An MLSA-based online scheme for the rapid identification of *Stenotrophomonas* isolates

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An online scheme to assign Stenotrophomonas *isolates to genomic groups was developed using the multilocus sequence analysis (MLSA), which is based on the DNA sequencing of selected fragments of the housekeeping genes ATP synthase alpha subunit (*atp*A), the recombination repair protein (*rec*A), the RNA polymerase alpha subunit (*rpo*A) and the excision repair beta subunit (*uvr*B). This MLSA-based scheme was validated using eight of the 10* Stenotrophomonas *species that have been previously described. The environmental and nosocomial* Stenotrophomonas *strains were characterised using MLSA, 16S rRNA sequencing and DNA-DNA hybridisation (DDH) analyses. Strains of the same species were found to have greater than 95% concatenated sequence similarity and specific strains formed cohesive readily recognisable phylogenetic groups. Therefore, MLSA appeared to be an effective alternative methodology to amplified fragment length polymorphism fingerprint and DDH techniques. Strains of* Stenotrophomonas *can be readily assigned through the open database resource that was developed in the current study (www.steno.lncc.br/).*

Key words: *Stenotrophomonas* - MLSA - online scheme

The genus *Stenotrophomonas* is widespread in the environment and exists as a free-living and plant-associated organism as well as an opportunistic pathogen (Falagas et al. 2008, Nyc & Matejková 2010). Therefore, *Stenotrophomonas* displays great metabolic versatility and intraspecific heterogeneity (Ryan et al. 2009). The species *Stenotrophomonas maltophilia* is an important nosocomial pathogen (Nicodemo & Paez 2007) that is correlated with a high mortality rate among immunocompromised patients (Araoka et al. 2010). The impact of *S. maltophilia* as a multidrug-resistant pathogen has significantly increased (Hauben et al. 1999, Sanchez et al. 2009), which highlights the importance of rapid and reliable isolate identification. Moreover, endophytic *Stenotrophomonas* spp have an important role in plant development in a variety of economically important agricultural species (Vega et al. 2005) by producing hormones, growth factors and N_2 for their hosts. Some strains of this genus are used as biocontrol agents against pathogenic fungi and yeasts in plants (Minkwitz & Berg 2001). In addition, some *Stenotrophomonas rhizophila* strains can degrade xenobiotic compounds and are used in the bioremediation of contaminated soils (Juhasz & Naidu 2000).

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Ten species of *Stenotrophomonas* have been described as follows: *S. maltophilia* (Palleroni & Bradbury 1993), which was identified in soils, plants and in nosocomial infections (Ryan et al. 2009), *Stenotrophomonas nitritireducens*, which was isolated from ammonia-supplied biofilters (Finkmann et al. 2000), *S. rhizophila,* which was isolated from the rhizosphere of rape plants (Wolf et al. 2002), *Stenotrophomonas acidaminiphila* (Assih et al. 2002), which was originally found in a lab-scale methanogenic reactor that treated industrial wastewater, *Stenotrophomonas koreensis* (Yang et al. 2006), which was isolated from compost in Daejeon (South Korea), and *Stenotrophomonas terrae* and *Stenotrophomonas humi*, which were described to encompass nitrate-reducing isolates that were obtained from soil samples in Ghent, Belgium (Heylen et al. 2007). The *Stenotrophomonas chelatiphaga* strain was isolated from sewage sludge in Kazan City, Russian Federation (Kaparullina et al. 2009). The *Stenotrophomonas pavanii* strain was isolated from sugar cane cultivars in Brazil (Ramos et al. 2011). Recently, the *Stenotrophomonas ginsengisoli* strain was isolated from the soil of a ginseng field in South Korea (Kim et al. 2010).

The precise identification and classification of *Stenotrophomonas* remains a problem. Band pattern methods [e.g., amplified fragment length polymorphism (AFLP)] and DNA-DNA hybridisation (DDH) techniques have been used to underpin the taxonomy of this group. However, the data that are generated by these tools are often difficult to reproduce and are available only in a few laboratories (Coenye et al. 2004a). In addition, the data that are generated by these methods cannot be used

to build an online electronic classification. These limitations can be overcome by the use of multilocus sequence typing (MLST), which uses the DNA sequence variation in seven or more housekeeping genes to characterise bacterial strains based on their unique allelic profiles (Maiden et al. 1998, Turner & Feil 2007). A simplified approach to MLST, which is widely applied in microorganism identification, uses only three-five housekeeping genes (Hanage et al. 2006, Brady et al. 2008) and is known as multilocus sequence analysis (MLSA). MLSA differs from MLST in the way the data are analysed. In MLSA, the sequence concatenation is used instead of the allelic profiles and the dataset includes multiple species collections. MLSA has been successfully used for species identification in several genera such as *Vibrio* (Thompson et al. 2005), *Enterococcus* (Naser et al. 2005) and the viridans group of *Streptococcus* (Bishop et al. 2009). The online scheme that is proposed in the current study is based on the DNA sequencing of selected fragments of the genes coding for the ATP synthase alpha subunit (*atp*A), the recombination repair protein (*rec*A), the RNA polymerase alpha subunit (*rpo*A) and the excision repair beta subunit (*uvr*B). This scheme allows for the rapid identification of *Stenotrophomonas* isolates through assignment to the genomic groups of the genus (Hauben et al. 1999, Coenye et al. 2004b).

MATERIALS AND METHODS

Bacterial strains and the isolation of DNA - All of the type strains of the genus *Stenotrophomonas* that were used for MLSA were deposited in the BCCM™/LMG Bacteria Collection and in our own research collection at the University of São Paulo (Table I). The genomic bacterial DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA: Cat. A 1120), according to the manufacturer's instructions.

TABLE I

Strains used in this study

Strain number	*Stenotrophomonas* species	Source	MLSA cluster	Genomic group	Reference
LMG 958^T	*Stenotrophomonas maltophilia*	Oral cancer	MLSA 1	6	Pallerony and Bradbury (1993)
LMG 22072	*Stenotrophomonas maltophilia*	HIV positive patient	MLSA 1	ND	Drancourt et al. (1997)
LMG 22075^T	*Stenotrophomonas rhizophila*	Rhizosphere of rape	MLSA 1	ND	Wolf et al. (2002)
LMG 10996	*Stenotrophomonas* sp.	Leg, ulcer	MLSA 1	2	-
LMG 978 ^{T}	*Pseudomonas beteli*	Piper beetle	MLSA 1	ND	-
LMG 980 ^{T}	*Pseudomonas hibiscicola*	*Hibiscus rosa-sinensis*	MLSA 1	ND	-
LMG 10874	*Stenotrophomonas* sp.	Human blood culture	MLSA 1	4	-
LMG 10878	*Stenotrophomonas* sp.	Patient with otitis, pus	MLSA 1	4	-
LMG 11002	*Stenotrophomonas* sp.	Mamilla	MLSA 1	3	-
LMG 10991	*Stenotrophomonas* sp.	Leg, pus	MLSA 1	2	-
LMG25348^T	*Stenotrophomonas pavanii*	Sugarcane	MLSA 1	-	Ramos et al. (2011)
ICB 194	*Stenotrophomonas* sp.	Sugarcane	MLSA 1	ND	-
LMG 10851	*Stenotrophomonas* sp.	Human blood culture	MLSA 2	5	-
LMG 10877	*Stenotrophomonas* sp.	Patient with sinusitis, pus	MLSA 2	5	-
LMG 11089	*Stenotrophomonas* sp.	Rhizosphere tuberous root	MLSA 2	5	-
LMG 23959 ^{T}	*Stenotrophomonas humi*	Soil	MLSA 3	ND	Heylen et al. (2007)
LMG 23958 ^{T}	*Stenotrophomonas terrae*	Soil	MLSA 3	ND	Heylen et al. (2007)
LMG 6608	*Stenotrophomonas* sp.	Rhizosphere	MLSA 4	9	-
LMG 11087	*Stenotrophomonas* sp.	Rhizosphere	MLSA 4	9	-
LMG 22073^T	*Stenotrophomonas acidaminiphila*	UASB reactor	MLSA 5	ND	Assih et al. (2002)
LMG 22074^T	*Stenotrophomonas nitritireducens*	Biofilter	MLSA 5	ND	Finkmann et al. (2000)
LMG 10881	*Stenotrophomonas* sp.	Fish	MLSA 5	ND	-
LMG 10882	*Stenotrophomonas* sp.	Sewage	MLSA 5	ND	-
LMG 10883	*Stenotrophomonas* sp.	Frozen fruit	MLSA 5	1	-
DSMZ 17805^T	*Stenotrophomonas koreensis*	Compost	MLSA ND	-	Yang et al. (2006)
KT2440	*Pseudomonas putida*	-	Outgroup	-	-
ATCC 33913	*Xanthomonas campestris*	-	Outgroup	-	-
9a5c	*Xylella fastidiosa*	-	Outgroup	-	-

HIV: human immunodeficiency virus; MLSA: multilocus sequence analysis; ND: not determined; UASB: upflow anaerobic sludge blanket.

The amplification and sequencing of housekeeping and 16S rRNA genes - Approximately 50 ng of DNA were used as the PCR template for the amplification of selected fragments of the genes *atp*A, *rec*A, *rpo*A and *uvr*B. The primers that were used in the current study and the respective annealing temperatures are listed in Table II. Because the complete *Stenotrophomonas* genome sequences are only available for two of the *S. maltophilia* strains (NC 011071 and NC 010943) (Crossman et al. 2008), the primer design (using the software Kodon, Applied Maths) included gene sequences of *Xanthomonas axonopodis*, *Xanthomonas campestris*, *Xylella fastidiosa*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas syringae*, *Mesorhizobium loti*, *Sinorhizobium meliloti*, *Brucella melitensis*, *Brucella suis*, *Ralstonia solanacearum* and *Agrobacterium tumefaciens*.

The polymerase chain reaction (PCR) for the housekeeping genes was composed of 38.2 µL of sterile MilliQ water, 1.5 µL of $MgCl_2$ (1.5 mmol·µL^{-1}), 5.0 µL of PCR buffer (10X), 0.4 µL of dNTPs (0.2 mmol·µL^{-1} each), 1.2 µL of the forward primer (20 µmol·µL^{-1}), 1.2 µL of the reverse primer 20 µmol^{-1}·µL, 0.4 µL of the *Taq* DNA Polymerase (2 U·µL^{-1}) and 2.0 µL of the template DNA (0.05 µg·µL^{-1}). The thermal program consisted of one cycle of 5 min at 95ºC, three cycles of 1 min at 95ºC, 2 min 15 s at 55ºC and 1 min 15 s at 72ºC, 30 cycles of 30 s at 95ºC, 1 min 15 s at 55ºC and 1 min 15 s at 72ºC and a final extension cycle of 7 min at 72ºC. The amplification of 16S rRNA was performed using 30-50 ng of DNA in 50-µL reactions containing 2 mmol·µL^{-1} $MgCl_2$, 200 µmol·µL^{-1} dNTPs (each), 0.3 µmol·µL^{-1} of each of the universal primers 27f (5'AGAGTTGATCCTGGCTCAG3') and 1525r (5'AAGGAGGTGWTCCARCC3') and 2U of the *Taq* DNA polymerase (Invitrogen) in the recommended buffer. The reaction mixtures were incubated in a thermal cycler Eppendorf Master Cycler Gradient (Eppendorf AG, Hamburg, Germany) at 94ºC for 2 min and then cycled 30 times at 94ºC for 1 min, 55ºC (annealing temperature) for 1 min and 72ºC for 3 min. A final extension at 72ºC for 10 min was used. All of the PCR products were purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Uppsala, cat. 28-9034-70). Subsequently, 5.0 µL of the purified PCR product was mixed with 4.0 µL of the solution from the DYEnamicTM ET dye terminator kit MegaBACE™ 1000 (GE Healthcare) and 1.0 µL of the sequencing primer (0.5 µmol·µL^{-1}). The thermal program consisted of 30 cycles of 20 s at 95ºC, 15 s at 55º and 60 s at 60º. The sequencing products were purified according to the manufacturer's instructions.

The comparison between the housekeeping genes and 16S rRNA and the phylogenetic analysis - *Stenotrophomonas* bacteria were further characterised by partial sequencing of *atp*A (600 nt), *rec*A (420 nt), *rpo*A (690 nt) and *uvr*B (1219 nt). The Chromas Pro 1.34 software was used to obtain the consensus sequences for the housekeeping genes and 16S rRNA. At least three reads were performed to obtain the consensus sequences. The consensus sequences were aligned using ClustalW (Altschul et al. 1990). The phylogenetic trees were created based on the maximum-parsimony (MP) and neighbour-joining (NJ) methods (Saitou & Nei 1987) using the software MEGA 4. We also separately analysed each of the housekeeping gene sequences and compared the resulting topologies with the topology that was obtained from the concatenated gene tree. The robustness of each topology was checked using 1,000 bootstrap replications. The type strain sequences of *P. putida*, *X. campestris* and *X. fastidiosa* were considered outliers because of their phylogenetic similarities with those of *Stenotrophomonas* spp.

Split tree decomposition analysis and the Phi test were performed with SplitsTree4 (Huson 1998). The guanine-cytosine content, the ratio of the mean synonymous substitutions per the synonymous site to the mean non-synonymous substitutions per the non-synonymous site (*ds*/*dn*) and the recombination tests were calculated using the software package START (pubmlst.org/software/analysis/start/) (Jolley et al. 2001).

DDH - DDH experiments (Supplementary data) were performed to confirm the sequence data for the *Stenotrophomonas* isolates. A modification of the microplate

TABLE II

Amplification and sequencing primers used in this study

Primer	Sequence (5'-3')	Position	GC (%)	PCR annealing temperature (°C)
*atp*A-03-F	CGCATCCTBGARGTKCCG	283	64.8	68
*atp*A-07-R	GCRCGCTCSAGCARRCG	892	73.5	68
*rec*A-05-F	GGYGAGATGGGMGAYCAG	466	63.9	60
*rec*A-07-R	TTKCCYTGSCCGATSCGCTC	889	65	60
*rpo*A-01-F	TKAAGGATGTGGCRATCC	254	52.6	62
*rpo*A-04-R	GGCCARTTCTCCARCTTCA	944	55	62
*uvr*B-01-F	GARYAYATYGARCARATGCG	381	45	58
*uvr*B-02-R	CCYTCYTTRTCDGCRTC	1600	54.9	58

F: forward; GC: guanine-cytosine content; PCR: polymerase chain reaction; R: reverse.

method of Ezaki et al. (1989) that was described by Willems et al. (2001) was used. The hybridisation temperature was 45ºC (calculated with the correction for the presence of 50% formamide).

Nucleotide sequence accession numbers - The gene sequences of the strains that were received from Laboratory of Microbiology/Belgian Co-ordinated Collections of Micro-organisms, Pasteur Institute, German Collection of Microorganisms and Cell Cultures and the University of São Paulo Biomedical Sciences Institute collections were previously deposited in the GenBank. The sequence data are also available at our website www.steno.lncc.br.

RESULTS

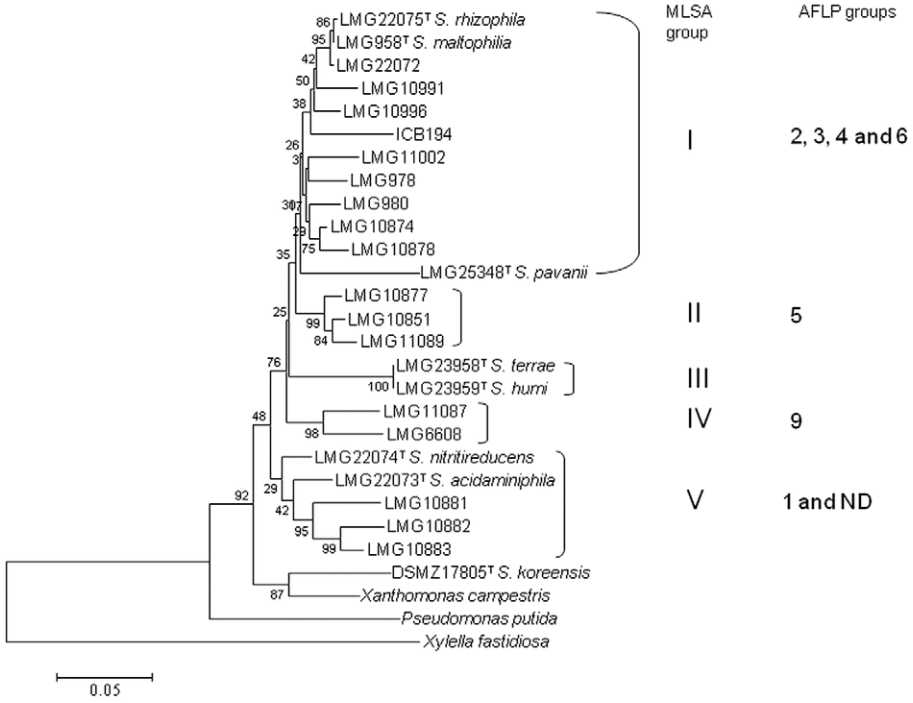
A broad collection of *Stenotrophomonas* strains was molecularly analysed to evaluate the usefulness of MLSA. The 16S rRNA results (data not shown) confirm that all of the strains and isolates that were used in the current work belong to the genus *Stenotrophomonas*, with the exception of the strains LMG 978^T (*Pseudomonas beteli*) and LMG 980^T (*Pseudomonas hibiscicola*). The genes that were analysed in the current study are typical housekeeping genes, which were shown by the relatively high *ds/d*n ratios and apparently have not undergone recombination (Table III). The NJ and MP methods were used to obtain the phylogenetic trees that were based on the concatenated sequences of *atp*A, *rec*A, *rpo*A and *uvr*B. The strain clusters that were generated were consistent with the former taxonomic studies in *Stenotrophomonas* spp strains. Overall, we found five *Stenotrophomonas* genomic groups (Figure), which were named MLSA I-V, that correspond to the AFLP groups that were described by Hauben et al. (1999). The topological analysis for each of the housekeeping gene sequences yielded essentially the same strain clusters with a few differences in the relative position of some clusters (Supplementary data). The DNA-DNA relatedness experiments showed 70% DNA-DNA binding for three strains (Supplementary data), which may indicate the presence of new species or a single species that is composed of heterogenic strains. Our results are clearly below the 70% DNA-DNA binding cut-off value for species delineation (Wayne et al. 1987).

TABLE III

Summary of gene features

Genes	Length (nt)	*ds*/*d*n	GC (%)	Recombination
*atp*A	316	7.2	63.4 ± 0.8	> 0.05
*rec*A	379	59.1	58.0 ± 1.4	> 0.05
*rpo*A	314	11.7	66 ± 3.3	> 0.05
*uvr*B	182	4.3	61.3 ± 2.9	> 0.05

ds/*d*n: the ratio of the mean synonymous substitutions per the synonymous site to the mean non-synonymous substitutions per the non-synonymous site; GC: guanine-cytosine content.



Consensus Neighbour-Joining phylogenetic tree based on the concatenated genes sequences of *rec*A, *rpo*A, *uvr*B and *atp*A. Values of bootstrap after 1,000 repetitions are shown at the nodes. *Pseudomonas putida*, *Xanthomonas campestris* and *Xylella fastidiosa* were used as out-groups. AFLP: amplified fragment length polymorphism; MLSA: multilocus sequence analysis; ND: not determined.

The MLSA I group encompassed three type strains: LMG 958^T (*S. maltophilia*), LMG 22075^T (*S. rhizophila*) and LMG 25348^T (*S. pavanii*). These strains have been grouped based on the 16S rRNA sequence analysis (Heylen et al. 2007, Ramos et al. 2011). The MLSA I group included nine other strains that were already described as members of the AFLP genomic groups 2, 3, 4 and 6 that were characterised by Hauben et al. (1999). The strains LMG 10996 and LMG 10991 also showed a high degree of similarity (84%) based on DDH results, consistent with previously reported levels (Hauben et al. 1999). The LMG 10996 strain in this group appears to be a putative new species considering our preliminary data of phenotypic tests (data not shown) and DDH results (Supplementary data). The MLSA II group contained three strains that belonged to Hauben's AFLP group 5 (LMG 10877, LMG 10851 and LMG 11089). These strains share 98.8% of similarity in their 16S rRNA sequences (Hauben et al. 1999). The comparison of two of these strains (LMG 10877 and LMG 10851) using the DDH technique revealed only 58% homology (Hauben et al. 1999).

The MLSA III group harboured the two strains *S. humi* and *S. terrae*, which have 44.2% DDH similarity (Heylen et al. 2007). These strains displayed high similarity levels in the 16S rRNA sequences (Heylen et al. 2007). The MLSA IV group is formed by two strains (LMG 6608 and LMG 11087). These strains showed 74% and 99% homology based on the DDH and the 16S rRNA sequencing, respectively. The MLSA IV group corresponded to Hauben's genomic group 9 (Hauben et al. 1999).

The MLSA V group included the two closely related strains *S. acidaminiphila* (LMG 22073^T) and *S. nitritireducens* (LMG 22074^T), with nearly 65.8% DDH similarity between them (Assih et al. 2002). These two species have indistinguishable phenotypes. The MLSA V group also included the strains LMG 10883, which is a member of Hauben's AFLP genomic group 1, and LMG 10881 and LMG 10882, which are not members of the Hauben's AFLP genomic groups. The strains LMG 10883 and LMG 10882 were reported by Hauben et al. (1999) with a DNA-DNA binding value of 6% between both strains. However, Coenye et al. (2004b) repeated the experiment and obtained a value of 83%. These results confirm our data using the MLSA method.

DISCUSSION

The comparative genomic analysis of clinical and environmental isolates of *Stenotrophomonas* is of great interest. The analysis facilitates the rapid and reliable identification of these bacteria and contributes to our understanding of the adaptation strategies of this genus to different niches (Ryan et al. 2009). However, the precise identification and classification of *Stenotrophomonas* remains a problem. The band pattern methods (e.g., AFLP) and DDH techniques have been used to underpin the taxonomy of *Stenotrophomonas*. The data that are generated by these tools are often difficult to reproduce and are available only in relatively few laboratories (Coenye et al. 2004a). Moreover, this category of data cannot be used to build an online electronic classification.

Here, we proposed a rapid and cost-effective online scheme for molecular screening of the *Stenotrophomonas* isolates that was based on MLSA typing. Our scheme allowed the assignment of bacterial isolates and strains into well-characterised genomic groups to estimate genetic distances, to measure the intraspecific diversity and to identify putative new species.

The results of the MLSA analysis confirm that there is significant diversity within *Stenotrophomoas* spp. More importantly, our MLSA groups were in close agreement with the genomic groups that were classified by Hauben et al. (1999). We used AFLP fingerprint, 16S rRNA sequencing and DDH analyses. The results indicate that the partial genomic sequencing of housekeeping genes and ribosomal rRNAs provide sufficient information for the proper classification of these species.

Further studies that aim to refine and enhance the current MLSA scheme are currently under way in our laboratory. These studies will increase the number of loci and the length of sequence reads and will provide additional phenotypic information (e.g., multidrug resistance, adhesins, virulence factors) that are related to strains and isolates that are included in our database. Altogether, this new information should provide a reliable system of classification for this genus.

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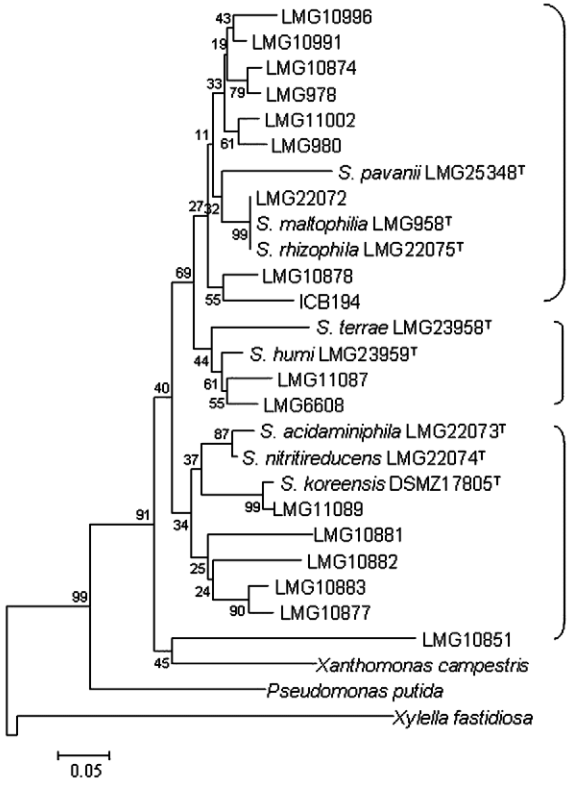
TABLE

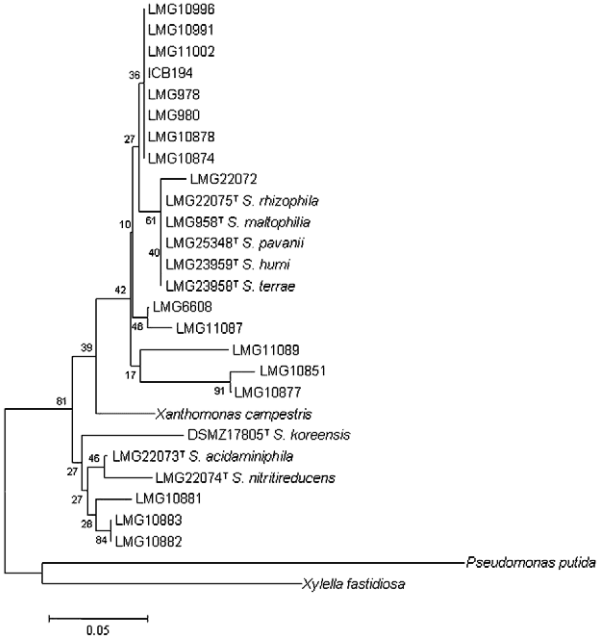
Percentage DNA-DNA hybridization between *Stenotrophomonas* strains

	1[a]	2[a]	3[a]	4[a]	5[a]	6[a]	7[a]	8[a]	9[a]	10[a]	11[a]	12[b]	13[b]	14[b]	15[b]	16[b]	17[b]	18[b]	19[b]
1 LMG 10996	**100**																		
2 LMG 10991	84	**100**																	
3 ICB 194	64.8	-	**100**																
4 LMG 25348^T *S. pavanii*	58.1	-	-	**100**															
5 LMG 22073^T *S. acidaminiphila*	17.7	-	-	-	**100**														
6 LMG 23369^T *S. koreensis*	12.3	-	-	-	-	**100**													
7 LMG 958^T *S. maltophilia*	56.6	-	77.1	55.1	-	-	**100**												
8 LMG 22074^T *S. nitritireducens*	26.3	-	-	31.2	66.9	-	-	**100**											
9 LMG 23958^T *S. terrae*	19.3	-	-	-	-	-	-	-	**100**										
10 LMG 23959^T *S. humi*	16	-	-	-	-	-	-	-	-	**100**									
11 LMG 24537^T *S. rhizophila*	24.5	-	-	30.7	-	-	-	-	-	-	**100**								
12 LMG 11004	65	89	-	-	-	-	-	-	-	-	-	**100**							
13 LMG 11000	71	-	-	-	-	-	-	-	-	-	-	-	**100**						
14 LMG 11111	-	76	-	-	-	-	-	-	-	-	-	-	-	**100**					
15 LMG 978	-	-	-	-	-	-	54	-	-	-	-	-	-	-	**100**				
16 LMG 980	53	-	-	-	-	-	55	-	-	-	-	-	-	-	-	**100**			
17 LMG 10874	-	-	-	-	-	-	60	-	-	-	-	-	-	-	-	-	**100**		
18 LMG 10878	-	-	-	-	-	-	49	-	-	-	-	-	-	-	-	-	-	**100**	
19 LMG 10877	-	-	-	-	-	-	51	-	-	-	-	-	-	-	-	-	-	-	**100**

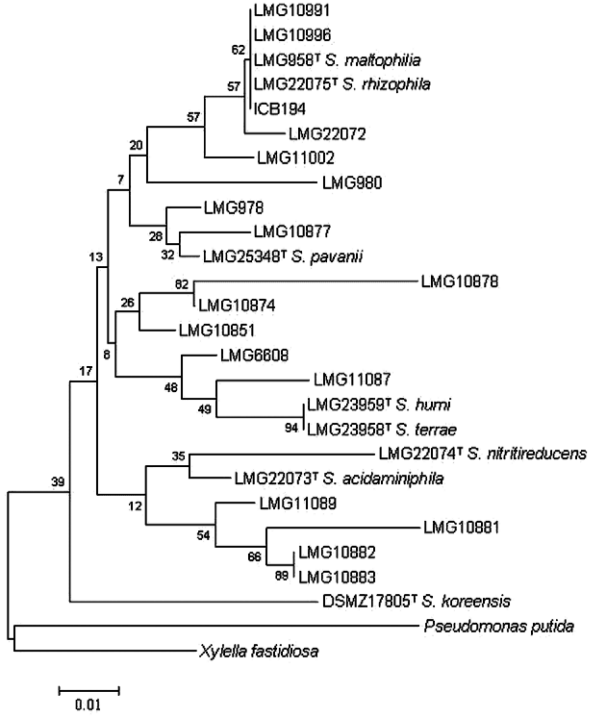
a: data from our study; *b*: data from Hauben et al. (1999).



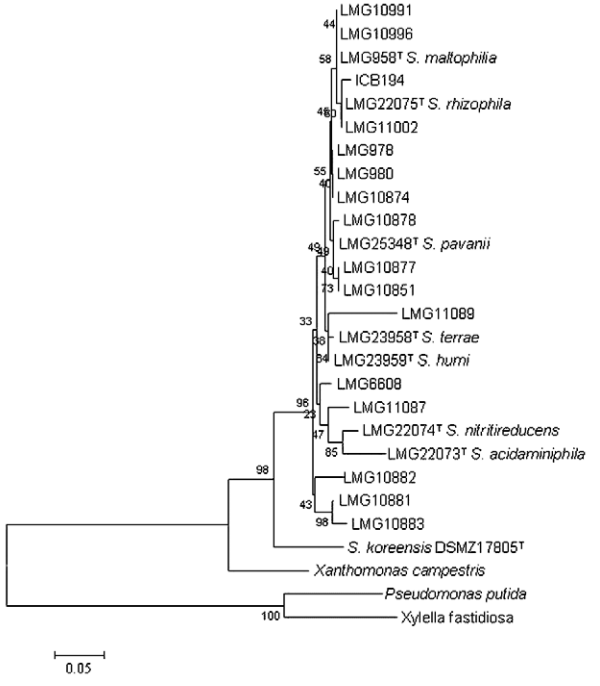
Neighbour-Joining phylogenetic tree based on the *atp*A gene sequences. Values of bootstrap after 1,000 repetitions are shown at the nodes. *Pseudomonas putida*, *Xanthomonas campestris* and *Xylella fastidiosa* were used as out-groups.



Neighbour-Joining phylogenetic tree based on the *rec*A gene sequences. Values of bootstrap after 1,000 repetitions are shown at the nodes. *Pseudomonas putida* and *Xylella fastidiosa* were used as out-groups.



Neighbour-Joining phylogenetic tree based on the *rpo*A gene sequences. Values of bootstrap after 1,000 repetitions are shown at the nodes. *Pseudomonas putida*, *Xanthomonas campestris* and *Xylella fastidiosa* were used as out-groups.



Neighbour-Joining phylogenetic tree based on the *uvr*B gene sequences. Values of bootstrap after 1,000 repetitions are shown at the nodes. *Pseudomonas putida*, *Xanthomonas campestris* and *Xylella fastidiosa* were used as out-groups.