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Phylogenomic analysis of the genus *Rosenbergiella* and description of *Rosenbergiella gaditana* sp. nov., *Rosenbergiella metrosideri* sp. nov., *Rosenbergiella epipactidis* subsp. *epipactidis* subsp. nov., *Rosenbergiella epipactidis* subsp. *californiensis* subsp. nov., *Rosenbergiella epipactidis* subsp. *japonicus* subsp. nov., *Rosenbergiella nectarea* subsp. *nectarea* subsp. nov. and *Rosenbergiella nectarea* subsp. *apis* subsp. nov., isolated from floral nectar and insects

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Abstract

The genus *Rosenbergiella* is one of the most frequent bacterial inhabitants of flowers and a usual member of the insect microbiota worldwide. To date, there is only one publicly available *Rosenbergiella* genome, corresponding to the type strain of *Rosenbergiella nectarea* (8N4^T), which precludes a detailed analysis of intra-genus phylogenetic relationships. In this study, we obtained draft genomes of the type strains of the other *Rosenbergiella* species validly published to date (*R. australiborealis*, *R. collisarenosi* and *R. epipactidis*) and 23 additional isolates of flower and insect origin. Isolate S61^T, retrieved from the nectar of an *Antirrhinum* sp. flower collected in southern Spain, displayed low average nucleotide identity (ANI) and *in silico* DNA–DNA hybridization (isDDH) values when compared with other *Rosenbergiella* members (≤86.5 and ≤29.8%, respectively). Similarly, isolate JB07^T, which was obtained from the floral nectar of *Metrosideros polymorpha* plants in Hawaii (USA) had ≤95.7% ANI and ≤64.1% isDDH with other *Rosenbergiella* isolates. Therefore, our results support the description of two new *Rosenbergiella* species for which we propose the names *Rosenbergiella gaditana* sp. nov. (type strain: S61^T=NCCB 100789^T=DSM 111181^T) and *Rosenbergiella metrosideri* sp. nov. (JB07^T=NCCB 100888^T=LMG 32616^T). Additionally, some *R. epipactidis* and *R. nectarea* isolates showed isDDH values<79% with other conspecific isolates, which suggests that these species include subspecies for which we propose the names *Rosenbergiella epipactidis* subsp. *epipactidis* subsp. nov. (S256^T=CECT 8502^T=LMG 27956^T), *Rosenbergiella epipactidis* subsp. *californiensis* subsp. nov. (FR72^T=NCCB 100898^T=LMG 32786^T), *Rosenbergiella epipactidis* subsp. *japonicus* subsp. nov. (K24^T=NCCB 100924^T=LMG 32785^T), *Rosenbergiella nectarea* subsp. *nectarea* subsp. nov. (8N4^T = DSM 24150^T = LMG 26121^T) and *Rosenbergiella nectarea* subsp. *apis* subsp. nov. (B1A^T=NCCB 100810^T= DSM 111763^T), respectively. Finally, we present the first phylogenomic analysis of the genus *Rosenbergiella* and update the formal description of the species *R. australiborealis*, *R. collisarenosi*, *R. epipactidis* and *R. nectarea* based on new genomic and phenotypic information.

INTRODUCTION

The genus *Rosenbergiella* (order *Enterobacterales*, class *Gammaproteobacteria*) is one of the most frequent bacterial inhabitants of flowers from phylogenetically diverse plants worldwide and it is also frequently isolated from insects and other flower visitors [1–10]. In recent years, there is a growing interest in studying the members of this genus due to their ability to withstand the harsh conditions of floral nectar, which typically include high osmotic pressure, low availability of nitrogen, and presence of toxins of plant origin [2, 11–15]. Furthermore, it has been shown that *Rosenbergiella* species can modify the chemical properties of nectar and, eventually, alter the behaviour of flower visitors, including the natural enemies of some pests [16].

To date, the genus *Rosenbergiella* is classified within the family *Enterobacteriaceae*, even when its closest phylogenetic relatives are the genera *Phaseolibacter* and *Tatumella* [17, 18], both of which belong to the family *Erwiniaceae* since the major taxonomic

revision of the 'Enterobacteriales' carried out by Adeolu *et al.* [19]. The reclassification of *Rosenbergiella* within the family *Erwiniaceae* has been recently proposed based on phylogenomic data [20]. However, a detailed analysis of the phylogenetic relationships between *Rosenbergiella* species is pending, as genomic information is only available for the type species *Rosenbergiella nectarea* [21], and not for the other three current members of the genus, namely *Rosenbergiella australiborealis*, *Rosenbergiella collisarenosi* and *Rosenbergiella epipactidis* (https://lpsn.dsmz.de/genus/rosenbergiella, last accessed on 29 November 2022).

In this study we analysed different *Rosenbergiella* isolates retrieved from floral nectar and insects from four continents. The results of multi-locus sequence analysis and overall genome relatedness indices demonstrated the existence of novel species and subspecies among nectar and insect isolates, for which we propose the names *Rosenbergiella gaditana* sp. nov., *Rosenbergiella metrosideri* sp. nov., *Rosenbergiella epipactidis* subsp. *epipactidis* subsp. nov., *Rosenbergiella epipactidis* subsp. *californiensis* subsp. nov., *Rosenbergiella epipactidis* subsp. *japonicus* subsp. nov., *Rosenbergiella nectarea* subsp. *nectarea* subsp. nov. and *Rosenbergiella nectarea* subsp. *apis* subsp. nov. Furthermore, we performed the first phylogenomic analysis of the genus *Rosenbergiella*. Finally, the formal descriptions of *R. australiborealis*, *R. collisarenosi*, *R. epipactidis* and *R. nectarea* are updated based on new genomic data obtained for the respective type strains and phenotypic information acquired from the study of a broad collection of nectar and insect isolates.

ISOLATION AND ECOLOGY

Forty-six bacterial isolates were analysed in this study (Table 1). Forty-one of these isolates were obtained between 2011 and 2018 from floral nectar samples of different plant species collected in Belgium, France, Japan, South Africa, Spain and the United States. The other five isolates were retrieved from the mouth, gut or crop of honeybees (*Apis mellifera*) and a bumble bee (*Bombus* sp.) sampled on the Stanford University campus (Stanford, CA, USA) in 2018. Bacterial isolates were obtained from floral nectar and bees following the procedures described in Álvarez-Pérez *et al.* [22]. Briefly, nectar samples were diluted in 500 µl of saline solution (0.85% w/v NaCl, Merck Millipore) and a 25 µl aliquot of each was streaked on tryptone soy agar (TSA; Merck Millipore). Immediately after being captured, bees were kept individually in sterile containers and anaesthetized by placing them inside a freezer (at −20 °C) or a polystyrene box with ice for 10 min, after which they were allowed to feed on sterile sugar water (20% w/v sucrose, Merck Millipore) and then dissected to extract their gut. Insect guts were ground inside a microtube containing 1 ml of saline solution using a disposable pellet pestle. Two-microliter aliquots of the remaining sugar water and 10 µl aliquots of homogenized gut samples were streaked on TSA plates. All cultures were incubated at 25 °C for 7 days, and a colony of each phenotypically distinct type was picked and subcultured on TSA to obtain pure cultures. All studied isolates were grown on TSA at 25 °C and stored at −80 °C in brain heart infusion (BHI) broth (Becton Dickinson) containing 25% glycerol (Merck Millipore) until further use.

Studied nectar isolates included the type strains of *R. australiborealis*, *R. collisarenosis* and *R. epipactidis* (S264^T=CdVSA 20.1^T, S260^T=8.8A^T and S256^T=2.1A^T, respectively), for which a genome assembly was not available until this study, and three additional *R. australiborealis* isolates and two *R. nectarea* isolates that had been previously characterized by Lenaerts *et al.* [18]. Sequence analysis of the 16S rRNA gene (≥1436 bp; see amplification and sequencing conditions in Table S1 (available in the online version of this article) obtained for the remaining 38 unclassified isolates showed that these had the highest sequence identity (≥99.5%) with members of genus *Rosenbergiella* (Table S2).

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Abbreviations: ANI, average nucleotide identity; BHI, brain heart infusion; BI, Bayesian inference; CI, confidence interval; GSI, gene support index; isDDH, *in silico* DNA–DNA hybridization; ML, maximum-likelihood; NJ, neighbour-joining; TSA, tryptone soy agar; UBCG2, up-to-date bacterial core genes 2.
Partial 16S rRNA, *atpD*, *gyrB* and *rpoB* gene sequences obtained for the type strains of the new taxa described in this study have been deposited in the GenBank/EMBL/DDBJ data bases under the following accession numbers: MT341811, MT354635, MT354674 and MT354713 for S61^T; MT341875, MT354641, MT354680 and MT354719 for JB07^T; KF876184, KF876195, KF876204 and KF876212 for S256^T; MT341873, MT354639, MT354678 and MT354717 for FR72^T; MT341879, MT354645, MT354684 and MT354723 for K24^T; HQ284827, JN808189, JF745806 and JF745805 for 8N4^T; and MT341812, MT354655, MT354694 and MT354733 for B1A^T. Draft genome assemblies of S61^T, JB07^T, S256^T, FR72^T, K24^T, 8N4^T and B1A^T, have been deposited in the GenBank/ENA/DDBJ databases under the accession numbers GCA_018494065.1, GCA_022602565.1, GCA_018494055.1, GCA_022602615.1, GCA_022602435.1, GCA_900111105.1 and GCA_018494105.1, respectively. Accession numbers for the partial nucleotide sequences and draft genome assemblies obtained for other isolates characterized in this study are indicated in Figures 1 and 2 and Tables S2–S6. Three supplementary figures and nine supplementary tables are available with the online version of this article.

Table 1. Overview of the *Rosenbergiella* isolates analysed in this study

Rosenbergiella **(sub)species**	**Isolate***	**Source**	**Sampling location**	**Year of isolation**	**Isolate donor(s)†**
R. australiborealis	S264^{T}=CdVSA 20.1^{T}= CECT 8500^{T}= LMG 27954$^{T\#}$	Floral nectar of *Protea roupelliae* (Proteaceae)	Mount Gilboa, KwaZulu-Natal, South Africa	2011	CdV, SAP
	S262 = SAP 86.2B$^{\#}$	Floral nectar of *Narcissus papyraceus* (Amaryllidaceae)	Hinojos, Huelva, Spain	2011	CMH, SAP
	S265 = CdVSA 21.1$^{\#}$	Floral nectar of *Protea roupelliae* (Proteaceae)	Mount Gilboa, KwaZulu-Natal, South Africa	2011	CdV, SAP
	S266 = CdVSA 50.1$^{\#}$	Floral nectar of *Protea subvestita* (Proteaceae)	Sani Pass, KwaZulu-Natal, South Africa	2011	CdV, SAP
R. collisarenosi	S260^{T}=8.8A^{T}=CECT 8501^{T}= LMG 27955$^{T\#}$	Floral nectar of *Epipactis palustris* (Orchidaceae)	Ter Yde, Oostduinkerke, West Flanders, Belgium	2012	BL, HJ
	JB25	Floral nectar of *Metrosideros polymorpha* (Myrtaceae)	Hawai'i Volcanoes National Park, Hawaii, USA	2013	RRJ
	S147	Floral nectar of *Phlomis purpurea* (Lamiaceae)	P.N. S^{a} Hornachuelos, Córdoba, Spain	2011	SAP
	S294	Floral nectar of *Buddleja davidii* (Scrophulariaceae)	Mechelen, Antwerp province, Belgium	2017	SAP
	S99 = SAP 817.2B	Floral nectar of *Iris xiphium* (Iridaceae)	Hinojos, Huelva, Spain	2011	SAP
R. epipactidis subsp. *californiensis* subsp. nov.	FR72^{T}=NCCB 100898^{T}=LMG 32786^{T}	Floral nectar of *Diplacus (Mimulus) aurantiacus* (Phrymaceae)	Jasper Ridge Biological Preserve, Stanford, California, USA	2017	KT, TF
	B3-15	Crop of *Apis mellifera*	Stanford campus, California, USA	2018	TF, SAP
	JR114	Floral nectar of *Diplacus (Mimulus) aurantiacus* (Phrymaceae)	Jasper Ridge Biological Preserve, Stanford, California, USA	2018	TF, SAP
	S55	Floral nectar of *Antirrhinum* sp. (Plantaginaceae)	Barbate, Cádiz, Spain	2011	SAP
R. epipactidis subsp. *epipactidis* subsp. nov.	S256^{T}= 2.1A^{T}=CECT 8502^{T}=LMG 27956$^{T\#}$	Floral nectar of *Epipactis palustris* (Orchidaceae)	Dune du Perroquet, Bray-Dunes, France	2012	BL, HJ
	JB02	Floral nectar of *Metrosideros polymorpha* (Myrtaceae)	Hawai'i Volcanoes National Park, Hawaii, USA	2013	RRJ
	JB21	Floral nectar of *Metrosideros polymorpha* (Myrtaceae)	Hawai'i Volcanoes National Park, Hawaii, USA	2013	RRJ
	K1916	Floral nectar of *Diplacus (Mimulus) aurantiacus* (Phrymaceae)	Jasper Ridge Biological Preserve, Stanford, California, USA	2017	KT, TF
	K264	Floral nectar of *Diplacus (Mimulus) aurantiacus* (Phrymaceae)	Jasper Ridge Biological Preserve, Stanford, California, USA	2017	KT, TF
	K265	Floral nectar of *Diplacus (Mimulus) aurantiacus* (Phrymaceae)	Jasper Ridge Biological Preserve, Stanford, California, USA	2017	KT, TF
	K371	Floral nectar of *Diplacus (Mimulus) aurantiacus* (Phrymaceae)	Jasper Ridge Biological Preserve, Stanford, California, USA	2017	KT, TF
	K372	Floral nectar of *Diplacus (Mimulus) aurantiacus* (Phrymaceae)	Jasper Ridge Biological Preserve, Stanford, California, USA	2017	KT, TF

Continued

Table 1. Continued

Rosenbergiella **(sub)species**	**Isolate***	**Source**	**Sampling location**	**Year of isolation**	**Isolate donor(s)†**
	S50	Floral nectar of *Antirrhinum* sp. (Plantaginaceae)	Barbate, Cádiz, Spain	2011	SAP
	S67	Floral nectar of *Antirrhinum* sp. (Plantaginaceae)	Barbate, Cádiz, Spain	2011	SAP
	S68	Floral nectar of *Antirrhinum* sp. (Plantaginaceae)	Barbate, Cádiz, Spain	2011	SAP
	S76	Floral nectar of *Lathyrus* sp. (Fabaceae)	Barbate, Cádiz, Spain	2011	SAP
R. epipactidis subsp. *japonicus* subsp. nov.	K24^T=NCCB 100924^T=LMG 32785^T	Floral nectar of *Eurya japonica* (Pentaphylacaceae)	Takaike Kozagawacho Higashimurogun, Wakayama prefecture, Japan	2016	KT, TF
R. gaditana sp. nov.	S61^T=DSM 111181^T=NCCB 100789^T	Floral nectar of *Antirrhinum* sp. (Plantaginaceae)	Barbate, Cádiz, Spain	2011	SAP
	S284	Floral nectar of *Echium* sp. (Boraginaceae)	Madrid, Spain	2017	SAP
	S290	Floral nectar of *Echium* sp. (Boraginaceae)	Madrid, Spain	2017	SAP
R. metrosideri sp. nov.	JB07^T=NCCB 100888^T=LMG 32616^T	Floral nectar of *Metrosideros polymorpha* (Myrtaceae)	Hawai'i Volcanoes National Park, Hawaii, USA	2013	RRJ
R. nectarea subsp. *apis* subsp. nov.	B1A^T = DSM 111763^T= NCCB 100810^T	Mouth of *Apis mellifera*	Stanford campus, California, USA	2018	TF, SAP
	B3A	Mouth of *Apis mellifera*	Stanford campus, California, USA	2018	TF, SAP
	B4A	Gut of *Bombus* sp.	Stanford campus, California, USA	2018	TF, SAP
	B5A	Mouth of *Apis mellifera*	Stanford campus, California, USA	2018	TF, SAP
	S255=1.12A$^\#$	Floral nectar of *Epipactis palustris* (Orchidaceae)	Dune Dewulf, Ghyvelde, France	2012	BL, HJ
	S258=2.6A$^\#$	Floral nectar of *Epipactis palustris* (Orchidaceae)	Dune du Perroquet, Bray-Dunes, France	2012	BL, HJ
R. nectarea subsp. *nectarea* subsp. nov.	FNA5	Floral nectar of *Diplacus (Mimulus) aurantiacus* (Phrymaceae)	Jasper Ridge Biological Preserve, Stanford, California, USA	2017	KT, TF
	FR67	Floral nectar of *Diplacus (Mimulus) aurantiacus* (Phrymaceae)	Jasper Ridge Biological Preserve, Stanford, California, USA	2017	KT, TF
	K1039	Floral nectar of *Diplacus (Mimulus) aurantiacus* (Phrymaceae)	Jasper Ridge Biological Preserve, Stanford, California, USA	2017	KT, TF
	K353	Floral nectar of *Diplacus (Mimulus) aurantiacus* (Phrymaceae)	Jasper Ridge Biological Preserve, Stanford, California, USA	2017	KT, TF
	M26	Floral nectar of *Symphytum officinale* (Boraginaceae)	Pulderbos, Antwerp province, Belgium	2013	BL, HJ
	S292	Floral nectar of *Symphytum officinale* (Boraginaceae)	Mechelen, Antwerp province, Belgium	2017	SAP

Continued

Table 1. Continued

Rosenbergiella (sub)species	Isolate*	Source	Sampling location	Year of isolation	Isolate donor(s)†
	S321	Floral nectar of *Linaria vulgaris* (Scrophulariaceae)	Sint-Katelijne-Waver, Antwerp province, Belgium	2017	SAP
	S323	Floral nectar of *Linaria vulgaris* (Scrophulariaceae)	Sint-Katelijne-Waver, Antwerp province, Belgium	2017	SAP
	S324	Floral nectar of *Linaria vulgaris* (Scrophulariaceae)	Sint-Katelijne-Waver, Antwerp province, Belgium	2017	SAP
	ST23	Floral nectar of *Linaria vulgaris* (Scrophulariaceae)	Leuven-Heverlee, Flemish Brabant, Belgium	2013	BL, HJ

*T, type strain. A hash symbol (#) after the isolate's name indicates that this was included in previous taxonomic studies [18].
†Isolate donors: BL, Prof. Bart Lievens (KU Leuven, Belgium), CdV, Dr. Clara de Vega (Universidad de Sevilla, Spain); HJ, Prof. Hans Jacquemyn (KU Leuven, Belgium); KT, Dr. Kaoru Tsuji (Kyoto University, Japan); RRJ, Prof. Robert R. Junker (Philipps-University Marburg, Germany); SAP, Dr. Sergio Álvarez-Pérez (Complutense University of Madrid, Spain); TF, Prof. Tadashi Fukami (Stanford University, California, USA).

ATPD, *GYRB* AND *RPOB* GENE PHYLOGENY

Previous reports have confirmed that the phylogenetic analysis of the housekeeping genes coding for the ATP synthase β-chain (*atpD*), the DNA gyrase subunit B (*gyrB*) and the RNA polymerase β-subunit (*rpoB*) provides a better taxonomic resolution for *Rosenbergiella* than the 16S rRNA gene [17, 18]. Accordingly, sequence similarity between the 46 isolates included in this study and the type strains of *Rosenbergiella* species was assessed (see results in Tables S3–S5), and a phylogenetic tree was built from a concatenation of partial sequences of the three protein-encoding genes (*atpD*, *gyrB* and *rpoB*, in this order; 1515 bp in total) obtained as indicated in Table S1. The *atpD*, *gyrB* and *rpoB* sequences available at the GenBank/ENA/DDBJ databases for the *R. nectarea* isolates analysed by Halpern *et al.* [17] (8N4^T, 9N2 and 10N3), the type strain of *Phaseolibacter flectens* (ATCC 12775^T) and the type strain of *Tatumella citrea* (LMG 22049^T, used as outgroup) were also included in the phylogenetic analyses. Nucleotide sequences were aligned using MUSCLE [23], and the resulting alignments were trimmed with BioEdit version 7.0.9.0 [24] to ensure that all sequences had the same start and endpoint. A maximum-likelihood (ML) tree was then obtained using PhyML version 3.0 with smart model selection [25, 26] under a general time reversible substitution model with gamma distributed rate variation among sites and a proportion of invariable sites (GTR+G+I; gamma shape parameter=0.535 and p-invariant sites=0.480), with four substitution rate categories, starting trees generated by BioNJ, and the nearest-neighbour interchange tree search algorithm. Alternative trees were built using the neighbour-joining (NJ) algorithm and Bayesian inference (BI) (Figures S1 and S2, respectively). All phylogenetic trees were visualized and edited with MEGA X [27].

Most studied isolates formed well-supported clades in the ML tree (>90% bootstrap support) with the type strains of previously validated *Rosenbergiella* species (Fig. 1). However, there were three nectar isolates (S61^T, S284 and S90) forming a clade clearly clustering apart from any type strain, and which, therefore, may represent a new species of *Rosenbergiella*. The phylogenetic position of this clade was uncertain, as it branched with *R. australiborealis* and *R. collisarenosi* in the ML tree (Fig. 1), with *R. nectarea* and *R. epipactidis* in the NJ tree (Fig. S1), and independently in the BI tree (Fig. S2). Furthermore, isolate JB07^T, which was identified as a second potentially new *Rosenbergiella* species in (phylo)genomic analyses (see below), clustered with *R. epipactidis* isolates in all trees built from *atpD+gyrB+rpoB* sequences (Figs. 1, S1 and S2, available in the online Supplementary Material). Nevertheless, as the *R. epipactidis* clade included several subclades whose relative position and node support varied from tree to tree (compare Figs. 1, S1 and S2), the closest phylogenetic relatives to JB07^T could not be determined. Therefore, it was concluded that housekeeping gene analysis might not be enough for resolving some *Rosenbergiella* species complexes. Finally, a group of six isolates from floral nectar and insects (B1A^T, B3A, B4A, B5A, S255=1.12A and S258=2.6A) was closely related to the clade including *R. nectarea* 8N4^T, but clustered apart from it (82% bootstrap support in the ML tree (Fig. 1), 98% bootstrap support in the NJ tree (Fig. S1) and clade credibility value of 100% in the BI tree (Fig. S2)), suggesting that it may represent a third new *Rosenbergiella* species or a subspecies of *R. nectarea*.

GENOME FEATURES

The type strains of *R. australiborealis* (S264^T), *R. collisarenosi* (S260^T) and *R. epipactidis* (S256^T), and 23 additional isolates selected as representative isolates of the different clades identified in the different phylogenetic trees built from a concatenation of housekeeping gene sequences (see above), were used for detailed analysis of genome features. DNA extraction from these 26 isolates was performed using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific), following the manufacturer's instructions. Total genomic DNA was sequenced using a MiSeq platform (Illumina) in paired-end mode. Raw reads were adapter

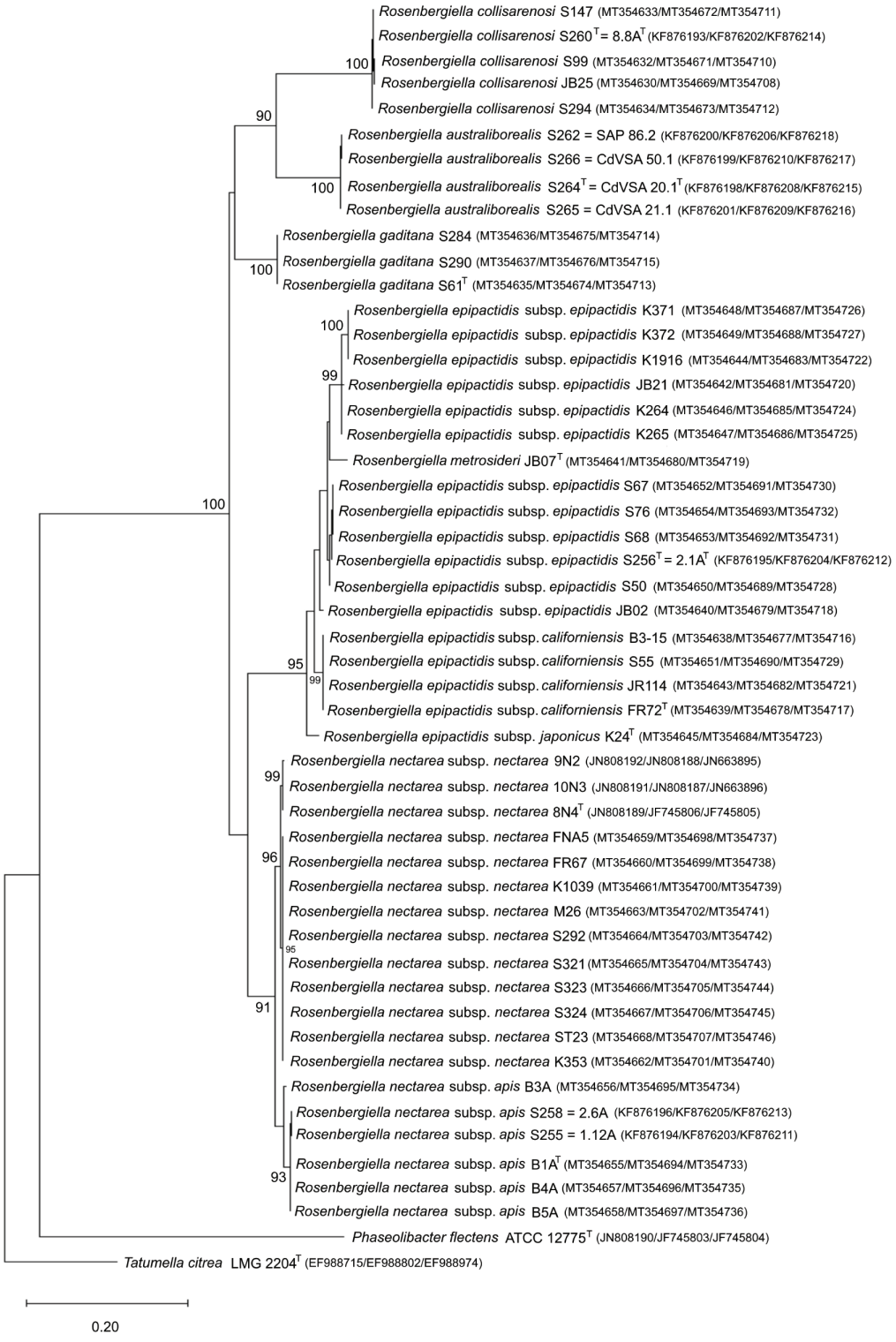

Fig. 1. Maximum-likelihood consensus tree, based on a concatenation of *atpD*, *gyrB* and *rpoB* gene sequences (1515 bp in total), showing the relationships of the *Rosenbergiella* isolates included in this study with other members of genus *Rosenbergiella* and *Phaseolibacter flectens* ATCC 12775^T and *Tatumella citrea* LMG 22049^T (outgroup). The bar at the bottom of the tree indicates a length corresponding to 0.2 nucleotide substitutions per site. Node support values (bootstrap percentages, based on 1000 replicates) ≥90% are shown next to the corresponding nodes. GenBank/ENA/DDBJ accession numbers are indicated between parentheses (*atpD*/*gyrB*/*rpoB*).

trimmed using the BBTools package BBDuk (https://sourceforge.net/projects/bbmap/, last accessed on 10 June 2022), and *de novo* assembly was performed using Spades version 3.9.0 [28]. Sequencing of the genomic DNA obtained from target isolates yielded between 2666284 and 4961112 reads, with the average read length ≥126.22 bp in all cases. After trimming and quality filtering, reads were assembled into draft genomes with an average coverage ranging from 97.6× to 195.9×, as determined using SAMtools version 1.10 [29] in combination with Qualimap version 2.2.1 [30] for report generation. Sequence length of the shortest contig at 50% of the total genome length (*N50*), which was determined using QUAST version 5.0.2 [31], ranged from 108.0 to 510.1 kb (Table S6). All genomes were estimated to be 100% complete by CheckM version 1.1.3, using the taxonomic-specific workflow and selecting the *Gammaproteobacteria* marker set [32]. These quality values agree with the current minimal standards for the use of genome data in bacterial taxonomy [33]. Additionally, the 16S rRNA, *atpD*, *gyrB* and *rpoB* sequences extracted from the annotated genome assemblies (see below) displayed ≥99.5% sequence similarity to the sequences obtained for the same gene markers by Sanger sequencing (Table S7), thus validating the authenticity of the *Rosenbergiella* draft genome sequences determined in this study. The genome sequence of *R. nectarea* 8N4^T (accession no. GCA_900111105.1) was downloaded from the NCBI database using genomepy version 0.7.2 [34] and incorporated in subsequent analyses.

Genome sizes of the studied isolates was found to vary between 2.98 Mbp (*R. australiborealis* S264^T) and 3.58 Mbp (*R. collisarenosi* isolate JB25), and the G+C content ranged from 45.3mol% (*R. australiborealis* isolate S262) to 48.4mol% (*R. collisarenosi* isolates JB25 and S99), as determined by QUAST (Table S6). These values are similar to the genome size and G+C content of *R. nectarea* 8N4^T determined in a previous study (3.29 Mbp and 47.4mol%, respectively) [21]. Annotation of *Rosenbergiella* genomes with Prokka version 1.11 [35] predicted between 2850 and 3308 coding regions (CDS) per genome (Table S6).

Pairwise average nucleotide identity (ANI) values between *Rosenbergiella* genomes were determined using Pyani version 0.2.11 [36], and *in silico* DNA–DNA hybridization (isDDH) was carried out using the Genome-to-Genome Distance Calculator version 3.0 (http://ggdc.dsmz.de; last accessed on 10 June 2022) [37]. Most studied isolates showed ANI and isDDH estimates above the current generally accepted thresholds for species delimitation (95–96 and 70 %, respectively [33, 38]) with only one of the type strains of *Rosenbergiella* species validly published to date, but below such threshold when compared with the type strains of other species (Tables 2 and 3). In this way, isolates S262 and S265 were identified as members of *R. australiborealis* (ANI ≥99.4% and isDDH ≥95.5% with the type strain S264^T), isolates JB25, S99 and S294 as members of *R. collisarenosi* (ANI ≥99.2% and isDDH ≥93.2% with the type strain S264^T), isolates FR72^T, JB02, JB21, K24^T, K265, K371, K372, S50 and S68 as members of *R. epipactidis* (ANI ≥97.0% and isDDH between 71.6 and 97.1% with the type strain S256^T), and isolates B1A^T, B3A, B5A, FNA5, FR67, S255 and S258 as members of *R. nectarea* (ANI ≥96.5 % with the type strain 8N4^T; isDDH estimates with respect to 8N4^T ranged from 69.7 to 89.8 %, but the respective model-based confidence intervals (CIs) included in all cases values >70%). Isolate JB07^T showed 95.3% ANI with *R. epipactidis* S256^T, and 94.9–95.7 % ANI with the other nine isolates identified as members of *R. epipactidis*. However, pairwise isDDH estimates between JB07^T and *R. epipactidis* isolates ranged from 57 to 64.1% (61.4% with respect to S256^T), with all model-based confidence intervals falling below the threshold for species delineation (i.e., 70%). Similarly, isolate S61^T displayed low ANI and isDDH values with all other studied isolates (≤86.5% and ≤29.8%, respectively). Therefore, it was concluded that isolates S61^T and JB07^T constitute novel species of *Rosenbergiella*, for which we propose the names *Rosenbergiella gaditana* sp. nov. and *Rosenbergiella metrosideri* sp. nov.

On the other hand, the broad ranges of isDDH values observed between isolates identified as *R. epipactidis* or *R. nectarea* with respect to their respective type strains (see above) suggested that these two species might contain different subspecies. In this regard, Meier-Kolthoff *et al.* [37] proposed to set the threshold for subspecies delineation in bacterial taxonomy at 79–80% isDDH. Considering the 79% cut-off value for subspecies delineation on the basis of isDDH estimates, two clearly different groups of isolates were identified for *R. nectarea* (Table 3): 1) a first group (RN1) that included the type strain of *R. nectarea* (8N4^T) and isolates FNA5 and FR67, all of which were of nectar origin and had pairwise isDDH values between 89.7 and 100%; and 2) a second group (RN2) that included three isolates from honeybees (B1A^T, B3A and B5A) and two isolates from floral nectar (S255 and S258), which displayed pairwise isDDH values between 84.6 and 100%. All pairwise isDDH values between members of the RN1 and RN2 groups were ≤72.8%. Therefore, these two groups of *R. nectarea* isolates seem to represent different subspecies, for which we propose the names *Rosenbergiella nectarea* subsp. *nectarea* subsp. nov. (RN1 group, with 8N4^T as the type strain) and *Rosenbergiella nectarea* subsp. *apis* subsp. nov. (RN2 group, with B1A^T as the type strain).

Different groups of isolates based on isDDH values could also be identified for *R. epipactidis*, albeit some of such groups had fuzzy boundaries (Table 3): 1) a first group (RE1) included the type strain of the species (S256^T) and isolates JB21, K265, K371, K372, S50 and S68, all of which were of nectar origin and had pairwise isDDH estimates between 77.3 and 100%, with the respective model-based CI including in all cases values>79%; 2) a second group (RE2) included only isolate FR72^T, obtained from the floral nectar of *Diplacus (Mimulus) aurantiacus* collected in California, USA; and 3) a third group (RE3) included only isolate K24^T, obtained from the floral nectar of *Eurya japonica* collected in Japan. The affiliation of isolate JB02, obtained from the floral nectar of *Metrosideros polymorpha* collected in Hawaii (USA), was less clear, as this isolate showed isDDH estimates of 76.2–77.7% (IC included in most cases values>79%; the only exception was the comparison between JB02 and JB21, for which the IC of the isDDH estimate was 73.1–78.9%), 69.8% (IC=66.8–72.7%) and 70.9% (IC=67.9–73.8%) with the members of the groups RE1, RE2 and RE3, respectively; nevertheless, isolate JB02 was tentatively classified within group RE1 based on the

Table 2. Pairwise average nucleotide identity (ANI) values for the *Rosenbergiella* isolates analysed in this study and the type strain of *Rosenbergiella nectarea* (8N4^T)*

	R. australiborealis			*R. collisarenosi*				*R. epipactidis*										*R. gaditana* S61^T	*R. metrosideri* JB07^T	*R. nectarea*							
								subsp. *californiensis*	subsp. *epipactidis*								subsp. *japonicus*			subsp. *nectarea*				subsp. *apis*			
	S262	S264^T	S265	JB25	S260^T	S294	S99	FR72^T	JB02	JB21	K265	K371	K372	S256^T	S50	S68	K24^T			8 N4^T	FNA5	FR67	B1A^T	B3A	B5A	S255	S258
S262	100	99.4	99.4	84.1	84.3	84.1	84.2	83.6	83.7	83.7	83.6	83.6	83.6	83.8	83.5	83.8	83.7	83.5	83.7	83.5	83.5	83.5	83.6	83.5	83.6	83.5	84.7
S264^T	99.4	100	99.5	84.1	84.0	84.0	84.1	83.5	83.6	83.7	83.5	83.5	83.5	83.5	83.5	83.5	83.5	83.4	83.4	83.3	83.4	83.4	83.5	83.3	83.5	83.4	84.4
S265	99.4	99.5	100	84.2	84.1	84.1	84.2	83.6	83.8	83.7	83.6	83.5	83.5	83.8	83.7	83.8	83.9	83.4	83.6	83.7	83.6	83.6	83.6	83.6	83.6	83.6	84.6
JB25	84.1	84.1	84.2	100	99.2	99.3	99.2	84.4	84.7	84.9	85.0	84.9	84.9	84.6	84.9	84.6	84.9	83.7	86.1	84.4	84.5	84.5	84.3	84.3	84.4	84.5	84.5
S260^T	84.3	84.0	84.1	99.2	100	99.7	99.6	83.7	83.8	83.8	83.8	83.9	83.9	84.0	83.7	83.7	84.0	83.7	83.6	84.4	84.5	84.5	84.5	84.7	84.5	84.7	84.6
S294	84.1	84.0	84.1	99.3	99.7	100	99.7	83.6	83.7	83.8	83.9	84.3	84.3	83.7	83.8	83.7	83.8	83.5	83.6	84.6	84.5	84.6	84.2	84.4	84.3	84.2	84.2
S99	84.2	84.1	84.2	99.2	99.6	99.7	100	83.8	83.8	83.8	83.8	83.8	83.8	83.8	83.8	83.9	84.1	83.6	83.7	84.7	84.8	84.8	84.5	84.6	84.5	84.4	84.4
FR72^T	83.6	83.5	83.6	84.4	83.7	83.6	83.8	100	96.7	96.7	96.9	96.8	96.8	97.0	96.9	97.0	96.5	85.0	95.3	87.7	87.7	87.7	87.5	87.6	87.5	87.6	87.6
JB02	83.7	83.6	83.8	84.7	83.8	83.7	83.8	96.7	100	97.3	97.4	97.4	97.4	97.6	97.6	97.6	96.8	85.1	95.1	87.7	87.8	87.8	87.7	87.8	87.7	87.7	87.7
JB21	83.7	83.7	83.7	84.9	83.8	83.8	83.8	96.7	97.3	100	99.6	99.6	99.6	97.6	97.8	97.6	96.8	85.0	95.7	87.8	87.8	87.8	87.7	87.7	87.7	87.7	87.7
K265	83.6	83.5	83.6	85.0	83.8	83.9	83.8	96.9	97.4	99.6	100	99.6	99.6	97.7	97.8	97.6	96.8	85.0	95.6	87.8	87.8	87.8	87.8	87.8	87.8	87.8	87.8
K371	83.6	83.5	83.5	84.9	83.9	84.3	83.8	96.8	97.4	99.6	99.6	100	100	97.6	97.8	97.6	96.9	85.0	95.6	87.9	87.8	87.8	87.8	87.8	87.8	87.8	87.8
K372	83.6	83.5	83.5	84.9	83.9	84.3	83.8	96.8	97.4	99.6	99.6	100	100	97.7	97.8	97.6	96.9	85.0	95.6	87.9	87.8	87.8	87.8	87.8	87.8	87.8	87.8
S256^T	83.8	83.5	83.8	84.6	84.0	83.7	83.8	97.0	97.6	97.6	97.7	97.6	97.7	100	97.9	99.7	97.1	85.1	95.3	88.1	88.1	88.1	87.8	87.9	87.8	87.8	87.8
S50	83.5	83.5	83.7	84.9	83.7	83.8	83.8	96.9	97.6	97.8	97.8	97.8	97.8	97.9	100	97.9	97.0	85.0	95.6	87.8	87.8	87.8	87.7	87.8	87.7	87.8	87.8
S68	83.8	83.5	83.8	84.6	83.7	83.7	83.9	97.0	97.6	97.6	97.6	97.6	97.6	99.7	97.9	100	97.0	85.2	95.3	88.1	88.1	88.1	87.8	87.9	87.8	87.8	87.8
K24^T	83.7	83.5	83.9	84.9	84.0	83.8	84.1	96.5	96.8	96.8	96.8	96.9	96.9	97.1	97.0	97.0	100	85.1	94.9	87.8	87.7	87.7	87.6	87.7	87.6	87.7	87.7
S61^T	83.5	83.4	83.4	83.7	83.7	83.5	83.6	85.0	85.1	85.0	85.0	85.0	85.0	85.1	85.0	85.2	85.1	100	84.9	86.5	86.5	86.5	86.3	86.4	86.3	86.4	86.4
JB07^T	83.7	83.4	83.6	86.1	83.6	83.6	83.7	95.3	95.1	95.7	95.6	95.6	95.6	95.3	95.6	95.3	94.9	84.9	100	87.4	87.4	87.4	87.3	87.4	87.3	87.3	87.3
8 N4^T	83.5	83.3	83.7	84.4	84.4	84.6	84.7	87.7	87.7	87.8	87.8	87.9	87.9	88.1	87.8	88.1	87.8	86.5	87.4	100	98.9	98.9	96.6	96.7	96.6	96.5	96.5
B1A^T	83.6	83.5	83.6	84.3	84.5	84.2	84.5	87.5	87.7	87.7	87.8	87.8	87.8	87.8	87.7	87.8	87.6	86.3	87.3	96.6	96.8	96.8	100	98.5	100	99.8	99.8
B3A	83.5	83.3	83.6	84.3	84.7	84.4	84.6	87.6	87.8	87.7	87.8	87.8	87.8	87.9	87.8	87.9	87.7	86.4	87.4	96.7	96.9	96.9	98.5	100	98.5	98.4	98.4
B5A	83.6	83.5	83.6	84.4	84.5	84.3	84.5	87.5	87.7	87.7	87.8	87.8	87.8	87.8	87.7	87.8	87.6	86.3	87.3	96.6	96.8	96.8	100	98.5	100	99.8	99.8
FNA5	83.5	83.4	83.6	84.5	84.5	84.5	84.8	87.7	87.8	87.8	87.8	87.8	87.8	88.1	87.8	88.1	87.7	86.5	87.4	98.9	100	100	96.8	96.9	96.8	96.7	96.7
FR67	83.5	83.4	83.6	84.5	84.5	84.6	84.8	87.7	87.8	87.8	87.8	87.8	87.8	88.1	87.8	88.1	87.7	86.5	87.4	98.9	100	100	96.8	96.9	96.8	96.7	96.7
S255	83.5	83.4	83.6	84.5	84.7	84.2	84.4	87.6	87.7	87.7	87.8	87.8	87.8	87.8	87.8	87.8	87.7	86.4	87.3	96.5	96.7	96.7	99.8	98.4	99.8	100	100
S258	84.7	84.4	84.6	84.5	84.6	84.2	84.4	87.6	87.7	87.7	87.8	87.8	87.8	87.8	87.8	87.8	87.7	86.4	87.3	96.5	96.7	96.7	99.8	98.4	99.8	100	100

*ANI values are expressed as percentages. ANI values greater than 96% (highlighted in grey) denote that the compared isolates belong to the same species. The (sub)species affiliation of tested isolates is shown in columns.

Table 3. Pairwise *in silico* DNA–DNA hybridization (isDDH) values for the *Rosenbergiella* isolates analysed in this study and the type strain of *Rosenbergiella nectarea* (8N4^T)*

	R. australiborealis			*R. collisarenosi*				*R. epipactidis*										*R. gaditana* S61^T	*R. metrosideri* JB07^T	*R. nectarea*							
								subsp. *californiensis*	subsp. *epipactidis*								subsp. *japonicus*			subsp. *nectarea*			subsp. *apis*				
	S262	S264^T	S265	JB25	S260^T	S294	S99	FR72^T	JB02	JB21	K265	K371	K372	S256^T	S50	S68	K24^T			8 N4^T	FNA5	FR67	B1A^T	B3A	B5A	S255	S258
S262	**100**	**95.5**	**95.0**	20.5	20.5	20.5	20.5	20.5	20.5	20.6	20.4	20.4	20.4	20.5	20.4	20.5	20.6	21.1	20.5	20.6	20.6	20.5	20.6	20.6	20.7	20.6	21.6
S264^T	**95.5**	**100**	**96.2**	20.4	20.3	20.4	20.4	20.3	20.3	20.4	20.2	20.2	20.2	20.3	20.3	20.3	20.3	21.0	20.2	20.4	20.4	20.4	20.5	20.4	20.6	20.4	21.4
S265	**95.0**	**96.2**	**100**	20.5	20.3	20.4	20.5	20.4	20.6	20.5	20.3	20.3	20.3	20.4	20.4	20.5	20.7	21.1	20.4	20.7	20.7	20.6	20.6	20.7	20.6	20.6	21.5
JB25	20.5	20.4	20.5	**100**	**93.2**	**93.8**	**92.8**	21.9	22.1	22.3	22.4	22.3	22.3	22.0	22.2	21.9	22.1	21.6	23.2	21.7	21.8	21.8	21.5	21.6	21.6	21.7	21.7
S260^T	20.5	20.3	20.3	**93.2**	**100**	**98.0**	**96.7**	20.9	21.1	21.1	21.1	21.1	21.1	21.2	21.0	21.1	21.1	21.5	20.8	21.5	21.5	21.5	21.5	21.7	21.6	21.8	21.7
S294	20.5	20.4	20.4	**93.8**	**98.0**	**100**	**97.1**	21.0	21.0	21.1	21.2	21.5	21.5	21.1	21.0	21.1	21.0	21.4	20.8	21.7	21.6	21.6	21.4	21.5	21.5	21.5	21.5
S99	20.5	20.4	20.5	**92.8**	**96.7**	**97.1**	**100**	21.1	21.3	21.1	21.1	21.2	21.2	21.1	21.2	21.3	21.3	21.6	20.9	21.8	21.8	21.8	21.6	21.7	21.7	21.7	21.7
FR72^T	20.5	20.3	20.4	21.9	20.9	21.0	21.1	**100**	**69.8$^\#$**	**69.9$^\#$**	**70.8$^\#$**	**70.7$^\#$**	**70.7$^\#$**	**71.6$^\#$**	**70.6$^\#$**	**71.2$^\#$**	**68.3$^\#$**	26.5	60.7	32.1	32.1	32.0	31.7	32.1	31.8	31.8	31.8
JB02	20.5	20.3	20.6	22.1	21.1	21.0	21.3	**69.8$^\#$**	**100**	**76.2$^\#$**	**76.8**	**76.5**	**76.6**	**77.7**	**76.9**	**77.4**	**70.9$^\#$**	26.8	59.6	31.9	32.3	32.1	31.8	32.1	32.0	31.9	32.0
JB21	20.6	20.4	20.5	22.3	21.1	21.1	21.1	**69.9$^\#$**	**76.2$^\#$**	**100**	**97.0**	**96.7**	**96.6**	**77.4**	**79.5**	**77.3**	**70.6$^\#$**	26.6	63.7	32.2	32.4	32.3	32.0	32.2	32.2	32.0	32.1
K265	20.4	20.2	20.3	22.4	21.1	21.2	21.1	**70.8$^\#$**	**76.8**	**97.0**	**100**	**96.8**	**96.7**	**78.0**	**79.4**	**77.4**	**71.0$^\#$**	26.6	63.5	32.4	32.4	32.4	32.3	32.3	32.3	32.3	32.3
K371	20.4	20.2	20.3	22.3	21.1	21.5	21.2	**70.7$^\#$**	**76.5**	**96.7**	**96.8**	**100**	**99.9**	**77.7**	**79.4**	**77.3**	**71.3$^\#$**	26.6	64.1	32.5	32.5	32.4	32.2	32.3	32.3	32.3	32.3
K372	20.4	20.2	20.3	22.3	21.1	21.5	21.2	**70.7$^\#$**	**76.6**	**96.6**	**96.7**	**99.9**	**100**	**77.8**	**79.3**	**77.3**	**71.2$^\#$**	26.6	64.1	32.4	32.5	32.3	32.1	32.2	32.3	32.2	32.2
S256^T	20.5	20.3	20.4	22.0	21.2	21.1	21.1	**71.6$^\#$**	**77.7**	**77.4**	**78.0**	**77.7**	**77.8**	**100**	**79.6**	**97.1**	**72.8$^\#$**	26.7	61.4	32.9	32.9	32.9	32.3	32.5	32.4	32.4	32.4
S50	20.4	20.3	20.4	22.2	21.0	21.0	21.2	**70.6$^\#$**	**76.9**	**79.5**	**79.4**	**79.4**	**79.3**	**79.6**	**100**	**79.5**	**70.7$^\#$**	26.7	64.0	32.2	32.3	32.2	32.0	32.1	32.1	32.1	32.1
S68	20.5	20.3	20.5	21.9	21.1	21.1	21.3	**71.2$^\#$**	**77.4**	**77.3**	**77.4**	**77.3**	**77.3**	**97.1**	**79.5**	**100**	**72.2$^\#$**	26.8	60.7	33.0	33.0	32.9	32.3	32.4	32.4	32.4	32.4
K24^T	20.6	20.3	20.7	22.1	21.1	21.0	21.3	**68.3$^\#$**	**70.9$^\#$**	**70.6$^\#$**	**71.0$^\#$**	**71.3$^\#$**	**71.2$^\#$**	**72.8$^\#$**	**70.7$^\#$**	**72.2$^\#$**	**100**	26.7	57.0	32.4	32.4	32.3	32.1	32.4	32.3	32.2	32.3
S61^T	21.1	21.0	21.1	21.6	21.5	21.4	21.6	26.5	26.8	26.6	26.6	26.6	26.6	26.7	26.7	26.8	26.7	**100**	26.1	29.8	29.8	29.8	29.4	29.4	29.4	29.5	29.5
JB07^T	20.5	20.2	20.4	23.2	20.8	20.8	20.9	60.7	59.6	63.7	63.5	64.1	64.1	61.4	64.0	60.7	57.0	26.1	**100**	31.3	31.4	31.4	31.2	31.4	31.4	31.2	31.2
8 N4^T	20.6	20.4	20.7	21.7	21.5	21.7	21.8	32.1	31.9	32.2	32.4	32.5	32.4	32.9	32.2	33.0	32.4	29.8	31.3	**100**	**89.7**	**89.8**	**70.0**	**71.3**	**70.0**	**69.7**	**69.7**
B1A	20.6	20.5	20.6	21.5	21.5	21.4	21.6	31.7	31.8	32.0	32.3	32.2	32.1	32.3	32.0	32.3	32.1	29.4	31.2	**70.0**	**71.4**	**71.5**	**100**	**85.9**	**99.9**	**98.3**	**98.3**
B3A	20.6	20.4	20.7	21.6	21.7	21.5	21.7	32.1	32.1	32.2	32.3	32.3	32.2	32.5	32.1	32.4	32.4	29.4	31.4	**71.3**	**72.8**	**72.8**	**85.9**	**100**	**86.0**	**84.6**	**84.7**
B5A	20.7	20.6	20.6	21.6	21.6	21.5	21.7	31.8	32.0	32.2	32.3	32.3	32.3	32.4	32.1	32.4	32.3	29.4	31.4	**70.0**	**71.5**	**71.5**	**99.9**	**86.0**	**100**	**98.3**	**98.3**
FNA5	20.6	20.4	20.7	21.8	21.5	21.6	21.8	32.1	32.3	32.4	32.4	32.5	32.5	32.9	32.3	33.0	32.4	29.8	31.4	**89.7**	**100**	**100**	**71.4**	**72.8**	**71.5**	**71.2**	**71.2**
FR67	20.5	20.4	20.6	21.8	21.5	21.6	21.8	32.0	32.1	32.3	32.4	32.4	32.3	32.9	32.2	32.9	32.3	29.8	31.4	**89.8**	**100**	**100**	**71.5**	**72.8**	**71.5**	**71.3**	**71.2**
S255	20.6	20.4	20.6	21.7	21.8	21.5	21.7	31.8	31.9	32.0	32.3	32.3	32.2	32.4	32.1	32.4	32.2	29.5	31.2	**69.7**	**71.2**	**71.3**	**98.3**	**84.6**	**98.3**	**100**	**99.9**
S258	21.6	21.4	21.5	21.7	21.7	21.5	21.7	31.8	32.0	32.1	32.3	32.3	32.2	32.4	32.1	32.4	32.3	29.5	31.2	**69.7**	**71.2**	**71.2**	**98.3**	**84.7**	**98.3**	**99.9**	**100**

*isDDH values are expressed as percentages. Values greater than 70% denote that the compared isolates belong to the same species (these are highlighted in grey; values in bold type indicate that the 95% confidence interval for the isDDH estimate includes values ≥70%. Values marked with a hash symbol (#) denote that the confidence interval for the isDDH falls entirely below 79%, which is the cut-off for subspecies delineation [37]. The (sub)species affiliation of tested isolates is shown in columns.

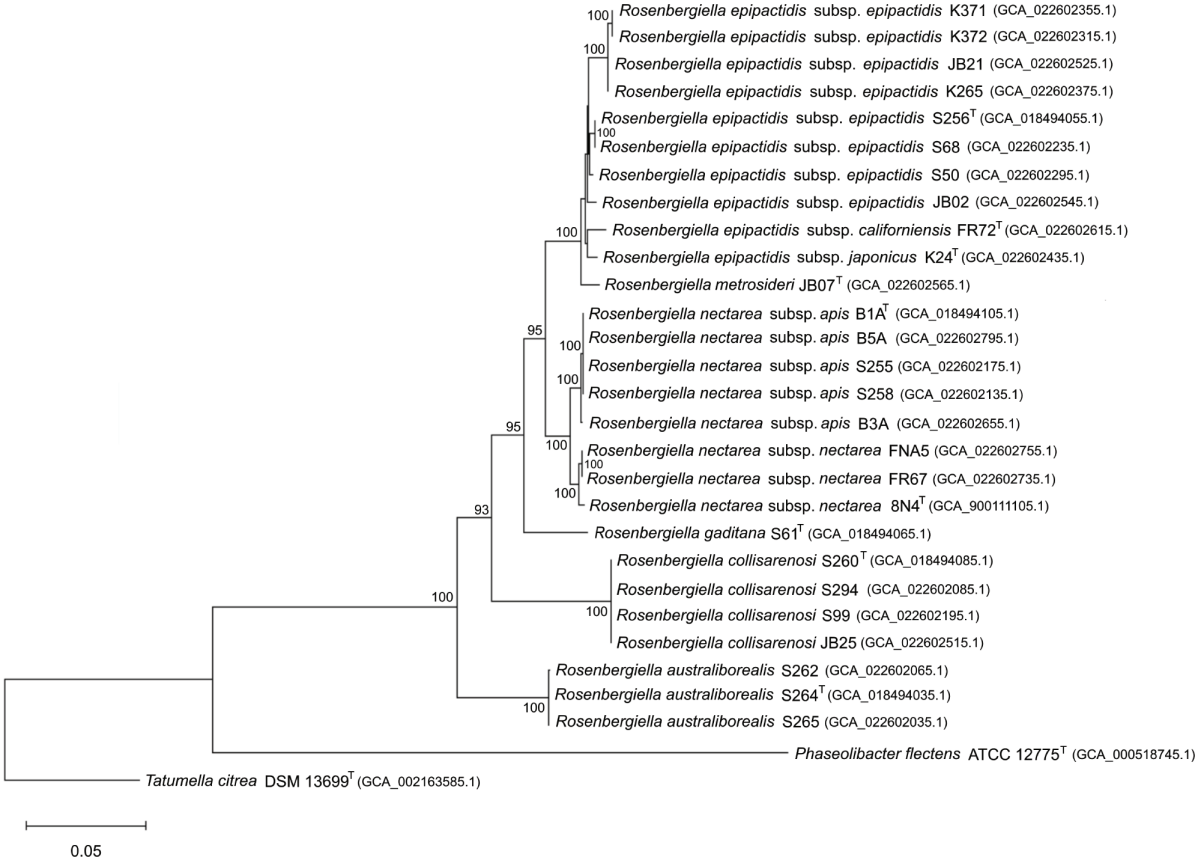

Fig. 2. Phylogenomic tree of the genus *Rosenbergiella*, the closely related species *Phaseolibacter flectens* and the outgroup species *Tatumella citrea*, generated using an alignment of 49 core gene sequences (Table S8). Evolutionary distances are in the units of number of base substitutions per site. Bootstrap support values (based on 1000 replicates) ≥90% are shown next to the corresponding nodes. GenBank/ENA/DDBJ accession numbers for the genomes used in this analysis are indicated between parentheses.

closer isDDH estimates with the members of that group. The pairwise isDDH estimates between members of the RE1, RE2 and RE3 groups ranged between and 68.3 and 72.8%, with all CI intervals falling entirely below the 79% cut-off set for subspecies delineation based on isDDH values. Therefore, it was concluded that these three groups of *R. epipactidis* isolates might represent different subspecies, for which we propose the names *Rosenbergiella epipactidis* subsp. *epipactidis* subsp. nov. (RE1 group, with S256^T as the type strain), *Rosenbergiella epipactidis* subsp. *californiensis* subsp. nov. (RE2 group, with FR72^T as the type strain) and *Rosenbergiella epipactidis* subsp. *japonicus* subsp. nov. (RE3 group, with K24^T as the type strain).

PHYLOGENOMIC ANALYSIS

Analysis of the core genome and pangenome of the type strains and representative isolates of all *Rosenbergiella* species validly described so far, the new *Rosenbergiella* species and subspecies identified in this study (*R. gaditana* sp. nov., *R. metrosideri* sp. nov., *R. epipactidis* subsp. *epipactidis* subsp. nov., *R. epipactidis* subsp. *californiensis* subsp. nov., *R. epipactidis* subsp. *japonicus* subsp. nov., *R. nectarea* subsp. *nectarea* subsp. nov. and *R. nectarea* subsp. *apis* subsp. nov.), and the type strains of *P. flectens* and *T. citrea* (accession nos. GCA_000518745.1 and GCA_002163585.1, respectively) was performed with Roary version 3.13.0 [39], using the annotated assemblies in GFF3 format produced by Prokka as input files. A minimum of 95% of identity for BLASTp searching was set for this analysis, and we considered as core genome those genes present in 99% of the studied genomes. The alignment of the 49 core gene sequences yielded by Roary (Table S8) was then used to generate an ML tree using IQ-TREE version 1.6.12 [40], with 1000 ultrafast bootstrap replicates [41] and 1000 replicates for the Shimodaira–Hasegawa (SH)-like approximate likelihood ratio test. ModelFinder [42], as implemented in IQ-TREE, was used to find the best-fit nucleotide substitution model based on the Bayesian information criterion and resulted in the selection of a general time reversible model with empirical base frequencies, a proportion of invariable sites, and gamma distributed rates across sites (GTR+F+I+G4; gamma shape parameter=0.580, p-invariant sites=0.591).

Table 4. Metabolic and physiological characteristics of the Rosenbergiella *gaditana* sp. nov., Rosenbergiella *metrosideri* sp. nov., *Rosenbergiella epipactidis* subsp. *californiensis* subsp. nov., *Rosenbergiella epipactidis* subsp. *epipactidis* subsp. nov., *Rosenbergiella epipactidis* subsp. *japonicus* subsp. nov., *Rosenbergiella nectarea* subsp. *nectarea* subsp. nov. and *Rosenbergiella nectarea* subsp. *apis* subsp. nov. in comparison with other *Rosenbergiella* species and *Phaseolibacter flectens*

All isolates were positive for motility, catalase production, growth in microaerobiosis and anaerobiosis, growth in MacConkey agar, and fermentation of glucose. All isolates tested negative for oxidase, haemolysis, gelatin liquefaction, production of DNase, indole, H_2S, ornithine decarboxylase and urease, and fermentation of adonitol, mannitol, raffinose, rhamnose and sorbitol. Results for the different phenotypes can be positive (+), weakly positive (w), negative (–), or not determined (ND). When different possibilities are reported, the number of isolates displaying each result is shown in parentheses and the result obtained for the type strain is indicated by an asterisk. Detailed results are presented in Table S7.

Characteristic	*R. gaditana* sp. nov. (*n*=3)	*R. metrosideri* sp. nov. (*n*=1)	*R. australiborealis* (*n*=4)	*R. collisarenosi* (*n*=5)	*R. epipactidis* subsp. *epipactidis* subsp. nov. (*n*=12)	*R. epipactidis* subsp. *californiensis* subsp. nov. (*n*=4)	*R. epipactidis* subsp. *japonicus* subsp. nov. (*n*=1)	*R. nectarea* subsp. *nectarea* subsp. nov. (*n*=10)	*R. nectarea* subsp. *nectarea* subsp. nov. (*n*=3)†	*R. nectarea* subsp. *apis* subsp. nov. (*n*=6)	*Phaseolibacter flectens* (*n*=2)†
Growth at:											
4 °C	– (2*)/w (1)	w	w	– (1)/w (4*)	– (1)/w (11*)	w	w	– (3)/w (7)	+	w	+
12 °C	w	w	w	w	w	w	w	w	ND	w	ND
25 °C	+	+	+	+	+	+	+	+	+	+	+
30 °C	+	+	+	+	+	+	+	+	+	+	+
37 °C	w	+	+	w	w (6*)/+ (6)	w (3*)/+ (1)	w	w	–	w (4*)/+ (2)	+
41 °C	–	w	w	– (1)/w (4*)	– (4)/w (8*)	w	w	– (3)/w (7)	–	w	+
NaCl tolerance:											
0 % (w/v)	+	+	+	+	+	+	+	+	w	+	ND
1 % (w/v)	+	+	+	+	+	+	+	+	+	+	ND
3 % (w/v)	w (1*)/+ (2)	+	w	+	+	+	+	+	+	+	ND
5 % (w/v)	w	+	– (1)/w (3*)	w (3*)/+ (2)	w (6)/+ (6*)	w	w	w (2)/+ (8)	+	w	ND
7.5 % (w/v)	w	w	– (2)/w (2*)	w	w (11*)/+ (1)	w	w	w	–	w	ND
10 % (w/v)	– (2)/w (1*)	w	–	–	– (4)/w (8*)	w	w	– (7)/w (3)	–	– (3)/w (3*)	+
Sucrose tolerance:											
0 % (w/v)	+	+	+	+	+	+	+	+	w	+	+
10 % (w/v)	+	+	+	+	+	+	+	+	+	+	+
20 % (w/v)	+	+	+	+	+	+	+	+	+	+	+
30 % (w/v)	+	+	+	+	+	+	+	+	+	+	+
40 % (w/v)	+	+	+	+	+	+	+	w (1)/+ (9)	+	+	+
50 % (w/v)	–	w	–	w (1)/+ (4*)	w (1*)/+ (11)	+	–	– (1)/w (4)/+ (5)	+	+	+
60 % (w/v)	–	–	–	–	–	–	–	–	+	–	+
Arginine dihydrolase	–	–	–	–	–	–	–	–	+	–	–

Continued

Table 4. Continued

Characteristic	*R. gaditana* sp. nov. (*n*=3)	*R. metrosideri* sp. nov. (*n*=1)	*R. australiborealis* (*n*=4)	*R. collisarenosi* (*n*=5)	*R. epipactidis* subsp. *epipactidis* subsp. nov. (*n*=12)	*R. epipactidis* subsp. *californiensis* subsp. nov. (*n*=4)	*R. epipactidis* subsp. *japonicus* subsp. nov. (*n*=1)	*R. nectarea* subsp. *nectarea* subsp. nov. (*n*=10)	*R. nectarea* subsp. *nectarea* subsp. nov. (*n*=3)†	*R. nectarea* subsp. *apis* subsp. nov. (*n*=6)	*Phaseolibacter flectens* (*n*=2)†
Citrate utilization	+	+	+	– (1)/+ (4*)	– (2)/+ (10*)	+	+	– (1)/+ (9)	–*/+	+	– (1)/+ (1*)
β-Galactosidase	–	–	–	+	– (3)/+ (9*)	+	+	– (9)/+ (1)	–/+*	– (1)/+ (5*)	–
Lysine decarboxylase	+	+	+	+	+	+	+	+	–	+	–
Nitrate reduction to nitrogen	–	–	–	–	–	–	–	–	–	–	+
Sodium malonate utilization	–	–	–	–	–	– (3*)/w (1)	–	–	ND	–	ND
Tryptophan deamination	–	–	–	–	–	–	–	–	–	–	+
Voges–Proskauer	–	–	–	– (3*)/+ (2)	– (5)/+ (7*)	– (3*)/+ (1)	–	– (7)/+ (3)	+	– (3*)/+ (3)	+
Fermentation of:											
Arabinose	+	–	w	+	+	+	+	w (1)/+ (9)	+	+	– (1*)/+ (1)
Inositol	–	–	–	– (4*)/w (1)	– (11*)/w (1)	–	–	–	–	–	–
Lactose	–	–	–	–	–	– (2*)/w (2)	–	–	–	–	–
Salicin	w	+	+	+	w (4*)/+ (8)	+	–	– (5)/+ (5)	w	w (2)/+ (4*)	–
Sucrose	w	w	+	w (2)/+ (3*)	w (2)/+ (10*)	– (1)/w (3*)	–	– (5)/w (2)/+ (3)	+	+	+
Xylose	w (2*)/+ (1)	+	+	+	w (1)/+ (11*)	+	+	+	+	+	w

†Results obtained by Halpern *et al.* [17, 45] and/or Lenaerts *et al.* [18].

The ML tree built from the alignment of core genes confirmed the close relatedness between *R. epipactidis* and *R. metrosideri* (100 % bootstrap node support; Fig. 2) representing two species that could not be resolved by housekeeping gene analysis (Figs. 1, S1 and S2). Furthermore, *R. australiborealis* was identified as the most basal species in the genus, whereas the other (sub)species branched successively in the following order: *R. collisarenosi*, *R. gaditana*, the two subspecies of *R. nectarea* proposed in this study, *R. metrosideri*, and the three subspecies of *R. epipactidis*. Similar results were obtained in an alternative phylogenomic tree generated using the up-to-date bacterial core genes 2 (UBCG2) pipeline version 3 [43] (Figure S3). Notably, whereas the clade formed by *R. metrosideri* and the three subspecies of *R. epipactidis* had a 100% bootstrap support (Fig. 2) and a gene support index (GSI; defined as the number of individual gene trees that present the same node in the UBCG2 tree) of 54 (Fig. S3), the clade containing *R. epipactidis* subsp. *epipactidis*, *R. epipactidis* subsp. *californiensis* and *R. epipactidis* subsp. *japonicus* was only moderately supported (71% bootstrap node support in the core genome tree and GSI of 23 in the UBCG2 tree), thus confirming the fuzzy (sub)species boundaries in the *R. epipactidis/R. metrosideri* clade suggested by the analysis of ANI and isDDH values.

PHYSIOLOGY

All 46 isolates included in this study were physiologically and biochemically characterized following standard methods. Unless otherwise indicated, all phenotypic tests were performed at 25 °C. Growth at 4, 12, 25, 30, 37 and 41 °C, Gram-staining, and tests for oxidase, catalase, growth in anaerobiosis, and microaerobiosis, haemolysis and DNase were performed as described in Álvarez-Pérez *et al.* [44] and Lenaerts *et al.* [18]. Production of acetoin (Vogues–Proskauer reaction), arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, β-galactosidase, indole, H_2S and urease, utilization of citrate and sodium malonate as sole carbon sources, tryptophan deamination, gelatin liquefaction, and fermentation of adonitol, arabinose, glucose, inositol, lactose, mannitol, raffinose, rhamnose, salicin, sorbitol, sucrose and xylose were evaluated using the Microbact Gram-negative identification system 12A and 24E (Oxoid), according to the manufacturer's instructions. Tests for motility were performed in glass tubes containing a medium composed of 10 g l^{-1} tryptone (Oxoid), 5 g l^{-1} NaCl (Merck Millipore) and 5 g l^{-1} agar (Difco). Tolerance to 0–10% w/v of NaCl in lysogeny broth (LB) agar (Difco) and 0–60% w/v sucrose (Sigma–Aldrich) in LB was determined as described in Álvarez-Pérez *et al.* [22].

Table 4 summarizes the main traits of *R. gaditana*, *R. metrosideri* and the different subspecies of *R. epipactidis* and *R. nectarea* in comparison with the results obtained for other *Rosenbergiella* species and *P. flectens* in this and previous studies (detailed results are presented in Table S9) [17, 18, 45]. The isolates of all *Rosenbergiella* (sub)species and *P. flectens* are Gram-negative coccobacilli, catalase-positive, oxidase-negative, non-haemolytic, and grow well under microaerobic as well as aerobic conditions, and more scarcely in anaerobiosis. Profuse growth of all isolates was observed at 25 and 30 °C, but most isolates also grew to some extent at 4, 12 and/or 37 °C. In addition, some isolates of all species except *R. gaditana* grew at 41 °C, although growth was very scarce in most cases. All tested isolates tolerated sucrose concentrations up to 40% and all isolates of *R. epipactidis* subsp. *californiensis* and *R. nectarea* subsp. *apis* grew at 50% sucrose. The type strain of *R. metrosideri* grew weakly at 50% sucrose, and isolates of *R. collisarenosi*, *R. epipactidis* subsp. *epipactidis* and *R. nectarea* subsp. *nectarea* displayed variable growth (none, weak or profuse) at that sugar concentration. Moreover, it has been reported that some strains of *R. nectarea* and *P. flectens*, including their respective type strains, tolerate 60 % sucrose [17, 45]. In addition, most *Rosenbergiella* isolates grew profusely in LB agar containing between 0 and 3% NaCl, and most of them also displayed some growth in media containing 5 and 7.5% NaCl. *Phaseolibacter flectens* tolerated 10% NaCl, whereas only a few *Rosenbergiella* isolates, including the type strains of *R. gaditana* (S61^T) and *R. metrosideri* (JB07^T) and some representatives of *R. epipactidis* (including the type strains *R. epipactidis* subsp. *epipactidis* S256^T, *R. epipactidis* subsp. *californiensis* FR72^T and *R. epipactidis* subsp. *japonicus* K24^T) and *R. nectarea* (including *R. nectarea* subsp. *apis* B1A^T but not *R. nectarea* subsp. *nectarea* 8N4^T), could grow at that salt concentration.

All *Rosenbergiella* isolates and *P. flectens* were positive for motility, growth in MacConkey agar, and fermentation of glucose. Negative results were observed in all cases for production of DNase, indole, H_2S, ornithine decarboxylase and urease, gelatin liquefaction, and fermentation of adonitol, mannitol, raffinose, rhamnose and sorbitol. *Rosenbergiella nectarea* subsp. *nectarea* yielded variable results for arginine dihydrolase (positive for the strains tested by Halpern *et al.* [17] and negative for those included in this study), whereas isolates of all other *Rosenbergiella* (sub)species and of *P. flectens* yielded negative results in this test. The *R. nectarea* and *P. flectens* isolates analysed in previous studies [17, 45] were negative for lysine decarboxylase, whereas the *R. nectarea* isolates tested in this study and isolates of all other *Rosenbergiella* species produced this enzyme. All *Rosenbergiella* isolates were unable to reduce nitrate to nitrogen and deaminate tryptophan, which differentiates the members of this genus from *P. flectens*. Most tested isolates were negative for utilization of sodium malonate (only *R. epipactidis* subsp. *californiensis* S55 yielded a weakly positive result), fermentation of inositol (weakly positive only for *R. collisarenosi* S294 and *R. epipactidis* subsp. *epipactidis* JB02), and fermentation of lactose (weakly positive only for *R. epipactidis* subsp. *californiensis* isolates B3-15 and JR114). In contrast, the results for other tests such as citrate utilization, β-galactosidase, Voges–Proskauer, and fermentation of arabinose, salicin, sucrose and xylose were more variable at the inter- and intra-(sub)species level (Tables 4 and S9).

EMENDED DESCRIPTION OF *ROSENBERGIELLA AUSTRALIBOREALIS* LENAERTS *ET AL*. 2017

The description of this taxon is the same as that given by Lenaerts *et al.* [18], with the following amendments.

Colonies grow well at 25, 30 and 37 °C, and more scarcely at 4, 12 and 41 °C. All isolates can utilize citrate and are positive for lysine decarboxylase. Sodium malonate is not utilized. Arginine dihydrolase, β-galactosidase, indole, H_2S, ornithine decarboxylase and urease are not produced. Nitrate is not reduced to nitrogen. Negative results for tryptophan deamination and the Voges–Proskauer reaction. All isolates can ferment arabinose (weak reaction in all cases), glucose, salicin, sucrose and xylose. Adonitol, inositol, lactose, mannitol, raffinose, rhamnose and sorbitol are not fermented. Sucrose is tolerated at concentrations ranging from 0 to 40% (w/v). Growth occurs in media containing 0 and 1% (w/v) NaCl, and most tested isolates, including the type strain, display some grow in LB agar supplemented with up to 7.5% (w/v) NaCl.

The type strain is S264^T (= CdVSA 20.1^T=CECT 8500^T=LMG 27954^T). The genome size of the type strain is 2.98 Mb, with G+C content of 45.4 mol%.

Genome sequence accession number for the type strain S264^T: GCA_018494035.1.

The 16S rRNA, *atpD*, *gyrB* and *rpoB* gene sequences of the type strain S264^T have been deposited in the GenBank/EMBL/DDBJ databases under the following accession numbers: KF876187, KF876198, KF876208 and KF876215, respectively.

EMENDED DESCRIPTION OF *ROSENBERGIELLA COLLISARENOSI* LENAERTS *ET AL.* 2017

The description of this taxon is the same as that given by Lenaerts *et al.* [18], with the following amendments.

Colonies grow well at 25 and 30 °C, and more scarcely at 12 and 37 °C. Most isolates, including the type strain, display some growth at 4 and 41 °C. The type strain and most other isolates can utilize citrate. All isolates are positive for production of β-galactosidase and lysine decarboxylase, and negative for sodium malonate utilization, tryptophan deamination, and the production of arginine dihydrolase, indole, H_2S, ornithine decarboxylase and urease. Voges–Proskauer reaction is variable (negative for the type strain). Nitrate is not reduced to nitrogen. All isolates can ferment arabinose, glucose, salicin, sucrose (weak reaction for some isolates) and xylose. Adonitol, lactose, mannitol, raffinose, rhamnose and sorbitol are not fermented. The type strain and most other isolates cannot ferment inositol. Sucrose is tolerated at concentrations ranging from 0 to 50% (w/v). Growth occurs in media containing up to 7.5% (w/v) NaCl, albeit weak growth is observed for most isolates, including the type strain, at ≥5% (w/v) NaCl.

The type strain is S260^T (=8.8A^T=CECT 8501^T=LMG 27955^T). The genome size of the type strain is 3.36 Mb, with G+C content of 48.2 mol%.

Genome sequence accession number for the type strain S260^T: GCA_018494085.1.

The 16S rRNA, *atpD*, *gyrB* and *rpoB* gene sequences of the type strain S260^T have been deposited in the GenBank/EMBL/DDBJ data bases under the following accession numbers: KF876186, KF876193, KF876202 and KF876214, respectively.

EMENDED DESCRIPTION OF *ROSENBERGIELLA EPIPACTIDIS* LENAERTS *ET AL*. 2017

The description of this taxon is the same as that given by Lenaerts *et al.* [18], with the following amendments.

Colonies grow well at 25 and 30 °C, and more scarcely at 12 and 37 °C. Most isolates, including the type strain, display some growth at 4 and 41 °C. The type strain and most other isolates can utilize citrate. All isolates are positive for the production lysine decarboxylase and negative for tryptophan deamination and the production of arginine dihydrolase, indole, H_2S, ornithine decarboxylase and urease. Variable results for production of β-galactosidase and the Voges–Proskauer reaction (positive in both cases for the type strain and most isolates). The type strain and most other isolates cannot utilize sodium malonate. Nitrate is not reduced to nitrogen. All isolates can ferment arabinose, glucose and xylose (weak reaction for some isolates). Adonitol, lactose, mannitol, raffinose, rhamnose and sorbitol are not fermented. Variable results are observed for fermentation of salicin and sucrose (positive for the type strain and most other isolates, albeit reactions are weakly positive in some cases) and inositol (negative for the type strain and most other isolates). Sucrose is tolerated at concentrations ranging from 0 to 50% (w/v), but the type strain grows weakly at 50% sucrose. Growth of the type strain and most isolates occurs in media containing up to 10% (w/v) NaCl, albeit weak growth is generally observed at ≥5 % NaCl.

The type strain is S256^T (= 2.1A^T=CECT 8502^T=LMG 27956^T). The genome size for the type strain is 3.21 Mb, with G+C content of 47.6 mol%.

Genome sequence accession number for the type strain S256^T: GCA_018494055.1.

The 16S rRNA, *atpD*, *gyrB* and *rpoB* gene sequences of the type strain S256^T have been deposited in the GenBank/EMBL/DDBJ databases under the following accession numbers: KF876184, KF876195, KF876204 and KF876212, respectively.

EMENDED DESCRIPTION OF *ROSENBERGIELLA NECTAREA* HALPERN *ET AL*. 2013

The description of this taxon is the same as that given by Halpern *et al.* [17], with the following amendments.

Colonies grow well at 25 and 30 °C, and more scarcely at 12 °C. The type strain and most isolates can grow weakly at 4 °C, and most isolates (excluding the type strain) also display some growth at 37 and 41 °C. Variable results for citrate utilization and lysine decarboxylase (negative in both cases for the type strain, positive for most isolates), production of β-galactosidase and arginine dihydrolase, and the Voges–Proskauer reaction (positive in all cases for the type strain, negative for most isolates). All isolates are negative for tryptophan deamination and the production of indole, H_2S, ornithine decarboxylase and urease. Nitrate is not reduced to nitrogen. All isolates can ferment arabinose (weak reaction for some isolates), glucose and xylose. Adonitol, inositol, lactose, mannitol, raffinose, rhamnose and sorbitol are not fermented. Variable results (positive, weakly positive, or negative) are observed for fermentation of salicin (weakly positive for the type strain) and sucrose (positive for the type strain). Sucrose is tolerated at concentrations ranging from 0 to 40% (w/v), and most isolates can grow at 50% sucrose. Some isolates, including the type strain, can grow at 60% sucrose. Growth of most isolates occurs in media containing up to 7.5% (w/v) NaCl, but the type strain displays no growth at ≥5% NaCl. Only some isolates can weakly grow in media containing 10% NaCl.

The type strain is 8N4^T (=DSM 24150^T=LMG 26121^T). The genome size of the type strain is 3.29 Mbp and its DNA G+C content is 47.4 mol% [21].

Genome sequence accession number for the type strain 8N4^T: GCA_900111105.1.

The 16S rRNA, *atpD*, *gyrB* and *rpoB* gene sequences of the type strain 8N4^T have been deposited in the GenBank/EMBL/DDBJ databases under the following accession numbers: HQ284827, JN808189, JF745806 and JF745805, respectively.

DESCRIPTION OF *ROSENBERGIELLA EPIPACTIDIS* SUBSP. *EPIPACTIDIS* SUBSP. NOV.

Rosenbergiella epipactidis subsp. *epipactidis* (epi.pac.ti'dis. N.L. gen. fem. n. *epipactidis*, referring to the genus name of the host plant (*Epipactis*) from which the subspecies was first isolated).

Characteristics are as described for *Rosenbergiella epipactidis*. The type strain for the subspecies is S256^T (=2.1A^T=CECT 8502^T=LMG 27956^T), isolated by Lenaerts *et al.* [18] from floral nectar of an orchid (*Epipactis palustris*, Orchidaceae) in Dune du Perroquet (Bray-Dunes, France) in 2012. The genome size of the type strain is 3.21 Mbp and its DNA G+C content is 47.6 mol%.

Genome sequence accession number for the type strain S256^T: GCA_018494055.1.

The 16S rRNA, *atpD*, *gyrB* and *rpoB* gene sequences of the type strain S256^T have been deposited in the GenBank/EMBL/DDBJ databases under the following accession numbers: KF876184, KF876195, KF876204 and KF876212, respectively.

DESCRIPTION OF *ROSENBERGIELLA EPIPACTIDIS* SUBSP. *CALIFORNIENSIS* SUBSP. NOV.

Rosenbergiella epipactidis subsp. *californiensis* (ca.li.for.ni.en'sis. N.L. masc. adj. *californiensis*, referring to the fact that most isolates of the subspecies have been obtained from plants and insects collected in California, USA).

This description is based on the characteristics of two isolates (FR72^T and JR114) obtained from the floral nectar of sticky-monkey flowers (*Diplacus (Mimulus) aurantiacus*, Phrymaceae) collected between 2017 and 2018 at Jasper Ridge Biological Preserve (Stanford, California, USA), one isolate (S55) found in the floral nectar of dragon flowers (*Antirrhinum* sp., Plantaginaceae) collected in 2011 in Barbate (Cádiz, Spain), and one isolate (B3-15) obtained from the honey crop of a honeybee (*Apis mellifera*) collected in 2018 at the Stanford University campus in Stanford (California, USA). Cells are Gram-negative coccobacilli that are facultative anaerobes and motile. After 72 h of aerobic incubation at 25 °C on TSA medium, colonies are circular (1–4 mm of diameter), pale yellow, translucent, convex, smooth and with entire margins. Catalase-positive, oxidase-, DNase- and gelatinase-negative. Colonies grow on MacConkey agar and Columbia blood agar without haemolysis activity. Growth occurs at 25 and 30 °C. Isolate S55 grows well at 37 °C, whereas FR72^T and the other isolates display limited growth at this temperature. Weak growth of all tested isolates is observed at 4, 12 and 41 °C. All isolates can use citrate as the sole carbon source and produce β-galactosidase and lysine decarboxylase, but they are negative for tryptophan deamination, and the production of arginine dihydrolase, indole, H_2S, ornithine decarboxylase and urease. Nitrate is not reduced to nitrogen. Variable results for the Voges–Proskauer reaction (negative for FR72^T, B3-15 and S55, positive for JR114) and sodium malonate utilization (negative for FR72^T, B3-15 and JR114, weakly positive for S55). All isolates can ferment arabinose, glucose, salicin, and xylose. Adonitol, inositol, mannitol, raffinose, rhamnose and sorbitol are not fermented. Variable results are observed for fermentation of lactose (negative reaction for FR72^T and isolate S55, weakly positive for isolates B3-15 and JR114) and sucrose (weakly positive reaction for FR72^T and isolates B3-15 and JR114, negative for isolate S55). All tested isolates can grow at decreased oxygen concentrations and in media containing 0–10 % w/v of NaCl, although growth at ≥5% NaCl is scarce. Sucrose is tolerated at concentrations ranging from 0 to 50% (w/v).

The type strain for the subspecies is FR72^T (=NCCB 100898^T=LMG 32786^T), isolated from the floral nectar of *Diplacus (Mimulus) aurantiacus* (Phrymaceae) collected in 2017 at Jasper Ridge Biological Preserve (Stanford, California, USA). The genome size of the type strain is 3.17 Mbp and its DNA G+C content is 47.6 mol%.

Genome sequence accession number for the type strain FR72^T: GCA_022602615.1.

The 16S rRNA, *atpD*, *gyrB* and *rpoB* gene sequences of the type strain FR72^T have been deposited in the GenBank/EMBL/DDBJ databases under the following accession numbers: MT341873, MT354639, MT354678 and MT354717, respectively.

DESCRIPTION OF *ROSENBERGIELLA EPIPACTIDIS* SUBSP. *JAPONICUS* SUBSP. NOV.

Rosenbergiella epipactidis subsp. *japonicus* (ja.po'ni.cus. N.L. masc. adj. *japonicus*, referring to the fact that the species was first isolated from plants collected in Japan).

This description is based on the characteristics of a single isolate, namely K24^T, which was found in the floral nectar of *Eurya japonica* (Pentaphylacaceae) collected in Japan in 2016. Cells are Gram-negative coccobacilli that are facultative anaerobes and motile. After 72 h of aerobic incubation at 25 °C on TSA medium, colonies are circular (1.5–2.5 mm of diameter), pale yellow, translucent, convex, smooth and with entire margins. Catalase-positive, oxidase-, DNase- and gelatinase-negative. Colonies grow on MacConkey agar and Columbia blood agar without haemolysis activity. Growth occurs at 25 and 30 °C. Weak growth is observed at 4, 12, 37 and 41 °C. Isolate K24^T can use citrate as the sole carbon source and produce lysine decarboxylase and β-galactosidase but yields a negative result in the Voges–Proskauer reaction, tryptophan deamination, utilization of sodium malonate, and production of arginine dihydrolase, indole, H_2S, ornithine decarboxylase and urease. Nitrate is not reduced to nitrogen. K24^T can ferment arabinose, glucose and xylose. Adonitol, inositol, lactose, mannitol, raffinose, rhamnose, salicin, sorbitol and sucrose are not fermented. K24^T can grow at decreased oxygen concentrations and in media containing 0–10 % (w/v) NaCl, although growth is weak at ≥5% NaCl. Sucrose is tolerated at concentrations ranging from 0 to 40% (w/v).

The type strain for the subspecies is K24^T (=NCCB 100924^T=LMG 32785^T), isolated from the floral nectar of *Eurya japonica* collected in 2016 at Takaike Kozagawacho Higashimurogun (Wakayama prefecture, Japan). The genome size of the type strain is 3.37 Mbp and its DNA G+C content is 47.3 mol%.

Genome sequence accession number for the type strain K24^T: GCA_022602435.1.

The 16S rRNA, *atpD*, *gyrB* and *rpoB* gene sequences of the type strain K24^T have been deposited in the GenBank/EMBL/DDBJ databases under the following accession numbers: MT341879, MT354645, MT354684 and MT354723, respectively.

DESCRIPTION OF *ROSENBERGIELLA NECTAREA* SUBSP. *NECTAREA* SUBSP. NOV.

Rosenbergiella nectarea subsp. *nectarea* (nec.ta're.a. L. fem. adj. *nectarea*, from nectar, referring to the source of the type strain).

Characteristics are as described for *Rosenbergiella nectarea*. The type strain for the subspecies is 8N4^T (=DSM 24150^T=LMG 26121^T), isolated by Halpern *et al.* [17] from floral nectar of an almond tree (*Prunus dulcis*, Rosaceae) in northern Israel. The genome size of the type strain is 3.29 Mbp and its DNA G+C content is 47.4 mol% [21].

Genome sequence accession number for the type strain 8N4^T: GCA_900111105.1.

The 16S rRNA, *atpD*, *gyrB* and *rpoB* gene sequences of the type strain 8N4^T have been deposited in the GenBank/EMBL/DDBJ databases under the following accession numbers: HQ284827, JN808189, JF745806 and JF745805, respectively.

DESCRIPTION OF *ROSENBERGIELLA NECTAREA* SUBSP. *APIS* SUBSP. NOV.

Rosenbergiella nectarea subsp. *apis* (a'pis. L. gen. fem. n. *apis* of/from a honeybee, the genus name of the honeybee *Apis mellifera*, referring to the fact that the subspecies was first identified from isolates obtained from this insect host).

This description is based on the characteristics of four isolates (B1A^T, B3A, B4A and B5A) obtained from the mouth of honeybees (*Apis mellifera*) and the gut of a bumble bee (*Bombus* sp.), collected in July 2018 on the Stanford University campus in Stanford, California, USA, and two isolates (S255=1.12A and S258=2.6A) obtained from the floral nectar of orchids (*Epipactis palustris*, Orchidaceae) collected at different locations of France in 2012. Cells are Gram-negative coccobacilli that are facultative anaerobes and motile. After 72 h of aerobic incubation at 25 °C on TSA medium, colonies are circular (1–4 mm of diameter), pale yellow, translucent, convex, smooth and with entire margins. Catalase-positive, oxidase-, DNase- and gelatinase-negative. Colonies grow on MacConkey agar and Columbia blood agar without haemolysis activity. Growth occurs at 25 and 30 °C. Isolates S255 and S258 grow well at 37 °C, whereas B1A^T and the other isolates display limited growth at this temperature. Weak growth of all tested isolates is observed at 4, 12 and 41 °C. All isolates can use citrate as the sole carbon source and produce lysine decarboxylase, but they are negative for tryptophan deamination, sodium malonate utilization, and the production of arginine dihydrolase, indole,

H_2S, ornithine decarboxylase and urease. Nitrate is not reduced to nitrogen. Variable results for production of β-galactosidase (negative for B3A, positive for the type strains and the other isolates) and the Voges–Proskauer reaction (negative for B1A^T, B4A and B5A, positive for the other isolates). All isolates can ferment arabinose, glucose, salicin (weakly positive reaction for S255 and S258), sucrose and xylose. Adonitol, inositol, lactose, mannitol, raffinose, rhamnose and sorbitol are not fermented. All tested isolates can grow at decreased oxygen concentrations and in media containing 0 to 7.5% w/v NaCl, although growth at ≥3% NaCl is scarce. Isolates B1A^T, S255 and S258 also show weak growth in LB agar supplemented with 10% NaCl. Sucrose is tolerated at concentrations ranging from 0 to 50% (w/v).

The type strain for the subspecies is B1A^T (=DSM 111763^T=NCCB 100810^T), isolated from the mouth of a honeybee (*Apis mellifera*) collected in July 2018 at Stanford campus (Stanford, California, USA). The genome size of the type strain is 3.4 Mbp and its DNA G+C content is 47.3 mol%.

Genome sequence accession number for the type strain B1A^T: GCA_018494105.1.

The 16S rRNA, *atpD*, *gyrB* and *rpoB* gene sequences of the type strain B1A^T have been deposited in the GenBank/EMBL/DDBJ databases under the following accession numbers: MT341812, MT354655, MT354694 and MT354733, respectively.

DESCRIPTION OF *ROSENBERGIELLA GADITANA* SP. NOV.

Rosenbergiella gaditana (ga.di.ta'na. L. fem. adj. *gaditana*, referring to the fact that the species was first isolated from plants collected in the Spanish province of Cádiz).

This description is based on the characteristics of three isolates which were found in the floral nectar of dragon flowers (*Antirrhinum* sp., Plantaginaceae) collected in Barbate in 2011 (Cádiz, Spain; isolate S61^T) and viper's bugloss flowers (*Echium* sp., Boraginaceae) collected in Madrid (Madrid, Spain; isolates S284 and S290) in 2017. Cells are Gram-negative coccobacilli that are facultative anaerobes and motile. After 72 h of aerobic incubation at 25 °C on TSA medium, colonies are circular (1–4 mm of diameter), pale yellow, translucent, convex, smooth and with entire margins. Catalase-positive, oxidase-, DNase- and gelatinase-negative. Colonies grow on MacConkey agar and Columbia blood agar without haemolysis activity. Growth occurs at 25 and 30 °C, but not at 41 °C. Weak growth of all isolates is observed at 12 and 37 °C, and of isolate S290 at 4 °C (S61^T and S284 cannot grow at this latter temperature). All isolates can use citrate as the sole carbon source and produce lysine decarboxylase but yield a negative result in the Voges–Proskauer reaction, tryptophan deamination, utilization of sodium malonate, and production of arginine dihydrolase, β-galactosidase, indole, H_2S, ornithine decarboxylase and urease. Nitrate is not reduced to nitrogen. All isolates can ferment arabinose, glucose, salicin and sucrose (weakly positive result in both cases for all isolates), and xylose (weakly positive result for S61^T and S290). Adonitol, inositol, lactose, mannitol, raffinose, rhamnose and sorbitol are not fermented. The three tested isolates can grow at decreased oxygen concentrations and in media containing 0–7.5% (w/v) NaCl, although growth is weak for isolate S61^T at ≥3% NaCl and for the other isolates at ≥5% NaCl. Isolate S61^T shows weak growth in LB agar supplemented with 10% NaCl, whereas the other isolates cannot grow at this salt concentration. Sucrose is tolerated at concentrations ranging from 0 to 40% (w/v).

The type strain is S61^T (= DSM 111181^T=NCCB 100789^T), isolated from floral nectar of *Antirrhinum* sp. collected in April 2011 in Barbate (Cádiz, Spain). The genome size of the type strain is 3.07 Mbp and its DNA G+C content is 46.3 mol%.

Genome sequence accession number for the type strain S61^T: GCA_018494065.1.

The 16S rRNA, *atpD*, *gyrB* and *rpoB* gene sequences of the type strain S61^T have been deposited in the GenBank/EMBL/ DDBJ databases under the following accession numbers: MT341811, MT354635, MT354674 and MT354713, respectively.

DESCRIPTION OF *ROSENBERGIELLA METROSIDERI* SP. NOV.

Rosenbergiella metrosideri (me.tro.si'de.ri. N. L. gen. n. *metrosideri*, of *Metrosideros*, the genus name of the host plant from which the species was first isolated).

This description is based on the characteristics of a single isolate, namely JB07^T, which was found in the floral nectar of *Metrosideros polymorpha* (Myrtaceae) collected in Hawai'i Volcanoes National Park (Hawaii, USA) in 2013. Cells are Gram-negative coccobacilli that are facultative anaerobes and motile. After 72 h of aerobic incubation at 25 °C on TSA medium, colonies are circular (1.5–2.5 mm of diameter), pale yellow, translucent, convex, smooth and with entire margins. Catalase-positive, oxidase-, DNase- and gelatinase-negative. Colonies grow on MacConkey agar and Columbia blood agar without haemolysis activity. Growth occurs at 25, 30 and 37 °C. Weak growth is observed at 4, 12 and 41 °C. Isolate JB07^T can use citrate as the sole carbon source and produce lysine decarboxylase but yields a negative result in the Voges–Proskauer reaction, tryptophan deamination, utilization of sodium malonate, and production of arginine dihydrolase, β-galactosidase, indole, H_2S, ornithine decarboxylase and urease. Nitrate is not reduced to nitrogen. JB07^T can ferment glucose, salicin, sucrose (weakly positive result) and xylose. Adonitol, arabinose, inositol, lactose, mannitol, raffinose, rhamnose and sorbitol are not fermented. JB07^T can grow at decreased

oxygen concentrations and in media containing 0–10% (w/v) NaCl, although growth is weak at ≥7.5% NaCl. Sucrose is tolerated at concentrations ranging from 0 to 40% (w/v), and weak growth is observed at 50% sucrose.

The type strain is JB07^T (= NCCB 100888^T=LMG 32616^T), isolated from floral nectar of *Metrosideros polymorpha* collected in 2013 in Hawai'i Volcanoes National Park, Hawaii, USA. The genome size of the type strain is 3.34 Mbp and its DNA G+C content is 47.2 mol%.

Genome sequence accession number for strain JB07^T: GCA_022602565.1.

The 16S rRNA, *atpD*, *gyrB* and *rpoB* gene sequences of the type strain JB07^T have been deposited in the GenBank/EMBL/DDBJ databases under the following accession numbers: MT341875, MT354641, MT354680 and MT354719, respectively.

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Author contribution
Conceptualization: S.A.P. and B.L. Resources: all authors. Investigation, formal analysis, and data curation: S.A.P. and K.V. Writing – original draft preparation: S.A.P. Writing – review and editing: all authors. Supervision: B.L. Funding: S.A.P., C.d.V. and T.F.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Ethics approval was not required. All bacterial isolates used in this study were collected following local regulations and, when required, the specifications of the Nagoya Protocol on Access and Benefit Sharing (ABS).

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