic species, this medium was also supplemented with 1 % Tween 80 (Merck). After checking colony morphology, a cell mass of 30 mg wet weight was scraped from the plate and used for DNA isolation. DNA was isolated by the guanidium thiocyanate method (Pitcher *et al*., 1989) with the modification of a combined lysozyme and mutanolysin treatment (Björkroth & Korkeala, 1996).

Selection of restriction enzymes for ribotyping. Initially, 15 restriction enzymes, *Bam*HI, *Bgl*II, *Bss*HII, *Bst*EII, *Cla*I, *Dpn*II, *Dra*I, *Eco*RI, *Hin*dIII, *Sac*II, *Sgr*AI, *Sma*I, *Spe*I, *Sph*I and *Ssp*I (New England Biolabs), were tested for ribotyping of *Corynebacterium–Turicella* by using two strains, *Corynebacterium auris* 328^T and *T. otitidis* 272.

Ribotyping. Restriction endonuclease treatment of 2 µg DNA was done as specified by the manufacturer (New

Table 1. Strains used in the study

Strains were obtained from the Culture Collection of the Department of Medical Microbiology, University of Zürich (DMMZ) or the Swiss Culture Collection for Bacteria (LA).

Taxon	Strain	Taxon	Strain
C. accolens	DMMZ 1882^T	*C. jeikeium*	DMMZ 1111^T
	DMMZ 1883		DMMZ 1949
	DMMZ 1351	*C. macginleyi*	DMMZ 1352^T
C. afermentans subsp. *afermentans*	DMMZ 545^T		DMMZ 228
	DMMZ 1305	*C. minutissimum*	LA 3512^T
	DMMZ 1829		DMMZ 693
	DMMZ 1768		DMMZ 790
C. afermentans subsp. *lipophilum*	DMMZ 872^T	*C. mucifaciens*	DMMZ 2278^T
	DMMZ 1620		DMMZ 216
	DMMZ 1764		DMMZ 300
C. amycolatum	DMMZ 455^T	*C. propinquum*	DMMZ 1319
	DMMZ 2427		DMMZ 1814
	DMMZ 2460		DMMZ 2068
C. argentoratense	DMMZ 1336^T	*C. pseudodiphtheriticum*	LA 3518
	DMMZ 1187		DMMZ 2120
	DMMZ 1619		DMMZ 2283
C. auris	DMMZ 328^T	*C. pseudotuberculosis*	DMMZ 960
	DMMZ 355		DMMZ Ctb4
	DMMZ 399		DMMZ 962
C. confusum	DMMZ 2439^T	*C. riegelii*	DMMZ 2415^T
	DMMZ 3259		DMMZ 2582
	DMMZ 3364		DMMZ 3128
C. coyleae	DMMZ 214^T	*C. striatum*	LA 3520^T
	DMMZ 1075		DMMZ 1527
	DMMZ 1415		DMMZ 1728
C. diphtheriae	DMMZ 2401^T	*C. ulcerans*	DMMZ 957
	DMMZ 1188		DMMZ 958
	DMMZ 1314		DMMZ 959
C. durum	DMMZ 2705^T	*C. urealyticum*	DMMZ 34
	DMMZ 2740		DMMZ 874
C. falsenii	DMMZ 2537^T		DMMZ 1028
	DMMZ 2569	*C. xerosis*	DMMZ 1504^T
	DMMZ 2570		DMMZ 2543
C. glucuronolyticum	DMMZ 838^T		DMMZ 3296
	DMMZ 842	*T. otitidis*	DMMZ 234^T
	DMMZ 944		DMMZ 262
C. imitans	DMMZ 2023^T		DMMZ 272

England Biolabs). Southern transfer and hybridization were performed as outlined previously (Björkroth & Korkeala, 1996). The rDNA probe for ribotyping was labelled by reverse transcription (AMV-RT; Promega) by using Dig DNA label (Boehringer Mannheim) as described before (Blumberg *et al*., 1991). A lambda *Hin*dIII digest (Boehringer) was applied three times in each gel to allow normalization between the different electrophoresis runs.

Ribotyping data management. Membranes were scanned by a Hewlett Packard ScanJet 4c/T scanner. Numerical analysis of banding patterns was performed using the GELCOMPAR software version 4.0 (Applied Maths). The similarity between all pairs was expressed by Dice coefficient correlation and UPGMA (unweighted pair group method using arithmetic averages) clustering was used for construction of the dendrogram.

RESULTS AND DISCUSSION

Of the 15 restriction enzymes tested, *Bst*EII, *Hin*dIII, *Sma*I and *Sph*I digestion resulted in good cleavage of DNA from the test strains with revealing ribotypes. These four enzymes were selected for use for the remaining 72 strains. Since the use of *Hin*dIII resulted in incomplete digestion with many of the strains, numerical analysis of the strains was performed for *Bst*EII, *Sma*I and *Sph*I ribotypes.

*Bst*EII ribotypes generally had fewer bands (two to 11) (Fig. 1) than the ribotypes obtained with the two other restriction enzymes (Figs 2 and 3). In particular, *Bst*EII cleavage resulted in patterns with few high-molecular-mass fragments. For most species, all strains tested clustered together (e.g. *Corynebacterium falsenii*, *Corynebacterium xerosis*, *Corynebacterium amycolatum*, *Corynebacterium riegelii*, *C. auris*, *T. otitidis*, etc.), whereas, for example, the three strains of *Corynebacterium confusum* (Funke *et al*., 1998) and the three strains of *Corynebacterium propinquum* did not (Fig. 1). When only a few high-molecular-mass fragments with only slight differences in location on a membrane are formed, the numerical analysis is prone to error. This can be seen in the interpretation of *Corynebacterium afermentans* subsp. *afermentans* 1620 and *T. otitidis* 272 patterns (Fig. 1). These patterns were recognized as identical; however, when inspected visually, the two low-molecular-mass fragments do not share the same location. This problem could be circumvented by extended gel running times. It is interesting to note that many phylogenetically closely related species, like *Corynebacterium jeikeium* and *C. falsenii* (Sjödén *et al*., 1998), *C. xerosis* and *C. amycolatum* (Pascual *et al*., 1995; Ruimy *et al*., 1995), *Corynebacterium mucifaciens* and *C. afermentans* (Funke *et al*., 1997b), *Corynebacterium coyleae* and *C. afermentans* (Funke *et al*., 1997c), *Corynebacterium accolens* and *Corynebacterium macginleyi* (Ruimy *et al*., 1995) and *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* (Pascual *et al*., 1995; Ruimy *et al*., 1995) clustered together. This observation was made exclusively in *Bst*EII patterns and it may be due to the presence of fewer cleavage sites in the area where the rRNA genes are located, resulting in homogeneous patterns within phylogenetic neighbours.

*Sma*I ribotyping patterns of *Corynebacterium* species showed between five and 12 bands (Fig. 2). Interestingly, all species tested except the three *C. urealyticum* strains showed a common band of approximately 1800 bp. This fragment may be cleaved from within the rRNA gene itself, in an area constant for the coryneform species. Again, most of the strains belonging to the same species clustered together and only a few species, like *Corynebacterium minutissimum*, presented heterogeneous patterns.

*Sph*I ribotyping patterns of corynebacteria showed between two and nine bands (Fig. 3). In general, heterogeneity of these ribotyping patterns was greater than for the other two restriction enzymes. Strains belonging to the species *C. xerosis*, *C. urealyticum*, *C. minutissimum*, *C. afermentans* subsp. *lipophilum* and *Corynebacterium durum* did not cluster together. However, all strains belonging to each of these species, with the exception of *C. minutissimum*, did cluster together when at least one of the three enzymes was used for ribotyping. Therefore, it is concluded that a combination of the three enzymes is essential in order to identify these medically relevant *Corynebacterium* species. Since each enzyme provides a different characterization result, no general threshold similarity value can be set within a species. Most of the species exhibited similarity values of 50 to 100% between strains when clustered together (Figs 1–3).

So far, ribotyping of *Corynebacterium* species has been used mainly for strain tracking or large-scale epidemiological investigations. The majority of studies (applying restriction enzyme *Bst*EII) have focussed on *Corynebacterium diphtheriae* (De Zoysa *et al*., 1995; Popovic *et al*., 1996; Riegel *et al*., 1997) as the most significant pathogen within the genus *Corynebacterium*. Further studies used ribotyping (restriction enzymes *Hin*dIII, *Pvu*II and *Bst*EII) to demonstrate nosocomial transmission of *C. jeikeium* (Pitcher *et al*., 1990) as well as transmission of *Corynebacterium imitans* between non-hospitalized persons (Funke *et al*., 1997a). However, ribotyping (with restriction enzymes *Pvu*II and *Eco*RI) has also been applied successfully in taxonomic descriptions of *C. afermentans* (Riegel *et al*., 1993b) and *C. propinquum* (Riegel *et al*., 1993a). These authors demonstrated clearly that both *C. afermentans* and *C. propinquum* could be identified by ribotyping when multiple restriction enzymes were applied. In a comprehensive study, Soto *et al*. (1991) observed, based on numerical analysis of rRNA gene patterns (restriction enzymes *Hin*dIII and *Eco*RI), that the former CDC coryneform group D-2 bacteria (synonymous with *C. urealyticum*) clustered together and represented a taxon separate from the other established corynebacteria. For the non-medical corynebacteria, Liebl *et al*. (1991) demonstrated that ribotyping (applying restriction

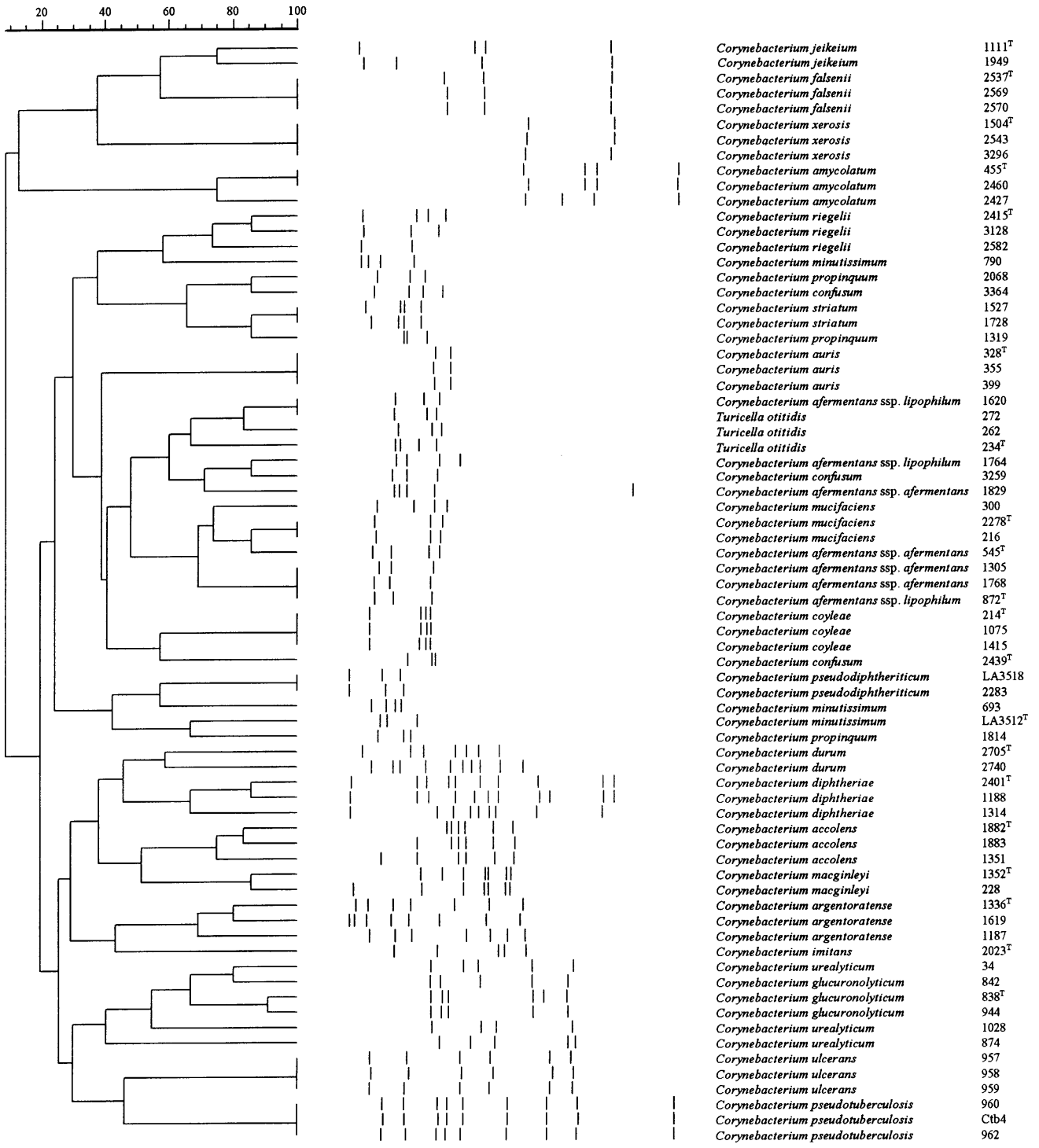

Fig. 1. *Bst*EII dendrogram and ribotypes; left, high molecular mass (23 kbp) and right, low molecular mass (500 bp).

enzymes *Bam*HI, *Hin*dIII or *Bgl*I) leads to the correct identification of *Corynebacterium glutamicum* strains.

It remains unclear to us why, despite of the promising results of Liebl *et al.* (1991) and Soto *et al.* (1991), the application of ribotyping for *Corynebacterium* identification has not been investigated in more detail before. It is possible that the availability of sophisticated scanning hardware and statistical cluster analysis software, together with a commercial automated system for ribotyping (Qualicon), will lead to increased interest in this method. Obviously, the

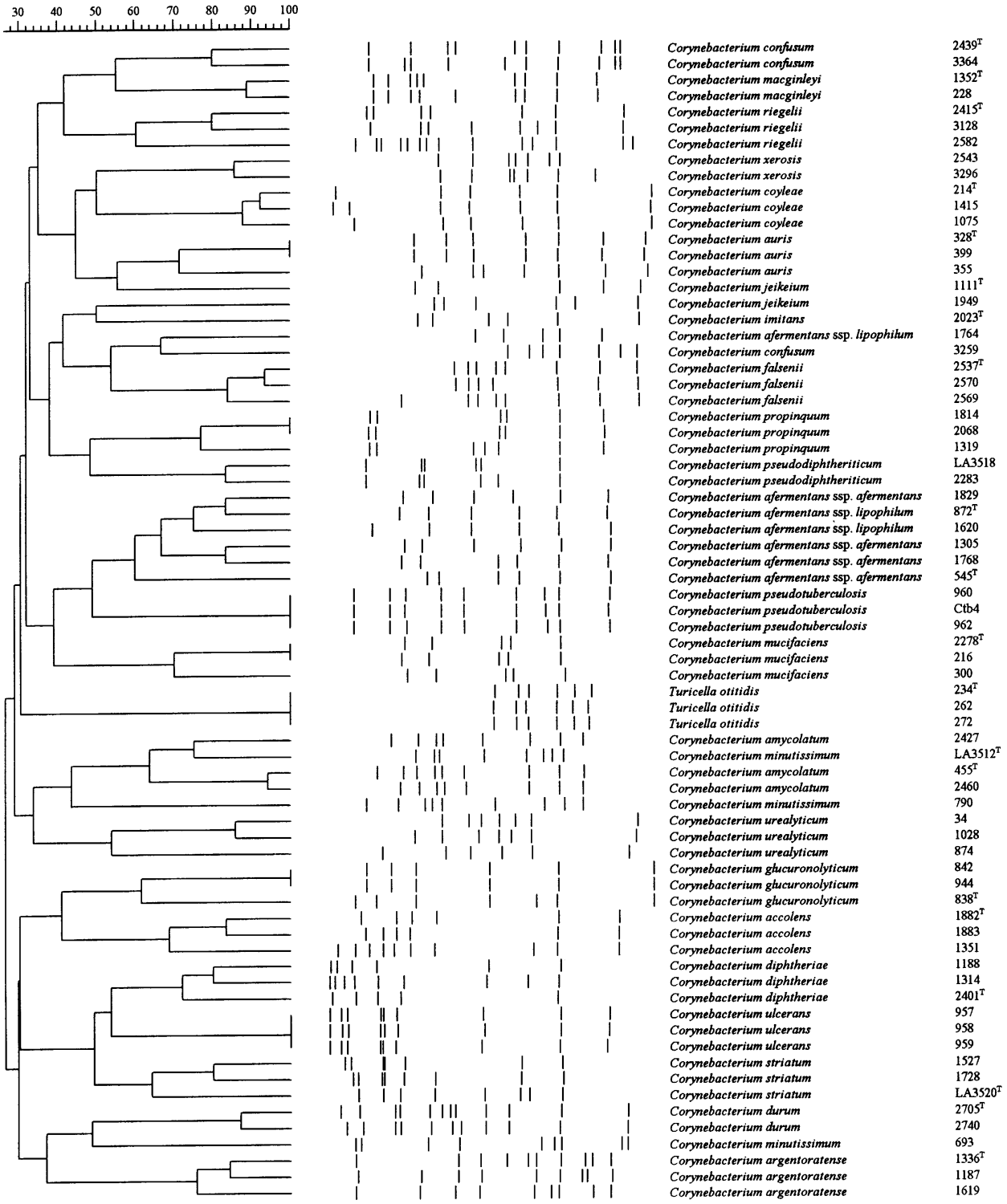

Fig. 2. *Sma*I dendrogram and ribotypes; left, high molecular mass (23 kbp) and right, low molecular mass (500 bp).

application of ribotyping to identification of *Corynebacterium* species is only possible after the creation of a comprehensive database, as in the present study. Once this has been done, it is not particularly time-consuming or labour-intensive to screen individual unidentified *Corynebacterium* strains for their identity. We acknowledge that it may have been desirable to test many more strains of each of the 26 species or subspecies included, but the primary goal of our study was to demonstrate the general applicability of our

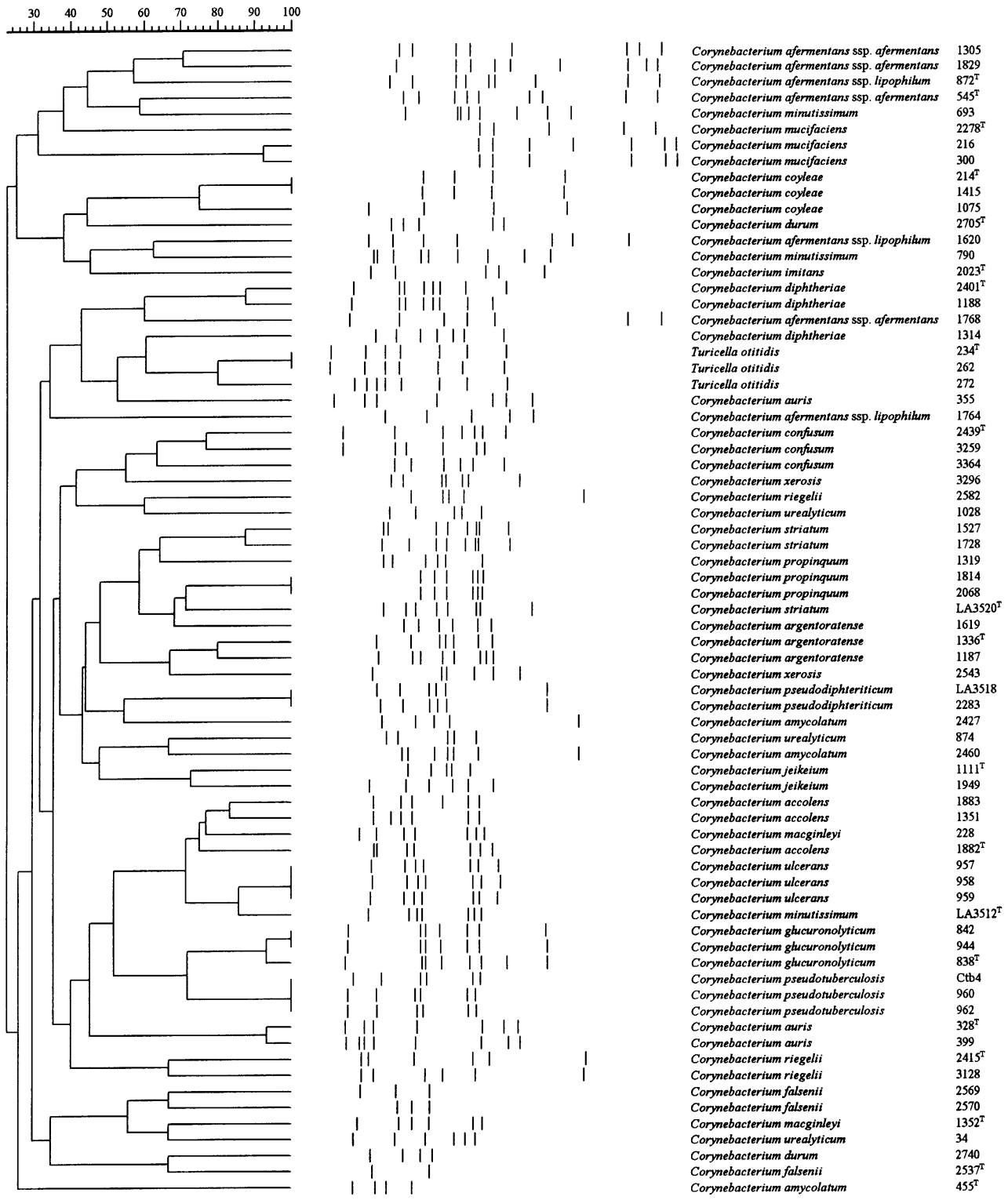

Fig. 3. *Sph*I dendrogram and ribotypes; left, high molecular mass (23 kbp) and right, low molecular mass (500 bp).

approach for the medically relevant members of the genus *Corynebacterium*. In our view, ribotyping is a complementary method for screening unidentified corynebacteria.

Other established methods used for identifying new *Corynebacterium* species include analysis of the 16S–23S rRNA intragenic spacer region (Aubel *et al.*, 1997) and the use of whole-cell protein profiles (Sjödén

et al., 1998). Species delineation within the genus *Corynebacterium* has often been achieved by sequencing of full 16S rRNA genes (Pascual *et al*., 1995; Ruimy *et al*., 1995), because the genus exhibits an enormous phylogenetic depth. Compared to other genera, 16S rDNA divergence rates are relatively high within the genus *Corynebacterium* (Pascual *et al*., 1995; Ruimy *et al*., 1995), which allows new *Corynebacterium* species to be established on the basis of 16S rRNA gene sequences. However, if sequencing facilities are not available, we consider ribotyping as a useful initial approach to test whether an unidentified *Corynebacterium* strain represents a new species.

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