Intragenetic relationships among *Micromonospora* species deduced from *gyrB*-based phylogeny and DNA relatedness

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The phylogenetic structure of genus *Micromonospora* within actinomycetes was examined by analysing the *gyrB* sequences of 15 validly described species and four subspecies. All but one of the *Micromonospora* strains formed a tight cluster, as had previously been demonstrated by a 16S rDNA-based phylogenetic analysis. However, the intragenetic relationships deduced from the *gyrB*-based phylogeny were different from those based on their 16S rDNA sequences. To examine which phylogeny would be more relevant for classifying genus *Micromonospora*, DNA–DNA hybridization experiments were performed. The *gyrB*-based classification agrees with the results of the DNA–DNA hybridization studies, indicating that this classification method is useful for analysing the phylogenetic relationships of high G+C Gram-positive bacteria at the level of the genomic species. Genus *Micromonospora* was reclassified into the following 14 species: *Micromonospora echinospora*, *Micromonospora pallida*, *Micromonospora nigra*, *Micromonospora purpureochromogenes*, *Micromonospora aurantiaca*, *Micromonospora carbonacea*, *Micromonospora chalcone*, *Micromonospora chersina*, *Micromonospora coerulescens*, *Micromonospora gallica*, *Micromonospora halophytica*, *Micromonospora inositol*, *Micromonospora olivasterospora* and *Micromonospora rosaria*.

Keywords: *inositol*, *gyrB*, *Micromonospora*, genomic species, DNA–DNA hybridization, genetic distance

INTRODUCTION

Family *Micromonosporaceae* Krasil'nikov 1938, emend. Goodfellow, Stanton, Simpson and Minnikin 1990, emend. Stackebrandt, Rainey and Ward-Rainey 1997 contains nine genera, namely genera *Micromonospora*, *Actinoplanes*, *Catellatospora*, *Couchioplanes*, *Catenuloplanes*, *Dactylosporangium*, *Pilimelia* (Koch *et al.*, 1996b), *Spirilliplanes* (Tamura *et al.*, 1997) and *Verrucosipora* (Rheims *et al.*, 1998). Phylogenetically, this family forms one of the major sublines within the suborder Micromonosporineae in the order *Actinomycetales* (Stackebrandt *et al.*, 1997). Genus *Micromonospora* Ørskov 1923 is the type genus of the family *Micromonosporaceae*, and contains many interesting strains such as antibiotic producers (Luedemann

& Brodsky, 1964) and degraders of natural rubber (Jendrosseck *et al.*, 1997).

According to the Approved Lists of Bacterial Names (Skerman *et al.*, 1980), 12 species and seven subspecies are listed in genus *Micromonospora*. In 1989, Kawamoto reported that eight species and seven subspecies in this genus can be recognized as individual species or subspecies from their phenotypic and chemotaxonomic traits, but he raised questions concerning the validity of the five species, namely *Micromonospora purpurea*, *Micromonospora rhodorangea*, *Micromonospora brunnea*, *Micromonospora aurantiaca* and *Micromonospora gallica* (Kawamoto, 1989). The preservation of these type strains has been discontinued by National Collection of Type Cultures, UK, and it is impossible to obtain the type strain of *M. gallica* (Kawamoto, 1989). Consequently, the taxonomic positions of the other four species can only be re-examined.

A 16S rDNA sequence-based study on the taxonomy

The DDBJ accession numbers for the *gyrB* sequences of *Micromonospora* strains in this paper are given in Table 1.

Table 1. Strains studied and accession numbers for their *gyrB* sequences

Name	Strain	Accession no.
<i>Actinoplanes brasiliensis</i>	IFO 13938 ^T	AB014125
<i>Actinoplanes cyaneus</i>	IFO 14990 ^T	AB014126
<i>Actinoplanes missouriensis</i>	IFO 13243 ^T	AB014130
<i>Actinoplanes philippinensis</i>	IFO 13878 ^T	AB014132
<i>Actinoplanes regularis</i>	IFO 12514 ^T	AB014133
<i>Actinoplanes utahensis</i>	IFO 13244 ^T	AB014135
<i>Couchioplanes caeruleus</i> subsp. <i>caeruleus</i>	IFO 13939 ^T	AB014137
<i>Couchioplanes caeruleus</i> subsp. <i>azurea</i>	IFO 13993 ^T	AB014138
<i>Dactylosporangium matsuzakiense</i>	ATCC 31570 ^T	AB014104
<i>Micromonospora aurantiaca</i>	IFO 14068	AB015620
<i>Micromonospora aurantiaca</i>	IFO 16125 ^T	AB015621
<i>Micromonospora brunnea</i>	IFO 14069 ^T	AB014145
<i>Micromonospora carbonacea</i> subsp. <i>aurantiaca</i>	IFO 14107 ^T	AB014146
<i>Micromonospora carbonacea</i> subsp. <i>carbonacea</i>	IFO 14108 ^T	AB014147
<i>Micromonospora chalcea</i>	IFO 13503 ^T	AB014148
<i>Micromonospora chersina</i>	IFO 15963 ^T	AB015622
<i>Micromonospora coerulea</i>	IFO 13504 ^T	AB014151
<i>Micromonospora echinospora</i> subsp. <i>echinospora</i>	IFO 13149 ^T	AB014154
<i>Micromonospora echinospora</i> subsp. <i>ferruginea</i>	IFO 14109 ^T	AB014309
<i>Micromonospora echinospora</i> subsp. <i>pallida</i>	IFO 16070 ^T	AB014153
<i>Micromonospora halophytica</i> subsp. <i>halophytica</i>	IFO 14112 ^T	AB014157
<i>Micromonospora halophytica</i> subsp. <i>nigra</i>	IFO 16103 ^T	AB014156
<i>Micromonospora inositol</i>	ATCC 21773 ^T	AB014158
<i>Micromonospora olivasterospora</i>	IFO 14304 ^T	AB014159
<i>Micromonospora purpurea</i>	IFO 13150 ^T	AB014160
<i>Micromonospora purpureochromogenes</i>	IFO 13324 ^T	AB014161
<i>Micromonospora rhodorangea</i>	IFO 14863 ^T	AB014162
<i>Micromonospora rosaria</i>	IFO 13697 ^T	AB014163

of *Micromonosporaceae* has been performed by Koch *et al.* (1996a). The 16S rDNA-based phylogeny of genus *Micromonospora*, however, did not always agree with other taxonomic characteristics. We assume that the 16S rDNA sequences are not sufficiently divergent in genus *Micromonospora* to distinguish the different species.

Yamamoto & Harayama (1995) proposed that protein-encoding genes such as *gyrB* could be suitable for the phylogenetic classification and identification of closely related bacteria. They succeeded in discriminating among closely related strains of genus *Pseudomonas* (Yamamoto & Harayama, 1995). In subsequent works (Yamamoto & Harayama, 1996, 1998; Harayama & Yamamoto, 1996; Yamamoto *et al.*, 1999), they showed the *gyrB*-based grouping of genus *Acinetobacter* to be consistent with the results of DNA–DNA hybridization, indicating that the *gyrB* sequence would be useful for resolving bacterial strains at the genomic species level.

We thus applied the *gyrB*-based method for classifying the *Micromonospora* strains. In this study, we determine the *gyrB* sequences of the type strains of *Micromonospora* species and analyse their phylogenetic structures. DNA relatedness is also analysed for

these strains to confirm the phylogenetic structure deduced from the *gyrB* sequences.

METHODS

Bacterial strains, culture conditions and DNA preparation.

Table 1 gives the names of the strains, their numbers in the IFO or ATCC culture collections, and the DDBJ/EMBL/GenBank accession numbers of their *gyrB* sequences. Each strain was preserved by a liquid dried method in which its culture was dried without freezing. It was cultivated for 2–3 d at 30 °C in IFO medium numbers 227, 228 and 231 (Imai *et al.*, 1996) and in ATCC medium number 172 (Cote, 1996). Genomic DNA was extracted from the cells and purified by the method described elsewhere (Yoon *et al.*, 1996).

Determination of *gyrB* sequences. The protocol for determining the *gyrB* sequences was almost the same as that described by Yamamoto & Harayama (1995). The primers used are described next, the location of each primer sequence being indicated by the numbering system for the *Escherichia coli gyrB* sequence. A *gyrB* segment about 1.2 kb long was amplified by PCR, using universal primers UP1TL (5'-CAY GCn GGn AAr TTy GA-3'; positions 295–314) and UP2rTL (5'-TCn ACr TCn GCr TCn GTC AT-3'; positions 1486–1505). To determine the nucleotide sequence of both strands of the *gyrB* segment, shorter fragments were amplified by using the PCR-amplified 1.2 kb long *gyrB* segment as

Fig. 1. Unrooted tree based on the *gyrB* sequences for genus *Micromonospora*, and other members of family *Micromonosporaceae*. A neighbour-joining dendrogram based on the 1149 bp long *gyrB* sequences was constructed. Each number on the dendrogram is the percentage of occurrence in 1000 bootstrapped trees.

a template and two pairs of primers, either UP1M13r (5'-CAG GAA ACA GCT ATG ACC AyG snG GnG GnA ArT Tyr A-3'; positions 295–314) plus QTK-21 (5'-TGT AAA ACG ACG GCC AGT Ary TTn kyy TTn GTy TG-3'; positions 1003–1019) or GGTH-r (5'-TGT AAA ACG ACG GCC AGT GAn GGn GGn ACn CA-3'; positions 829–842) plus UP2r-21 (5'-TGT AAA ACG ACG GCC AGT rTC nAC rTC nGC rTC nGT CAT-3'; positions 1486–1505). The amplification reaction was subjected to 35 cycles with a denaturation step of 95 °C for 1 min, an annealing step of 63 °C for 1 min, and an extension step of 72 °C for 2 min. Sequences were determined by using the universal primers for the M13 phage vector.

Analysis of sequence data. The *gyrB* sequences and their deduced amino acid sequences were aligned by CLUSTAL W 1.7 (Thompson *et al.*, 1994), and the alignments were manually corrected. Phylogenetic analyses were performed by the PHYLIP version 3.5c package (Felsenstein, 1993). Distance matrices based on Kimura's 2-parameter model (Kimura, 1980) were produced by the DNADIST program, and the neighbour-joining tree was constructed by the NEIGHBOR program. The resulting trees were depicted by using the TreeView version 1.5 package (Page, 1996). The stability of the grouping was assessed by bootstrapping with the SEQBOOT, DNADIST, NEIGHBOR and CONSENSE programs. A total of 1000 bootstrapped trees was generated. The maximum-likelihood tree was constructed by the DNAML program under the assumption that the transition/transversion ratio was 2:000.

DNA–DNA hybridization. The DNA relatedness of each *Micromonospora* strain was estimated by DNA–DNA hybridization. DNA of the strain was hybridized with DNAs from all the other strains. The microplate-hybridization method developed by Ezaki *et al.* (1988, 1989) was applied to

determine DNA similarity. Heat-denatured sample DNA (1 µg) was immobilized in a microplate well (Immuno plate II; Nunc). After prehybridization for 30 min at 37 °C, hybridization was performed at 55 °C for 3 h with a probe DNA labelled by photobiotin acetate (Bresatec). After washing four times with 1 × SSC, the amount of remaining probe DNA was measured by the activity of β-galactosidase conjugated with streptavidin. In each experiment, five replicates were prepared, and the mean of two independent experiments was used for calculating DNA similarity by using the following equation: DNA similarity (%) = $100 \times (X - N) / (P - N)$, where *X* is the fluorescence intensity obtained with a sample DNA, *P* is that of self-hybridization and *N* is that obtained with calf thymus DNA.

RESULTS

Phylogenetic classification of the *Micromonospora* type strains

We determined the partial *gyrB* sequences of the type strains of 14 *Micromonospora* species and four subspecies which are validly described. These sequences were aligned with those of other members of family *Micromonosporaceae*, and their phylogenetic positions were determined (Fig. 1) by constructing a phylogenetic tree with either the neighbour-joining method (Fig. 1) or the maximum-likelihood method (data not shown). When the topologies of these trees were compared, they were congruent to each other, except for the branching positions of two deep branches, namely those of *Micromonospora olivasterospora* IFO 14304^T and *Micromonospora inositolata* ATCC 21773^T.

Table 2. Levels of DNA relatedness among the strains in three *gyrB* groups of genus *Micromonospora*

<i>gyrB</i> group	Strain	DNA relatedness (%) with IFO strain:									
		13149 ^T	13150 ^T	14863 ^T	14109 ^T	16070 ^T	14107 ^T	14108 ^T	13324 ^T	14069 ^T	14112 ^T
I	<i>M. echinospora</i> subsp. <i>echinospora</i> IFO 13149 ^T		100	110	90	47					
	<i>M. purpurea</i> IFO 13150 ^T	109		104	94	51					
	<i>M. rhodorangea</i> IFO 14863 ^T	91	90		66	47					
	<i>M. echinospora</i> subsp. <i>ferruginea</i> IFO 14109 ^T	92	89	78		49					
	<i>M. pallida</i> IFO 16070 ^T	44	41	49	34						
II	<i>M. carbonacea</i> subsp. <i>aurantiaca</i> IFO 14107 ^T							106			
	<i>M. carbonacea</i> subsp. <i>carbonacea</i> IFO 14108 ^T						80				
III	<i>M. purpureochromogenes</i> IFO 13324 ^T									65	21
	<i>M. brunnea</i> IFO 14069 ^T								72		25
	<i>M. halophytica</i> subsp. <i>halophytica</i> IFO 14112 ^T								12	15	

Table 3. Levels of DNA relatedness (%) between the type strains of thirteen validly described *Micromonospora* species

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. <i>M. pallida</i> IFO 16070 ^T		52	16	25	22	19	29	16	15	22	14	20	17
2. <i>M. echinospora</i> IFO 13149 ^T	45		16	19	11	11	23	14	18	20	19	23	11
3. <i>M. rosaria</i> IFO 13697 ^T	19	10		16	18	13	21	11	10	13	15	16	6
4. <i>M. inositol</i> ATCC 21773 ^T	15	13	11		42	11	14	14	19	25	18	21	17
5. <i>M. coerulea</i> IFO 13504 ^T	11	8	11	41		11	14	9	16	18	22	21	19
6. <i>M. olivasterospora</i> IFO 14304 ^T	10	8	13	21	18		20	11	14	13	19	15	15
7. <i>M. carbonacea</i> IFO 14107 ^T	13	15	16	20	17	12		13	7	14	19	17	14
8. <i>M. nigra</i> IFO 16103 ^T	14	17	13	23	15	8	26		17	19	19	19	18
9. <i>M. chalcone</i> IFO 13503 ^T	8	10	9	23	9	7	19	10		24	21	16	44
10. <i>M. chersina</i> IFO 15963 ^T	13	12	14	22	12	10	24	10	28		26	31	23
11. <i>M. halophytica</i> IFO 14112 ^T	11	14	12	14	28	9	28	14	24	23		33	11
12. <i>M. purpureochromogenes</i> IFO 13324 ^T	11	12	11	13	28	8	21	10	9	23	29		25
13. <i>M. aurantiaca</i> IFO 16125 ^T	5	5	6	12	16	5	14	6	34	12	16	15	

All the *Micromonospora* species besides strain IFO 14068 were tightly grouped in the phylogenetic tree. The *gyrB*-based phylogeny revealed three closely related groups: *gyrB* group I comprised *M. rhodorangea* IFO 14863^T, *M. purpurea* IFO 13150^T, *Micromonospora echinospora* subsp. *echinospora* IFO 13149^T, *Micromonospora echinospora* subsp. *ferruginea* IFO 14109^T and *Micromonospora echinospora* subsp. *pallida* IFO 16070^T; *gyrB* group II comprised *Micromonospora carbonacea* subsp. *aurantiaca* IFO 14107^T and *Micromonospora carbonacea* subsp. *carbonacea* IFO 14108^T; and *gyrB* group III comprised *Micromonospora brunnea* IFO 14069^T, *Micromonospora purpureochromogenes* IFO 13324^T and *Micromonospora halophytica* subsp. *halophytica* IFO 14112^T. The branches of these groupings were all supported by high bootstrap values (Fig. 1).

In addition to these branches, *Micromonospora olivasterospora* IFO 14304^T, *Micromonospora rosaria* IFO 13697^T, *Micromonospora coerulea* IFO 13504^T, *Micromonospora halophytica* subsp. *nigra* IFO 16103^T, *Micromonospora chersina* IFO 15963^T, *Micromono-*

spora chalcone IFO 13503^T, *Micromonospora aurantiaca* IFO 16125^T and *M. inositol* ATCC 21773^T formed independent branches (Fig. 1).

DNA–DNA hybridization studies on the type strains of genus *Micromonospora*

DNA–DNA hybridization experiments were then performed by using DNA from each *Micromonospora* species in order to confirm the relationships that had been deduced from the *gyrB* analysis. We first analysed the DNA relatedness among the species belonging to either *gyrB* group I, II or III. As presented in Table 2, four of the five strains in *gyrB* group I showed mutually DNA relatedness of more than 90%. The DNA relatedness between two strains in *gyrB* group II was more than 80%. Similarly, the DNA relatedness between two of the three strains in *gyrB* group III was more than 65%. To identify the DNA relatedness of all members of genus *Micromonospora*, DNA–DNA hybridization experiments were performed on the remaining species and on representatives of *gyrB*

groups I, II and III. DNA relatedness between these combinations was determined to be less than 70% as shown in Table 3. These results suggest that genus *Micromonospora* should be reclassified into 14 species.

DISCUSSION

Taxonomy of genus *Micromonospora*

The results of the DNA–DNA hybridization analysis showed *M. rhodorangea* IFO 14863^T, *M. echinospora* subsp. *ferruginea* IFO 14109^T and *M. purpurea* IFO 13150^T to be synonyms of *M. echinospora* IFO 13149^T. Similarly, *M. brunnea* IFO 14069^T was confirmed to be a synonym of *M. purpureochromogenes* IFO 13324^T as has been described by Szabo & Fernandez (1984). Based on the morphology of the spores, *M. carbonacea* has been divided into two subspecies, namely *Micromonospora carbonacea* subsp. *aurantiaca* and *Micromonospora carbonacea* subsp. *carbonacea* (Luedemann & Brodsky, 1965). However, the DNA relatedness between IFO 14107^T and IFO 14108^T shows that they are closely related and should not be divided into two subspecies. Kawamoto (1989) has raised a question concerning the taxonomic position of *M. aurantiaca* from its multiple spore formation, so we analysed the *gyrB* sequences of two *M. aurantiaca* strains, namely IFO 14068 (= KCC A-0232 = VKM Ac 613 = LIA 0741 = INA 9442) and IFO 16125^T (= DSM 43813^T = NRRL B-16091^T, = ATCC 27029^T = VKM Ac1936^T = INA 9442^T). Strain IFO 14068 has been classified as genus *Actinoplanes* from its 16S rRNA (T. Tamura, unpublished results) and *gyrB* sequences. We have also confirmed that VKM Ac 613 has the same 16S rRNA sequence as that of IFO 14068 (T. Tamura, unpublished results). However, another strain of *M. aurantiaca*, IFO 16125^T, was classified by its 16S rRNA and *gyrB* sequences to be in the genus *Micromonospora* (Fig. 1). The 16S rRNA sequences of VKM Ac 1936 and DSM 43813 were the same as that of IFO 16125^T (Koch *et al.*, 1996a; T. Tamura, unpublished results). We conclude from these data that the origins of IFO 14068 (= KCC A-0232 = VKM Ac613 = LIA 0741) and IFO 16025^T (= DSM 43813^T = NRRL B-16091^T, ATCC 27029^T = VKM Ac 1936^T) are different, only one of them being INA 9442.

Genus *Micromonospora* should consequently be reclassified into the following 14 species: *M. echinospora* (Luedemann and Brodsky 1964) Kasai *et al.* 2000; *M. pallida* (Luedemann and Brodsky 1964) Kasai *et al.* 2000; *M. nigra* (Weinstein *et al.* 1968) Kasai *et al.* 2000; *M. purpureochromogenes* (Waksman and Curtis 1916) Luedemann 1971; *M. aurantiaca* Sveshnikova *et al.* 1969; *M. carbonacea* Luedemann and Brodsky 1965; *M. chalicea* (Foulerton 1905) Ørskov 1923; *M. chersina* Tomita *et al.* 1992; *M. coerulea* Jensen 1932; *M. gallica* (Erikson 1935) Waksman 1961; *M. halophytica* Weinstein *et al.* 1968; *M. inositola* Kawamoto *et al.* 1974; *M. olivasterospora* Kawamoto *et al.* 1983; and *M. rosaria* Horan and Brodsky, 1986. The emended descriptions follow.

Emendation of *Micromonospora echinospora* Luedemann and Brodsky 1964

Micromonospora echinospora (e.chi'no.spo.ra. Gr. adj. *echinos* spiny appearance; Gr. n. *spora* seed; M.L. fem. n. *echinospora* spiny spore).

The following emended description is similar to the original description (Luedemann & Brodsky, 1964; Wagman *et al.*, 1974), except for the colour of the colony surface and menaquinone. No aerial mycelium. Spores spherical, 1.0–1.5 µm in diameter. Blunt spines on spore surface 0.1–0.2 µm long. Sporophores mostly solitary but occasionally in small clusters on the same hypha. Spores adhere firmly to the sporophore until mature. Colony raised. Convolute abundant growth. Diffusible pigment cannot be detected or is detected with slight amber colour. Melanin pigment is not produced. Colour of the colony surface is dark brown or purple to black, or reddish-brown to orange. Reverse colour of colony is dark reddish-orange. Substrate mycelia range from 0.4 to 0.8 µm in diameter and are sparsely branched and regular non-septate. Utilizes L-arabinose. Gelatin liquefied. Milk digested. Good growth between 27 and 37 °C. Major menaquinone is MK-10(H₄) or MK-10(H₆). Most strains produce the gentamicin antibiotic complex. Isolated from soil and aquatic environments.

Description of *Micromonospora pallida* comb. nov.

Micromonospora pallida (pal'lid.a. L. fem. adj. *pallida* pale).

The following description is based on the previously described characteristics (Luedemann & Brodsky, 1964; Kawamoto, 1989). Purple mycelial pigments are not present. The colony colour is pale, ranging from light ivory to light melon yellow, except where abundant sporulation occurs to impart a dark brown to black coloration. Nitrate reduction is positive. The carbohydrate utilization pattern shows that good growth occurs on D-fructose. Growth on D-ribose is slight, but abundant sporulation imparts a black colour to the colony. The menaquinone profile is different from that of other *Micromonospora* species. Its type is MK-12. The organism produces antibiotics of the gentamicin complex. The organism was isolated from a soil sample from Jamesville, NY, USA.

Description of *Micromonospora nigra* comb. nov.

Micromonospora nigra (ni'gra. L. fem. adj. *nigra* black).

The following description is based on the previously described characteristics (Weinstein *et al.*, 1968; Kawamoto, 1989). Growth on many natural agar media is initially orange in colour but, as sporulation progresses, turns olive brown and eventually black. No reddish-brown diffusible pigment is produced. The typical black sporulation layer is commonly found on plates containing D-fructose, D-galactose, β-lactose,

raffinose or D-trehalose. The organism produces antibiotics of the halomycin complex. The organism was isolated from a salt pool in Syracuse, NY, USA.

Probable evolution of 16S rRNA genes of *Micromonospora* strains

Yamamoto & Harayama (1998) have shown the example that nucleotide substitution in the variable region of 16S rDNA sequence might introduce errors in the phylogenetic analysis of closely related strains. According to their analysis, the correlation between the genetic distances calculated from the synonymous substitutions in *gyrB* and the substitution rates in the variable regions of 16S rDNA was quite low. They also showed that the branching patterns of the *gyrB*-based phylogenetic trees were similar to those of the 16S rDNA-based trees, if the latter trees were constructed by excluding the sequences of the variable regions. They assumed that many base substitutions in the variable regions of 16S rDNA would not be provoked by point mutations, but by other types of mutation such as gene conversion. In the case of *Micromonospora* strains, the phylogenetic tree based on *gyrB* was different from that based on the 16S rDNA sequences, even when the sequences in the variable regions were excluded (data not shown). This observation suggests that the evolutionary route of 16S rDNA in this genus might have been biased under some selective pressure. As described above, some of the *Micromonospora* strains were isolated as aminoglycoside antibiotics producers. Aminoglycoside antibiotics are known to bind directly to the A-site of 16S rRNA (Fourmy *et al.*, 1996). The 16S rRNA genes of aminoglycoside-antibiotic-producing-bacteria might have evolved under the pressure of their products.

Correlation between DNA homology values and genetic distances based on *gyrB* sequences

A comparison of the *gyrB*-based phylogeny with the DNA relatedness allows the conclusion that the phylogenetic structures deduced from the *gyrB* sequences are consistent with the results of DNA–DNA hybridization studies.

To evaluate the maximum genetic distance within a single species, we calculated the genetic distances among *Micromonospora* species based on Kimura's 2-parameter model. The genetic distance values were then compared with the values of DNA homology obtained from the DNA–DNA hybridization experiments shown in Table 2. The genetic distances among IFO 13149^T, IFO 13150^T and IFO 14863^T were 0, because the *gyrB* sequences of these members were completely identical. DNA homology values among these strains were 90–110%. The genetic distances among these three strains and IFO 14109^T were calculated to be 0.0088, and the DNA homology values among them were estimated to be 66–94%. The DNA homology values against IFO 14863^T were lower

Fig. 2. Correlation between the DNA relatedness and genetic distance calculated from the *gyrB* sequences. Genetic distances between all combinations of *Micromonospora* strains were calculated and plotted. The nucleotide substitution frequencies were corrected for multiple substitutions by using Kimura's 2-parameter model.

than those against other members, probably because the quality of the genomic DNA sample from IFO 14863^T might not have been particularly good. DNA homology values among IFO 16070^T and the other four members in group I were 34–51%, and their genetic distances were calculated to be 0.0412 between IFO 16070^T and IFO 14109^T and 0.0430 between IFO 16070^T and the other members. Moreover, the DNA homology values between *M. chalicea* IFO 13503^T and *M. aurantiaca* IFO 16125^T, and between *M. purpureochromogenes* IFO 13324^T and *M. brunnea* IFO 14069^T were 34–44% and 65–72%, respectively, while their genetic distances were calculated to be 0.0248 and 0.0141, respectively (Table 3). Thus, a genetic distance around 0.014 would correspond to the 70% value in DNA homology (Fig. 2). Although more data are required to establish the accurate relationship between the *gyrB* genetic distance and the DNA homology value, a *gyrB* genetic distance of about 0.014 may be the maximum range for a single species.

Use of the *gyrB* gene for the classification of bacteria

DNA–DNA hybridization studies are recognized as the standard by which to classify bacteria at the species level. However, the method is laborious and time-consuming. The data presented in this paper show that *gyrB*-based phylogenetic studies could classify high G+C Gram-positive bacteria at the species or sub-species levels.

We are currently constructing the Identification and Classification of Bacteria (ICB) database which is accessible at the Web site of <http://www.mbio.co.jp/> (Kasai *et al.*, 1998). More than 1000 *gyrB* data, of

which about 350 *gyrB* sequences originate from high G+C Gram-positive bacteria, are deposited in the ICB database. The database provides analytical tools for finding related strains by comparing the *gyrB* sequences.

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