Research Paper

Verification of the stability of identified species names using MALDI biotyper and the accuracy of identical species names through database updates

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Abstract The matrix-assisted laser desorption/ionization (MALDI) Biotyper calculates score values (SV) based on the similarity between spectra of isolates and those registered in the database, ranking species names from first to tenth. An SV ≥2.000 is generally sufficient for species-level identification; however, when multiple species exceed this threshold simultaneously, reduced identification accuracy may be a concern.

This study analyzed data from 41 species collected between January 2019 and September 2024 to determine the proportion of isolates with an SV ≥2.000 for the top-ranked species and to assess the stability of rankings from first to tenth. Spectra originally analyzed with Version 11 were reexamined using the Version 12 and 13 databases to assess the effects of database updates.

In Version 11, only *Staphylococcus haemolyticus* and four *Candida* species showed less than 90% of isolates with an SV ≥2.000 for the first-ranked species, whereas the remaining 36 species reached ≥90%. In contrast, only five species showed ≥80% stability across ranks 1−10, indicating species-specific variability. Database updates affected 32 species: 25 showed increased area under curve values, while seven decreased.

For *Serratia marcescens*, comparison of mixed-spectrum patterns with 16S rRNA phylogenetic analysis showed that closely related species were not simultaneously detected, highlighting methodological differences between mass spectrometry and molecular analysis. Stability depended on both the number of registered reference strains and the genus composition of species within the same genus. These findings underscore the need to consider species identity and SV stability when updating databases to improve MALDI Biotyper reliability for species identification.

Key words: MALDI-TOF MS, Score Value (SV), identified bacterial species, multiple bacterial species names, area under the curve (AUC)

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Introduction

In recent years, matrix-assisted laser desorption/ionization−time of flight mass spectrometry (MALDI-TOF MS) has been increasingly adopted in Japan for the identification of bacterial and fungal species[1−12]. MALDI-TOF MS rapidly identifies species by measuring ribosomal proteins (2,000−20,000 Da) that are highly expressed within microbial cells and analyzing their mass spectra. This technology has been reported to achieve species-level identification with accuracy comparable to 16S rRNA gene analysis[13−16].

Currently, approximately 400 MALDI-TOF MS instruments are in use in clinical microbiology laboratories across Japan, with the MALDI Biotyper (Bruker Daltonics) being the most widely used system. This device ranks species from first to tenth based on similarity scores, determined by matching the measured spectrum with reference spectra registered in the database. Typically, a score value (SV) of 2.000 or higher is considered sufficient for reliable species-level identification. However, even when the SV is ≥2.000, multiple species may appear among the top ranks, making unambiguous identification of the correct species difficult.

This study examined the proportion of identification results with an SV ≥ 2.000 obtained using the MALDI Biotyper, when multiple species were displayed as mixed results. Using clinical report names as the reference, we focused on three aspects: (1) the proportion of species names with an SV ≥ 2.000 among the first-ranked results in the Version 11 database; (2) the stability of the same species name across first- to tenth-ranked positions when SV ≥ 2.000; and (3) a comparison of identified species names across different database versions. Additionally, for the genus *Serratia*, in which species name stability decreased after database updates, we conducted detailed analyses of occurrence patterns in strains where multiple species appeared with an SV ≥ 2.000. We also examined interspecies relationships using a constructed phylogenetic tree. This paper reports the results of these analyses.

Materials and Methods

1. Data collection

Spectral data from 283,587 strains, analyzed with the MALDI Biotyper Microflex LT (Versions 5−8, 9, and 11), were used in this study. These strains were isolated from various clinical specimens, including urine and sputum, at the Takagi Hospital Clinical Microbiology and Genetic Testing Research Center between January 2019 and September 2024. The spectral data were classified according to the database version used: Versions 5−8 (August 2016−May 2021, 109,876 strains), Version 9 (June 2021−July 2022, 55,634 strains), and Version 11 (August 2022−September 2024, 118,077 strains). The exact timing of database updates for Versions 5−8 was unknown; therefore, these versions were grouped together. Measurements with the MALDI Biotyper were performed using one of the following methods: cell smear, formic acid, or ethanol−formic acid extraction[17]. The clinically reported species name was primarily determined based on the first-ranked species with an SV ≥ 2.000, supplemented by biochemical characteristics when necessary.

2. Selection of target species for analysis

Data aggregation was performed using Microsoft Access, employing an analysis system independently developed at our institution. Among the 955 species, only strains displaying multiple distinct species names with an SV ≥ 2.000 were extracted. A total of 41 species with measurement data from ≥30 strains in Version 11 and either Versions 5−8 or Version 9 were included in the analysis[18] (Table 1). Table 2 shows the number of registered strains for each database version of these 41 species.

3. Comparative evaluation of the proportion of first-ranked bacterial species with an SV ≥ 2.000 and the stability of the same species across first- to tenth-ranked positions

①Proportion of first-ranked species with an SV ≥ 2.000: Based on Version 11 data, we evaluated the proportion of cases in which the first-ranked species matched the clinically reported species, using a 90% cutoff.

②Stability of the same species name within the first- to tenth-ranked positions: The proportion of cases in which all species names from the first- to tenth-ranked positions matched the clinically reported species and had an SV ≥ 2.000 was calculated. Stability was considered acceptable if it was ≥80%. Version-to-version changes in SV stability across the top 10 ranks were assessed using the area under the curve (AUC), on a scale of 0−1.000. Using Version 11 as the baseline, fluctuations of ＜5% were considered “no change,” and those ≥5% were considered “change” for evaluation[19,20] (Table 3).

The results of analyses ① and ② are shown in Table 4, and Table 5 presents a comparison of percentage changes in SV stability for the same species across database versions.

4. Comparison of SV and identified species names across versions using measured spectral data (Serratia marcescens and Staphylococcus epidermidis)

Based on Version 11, among the nine species for which AUC fluctuated by ≥5% in both Versions 5−8 and Version 9, *S. epidermidis* was selected as a representative species in

Table 1. Bacterial species and number of strains examined by gram stain method

	Strain	Number of shares analyzed		Strain	Number of shares analyzed
Gram-positive cocci	*Staphylococcus aureus*	3,479	Gram-positive rod	*Corynebacterium striatum*	881
	Staphylococcus epidermidis	1,000		*Clostridioides difficile*	113
	Staphylococcus haemolyticus	477	Gram-negative rod	*Haemophilus parainfluenzae*	219
	Staphylococcus capitis	168		*Haemophilus influenzae*	139
	Staphylococcus caprae	117		*Pseudomonas aeruginosa*	1,820
	Staphylococcus lugdunensis	96		*Stenotrophomonas maltophilia*	725
	Staphylococcus hominis	89		*Acinetobacter baumannii* complex	402
	Enterococcus faecalis	838		*Klebsiella pneumoniae*	2,370
	Enterococcus faecium	749		*Escherichia coli*	2,366
	Enterococcus raffinosus	73		*Enterobacter cloacae* complex	948
	Enterococcus avium	69		*Proteus mirabilis*	838
	Streptococcus agalactiae	750		*Serratia marcescens*	370
	Streptococcus anginosus	299		*Citrobacter koseri*	258
	Streptococcus constellatus	192		*Klebsiella aerogenes*	258
	Streptococcus pneumoniae	77		*Providencia stuartii*	203
	Aerococcus urinae	91		*Klebsiella oxytoca*	198
	Rothia mucilaginosa	115		*Morganella morganii*	149
				Raoultella ornithinolytica	73
Gram-negative cocci	*Moraxella catarrhalis*	130	Yeast-like fungi	*Candida albicans*	2,029
	Neisseria subflava	191		*Candida glabrata*	1,247
				Candida tropicalis	387
				Candida dubliniensis	141

which both the number of registered strains and the AUC increased with version updates. In contrast, among the species in which AUC decreased in Version 11, *S. marcescens* was selected due to a particularly marked decrease. For these two species, spectral data from ＞30 strains of the same species name, preserved between April and October 2024, were collected. Species names from the first- to tenth-ranked positions and their corresponding SVs were compared between Version 11 and Versions 12 or 13. Table 6 shows the number of registered strains added to or removed from the database in Versions 12 and 13.

5. Detailed analysis of SV decrease in S. marcescens

S. marcescens showed a marked decrease in SV in Version 11 compared with Versions 5−8 and Version 9. Therefore, among the 13 *Serratia* species registered in Version 11, strains in which multiple species were displayed with an SV ≥ 2.000 were extracted, and the occurrence patterns of each strain were examined (Table 7).

The 13 species included *S. marcescens*, *Serratia entomophila*, *Serratia ficaria*, *Serratia fonticola*, *Serratia grimesii*, *Serratia liquefaciens*, *Serratia nematodiphila*, *Serratia odorifera*, *Serratia plymuthica*, *Serratia quinivorans*, *Serratia rubidaea*, *Serratia ureilytica*, and *Serratia proteamaculans*.

To analyze correlations among these co-appearing species, a molecular phylogenetic tree was constructed using the 16S rRNA gene sequences of type strains with the Ribosomal Database Project Release 11 Sequence Analysis Tools, and phylogenetic relationships were examined[21−23].

Results

This study analyzed clinically reported bacterial identification data, with a focus on two main objectives:

①Comparison of the proportion of isolates for which the first-ranked species had an SV ≥ 2.000, using Version 11 as the reference.

②Evaluation of the stability of the top 10 species ranked with an SV ≥ 2.000.

1. Species with a first-ranked SV ≥ 2.000 in ≥90% of isolates and ≥80% stability across the top 10 ranks:

Five species met these criteria: *Morganella morganii*

Table 2. Number of registered strains by version based on Gram stain

	Strain	Version				Strain	Version		
		5−8	9	11			5−8	9	11
Gram-positive cocci	*S. aureus*	14	14	27	Gram-positive rod	*C. striatum*	9	9	9
	S. caprae	8	8	8		*C. difficile*	10	10	10
	S. capitis	7	7	7	Gram-negative rod	*H. influenzae*	11	11	11
	S. epidermidis	10	13	19		*H. parainfluenzae*	27	27	27
	S. haemolyticus	12	12	10		*A. baumannii* complex	46−48	46	46
	S. hominis	7−8	8	8		*P. aeruginosa*	9	9	9
	S. lugdunensis	7	7	7		*S. maltophilia*	10	10	10
	E. avium	8	8	8		*C. koseri*	10	10	10
	E. faecalis	11	11	11		*E. cloacae* complex	20−21	21	24
	E. faecium	10	10	10		*E. coli*	14	14	14
	E. raffinosus	2−4	4	6		*K. aerogenes*	7	7	7
	S. agalactiae	9	9	9		*K. oxytoca*	8	8	8
	S. anginosus	9	9	9		*K. pneumoniae*	11−12	11	11
	S. constellatus	6	6	7		*M. morganii*	16−17	16	16
	S. pneumoniae	30−31	31	31		*P. mirabilis*	9	9	9
	A. urinae	7	7	7		*P. stuartii*	8	8	8
	R. mucilaginosa	9	9	9		*R. ornithinolytica*	13	13	13
						S. marcescens	8	8	8
Gram-negative cocci	*M. catarrhalis*	10	10	10	Yeast-like fungi	*C. albicans*	25−31	31	31
	N. subflava	2−7	7	7		*C. dubliniensis*	14−16	16	16
						C. glabrata	11−15	15	15
						C. tropicalis	9−18	18	19

(Fig. 1), *Haemophilus influenzae*, *Staphylococcus aureus*, *Citrobacter koseri*, and *Proteus mirabilis*.

Both *M. morganii* and *H. influenzae* showed no changes in SV stability across all database versions.

In contrast, while *S. aureus*, *C. koseri*, and *P. mirabilis* maintained a first-ranked SV ≥ 2.000 in ≥90% of cases and demonstrated ≥80% stability across the top 10 ranks, their identification results changed with version updates.

2. Species with a first-ranked SV ≥ 2.000 in ≥90% of isolates, but <80% SV stability across the top 10 ranks, and no significant changes in AUC values due to version updates:

Seven species fell into this category: *Enterococcus faecalis*, *Staphylococcus caprae*, *Rothia mucilaginosa*, *Stenotrophomonas maltophilia*, *Escherichia coli* (Fig. 2), *Klebsiella aerogenes*, and *Providencia stuartii*.

3. Species with a first-ranked SV ≥ 2.000 in ≥90% of cases, but <80% SV stability across the top 10 ranks, showing changes in SV or stability after database updates:

Twenty-four species were identified in this category, including:

Enterococcus avium, *Enterococcus faecium*, *Enterococcus raffinosus*, *S. epidermidis*, *Staphylococcus capitis*, *Staphylococcus hominis*, *Staphylococcus lugdunensis*, *Streptococcus agalactiae*, *Streptococcus anginosus*, *Streptococcus constellatus*, *Streptococcus pneumoniae*, *Aerococcus urinae*, *Corynebacterium striatum*, *Clostridioides difficile*, *Moraxella catarrhalis*, *Neisseria subflava*, *Acinetobacter baumannii* complex, *Pseudomonas aeruginosa*, *Enterobacter cloacae* complex, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *S. marcescens*, *Raoultella ornithinolytica*, and *Haemophilus parainfluenzae* (Fig. 3 and 4).

Among these, seven species showed an AUC value that was ≥5% higher in Versions 5−8 or Version 9 compared to Version 11: *S. capitis*, *S. anginosus*, *M. catarrhalis*, *N. subflava*, *C. striatum*, *S. marcescens*, and *H. parainfluenzae*.

Specifically, for *S. marcescens*, the AUC dropped significantly from 0.436 (Versions 5−8) and 0.404 (Version 9) to 0.244 in Version 11 (±5% range: 0.232−0.256), indicating a marked decline beyond the acceptable range.

4. Species with a first-ranked SV ≥ 2.000 in <90% of cases, and stability of SV ≥ 2.000 across the top 10 ranks

Table 3. Area under the curve (AUC, 0−1.000) for each version and the range for version 11 ± 5%

Strain	Changes in the number of bacterial strains in each version	Version			Version11 ± 5%
		5−8	9	11	
S. aureus	Yes	**0.937**	0.965	0.988	0.939−1.000
S. caprae	None	0.247	N	0.259	0.246−0.272
S. capitis	None	**0.216**	**0.291**	0.272	0.258−0.285
S. epidermidis	Yes	**0.202**	**0.214**	0.295	0.280−0.309
S. haemolyticus	Yes	**0.164**	**0.160**	0.179	0.170−0.188
S. hominis	Yes	**0.480**	N	0.598	0.568−0.628
S. lugdnensis	None	**0.235**	N	0.269	0.256−0.282
E. faecalis	None	0.774	0.775	0.811	0.770−0.851
E. faecium	None	0.769	**0.736**	0.786	0.747−0.825
E. avium	None	**0.358**	N	0.578	0.549−0.607
E. raffinosus	Yes	**0.118**	N	0.441	0.419−0.463
S. agalactiae	None	**0.724**	0.775	0.771	0.732−0.809
S. anginosus	None	**0.243**	**0.212**	0.225	0.214−0.236
S. constellatus	Yes	**0.150**	**0.149**	0.241	0.229−0.253
S. pneumoniae	Yes	**0.750**	N	0.827	0.786−0.868
A. urinae	Yes	**0.229**	N	0.339	0.332−0.356
R. mucilaginosa	None	0.271	N	0.284	0.270−0.298
M. catarrhalis	None	**0.771**	N	0.685	0.651−0.720
N. subflava	Yes	**0.771**	N	0.685	0.651−0.719
C. striatum	None	0.253	**0.288**	0242	0.231−0.255
C. difficile	None	**0.178**	N	0.330	0.313−0.347
A. baumnnii complex	None	**0.590**	**0.430**	0.820	0.779−0.861
P. aeruginosa	None	**0.736**	0.756	0.777	0.738−0.816
S. maltophilia	None	0.406	0.424	0.418	0.397−0.439
C. koseri	None	**0.892**	0.972	0.980	0.931−1.000
E. cloacae complex	Yes	**0.647**	0.834	0.824	0.783−0.866
E. coli	None	0.908	0.924	0.946	0.899−0.993
K. aerogenes	None	0.671	0.686	0.690	0.656−0.725
K. oxytoca	None	0.305	**0.258**	0.303	0.288−0.318
K. pneumoniae	Yes	**0.739**	**0.662**	0.795	0.755−0.835
M. morganii	Yes	0.978	0.983	1.000	0.950−1.000
P. mirabilis	None	**0.841**	**0.868**	0.978	0.929−1.000
P. stuartii	None	0.745	0.785	0.752	0.714−0.789
R. orunithinolytica	None	**0.523**	N	0.671	0.637−0.705
S. marcescens	None	**0.436**	**0.404**	0.244	0.232−0.256
H. influenzae	None	0.962	N	0.967	0.919−1.000
H. parainfluenzae	None	**0.560**	0.498	0.510	0.484−0.535
C. albicans	Yes	**0.101**	**0.157**	0.171	0.163−0.180
C. dublinensis	Yes	**0.028**	**0.032**	0.050	0.047−0.053
C. glabrata	Yes	**0.135**	**0.223**	0.163	0.155−0.172
C. tropicalis	Yes	**0.039**	**0.145**	0.135	0.129−0.142

N: no data.
※ No data indicates fewer than 30 shares.
Bold numbers indicate AUC values that show a change of ±5% or more compared to Version 11.

＜80%, showing changes in SV or rank stability after database updates:

Five species were included in this category: *Staphylococcus haemolyticus*, *Candida albicans*, *Candida dubliniensis*, *Candida glabrata*, and *Candida tropicalis* (Fig. 5 and 6).

Notably, among the *Candida* species, Version 11 yielded particularly low AUC values (0.050−0.171), in stark contrast to bacterial species (Table 3).

5. Correlation between the number of registered strains per version and changes in AUC:

Table 4. Comparison of identification accuracy by Microbial species, stability of identification accuracy, and changes in stability due to version changes

Percentage of clinical isolates matching Version 11 isolates with a score value (SV) of 2.000 or higher	Stability up to 10th place in Version 11	Changes due to version updates	Strain		
			Gram-positive bacteria	Gram-negative bacteria	Yeast-like fungi
Over 90%	Over 80% up to 10th place	No change		*M. morganii* *H. influenzae*	
		Changes	*S. aureus*	*C. koseri* *P. mirabilis*	
	Second place and below are below 80%	No change	*E. faecalis* *S. caprae* *R. mucilaginosa*	*S. maltophilia* *E. coli* *K. aerogenes* *P. stuartii*	
		Changes	*E. avium* *E. faecium* *E. raffinosus* *S. epidermidids* *S. capitis* *S. hominis* *S. lugdunensis* *S. agalactiae* *S. anginosus* *S. constellatus* *S. pneumoniae* *A. urinae* *C. striatum* *C. difficile*	*M. caarrhalis* *N. subflava* *A. baumannii* complex *P. aeruginosa* *E. cloacae* complex *K. oxytoca* *K. pneumoniae* *S. marcescens* *R. orunithinolytica* *H. parainfluenzae*	
Less than 90%	Second place and below are below 80%	Changes	*S. haemolyticus*		*C. albicans* *C. dubliniensis* *C. glabrata* *C. tropicalis*

※ Underlined species show an AUC decrease of ≥5% in Version 11 compared to Versions 5−8 or 9.

Changes in the number of registered strains between Versions 5−8, 9, and 11 were observed in 16 species, while 25 species showed no such changes. Among the 16 species with altered strain counts, 15 also showed corresponding changes in AUC values in Version 11, with *M. morganii* being the only exception.

Of these 15 species, 13 species (87%) showed an increase in AUC, while 2 species (13%) showed a decrease (*N. subflava* and *C. glabrata*).

Among the 25 species with no change in the number of registered stains per version, 17 showed changes in AUC, while 8 did not. In Version 11, AUC increased in 11 species (44%) and decreased in 6 species (24%) compared to Versions 5−8 or Version 9.

6. Detailed analysis of the *Serratia* genus:

A notable decrease in AUC for *S. marcescens* (0.244 in Version 11 vs. 0.436 in Versions 5−8 and 0.404 in Version 9) prompted further analysis of the *Serratia* genus.

Version 11 included 13 *Serratia* species, of which 9 were co-identified with an SV ≥ 2.000. The detection frequencies of these species are summarized in Table 7.

Fig. 7 and 8 illustrate the correlation between species co-identified (with SV ≥ 2.000) and their phylogenetic relationships based on 16S rRNA gene sequences. In these figures,

reference species are shown in square, Version 11 registered

Table 5. Percentage of bacterial species in each version that match the clinically reported species with an SV of 2.000 or higher

Strain	Version	1st place	2nd place	3rd place	4th place	5th place	6th place	7th place	8th place	9th place	10th place
S. aureus	5–8	98.7	97.4	96.8	96.2	95.1	95.1	93.9	91.9	88.7	83.1
	9	100	99.7	99.4	98.3	97.3	97.3	96.2	95.4	93	88.4
	11	99.8	99.5	99.2	99	98.7	98.7	98.6	98.5	98.2	98
S. capitis	5–8	88.4	**69.6**	**40.6**	**17.4**	**0**	**0**	**0**	**0**	**0**	**0**
	9	97.1	91.4	**68.6**	**34.3**	**0**	**0**	**0**	**0**	**0**	**0**
	11	96.9	**79.7**	**56.3**	**32.8**	**3.1**	**3.1**	**0**	**0**	**0**	**0**
S. epidermidis	5–8	84.9	**54.2**	**33.3**	**20.4**	**4.6**	**4.6**	**0.2**	**0**	**0**	**0**
	9	86.7	**58.1**	**37.1**	**20.5**	**5.2**	**5.2**	**1**	**0**	**0**	**0**
	11	93	**76.9**	**54.4**	**37**	**12.1**	**12.1**	**5.4**	**2.4**	**1.3**	**0**
S. haemolyticus	5–8	79	**42.5**	**20.5**	**12.5**	**3.5**	**3.5**	**2**	**0.5**	**0**	**0**
	9	79.3	**44.8**	**20.7**	**11.5**	**1.1**	**1.1**	**1.1**	**0**	**0**	**0**
	11	89.5	**52.6**	**16.3**	**12.1**	**3.7**	**3.7**	**1.6**	**0**	**0**	**0**
S. caprae	5–8	93.1	89.7	**55.2**	**8.6**	**0**	**0**	**0**	**0**	**0**	**0**
	11	91.5	74.6	**59.3**	**25.4**	**3.4**	**3.4**	**1.7**	**0**	**0**	**0**
S. hominis	5–8	95.5	90.9	88.6	**72.7**	**47.7**	**47.7**	**36.4**	**0**	**0**	**0**
	11	95.6	95.6	91.1	88.9	**68.9**	**68.9**	**53.3**	**35.6**	**0**	**0**
S. lugdunensis	5–8	94.7	**77.2**	**40.4**	**22.8**	**0**	**0**	**0**	**0**	**0**	**0**
	11	97.4	84.6	**53.8**	**33.3**	**0**	**0**	**0**	**0**	**0**	**0**
E. faecalis	5–8	97.9	97	95.5	94.8	88.2	88.2	84.5	**75.8**	**51.2**	**1.2**
	9	97.3	97.3	94	93.3	88.7	88.7	84.7	**76.7**	**52**	**2.7**
	11	99.2	98.3	98	97.2	94.4	94.4	91.9	80.4	**54.2**	**2.5**
E. faecium	5–8	99.2	98.4	97.2	95.6	90.5	90.5	87.3	**75.4**	**30.2**	**4.8**
	9	99.3	98.7	98	95.3	89.3	89.3	**79.9**	**64.4**	**20.1**	**1.3**
	11	99.1	98	97.1	96.3	93.4	93.4	91.1	81.3	**30.2**	**6**
E. avium	5–8	81.8	**78.8**	**63.6**	**54.5**	**30.3**	**30.3**	**18.2**	**0**	**0**	**0**
	11	94.4	91.7	91.7	88.9	**77.8**	**77.8**	**55.6**	**0**	**0**	**0**
E. raffinosus	5–8	**79.4**	**32.4**	**2.9**	**2.9**	**0**	**0**	**0**	**0**	**0**	**0**
	11	97.4	94.9	94.9	92.3	**30.8**	**30.8**	**0**	**0**	**0**	**0**
S. agalactiae	5–8	94.5	94.5	92.5	90.1	86	86	82.6	64.8	**32.4**	**0**
	9	98.5	98.5	97.8	96.3	94.8	94.8	89.6	**73.1**	**32.1**	**0**
	11	98.8	97.2	97.2	96.6	92.9	92.9	90.4	**73.4**	**31.3**	**0**
S. anginosus	5–8	88.8	**71.4**	**49**	**27.6**	**3.1**	**3.1**	**0**	**0**	**0**	**0**
	9	87.5	**66.1**	**41.1**	**17.9**	**0**	**0**	**0**	**0**	**0**	**0**
	11	94.48	**68.3**	**40.7**	**18.6**	**1.4**	**1.4**		**0**	**0**	**0**
S. constellatus	5–8	88.5	**53.8**	**7.7**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
	9	89.18	**56.75**	**2.7**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
	11	93.2	80.6	**64.1**	**2.9**	**0**	**0**	**0**	**0**	**0**	**0**
S. pneumoniae	5–8	97.5	82.5	80	80	**75**	**75**	**70**	**67.5**	**60**	**62.5**
	11	97.3	94.6	94.6	91.9	81.1	81.1	78.4	75.7	**70.3**	**62.2**
A. urinae	5–8	91.2	**73.5**	**50**	**14.7**	**0**	**0**	**0**	**0**	**0**	**0**
	11	94.7	91.2	82.5	**70.2**	**0**	**0**	**0**	**0**	**0**	**0**
R. mucilaginosa	5–8	84.2	81.6	**63.2**	**36.8**	**2.6**	**2.6**	**0**	**0**	**0**	**0**
	11	92.2	87	**64.9**	**40.3**	**0**	**0**	**0**	**0**	**0**	**0**

Table 5. Continued

Strain	Version	1st place	2nd place	3rd place	4th place	5th place	6th place	7th place	8th place	9th place	10th place
C. striatum	5−8	95.3	83.2	**55.8**	**19**	**0**	**0**	**0**	**0**	**0**	**0**
	9	96.7	85.8	**66.5**	**36.3**	**1.4**	**1.4**	**0**	**0**	**0**	**0**
	11	98.2	**79.2**	**41.8**	**22.8**	**0.5**	**0.5**	**0**	**0**	**0**	**0**
C. difficile	5−8	83.6	**53.7**	**35.8**	**4.5**	**0**	**0**	**0**	**0**	**0**	**0**
	11	95.7	89.1	**67.4**	**47.8**	**10.9**	**10.9**	**4.3**	**2.2**	**2.2**	**0**
N. subflava	5−8	100	95.7	82.9	**61.4**	**0**	**0**	**0**	**0**	**0**	**0**
	11	99.2	90.9	**78.5**	**66.9**	**0**	**0**	**0**	**0**	**0**	**0**
M. catarrhalis	5−8	96	93.3	92	86.7	80	80	**78.7**	**76**	**54.7**	**33.3**
	11	98.2	96.4	96.4	87.3	**74.5**	**74.5**	**72.7**	**52.7**	**20**	**12.7**
H. parainfluenzae	5−8	98.6	92.9	92.9	90	**65.7**	**65.7**	**37.1**	**17.1**	**0**	**0**
	9	100	95.8	87.5	83.3	**45.8**	**45.8**	**25**	**12.5**	**2.1**	**0**
	11	99	97	91.1	80.2	**46.5**	**46.5**	**33.7**	**15.8**	**0**	**0**
H. influenzae	5−8	100	100	100	98.5	97	97	92.4	92.4	92.4	92.4
	11	100	97.3	97.3	95.9	95.9	95.9	95.9	95.9	95.9	97.3
A. baumannii complex	5−8	99.5	84.7	**79.1**	**71.9**	**62.2**	**62.2**	**53.1**	**40.3**	**20.9**	**16.3**
	9	100	**74.2**	**66.7**	**52.7**	**38.7**	**38.7**	**23.7**	**15.1**	**10.8**	**9.7**
	11	100	95.6	93.8	92	86.7	86.7	**78.8**	**74.3**	**63.7**	**48.7**
P. aeruginosa	5−8	99.6	97.9	97.4	95.9	88.4	88.4	**79**	**59.6**	**29.4**	**0**
	9	99.5	97.3	96.8	96	89.9	89.9	81.1	**66.2**	**39.6**	**0**
	11	99.6	98.8	98.4	97.6	93.4	93.4	87.3	**72**	**36.7**	**0**
S. maltophilia	5−8	94.4	89.2	80.7	**70.6**	**24.9**	**24.9**	**13.8**	**4.8**	**1.5**	**0.7**
	9	96.3	91.9	80.9	**64**	**33.1**	**33.1**	**16.9**	**5.9**	**1.5**	**0.7**
	11	96.6	93.1	84.7	**74.4**	**25**	**25**	**12.8**	**5**	**0.9**	**0.3**
C. koseri	5−8	100	100	100	100	99.2	99.2	99.2	98.4	96	**0**
	9	100	100	98	98	98	98	98	98	96	88
	11	100	100	100	100	100	100	98.8	97.6	97.6	86.6
E. cloacae complex	5−8	98	94.8	90	83.7	**59.6**	**59.6**	**50.1**	**43**	**37.5**	**30.4**
	9	99.1	95.4	92.7	89.4	82.6	82.6	**78.4**	**74.8**	**71.6**	**67.9**
	11	99.5	96.9	95	91.9	81.4	81.4	**76.6**	**72.2**	**67.7**	**61.9**
E. coli	5−8	99.8	99.2	98.5	97.6	93.7	93.7	89.9	84.6	**78.7**	**72**
	9	99.8	99.8	99.3	98.8	95.4	95.4	92	88.6	82.2	**73.2**
	11	100	100	99.7	99.2	96.8	96.8	95.5	91.3	87.1	**79.5**
K. aerogenes	5−8	100	97.4	97.4	96.6	94.8	94.8	89.7	**0**	**0**	**0**
	9	100	98	98	98	98	98	96.1	**0**	**0**	**0**
	11	100	100	100	100	97.8	97.8	94.5	**0**	**0**	**0**
K. oxytoca	5−8	100	82.4	**71.8**	**31.8**	**8.2**	**8.2**	**2.4**	**0**	**0**	**0**
	9	98	86	**56**	**18**	**0**	**0**	**0**	**0**	**0**	**0**
	11	100	88.9	**76.2**	**34.9**	**1.6**	**1.6**	**0**	**0**	**0**	**0**
K. pneumoniae	5−8	99.4	97.6	94.4	91.2	83.3	83.3	**77**	**64.7**	**33.9**	**14.2**
	9	99.3	98.3	97	96	82.9	82.9	**65.8**	**35.6**	**3.7**	**0**
	11	100	99.7	99.6	97.9	89	89	**79.3**	**63.9**	**41.9**	**34.6**
M. morganii	5−8	100	100	100	100	98.2	98.2	98.2	98.2	92.7	92.7
	9	100	100	100	100	97.1	97.1	97.1	97.1	97.1	97.1
	11	100	100	100	100	100	100	100	100	100	100

Table 5. Continued

Strain	Version	1st place	2nd place	3rd place	4th place	5th place	6th place	7th place	8th place	9th place	10th place
P. mirabilis	5−8	99.4	98	97.4	96	95.5	95.5	92.6	87.8	**79**	**0**
	9	100	100	98.5	97.8	97	97	96.3	94	87.3	**0**
	11	100	100	99.7	99.1	98.9	98.9	98.3	96.9	94.9	90.9
P. stuartii	5−8	100	100	100	100	97.4	97.4	90.8	**59.2**	**0**	**0**
	9	100	100	100	100	100	100	100	85	**0**	**0**
	11	100	98.9	98.9	98.9	96.6	96.6	89.7	**72.4**	**0**	**0**
S. marcescens	5−8	99	89.2	84	**74.7**	**35.1**	**35.1**	**16**	**3.1**	**0**	**0**
	9	98.6	94.4	86.1	**66.7**	**22.2**	**22.2**	**11.1**	**2.8**	**0**	**0**
	11	100	**74**	**46.2**	**20.2**	**1.9**	**1.9**	**0**	**0**	**0**	**0**
R. ornithinolytica	5−8	97.1	88.6	**68.6**	**60**	**51.4**	**51.4**	**40**	**31.4**	**22.9**	**11.4**
	11	100	97.4	92.1	**78.9**	**65.8**	**65.8**	**57.9**	**50**	**36.8**	**26.3**
C. albicans	5−8	**43**	**26.1**	**15.2**	**9.9**	**2.4**	**2.4**	**1.5**	**0.5**	**0.3**	**0**
	9	**59.8**	**36.4**	**23.6**	**15**	**6.5**	**6.5**	**4.2**	**2.8**	**1.6**	**0.2**
	11	**60.5**	**39.8**	**26.6**	**17.6**	**7.4**	**7.4**	**4.9**	**3.4**	**2.5**	**1.3**
C. dubliniensis	5−8	**20**	**6.7**	**1.7**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
	9	**24.3**	**8.1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
	11	**36.4**	**11.4**	**2.3**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
C. glabrata	5−8	**63.4**	**39.6**	**21.4**	**9.7**	**0.2**	**0.2**	**0**	**0**	**0**	**0**
	9	**67.4**	**48.3**	**37.2**	**29.9**	**13.8**	**13.8**	**8.8**	**3.4**	**0.4**	**0**
	11	**54.2**	**38.8**	**30.1**	**19.7**	**7.8**	**7.8**	**3.1**	**1.1**	**0.7**	**0.2**
C. tropicalis	5−8	**21.5**	**8.1**	**3.7**	**3.7**	**0.7**	**0.7**	**0**	**0**	**0**	**0**
	9	**43.5**	**36.5**	**24.7**	**16.5**	**7.1**	**7.1**	**5.9**	**2.4**	**1.2**	**0**
	11	**53.9**	**33.5**	**25.1**	**13.2**	**3.6**	**3.6**	**2.4**	**0**	**0**	**0**

※Bold numbers indicate that the proportion of cases where the presented bacterial species matches the clinically reported species with an SV ≥ 2.000 is ＜80%.

Table 6. Number of strains added and removed for each genus in Versions 12 and 13

Genus	Version 12		Version 13		Total
	Additional	Delete	Additional	Delete	
Serratia spp.	0	0	2	0	2
Staphylococcus spp.	35	25	185	29	275

species are shown in underline, and species co-identified with the reference species (SV ≥ 2.000) are shown in circle.

The analysis revealed that species closely related based on 16S rRNA gene sequences were not always co-identified with an SV ≥ 2.000, while more distantly related species sometimes appeared with an SV ≥ 2.000. These findings indicate that MALDI-TOF MS identification results do not necessarily align with 16S rRNA-based phylogenetic relationships.

7. Evaluation of the impact on species identification and SVs in Versions 12 and 13:

For *S. marcescens*, the number of registered strains remained unchanged between Versions 11 and 12. However, in Version 13, two strains of *S. ficaria* were added.

This update affected the identified species and their SVs in 21 of 33 strains (63.6%) in Version 13. Of these, 12 strains showed SV ≥ 2.000. Notably, 18 of the 21 strains (85.7%) showed SV changes across the 7th and 10th ranked positions.

In the *Staphylococcus* genus, Version 12 added 7 species (35 strains) and removed 13 species (25 strains) compared to Version 11. Version 13 added 31 species (185 strains) and removed 13 species (29 strains).

For *S. epidermidis*, the number of strains registered in the database remained unchanged in Version 12, but 10 additional strains were added in Version 13. When testing 32 clinical *S. epidermidis* isolates, only 1 isolate (3.0%) showed changes in both the identified species and SV in Version 12, with no first-ranked SV ≥ 2.000 changes. In Version 13, all 32 isolates showed changes in identification and SV, with all first-ranked SVs ≥ 2.000 affected.

Table 7. Emergence patterns (%) for each *Serratia* species within the genus

	S. liquefaciens	*S. grimesii*	*S. odorifera*	*S. plymuthica*	*S. marcescens*	*S. ficaria*	*S. nematodiphila*	*S. ureilytica*	*S. entomophila*
S. liquefaciens	***	1.9	0.3	0.6	0	0	0	0	0
S. grimesii	1.9	***	0	0.3	0	0	0	0	0
S. odorifera	0.3	0	***	0	0	0.3	0	0	0
S. plymuthica	0.6	0.3	0	***	0	0	0	0	0
S. marcescens	0	0	0	0	***	10.2	3.0	71.5	0.3
S. ficaria	0	0	0.3	0	10.2	***	1.1	9.4	0
S. nematodiphila	0	0	0	0	3.0	1.1	***	0.8	0
S. ureilytica	0	0	0	0	71.5	9.4	0.8	***	0.3
S. entomophila	0	0	0	0	0.3	0	0	0.3	***

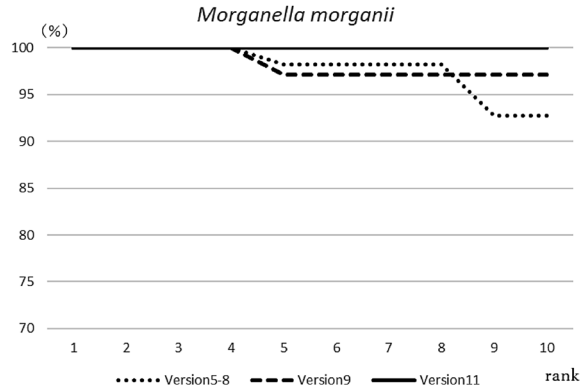

Fig. 1. Comparison of stability among Versions in *M. morganii.*

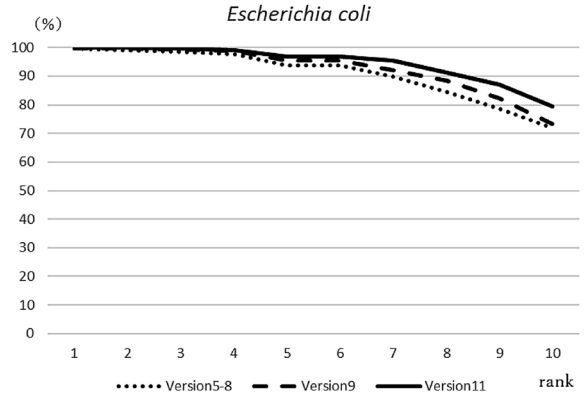

Fig. 2. Comparison of stability among Versions in *E. coli.*

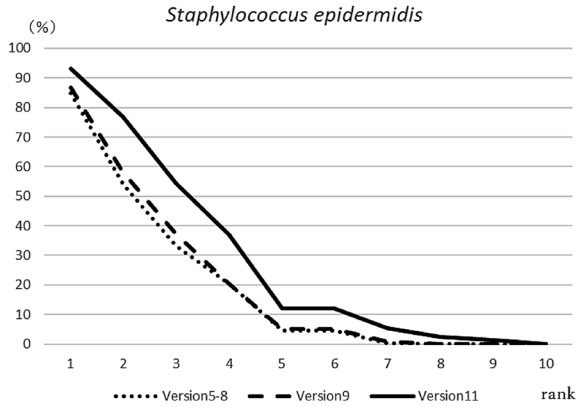

Fig. 3. Comparison of stability among Versions in *S. epidermidis.*

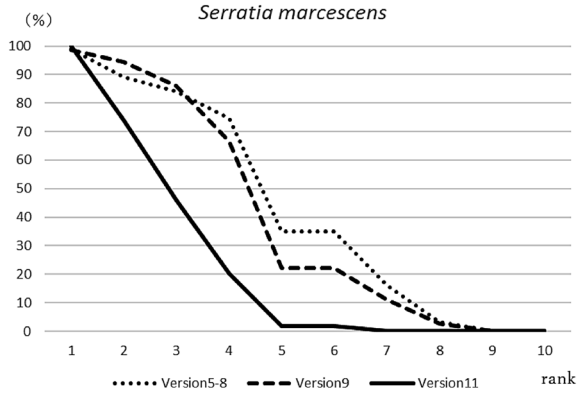

Fig. 4. Comparison of stability among Versions in *S. marcescens.*

4. Discussion

The MALDI Biotyper is a system that compares measured mass spectra with spectra of species registered in its database and presents the top 10 ranked species for each sample (ranks 1 through 10), along with their respective SVs. Generally, an SV of 2.000 or higher is considered to indicate high confidence in species-level identification. However, even when SV ≥ 2.000, multiple species may appear among the top ranks, which can make precise species assignment difficult. Similar limitations in distinguishing closely related species have been reported by Kanetake et al. for *Enterobacteriales*[24], by Cao et al. and Suzuki et al. for acid-fast bacteria[25,26], and by Rychert[27] and Rosiak et al.[28] for other species.

In this study, to compare differences among Biotyper database versions, we evaluated each version based on two factors: (1) the proportion of isolates for which the first-ranked species had an SV ≥ 2.000, and (2) the stability of

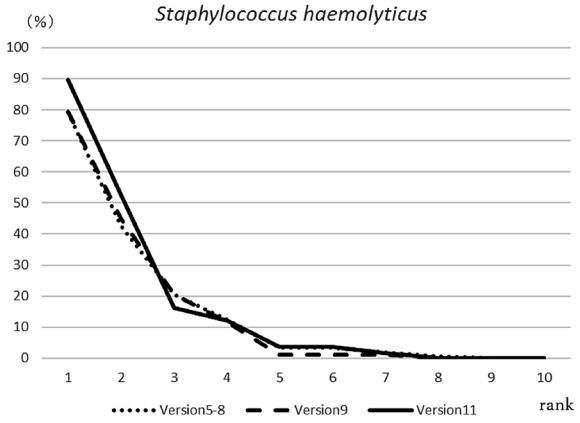

Fig. 5. Comparison of stability among Versions in *S. haemolyticus.*

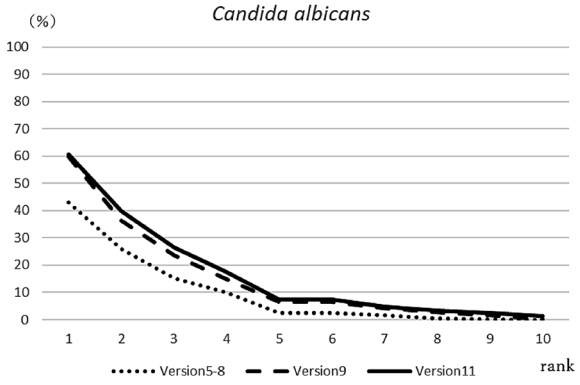

Fig. 6. Comparison of stability among Versions in *C. albicans.*

SVs across ranks 1 through 10.

Bacterial species that ranked first in the identification results of Version 11, with a ≥90% proportion of SV ≥ 2.000, accounted for 36 out of 41 species (88%). Of these, 5 species (12%) showed stable SVs (≥80%) across ranks 1 through 10. In contrast, 31 species (76%) had ≥90% of first-ranked isolates with an SV ≥ 2.000, but showed ＜80% stability across ranks 1 through 10. Although SV stability in the top 10 ranks was reasonably good overall, further improvements to the database are needed to enhance the stability of species identification in clinical microbiology.

Notably, *M. morganii*, which showed no change in AUC across versions, maintained an extremely high AUC of 0.978 in Versions 5−8, with a variation rate of ＜5%, likely explaining its stability. Among the five species for which the proportion of first-ranked SV ≥ 2.000 was ＜90%, only one was a bacterium (*S. haemolyticus*); the rest were fungi. For *S. haemolyticus*, Version 11 showed a higher AUC than Versions 5−8 or Version 9, despite having two fewer strains in the database. This suggests that changes in the number of registered strains of other species within the genus *Staphylococcus* may have had an indirect effect. For the *Candida* genus, Version 11 AUCs ranged from 0.050 to 0.171, significantly lower than those for bacterial species.

Lindsay et al.[29] and Yaman et al.[30] have reported high reliability of MALDI-TOF MS in species identification, but

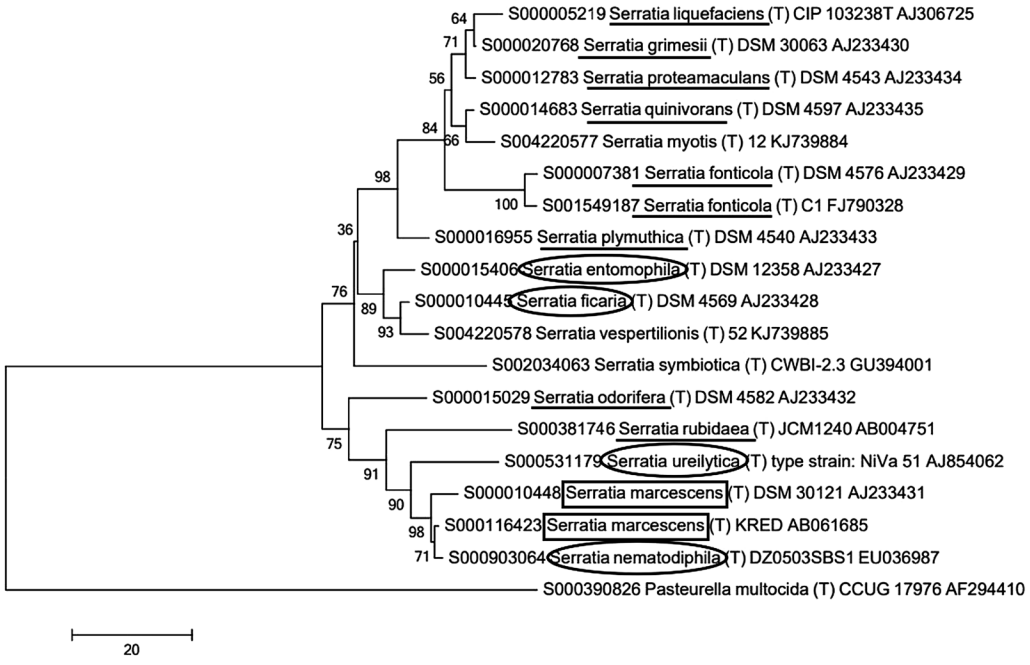

Fig. 7. Molecular phylogenetic tree of the genus Serratia based on the 16S rRNA gene: Taxonomic placement of registered bacterial species in MALDI Biotyper Version 11 and identified species using *S. marcescens* as a reference.

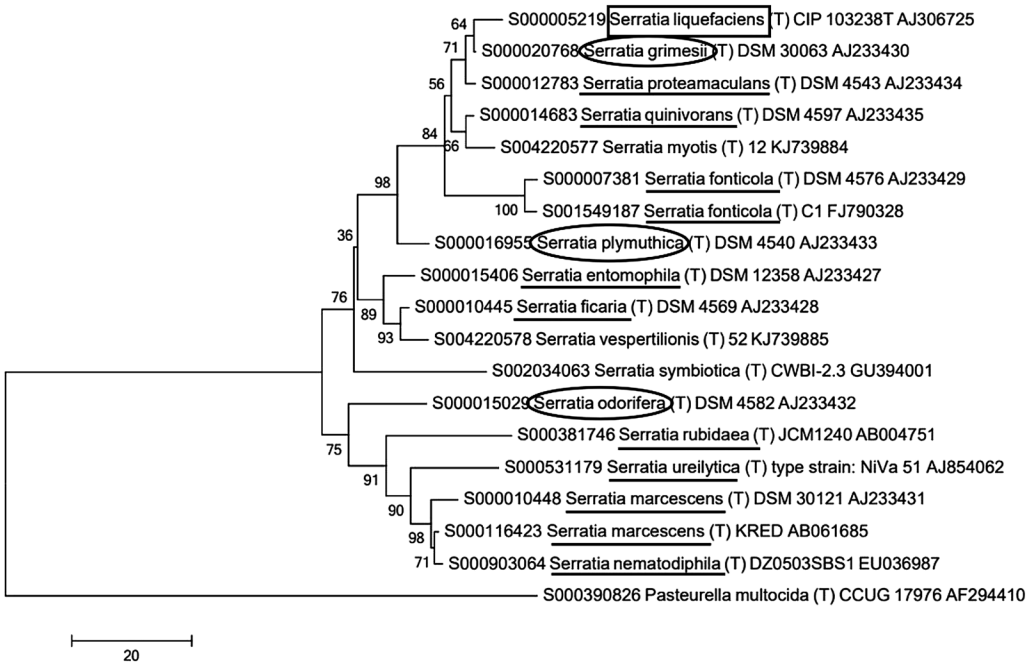

Fig. 8. Molecular phylogenetic tree of the genus *Serratia* based on 16S rRNA gene analysis: taxonomic placement of registered bacterial species in MALDI Biotyper Version 11 and identified species using *S. liquefaciens* as a reference.
Square: Reference strains.
Underline: Strains registered in Version 11.
Circle: Bacterial strains with SVs ≥ 2.000 among registered strains in Version 11.

Carolis et al.[31] and Fraser et al.[32] noted that for *Candida* species, weak spectral peaks or high levels of noise can result in poor-quality spectra. In our clinical experience, some *Candida* strains produce spectra that are difficult to obtain or interpret. These factors likely contribute to lower AUCs and reduced stability of species identification for *Candida*. Regarding the correlation between changes in the number of registered strains and AUC, using Version 11 as the reference: among the 25 species in which the number of registered strains remained unchanged across versions, changes in AUC were observed in 17 species. Of these, 11 species showed an increase in AUC in Version 11 compared to Versions 5−8 or Version 9, while 6 species showed a decrease. This indicates that even when the number of registered strains for a given species (e.g., within *Staphylococcus*) remains unchanged, additions or deletions of other species within the same genus in the database can affect the AUC values, which reflect the stability of species identification.

To illustrate this, we focused on *S. epidermidis* (which had an increase in registered strains) and *S. marcescens* (which had a significant drop in AUC from Versions 5−8/9 to Version 11). We reanalyzed the spectra originally obtained under Version 11 in Versions 12 and 13. For the *Staphylococcus* genus, Versions 12 and 13 included substantial additions and deletions of strains. In Version 12, because no new *S. epidermidis* strains were added, the data showed no major changes compared to Version 11. One exception was a single strain in which *Streptococcus dysgalactiae* appeared at rank 10 with an SV of 1.83, likely due to the addition of ten *S. dysgalactiae* strains in Version 12. In Version 13, ten additional *S. epidermidis* strains were added, and many of the top 10 ranks for each isolate showed species with an SV ≥ 2.000. No other species besides *S. epidermidis* appeared to cause instability. Overall, we concluded that species identification stability improved.

In the genus *Serratia*, the addition of two *S. ficaria* strains in Version 13 resulted in *S. ficaria* appearing among the top 10 ranked species, suggesting a potential negative impact on identification accuracy and stability. Furthermore, among *Serratia* strains where multiple species appeared in the top 10 ranks, species-specific mixed-display patterns were observed. To investigate the extent to which mixed-display patterns contribute to AUC decline in *Serratia* and how these patterns relate to 16S rRNA gene analysis, we constructed a molecular phylogenetic tree based on 16S rRNA sequences. In this tree, reference species are shown in square, species registered in Version 11 in underline, and

species that co-appeared with the reference species at SV ≥ 2.000 in circle. The results showed that the decline in AUC in Version 11 was partly due to the inclusion of an additional *S. nematodiphila* strain, which is phylogenetically very closely related to *S. marcescens*. This strain began to be presented at SV ≥ 2.000, causing mixed identifications. This suggests that genetic proximity contributes to the confusion. Meanwhile, *S. ficaria*, added in Version 13, occupied a taxonomic position distinct from *S. marcescens* in the phylogenetic tree; its closer relatives (*S. odorifera*, *S. rubidaea*) were not presented as co-species. Thus, while some distantly related species were nevertheless co-presented among the top ranks, there were also cases where species closely related by 16S rRNA were clearly distinguished by MALDI-TOF MS. These findings imply that, although MALDI-TOF MS identification is largely based on ribosomal and related proteins, its mechanism differs from 16S rRNA gene analysis and relies on its own algorithms and spectral features.

Previous reports are consistent with this interpretation. Rychert et al. found that 18 strains (1.6%) out of 1,146 were misidentified due to species mixing or selection of a single species[33], Sogawa et al.[34] reported that MALDI-TOF MS−based methods generally function comparably to conventional methods and are promising for clinical laboratories, but highlighted the need for pre-analytical steps and database improvements for certain pathogens. Strejcek et al.[35] similarly noted limitations in the concordance between 16S rRNA gene analysis and MALDI-TOF MS, suggesting the need to improve spectral reference databases. Cobo et al. and Li et al.[36,37] reported that while MALDI-TOF MS is reliable for most anaerobic bacteria, frequent database updates are required for accurate identification of rare, low-frequency, or newly discovered species. Furthermore, Cao et al.[25] evaluated the identification of the *Mycobacterium* genus and found that the clinical application of MALDI-TOF MS for identifying pathogenic mycobacteria at the species level remains unsatisfactory and requires improvement. However, specific methods for these improvements have not been adequately discussed.

In clinical diagnostic laboratories, identification tests must report a single species to clinicians; thus, the stability of identification accuracy is a critical factor. This study demonstrated that identification stability is influenced not only by the addition or removal of registered strains of the same species but also by the overall composition of registered species within the same genus. Specifically, species identification can be significantly affected in terms of the proportion of first-ranked species with an SV ≥ 2.000, the stability of SVs across the top 10 ranks, and the AUC. In particular, the addition of new species to the database may impact the identification of similar species and inadvertently reduce stability. Therefore, future database revisions should systematically include the following: (1) evaluations of both accuracy and stability of species identification; (2) registration design that considers phylogenetic relatedness and spectral similarity; and (3) assessments of version updates, including retrospective re-analysis. These measures should help ensure more consistent and reliable species identification results for clinical use.

Ethical Guidelines

This study does not require ethical approval as it utilizes bacterial strains cultured from human specimens. The authors have no ethical conflicts to disclose.

Conflict of Interest

The authors have no conflicts of interest to declare.

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