A Rapid MALDI-TOF MS Identification Database at Genospecies Level for Clinical and Environmental Aeromonas Strains

Cinzia Benagli1*, Antonella Demarta1, AnnaPaola Caminada1, Dominik Ziegler2, Orlando Petrini1, Mauro Tonolla1,3

1 Institute of Microbiology, Bellinzona, Switzerland, 2 Mabritec AG, Riehen, Switzerland, 3 Microbial Ecology, Microbiology Unit, Plant Biology Department University of Geneva, Genève, Switzerland

Abstract

The genus Aeromonas has undergone a number of taxonomic and nomenclature revisions over the past 20 years, and new (sub)species and biogroups are continuously described. Standard identification methods such as biochemical characterization have deficiencies and do not allow clarification of the taxonomic position. This report describes the development of a matrix-assisted laser desorption/ionisation–time of flight mass spectrometry (MALDI-TOF MS) identification database for a rapid identification of clinical and environmental Aeromonas isolates.

Introduction

Bacteria belonging to the genus Aeromonas are widely distributed in freshwater and brackish environments, and have long been recognized as etiologic agents for fish diseases [1]. They are included into the class Gammaproteobacteria, comprising Gram-negative, non-spore-forming rod-shaped bacteria, are facultative anaerobic oxidase- and catalase-positive, glucose-fermenting, resistant to the vibriostatic agent O/129, and generally motile [2].

Aeromonas play also a significant role as opportunistic pathogens for humans causing gastroenteritis, septicemia, pneumonia, meningitis, and wound infections in immunocompetent as well as in compromised patients. A. hydrophila, A. caviae and A. veronii (biovar sobria and biovar veronii), are clinically the most significant species [3].

So far, the genus Aeromonas comprises 21 validly proposed species: A. allosaccharophila, A. aquarorum, A. bestiarum, A. bivalvium, A. caviae (synonym: A. punctata) A. culicicola, A. enchelea (corresponds to HG 11), A. eucnepholia, A. hydrophila, A. jandaei, A. media, A. mollisaurum, A. popoffii, A. salmonicida, A. schuberti, A. sharmania, A. simiae, A. sobria, A. testa, A. trota (synonym: A. enteropelogenes), A. veronii (synonym: A. ichthiosmia). It has to be noted that within these proposed species the position of A. allosaccharophila, A. culicicola and A. sharmania has to be clarified since the first two might belong to A. veronii and the last one seems not belong to the genus Aeromonas at all [4,5].

Several phylogenetic studies on Aeromonas allowed the elevation of the genus name to the rank of family [2,6,7,8]. Nevertheless the taxonomy of this genus is rather complex and has been submitted to ongoing changes due to newly described species [9,10,11,12,13,14] and rearrangements of existing taxa [15,16,17,18,19,20]. One major problem in Aeromonas identification relies on the fact that some species are phenotypically very similar (e.g. A. caviae and A. media, A. veronii and A. sobria). Several molecular methods have been therefore applied as an alternative to the laborious DNA-DNA hybridization technique for resolving the Aeromonas taxonomy and even though the sequence analysis of ribosomal RNA genes allowed for the discrimination of the genospecies [6,21,22], other more discriminating housekeeping genes such as gyrB and fpoD are now increasingly used [8,23,24,25,26]. Nevertheless, sequencing and phylogenetic methods are costly, time consuming and therefore not appropriate for a rapid species identification in the diagnostic laboratory. A valid alternative to conventional methods of bacterial identification and classification, based on the characterization of biomarker molecules, but definitely more rapid and reliable is the mass spectrometry technique [27]; MALDI-TOF MS (matrix assisted laser desorption ionization mass spectrometry – time of flight) combined with a reliable database is a powerful method for the identification and comparison of microbial isolates based on protein fingerprints analysis of whole cells [28]. MALDI-TOF MS applications in microbiology are important for proteomic and natural product analyses [29]. This technique can be used to detect non-volatile and thermally unstable molecules from a few to several hundred kDa, the most applicable range used for the analysis is 2–20 kDa. The identification of microorganisms by MALDI-TOF MS is based on the detection of mass signals from biomarkers that are specific at genus, species or sub-group level.

All mass spectra were generated in positive linear mode by a MALDI-TOF MS [30]. The method relies on the fact that some species are phenotypically very similar (e.g. A. caviae and A. media, A. veronii and A. sobria). Several molecular methods have been therefore applied as an alternative to the laborious DNA-DNA hybridization technique for resolving the Aeromonas taxonomy and even though the sequence analysis of ribosomal RNA genes allowed for the discrimination of the genospecies [6,21,22], other more discriminating housekeeping genes such as gyrB and fpoD are now increasingly used [8,23,24,25,26]. Nevertheless, sequencing and phylogenetic methods are costly, time consuming and therefore not appropriate for a rapid species identification in the diagnostic laboratory. A valid alternative to conventional methods of bacterial identification and classification, based on the characterization of biomarker molecules, but definitely more rapid and reliable is the mass spectrometry technique [27]; MALDI-TOF MS (matrix assisted laser desorption ionization mass spectrometry – time of flight) combined with a reliable database is a powerful method for the identification and comparison of microbial isolates based on protein fingerprints analysis of whole cells [28]. MALDI-TOF MS applications in microbiology are important for proteomic and natural product analyses [29]. This technique can be used to detect non-volatile and thermally unstable molecules from a few to several hundred kDa, the most applicable range used for the analysis is 2–20 kDa. The identification of microorganisms by MALDI-TOF MS is based on the detection of mass signals from biomarkers that are specific at genus, species or sub-group level.

All mass spectra were generated in positive linear mode by scanning the sample spot with the laser beam, and after signal acquisition, the raw mass spectra are processed automatically by smoothing, baseline correction and peak recognition [30].
essential information used for microbial identification is contained in a peak list containing m/z values and intensities. This list is analysed by comparison to the database SARAMIS™ (Spectral Archive And Microbial Identification System), in which the identification at the species level is based on a percentage of confidence referred to reference spectra (SuperSpectra™) that contain family, genus and species specific m/z biomarkers, as described in the SARAMIS™ user manual. For the generation of one SuperSpectra™ some representatives isolates of one species from different locations (hospitals, reference centers and strain culture collections) are needed. Beside the FingerprintSpectra every isolate will be determined by accredited and published microorganism identification procedures. The SuperSpectra™ are generated based on measurements of well known microorganisms and contain sets of genus, species and strain biomarkers which are characteristic for the respective group of microorganisms. SuperSpectra™ are computed from typical strains covering more than 90% of the intraspecific diversity in most species.

Accuracy of the identification strongly relies upon the robustness of the database and the choice of reference isolates. This is especially important when considering genera comprising species of clinical and environmental origin presenting a high genetic diversity.

There are excellent precedents for the application of MALDI-TOF MS for taxonomic studies [31,32,33,34], as well as for routine diagnostic [35].

Previous studies proved the applicability of this technique for the identification of the Aeromonas species [36,37,38]. The major aim of this study was to establish a rapid and reliable species identification tool for the genus Aeromonas using the SARAMIS™ identification system based on a relatively high number of phylogenetically well characterized isolates of clinical and environmental origin.

Data Analysis

A database identification system was established analyzing 92 morphologically and genetically well characterized Aeromonas strains belonging to all known species of the genus. The resulting peak lists of these samples were exported to the SARAMIS™ software package (bioMérieux, France) and submitted to single-linkage cluster analysis to produce taxonomic trees. These trees were compared to a gyrB phylogenetic tree (Neighbour-Joining). Specific biomarkers containing sets of genus, species and strain characteristic masses were used for the creation of species-specific SuperSpectra™ recognizing the most frequently encountered species. 11 different SuperSpectra™ were created that allow identifications of: A. hydrophila, A. caviae, A. media, A. tecta, A. popoffii, A. eucrenophila, A. enchele, A. bestiarum, A. salmonicida, A. sobria and A. veronii).

Results and Discussion

The protein mass fingerprint analysis emerging from the MALDI-TOF MS data of 92 genetically well characterized Aeromonas strains provided a good separation at genospecies (Fig. 1) level comparable with the phylogenetic tree obtained by gyrB gene sequencing.

In fact both trees clustered the species A. veronii (A. veronii biovar sobria, A. veronii biovar sobria), A. caviae, and A. allosaccarophila together, confirming the hypothesis that this group in fact represents only one genospecies [18].

Interesting the m/z profiles analysis allowed to separate the two biovars veronii and sobria, furthermore the profile of the strain ATCC 51106 A. veronii biovar sobria was more closely related to that of A. allosaccarophila ATCC 51208 than to that of A. veronii biovar veronii, confirming the results obtained with the gyrB sequences.

Moreover MALDI-TOF MS analysis categorized in a single cluster A. encheleia and the unnamed Aeromonas sp. HG11 [23] and allowed the segregation in the different genospecies of the A. salmonicida/A. bestiarum/A. popoffii group.

A. salmonicida and A. bestiarum are difficult to separate on the basis of 16S rRNA (differ in only 2 nucleotide positions) [2] but they could be separated using gyrB as well as other housekeeping genes such as rpoD or tpoD.

At the subspecies level, A. salmonicida formed a very uniform group, with respective intraspecies substitution rates of 1.3 and
0.8% for *gyrB* and *rpoB*, rendering very difficult to classify strains at the subspecies level [41]. MALDI-TOF MS seemed to allow a better differentiation of the strains in study. The type strains of each subspecies were well differentiated and formed a defined group in the MALDI-TOF MS dendrogram (Fig. 1).

A branch in the MALDI-TOF MS dendrogram groups in one single cluster strains assigned to the species *A. aquariorum* and *A. hydrophila* subsp. *dhakensis* (Fig. 1). Data based on phylogenetic analysis by sequencing *gyrB*, *rpoD* and 16S rRNA [43], strongly suggested that strains of *A. hydrophila* subsp. *dhakensis* belongs in fact to the species *A. aquariorum*, confirming the results obtained with MALDI-TOF MS (Fig. 1).

Due to the reliable identification at species level, it was possible to create 11 different SuperSpectra™ for *A. hydrophila*, *A. caviae*,...
Table 1. Characteristic masses retained for the creation of SuperSpectra™.

| Characteristic | A. hydrophila | A. caviae | A. popoffi | A. tecta | A. eucrenophila | A. media | A. media | A. media | A. media | A. media | A. media | A. bestiarum | A. encheleia | A. salmonicida | A. veronii | A. veronii | A. veronii | A. sobria |
|----------------|--------------|-----------|------------|----------|----------------|---------|---------|---------|---------|---------|---------|--------------|-------------|---------------|----------|----------|----------|---------|
|                | 3332         | 3150      | 3683       | 3032     | 3150          | 3047    | 2007    | 2006    | 3150    | 3150    | 3150    | 3828         | 3772        | 3153          | 2241     | 3590     |
|                | 3871         | 3435      | 3827       | 3150     | 4258          | 3435    | 3156    | 2039    | 3844    | 3899    | 4346    | 3930         | 3606        | 3047          | 4172     |
|                | 4169         | 4302      | 4257       | 3970     | 4458          | 3665    | 3671    | 2071    | 3863    | 4259    | 4591    | 4189         | 4174        | 4170          | 4260     |
|                | 4256         | 4394      | 4322       | 4257     | 4514          | 4258    | 4265    | 2087    | 4257    | 4487    | 4699    | 4309         | 4262        | 4257          | 4348     |
|                | 4318         | 4974      | 4393       | 4458     | 4700          | 4317    | 4325    | 2093    | 4440    | 4600    | 5050    | 4393         | 4366        | 4309          | 4650     |
|                | 4445         | 5051      | 4879       | 4766     | 5051          | 4460    | 4468    | 2514    | 4591    | 4701    | 5584    | 5071         | 4504        | 4361          | 5052     |
|                | 4698         | 5187      | 5203       | 5075     | 5612          | 4591    | 4599    | 2614    | 4655    | 5007    | 5675    | 5186         | 4646        | 4490          | 6104     |
|                | 5003         | 5394      | 5665       | 5477     | 5743          | 4700    | 4707    | 6107    | 5070    | 5144    | 5700    | 5393         | 4704        | 4518          | 6307     |
|                | 5049         | 5687      | 6064       | 6876     | 6070          | 5462    | 5694    | 6313    | 5155    | 5351    | 5877    | 5590         | 4989        | 4670          | 6934     |
|                | 5706         | 5885      | 6329       | 7704     | 6306          | 6083    | 5903    | 8615    | 5603    | 6071    | 6085    | 6197         | 5161        | 5155          | 7184     |
|                | 6022         | 6213      | 6914       | 7943     | 6481          | 6305    | 6109    | 8923    | 5637    | 6307    | 6305    | 6859         | 6313        | 7234          | 7336     |
|                | 6304         | 7210      | 7194       | 8604     | 6833          | 6481    | 6315    | 9193    | 6305    | 6482    | 6480    | 7236         | 6867        | 7410          | 7920     |
|                | 7208         | 7410      | 7220       | 8963     | 7335          | 6861    | 6490    | 9220    | 6480    | 6951    | 6919    | 7408         | 9195        | 7749          | 8831     |
|                | 7347         | 7463      | 7492       | 9014     | 7888          | 7333    | 7206    | 9408    | 7566    | 7197    | 7195    | 7934         | 9384        | 8624          | 8941     |
|                | 7477         | 8606      | 7904       | 9535     | 8344          | 7369    | 7343    | 10318   | 7730    | 7335    | 7332    | 8160         | 9890        | 9042          | 9040     |
|                | 7746         | 8979      | 8060       | 10628    | 8706          | 7473    | 7684    | 10931   | 8343    | 8263    | 7658    | 8621         | 11164       | 10280         | 9231     |
|                | 8637         | 8998      | 9184       | 10953    | 9029          | 7915    | 8617    | 11376   | 9400    | 8607    | 8343    | 9040         | 11191       | 10904         | 11358    |
|                | 8913         | 9401      | 9399       | 11192    | 9186          | 8343    | 9197    | 12205   | 10136   | 9201    | 9400    | 11385        | 12216       | 11422         | 11735    |
|                | 9183         | 9949      | 9682       | 11399    | 9401          | 9185    | 9221    | 11273   | 9403    | 11166   | 12406   | 14213        | 13484       | 12304         | 12412    |
|                | 9398         | 11373     | 11329      | 11753    | 10311         | 11193   | 9412    | 10313   | 11348   | 12461   | 12461   |

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These results demonstrate that the mass spectral data of the strains contained sufficient protein information to distinguish between genera, species, and strains (Table 2).

Another mass spectrometry study of intact-cell with Aeromonas strains [37] also confirmed that the signals generated from the analysis of the protein masses could be used as specific biomarkers for the differentiation below the species level. For the vast majority of the species analyzed the identification was successful.

With A. testa and A. sobria we obtained a correct identification for all the strains, whereas for A. eucrenophila, A. salmonicida, and A. hydrophila only 1 strains for the first two and 2 strains for the last species could not be identified.

Identification of A. popoffii with the created SuperSpectra™ was possible only in 46% of the cases. These failure could be due to insufficient coverage of the specific SuperSpectra™ or lack of performance of the last.

The approach presented in this paper uses the technique MALDI-TOF MS to develop a rapid, sensitive and specific method to detect isolates of the genus Aeromonas.

Our work highlighted the importance of testing well characterized strains of different origins for producing high quality MALDI-TOF MS databases as rapid identification tools. In conclusion, we can affirm that MALDI-TOF MS is a rapid and relatively inexpensive method for the identification of Aeromonas species and constitutes a valid alternative to conventional methods of identification and classification.

**Supporting Information**

Table S1 Strains used in this study. (DOC)

**Author Contributions**

Conceived and designed the experiments: CB AD OP MT. Performed the experiments: CB. Analyzed the data: CB APC DZ MT. Contributed reagents/materials/analysis tools: OP. Wrote the paper: CB OP.

**References**

1. Austin BA, C (1996) Fish pathogens. The Genus Aeromonas. B. Austin, M. Alteweg, P. J. Gosling & S. Joseph ed: Chichester: Wiley. 197–244.
2. Martin-Carnahan A, Joseph SW (2005) Genus I. Aeromonas. In: D. J. Brenner NNR, J. T Staley, and, GMG (eds). Bergey’s manual of systematic bacteriology. 2nd ed. New York, NY.: Springer. 557–578.
3. Janda JM, Abbott SL (1998) Evolving concepts regarding the genus Aeromonas: an expanding Panorama of species, disease presentations, and unanswered questions. Clin Infect Dis 27: 332–344.
4. Collins MD, Martinez-Murcia AJ, Cai J (1993) Aeromonas enteropelogenes and Aeromonas ichthiosmia are identical to Aeromonas testa and Aeromonas serosa, respectively, as revealed by small-subunit rRNA sequence analysis. Int J Syst Bacteriol 43: 855–856.
5. Janda JM, Abbott SL (2004) The genus Aeromonas: taxonomy, pathogenicity, and infection. Clin Microbiol Rev 23: 35–73.
6. Martinez-Murcia AJ, Berlloch S, Collins MD (1992) Phylogenetic interrelationships of members of the genera Aeromonas and Plesiomonas as determined by 16S ribosomal DNA sequencing: lack of congruence with results of DNA-DNA hybridizations. Int J Syst Bacteriol 42: 412–421.
7. Ruimy R, Brettmayer V, Elbaze P, Lalay B, Boussemart O, et al. (1994) Phylogenetic analysis and assessment of the genera Vibrio, Photobacterium, Aeromonas, and Plesiomonas deduced from small-subunit rRNA sequences. Int J Syst Bacteriol 44: 416–426.
8. Yanez MA, Catalán V, Apaza D, Figueras MJ, Martinez-Murcia AJ (2003) Phylogenetic analysis of members of the genus Aeromonas based on gyrB gene sequences. Int J Syst Evol Microbiol 53: 1875–1878.
9. Palivy V, Kozowska A, Narayan NR, Patele M, Shouche YS (2002) Aeromonas culicola sp. nov., from the midgut of Culex quinquefasciatus. Int J Syst Evol Microbiol 52: 1723–1728.
10. Harf-Montel C, Fleche AL, Riegel P, Prevost G, Bermond D, et al. (2004) Aeromonas caimae sp. nov., isolated from monkey faeces. Int J Syst Evol Microbiol 54: 481–485.
11. Minana-Galbis D, Farfan M, Fuste MC, Loren JG (2004) Aeromonas molluscorum sp. nov., isolated from bivalve mussels. Int J Syst Evol Microbiol 54: 2073–2078.
12. Minana-Galbis D, Farfan M, Fuste MC, Loren JG (2004) Genetic diversity and population structure of Aeromonas hydrophila, Aer. bestiarum, Aer. salmonicida and Aer. popoffii by multilocus enzyme electrophoresis (MLEE). Environ Microbiol 6: 190–200.
13. Minana-Galbis D, Fuste MC, Loren JG (2007) Aeromonas bestiarum sp. nov., isolated from bivalve mussels. Int J Syst Evol Microbiol 57: 582–587.
14. Minana-Galbis D, Farfan M, Gaspar Loren J, Carmen Fuste M (2010) Proposal to assign Aeromonas diversa sp. nov. as a novel species designation for Aeromonas group 501. Syst Appl Microbiol 33: 15–19.
15. Pavan ME, Abbott SL, Zoronzopulos J, Janda JM (2000) Aeromonas salmonicida subsp. piscicida subsp. nov., a new pectinase-positive subspecies isolated from a heavily polluted river. Int J Syst Evol Microbiol 50 P 3: 1119–1129.
16. Huy G, Pearson M, Kompfer P, Denys R, Couckaret M, et al. (2007) Aeromonas hydrophila subsp. subsp. nov., isolated from septicaemic farmed frogs in Thailand. Int J Syst Evol Microbiol 53: 885–891.
17. Huy G, Kompfer P, Albert MJ, Kuhn I, Denys R, et al. (2002) Aeromonas hydrophila subsp. dhakensis subsp. nov., isolated from children with diarrhoea in Bangladesh, and extended description of Aeromonas hydrophila subsp. hydrophila (Chester 1901) Stanier 1943 (approved lists 1980). Int J Syst Evol Microbiol 52: 705–712.
18. Huy G, Couckaret M, Swings J (2005) Aeromonas culicola Pidiyar et al. 2002 is a later subjective synonym of Aeromonas oryzae Hickman-Brenner et al. 1987. Syst Appl Microbiol 28: 604–609.
19. Esteve C, Valera L, Gutierrez C, Ventosa A (2003) Taxonomic study of sucrose-positive Aeromonas jandaei-like isolates from faeces, water and eels: emendation of A. jandaei Carnahan et al. 1992. Int J Syst Evol Microbiol 53: 1411–1419.
20. Demartia A, Huy G, Toussa M, Swings J, Peduzzi R (2004) Polyphasic taxonomic study of “Aeromonas eucrenophila-like” isolates from clinical and environmental sources. Syst Appl Microbiol 27: 343–349.

| Table 2. Identification values at species level obtained with the created SuperSpectra™. |
|-----------------------------------------------|
| Aeromonas | hydrophila | 97 | 97 | 99% | 99% | 158 |
| Aeromonas | caviae | 176 | 6 | 7 | 9 | 197 |
| Aeromonas | media | 76 | 9 | 3 | 3 | 91 |
| Aeromonas | tecta | 12 | 12 | 12 |
| Aeromonas | popoffii | 13 | 6 | 19 |
| Aeromonas | eurecnophila | 21 | 3 | 1 | 25 |
| Aeromonas | encheleia | 8 | 1 | 9 |
| Aeromonas | bestiarum | 25 | 5 | 30 |
| Aeromonas | salmonicida | 41 | 1 | 1 | 44 |
| Aeromonas | veronii | 90 | 5 | 8 | 103 |
| Aeromonas | sobria | 21 | 21 | 21 |
| Aeromonas | spp | 23 | 23 |
| n | 641 | 35 | 13 | 52 | 741 |
| doi:10.1371/journal.pone.0048441.t002 | | | | | |
21. Martinez-Murcia AJ (1991) An automated RNA extraction procedure and application for 16S rRNA sequencing of Leuconostoc amelobiosum. Microbiologia 7: 106–112.

22. Martinez-Murcia AJ (1999) Phylogenetic positions of Aeromonas encheleia, Aeromonas popoffii, Aeromonas DNA hybridization group 11 and Aeromonas group 5. Int J Syst Bacteriol 49 Pt 4: 1403–1408.

23. Solor L, Yanez MA, Chacon MR, Aguilar-Arreola MG, Catalan V, et al. (2004) Phylogenetic analysis of the genus Aeromonas based on two housekeeping genes. Int J Syst Evol Microbiol 54: 1511–1519.

24. Martinez-Murcia AJ, Solor L, Saavedra MJ, Chacon MR, Guarro J, et al. (2005) Phenotypic, genotypic, and phylogenetic discrepancies to differentiate Aeromonas from Aeromonas lactamurans. Int Microbiol 8: 259–269.

25. Saavedra MJ, Figueras MJ, Martinez-Murcia AJ (2006) Updated phylogeny of the genus Aeromonas. Int J Syst Evol Microbiol 56: 2481–2487.

26. Saavedra MJ, Perea V, Fontes GC, Martins C, Martinez-Murcia A (2007) Phylogenetic identification of Aeromonas strains isolated from carcasses of pigs as new members of the species Aeromonas allosaccharophila. Antonie Van Leeuwenhoek 91: 159–167.

27. Fenselau C, Demirev PA (2001) Characterization of intact microorganisms by MALDI mass spectrometry. Mass Spectrom Rev 20: 157–171.

28. Lay JOJ (2001) MALDI-TOF mass spectrometry of bacteria. Mass Spectrom Rev 20: 172–194.

29. Welker M, Marsalek B, Sejnohova L, von Dohren H (2006) Detection and identification of oligopeptides in Microcystis (cyanobacteria) colonies: toward an understanding of metabolic diversity. Peptides 27: 2090–2103.

30. Hahn D, Mirza B, Benaghi C, Vogel G, Tonolla M (2011) Typing of nitrogen-fixing Frankia strains by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) mass spectrometry. Syst Appl Microbiol 34: 40–44.

31. Himse D, Vollmer T, Erhardt M, Welker M, Moore ER, et al. (2011) Differentiation of species of the Staphylococcus bovis/equinus-complex by MALDI-TOF Mass Spectrometry in comparison to sodA sequence analyses. Syst Appl Microbiol 34: 52–57.

32. Kumar S, Jakobsen IB, Nei M, Bioinformatics (2001) MEGA2:cmolecular evolutionary genetics analysis software. 17 1244–1245.

33. Kupfer M, Kuhnert P, Korczak BM, Peduzzi R, Demarta A (2006) Genetic relationships of Aeromonas strains inferred from 16S rRNA, gyrB and rpoB gene sequences. Int J Syst Evol Microbiol 56: 2743–2751.

34. Kumar S, Jakobsen IB, Nei M, Bioinformatics (2001) MEGA2:cmolecular evolutionary genetics analysis software. 17 1244–1245.