INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

RESEARCH ARTICLE

Nouioui *et al.*, *Int. J. Syst. Evol. Microbiol.* 2023;73:005713

DOI 10.1099/ijsem.0.005713

MICROBIOLOGY SOCIETY

OPEN ACCESS

Corynebacterium megadyptis sp. nov. with two subspecies, *Corynebacterium megadyptis* subsp. *megadyptis* subsp. nov. and *Corynebacterium megadyptis* subsp. *dunedinense* subsp. nov. isolated from yellow-eyed penguins

Imen Nouioui[1], Sarah C. Saunderson[2], Anne C. Midwinter[3], Melanie J. Young[4,5,6], Kate M. McInnes[4], Jim Watts[6] and Vartul Sangal[7,*]

Abstract

Novel *Corynebacterium* strains, 3B^T and 7B^T, were isolated from the oral cavities of young chicks of yellow-eyed penguins (hoiho), *Megadyptes antipodes*. A polyphasic taxonomic characterization of these strains revealed chemotaxonomic, biochemical and morphological features that are consistent with those of the genus *Corynebacterium*. The 16S rRNA gene sequence similarity values between the strains and their closest phylogenetic neighbour, *Corynebacterium ciconiae* CCUG 47525^T were 99.07%, values that are in line with their phylogenomic positions within the evolutionary radiation of the genus *Corynebacterium*. Digital DNA–DNA hybridization values and average nucleotide identities between the genome sequences of the two strains and related *Corynebacterium* species were well below the defined threshold values (70 and 95–96%, respectively) for prokaryotic species delineation. The genome size of these strains varied between 2.45–2.46 Mb with G+C content 62.7–62.9mol%. Strains 3B^T and 7B^T were Gram-stain positive bacilli that were able to grow in presence of 0–10% (w/v) NaCl and at temperature ranging between 20–37 °C. The major fatty acids (>15%) were $C_{16:0}$ and $C_{18:1}$ ω9*c*, and the mycolic acid profile included 32–36 carbon atoms. We propose that these strains represent a novel species, *Corynebacterium megadyptis* sp. nov. with 3B^T (=DSM 111184^T=NZRM 4755^T) as the type strain. Phylogenomically, strains 3B^T and 7B^T belong to two lineages with subtle differences in MALDI-TOF spectra, chemotaxonomic profiles and phenotypic properties. The fatty acid profile of strain 3B^T contains $C_{18:0}$ as a predominant type (>15%), which is a minor component in strain 7B^T. Strain 7B^T can oxidize *N*-acetyl-D-glucosamine, L-serine, α-hydroxy-butyric acid, L-malic acid, L-glutamic acid, bromosuccinic acid and L-lactic acid, characteristics not observed in strain 3B^T. Therefore, we propose that these strains represent two subspecies, namely *Corynebacterium megadyptis* subsp. *megadyptis* subsp. nov. (type strain, 3B^T=DSM 111184^T=NZRM 4755^T) and *Corynebacterium megadyptis* subsp. *dunedinense* subsp. nov. (type strain, 7B^T=DSM 111183^T=NZRM 4756^T).

INTRODUCTION

The genus *Corynebacterium* of the family *Corynebacteriaceae* [1] and order *Mycobacteriales* [2] encompassed 136 validly named species (LPSN database: https://lpsn.dsmz.de/genus/corynebacterium; assessed in June 2022) with the human pathogen *Corynebacterium diphtheriae* [3] as the type species [4]. Members of this taxon were isolated from diverse habitats such as soil, humans, animals, plants,

Author affiliations: [1]Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstraße 7B, 38124 Braunschweig, Germany; [2]Department of Microbiology and Immunology, School of Biomedical Sciences, University of Otago, PO Box 56, Dunedin 9054, New Zealand; [3]Molecular Epidemiology and Veterinary Public Health Laboratory (mEpiLab), Infectious Disease Research Centre, School of Veterinary Science, Massey University, Palmerston North 4410, New Zealand; [4]Science and Capability, Department of Conservation, 18-32 Manners St., Wellington 6011, New Zealand; [5]Department of Zoology, University of Otago, PO Box 56, Dunedin 9054, New Zealand; [6]Coastal Otago District Office, Department of Conservation, PO Box 5244 Moray Place, Dunedin, New Zealand; [7]Faculty of Health and Life Sciences, Northumbria University, Newcastle upon Tyne NE1 8ST, UK.

***Correspondence:** Vartul Sangal, vartul.sangal@northumbria.ac.uk

Keywords: *Corynebacterium megadyptis*; yellow-eyed penguin; diphtheritic stomatitis; *Megadyptes antipodes*; novel species.

Abbreviations: AAI, average amino-acid identity; ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization; ISP, International Streptomyces Project; NA, nutrient agar; TSA, trypticase soy agar.

The GenBank accession numbers for genome sequences of strains 3B^T and 7B^T are PQMV00000000 and PQMS00000000, respectively. 16S rRNA gene sequences are also available for these strains from the GenBank under the accession numbers MW647632 and MW647633, respectively.

One supplementary figure and Two supplementary tables are available with the online version of this article.

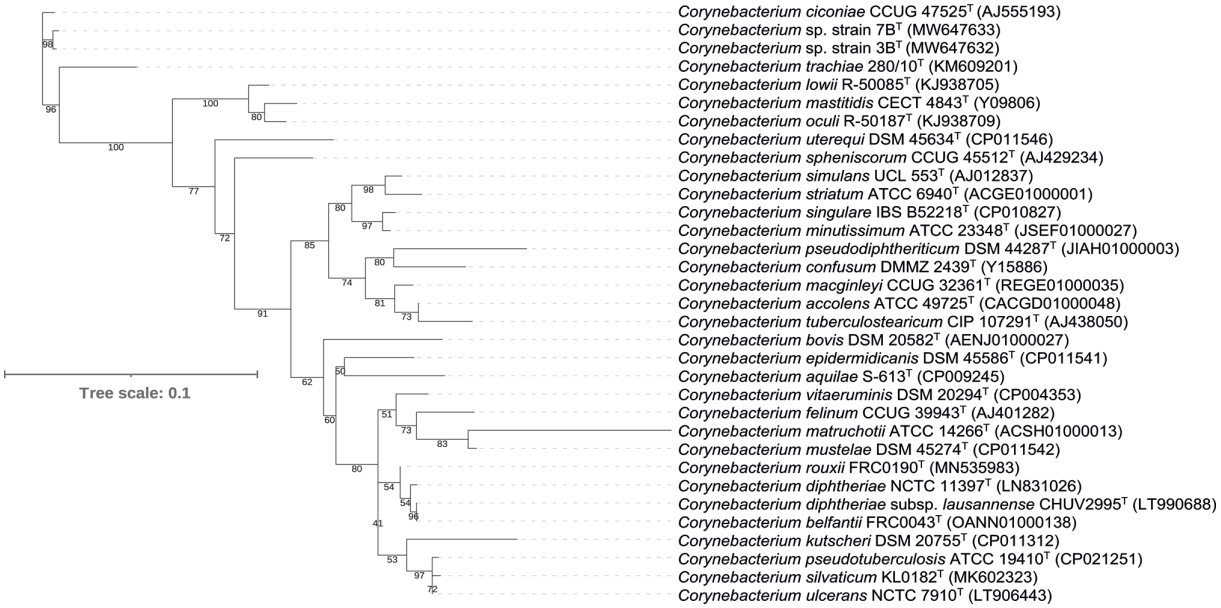

Fig. 1. A maximum-likelihood tree from the 16S rRNA gene alignment of related *Corynebacterium* strains identified using the EzBioCloud server [15]. The scale bar represents nucleotide substitutions per nucleotide site.

dairy products and marine samples. *Corynebacterium* strains are usually Gram-stain-positive, catalase-positive, non-spore-forming and non-motile chemoorganotrophs that do not form aerial mycelia [5]. Most species are facultative anaerobes, but some are aerobes [5].

Corynebacterial strains have been characterized as possessing a peptidoglycan cell wall rich in *meso*-diaminopimelic acid, and arabinose and galactose sugars. Major phospholipids include phosphatidylinositol, phosphatidylinositol dimannoside(s), phosphatidylglycerol trehalose dimycolates and unidentified glycolipids, while phosphatidylethanolamine was also present in some species. Members of this taxon had dihydrogenated menaquinones with eight [MK-8(H2)] and/or nine [MK-9(H2)] isoprene units [5]. The major fatty acids are straight chain saturated and monounsaturated types with $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ $\omega 9c$ as the major components. However, tuberculostearic acid (10-methyl $C_{18:0}$) may also be present in small to moderate amounts. The number of carbon atoms in mycolic acids range from 22 to 36; however, some *Corynebacterium* species lack mycolic acids. The G+C content ranges between 46–74mol% among corynebacterial species [5, 6].

The genus *Corynebacterium* encompasses species of industrial, medical and veterinary importance, such as the industrially important *Corynebacterium glutamicum*, which is used in manufacturing of several amino acids [7], and human pathogen *C. diphtheriae,* which is the causative agent of diphtheria [5, 8, 9]. More than 50% of *Corynebacterium* species have been found to be associated with clinical conditions in humans or animals [6]. In this study, we have characterized two novel *Corynebacterium* strains associated with diphtheritic stomatitis in yellow-eyed penguins [10] using phylogenomic and polyphasic taxonomic approaches.

ISOLATION AND ECOLOGY

Novel *Corynebacterium* strains were previously isolated from diphtheritic stomatitis lesions in the oral cavities of 2–14 day old yellow-eyed penguin chicks (locally known as hoiho) from the Otago Peninsula in New Zealand [10]. Diphtheritic stomatitis in yellow-eyed penguins is characterized by a thick cream-coloured fibrinopurulent exudate that covers the oral cavity and makes it difficult for young chicks to swallow food, resulting in inanition and significant weight loss due to malnutrition and ultimately death [11, 12]. These strains formed two distinct lineages based on the phylogenomic analyses [10]. In this study, two representative strains, $3B^T$ and $7B^T$, one from each lineage, were subjected to a polyphasic taxonomic characterization which confirmed their assignment to a new species.

16S rRNA GENE PHYLOGENY

The genome of $3B^T$ and $7B^T$ were previously sequenced by us and 16S rRNA gene sequences were extracted from the genome assemblies [10]. The strain identities were confirmed by PCR sequencing of 16S rRNA genes. Briefly, genomic DNA was extracted from biomass harvested from 3-day-old cultures prepared on DSMZ 535 medium and subjected to PCR amplification of a 16S

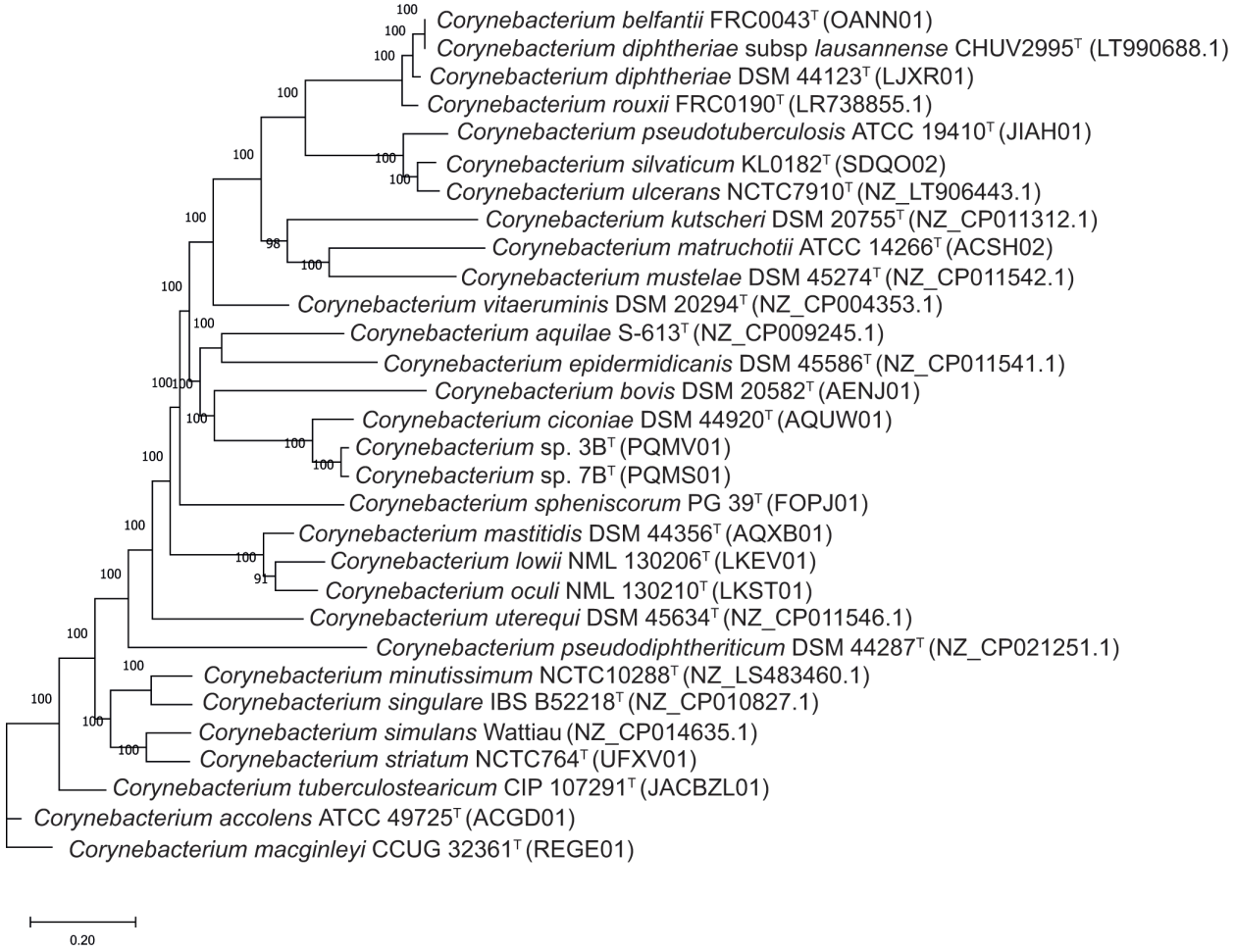

Fig. 2. A maximum-likelihood tree from the core genome alignment of related *Corynebacterium* strains. The scale bar represents nucleotide substitutions per nucleotide site.

rRNA gene following the protocol of Weisburg *et al.* [13]. The PCR products were purified and sequenced using the Sanger method [14]. These sequences were aligned to 16S rRNA sequences extracted from the genome assemblies for each respective strain and were 100% identical to them.

The 16S rRNA gene sequences of the strains were compared to those of the type strains of the genus *Corynebacterium* using the EzBioCloud server [15]. Pairwise 16S rRNA gene sequence similarity between strains 3B^T and 7B^T and their closest identified neighbour, *C. ciconiae* CCUG 47525^T were 99.07%. However, the similarity value was higher (99.5%) between strains 3B^T and 7B^T. The 16S rRNA gene sequences of both these strains and closely related species identified by using the EzBioCloud server were aligned using MUSCLE [16] and a maximum-likelihood (ML) tree was reconstructed using IQ-TREE following the GTR+F+I+G4 model with 100000 SH-aLRT and 100000 ultrafast bootstrap iterations [17]. The tree was visualized using iTOL version 6 [18]. Strains 3B^T and 7B^T formed a well-supported subclade closely related to *C. ciconiae* CCUG 47525^T (Fig. 1).

GENOME FEATURES

The widely accepted 16S rRNA similarity cut-off value of 98.7% for defining species needs to be carefully considered within the phylum *Actinomycetota*. Multiple species within the family *Geodermatophilaceae* [19] and within the genus *Mycobacterium* [20] share >99% 16S rRNA gene similarities. Therefore, we performed phylogenomic analyses to further confirm their species designation. The genome sequences of the type strains of the closely related species were also obtained from GenBank, where available (Table S1 available with the online version of this article). The quality of all genome assemblies were assessed using CheckM [21], showing 100% completeness and <0.5% potential contamination. All sequences were annotated using Prokka 1.13.7 [22] and compared using Roary version 3.12.0 with the identity cut-off value of 70% [23, 24]. An ML tree was reconstructed from the core genomic alignment using IQ-Tree as described above. The close phylogenetic relatedness between strains 3B^T and 7B^T and

Table 1. Phylogenomic variations between strains 3B^T, 7B^T and other phylogenomic relatives

Species	Strain	dDDH (%)		ANI (%)		AAI (%)	
		3B^T	7B^T	3B^T	7B^T	3B^T	7B^T
'*Corynebacterium megadyptis* subsp. *megadyptis*'	3B^T	–	66.1	–	95.63	–	97.46
'*Corynebacterium megadyptis* subsp. *dunedinense*'	7B^T	66.1	–	95.63	–	97.46	–
Corynebacterium accolens	ATCC 49725^T	20.5	20.5	77.40	77.23	61.6	61.62
Corynebacterium aquilae	S-613^T	22.5	22.8	79.00	78.97	63.85	63.81
Corynebacterium belfantii	FRC0043^T	21.9	22.5	78.71	78.00	63.45	63.62
Corynebacterium bovis	DSM 20582^T	21.1	20.9	77.50	78.11	62.03	61.84
Corynebacterium ciconiae	DSM 44920^T	25.5	25.4	83.40	83.30	88.07	88.03
Corynebacterium diphtheriae	DSM 44123^T	22.6	21.9	78.86	78.70	63.67	63.79
Corynebacterium diphtheriae subsp. *lausannense*	CHUV2995^T	23.4	23.8	78.63	78.33	63.44	63.67
Corynebacterium epidermidicanis	DSM 45586^T	22.8	22.1	78.14	78.35	64.19	64.22
Corynebacterium kutscheri	DSM 20755^T	24.7	24.3	79.79	79.34	63.24	63.18
Corynebacterium lowii	NML 130206^T	20.6	20.2	77.88	77.89	63.1	63.02
Corynebacterium macginleyi	CCUG 32361^T	20.5	20.5	76.88	77.02	61.45	61.52
Corynebacterium mastitidis	DSM 44356^T	20.4	21.2	77.84	78.07	63.12	63.25
Corynebacterium matruchotii	ATCC 14266^T	19.9	19.9	77.29	77.48	62.46	62.51
Corynebacterium minutissimum	NCTC10288^T	20.5	20.8	77.76	77.83	61.86	61.93
Corynebacterium mustelae	DSM 45274^T	25	25.6	78.02	78.04	63.35	63.25
Corynebacterium oculi	NML 130210^T	21.1	20.4	77.82	77.42	63.14	63.11
Corynebacterium pseudodiphtheriticum	DSM 44287^T	22.7	21.7	76.84	77.03	60.61	60.66
Corynebacterium pseudotuberculosis	ATCC 19410^T	24.5	24.6	78.37	78.64	63.8	63.91
Corynebacterium rouxii	FRC0190^T	24.3	23.7	79.21	78.99	63.95	64.21
Corynebacterium silvaticum	KL0182^T	22.2	23.2	78.08	78.86	63.88	63.95
Corynebacterium simulans	Wattiau	21.8	22.1	77.88	77.98	61.84	62
Corynebacterium singulare	IBS B52218^T	20.1	20.1	77.58	77.98	61.87	61.82
Corynebacterium spheniscorum	PG 39^T	21.7	21.8	77.20	77.45	63.63	63.37
Corynebacterium striatum	NCTC764^T	20.5	20.4	77.33	77.67	62.1	62.04
Corynebacterium tuberculostearicum	CIP 107291^T	20.5	20.2	77.90	77.62	61.93	62.08
Corynebacterium ulcerans	NCTC7910^T	24.2	23.2	77.92	77.97	63.77	63.91
Corynebacterium uterequi	DSM 45634^T	21.3	21.9	77.89	77.88	61.73	61.7
Corynebacterium vitaeruminis	DSM 20294^T	21.9	22	78.62	78.51	63.89	63.75

the type strain of *C. ciconiae* was confirmed in the genomic tree (Fig. 2), which is consistent with our previous study [10] and in concordance with the phylogenetic position of these strains within the evolutionary radiation of the genus *Corynebacterium*.

Phylogenomic comparisons, including digital DNA–DNA hybridization (dDDH), average nucleotide identity (ANI) and average amino acid identity (AAI) values, between the genome sequences of strains 3B^T and 7B^T and their closest relatives were carried out using the Genome-to-Genome Distance Calculator server [25], the ANI matrix calculator (http://enve-omics.ce.gatech.edu/g-matrix/) and the CompareM tool (https://github.com/dparks1134/CompareM), respectively (Table 1). Strains 3B^T and 7B^T have genome sizes of 2.45 Mb (number of contigs, 16; N50 value, 241725) and 2.46 Mb (number of contigs, 17; N50 value, 215111), with genomic G+C contents of 62.9 and 62.7 mol%, respectively. Their closest neighbour *C. ciconiae* DSM 44920^T has a genome size of 2.55 Mb with G+C content of 62.1 mol% (Table S1). The dDDH, ANI and AAI values between the genome sequences of

Table 2. Phenotypic properties distinguishing strains 3B^T and 7B^T from their closest phylogenomic neighbour *C. ciconiae* DSM 44920^T

All strains were able to metabolize: L-fucose, D-glucose, maltose and sucrose (sugars); acetoacetic acid, acetic acid, butyric acid, *N*-acetyl-neuraminic acid (organic acids); able to grow in the presence of up to 8% NaCl, potassium tellurite and Tween 40. All strains showed a weak reaction to sodium bromate. All strains were unable to metabolise: D-arabitol, cellobiose, D-fructose-6-phosphate, D-fucose *N*-acetyl-D-galactosamine, *β*-gentiobiose, D-glucose-6-ghosphate, 3-*O*-methyl-D-glucose, *β*-methyl-D-glucoside, inosine, myo-inositol, lactose, D-mannitol, *N*-acetyl-*β*-D-mannosamine, melibiose, raffinose, L-rhamnose, D-salicin, D-sorbitol, stachyose (sugars); D-aspartic acid, *β*-hydroxy-butyric acid, citric acid, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, glucuronamide, α-keto-glutaric acid, D-lactic acid methyl ester, D-malic acid, mucic acid, quinic acid, *p*-hydroxy-phenylacetic acid, L-pyroglutamic acid and D-saccharic acid (organic acids); L-arginine, L-histidine and glycine-proline (amino acids); the protein gelatin, and were sensitive to lincomycin, minocycline, troleandomycin, vancomycin (antibiotics); and unable to grow in the presence of guanidine hydrochloride, niaproof, sodium formate and tetrazolium blue.Symbols: –, negative reaction; w, weak reaction; +, positive reaction.

GENIII MicroPlate tests	3B^T	7B^T	DSM 44920^T
Sugars:			
N-Acetyl-D-glucosamine	–	+	–
Dextrin	+	w	–
D-Fructose	w	+	+
D-Galactose	w	+	–
L-Galactonic acid-γ-lactone	–	–	+
D-Mannose	+	+	w
Methyl pyruvate, glycerol	+	+	–
Trehalose, turanose	w	+	w
Amino acids:			
L-Alanine	–	–	+
L-Serine	–	+	–
Organic acids:			
L-Aspartic acid, γ-amino-n-butyric acid, α-keto-butyric acid	+	+	–
α-Hydroxy-butyric acid, L-malic acid	–	+	w
L-Glutamic acid, bromo-succinic acid, L-lactic acid	–	+	–
Propionic acid	+	+	w
Able to grow in the presence of:			
Lithium chloride	w	+	+
Pectin	–	+	–
1% Sodium lactate	–	+	–
Tetrazolium violet	–	–	+
Resistance to antibiotics:			
Fusidic acid, rifamycin SV	–	–	+
Nalidixic acid	–	+	–
Aztreonam	w	+	+

strains 3B^T and 7B^T and those of their closest neighbours were below the threshold of 70 and 95–96%, for prokaryotic species delineation (Table 1), respectively [26–30].

PHYSIOLOGY AND CHEMOTAXONOMY

For morphological and phenotypic characterization, pure cultures of strains 3B^T and 7B^T were obtained on Columbia horse blood agar plates after 24 h incubation at 37 °C. The purity of the cultures was confirmed by light microscopy, and they were maintained in 35% (w/v) glycerol at −20 °C. *C. ciconiae* DSM 44920^T, the closest phylogenomic neighbour, was obtained from

the German Collection of Microorganisms and Cell Cultures (DSMZ) for comparative analyses. Active cultures of these strains were maintained on DSMZ 535 medium.

The cultural properties of strains 3B^T and 7B^T were determined on trypticase soy agar (TSA; DSMZ 535), International *Streptomyces* Project (ISP) 2 (DSM 9879), GYM *Streptomyces* medium (DSMZ 65), nutrient agar (NA) and Columbia blood agar (Columbia agar supplemented with 5% defibrinated sheep blood; DSMZ 693) after 3 days of incubation at 37 °C [31]. The growth of these strains was evaluated under a wide range of temperatures (4, 10, 15, 25, 28, 30, 35, 37, 42 and 45 °C) and in the presence of different concentrations of sodium chloride (2%, 4%, 6%, 8%, 10%, 12%) using DSMZ 535 medium. These strains produced white-beige colonies on TSA, GYM, NA and Columbia blood agar media, but did not grow on ISP2 agar plates. Strain 3B^T showed poor growth on GYM and NA agar, but strain 7B^T grew well on both media. Both the strains grew very well on TSA and Columbia blood agar media and at temperatures between 28 and 37 °C. The optimum growth was observed at 30 °C on both media. Poor growth of strain 7B^T was observed at 15 °C but no growth was observed for strain 3B^T. Small, circular white-cream colonies, 1–2 mm in diameter were observed on Columbia blood agar plates after 16 h at 37 °C. Both strains were able to grow in the presence of up to 10% NaCl. Strain 3B^T was found to grow better than strain 7B^T at 8% NaCl. No growth was observed when these strains were cultured at 37 °C on DSMZ 535 medium under anaerobic conditions using an anaerobic bag system (Merck) for 5 days. The cells were Gram-stain-positive, rod-shaped and were confirmed to be acid-fast when 5-day-old cultures were stained by the Ziehl–Neelsen method [32].

The ability of strains 3B^T, 7B^T and *C. ciconiae* DSM 44920^T to metabolize a broad range of carbon and nitrogen sources and to grow in the presence of inhibitory compounds, antibiotics and different concentrations of sodium chloride were evaluated using GEN III MicroPlates and an Omnilog device (Biolog). The MicroPlates were inoculated with a bacterial suspension dissolved in an inoculation fluid C with a final transmittance of 85%. The OPM package for R, version 1.3.36 was used to analyse the resultant data [33]. These strains could be distinguished from their closest neighbour, *C. ciconiae* DSM 44920^T, in their abilities to utilize a wide range of carbon and nitrogen sources (Table 2). Unlike *C. ciconiae* DSM 44920^T, strains 3B^T and 7B^T were able to metabolize glycerol and methyl pyruvate (sugars) and L-aspartic acid, γ-amino-n-butyric acid and α-keto-butyric acid (organic acids; Table 2).

There are also some phenotypic differences between strains 3B^T and 7B^T (Table 2). Strain 7B^T was able to oxidize *N*-acetyl-D-glucosamine, L-serine, α-hydroxy-butyric acid, L-malic acid, L-glutamic acid, bromo-succinic acid and L-lactic acid. This strain could grow in the presence of 1% sodium lactate and was able to degrade pectin. None of these characteristics were observed for strain 3B^T. Strain 3B^T was not able to produce the *N*-acetyl-*β*-glucosaminidase enzyme that was reported positive for strain 7B^T [10].

Freeze-dried biomass of strains 3B^T, 7B^T and *C. ciconiae* DSM 44920^T harvested from 5-day-old cultures in DSMZ 535 broth incubated at 28 °C in a shaking incubator (200 r.p.m) was used for determining chemotaxonomic properties following standard chromatographic procedures. Diaminopimelic acid isomers of the peptidoglycan [34], whole-cell sugars [35] and polar lipid patterns [36] of the strains were determined, as previously described. Mycolic acids were extracted according to the procedure described by Minnikin and Goodfellow [37], and the carbon atom chains were determined by gas chromatography using Agilent 6890 N Network Gas Chromatograph. Cellular fatty acids extracts [38, 39], were analysed by gas chromatography and identified using the Standard Microbial Identification (MIDI) system version 4.5 and the ACTINO6 database [40].

The chemotaxonomic properties of strains 3B^T and 7B^T were coherent with their assignment to the genus *Corynebacterium* [5]. Similar to *C. ciconiae* DSM 44920^T, whole-organism hydrolysates of strains 3B^T and 7B^T contained *meso*-diaminopimelic acid and were rich in arabinose, galactose, glucose, mannose and ribose as cell-wall sugars. All three strains possessed diphosphatidylglycerol, phosphatidylinositol and phosphatidylglycerol as the major polar lipids, but several unidentified aminoglycolipids, aminolipids, glycolipids, lipids, phosphoaminoglycolipids and phosphoglycolipids were also detected (Fig S1). The predominant fatty acids (>15%) in strain 7B^T were $C_{18:1}$ ω9*c* and $C_{16:0}$ while additional $C_{18:0}$ was observed in strain 3B^T (Table S2). *C. ciconiae* DSM 44920^T also had $C_{18:1}$ *ω9c*, $C_{16.0}$ and $C_{18:0}$ as the major fatty acids. The mycolic acid profiles of strains 3B^T and 7B^T was composed of 32–36 carbon atoms, while strain DSM 44920^T was devoid of mycolic acids [41]. These results show that strains 3B^T and 7B^T are phenotypically and chemotaxonomically distinct from the closest relative *C. ciconiae* strain DSM 44920^T and should be classified as a new species.

In summary, strains 3B^T and 7B^T differ from their closest phylogenetic neighbour *C. ciconiae* DSM 44920^T in their abilities to utilize various carbon and nitrogen sources (Table 2) and in their chemotaxonomic characteristics including presence of polar lipids and proportion of fatty acids in the cell wall (Fig. S1 and Table S2). Although both strains 3B^T and 7B^T share >99% 16S rRNA gene similarity to *C. ciconiae* DSM 44920^T, phylogenomic characteristics including pairwise dDDH, ANI and AAI values clearly distinguished them to be a novel species (Fig. 2 and Table 1). Therefore, *Corynebacterium* isolates from yellow-eyed penguins are assigned to a novel species, *Corynebacterium megadyptis* sp. nov.

Strains 3B^T and 7B^T belong to two lineages in the core genome phylogeny and dDDH values between them was marginally below the species threshold of 70% [10]. However, pairwise ANI and AAI values were higher than the species threshold of 95% and only subtle differences were observed between the two lineages in the MALDI-TOF spectra [10]. Both the lineages are associated

with diphtheritic stomatitis in yellow-eyed penguins and genome content is highly conserved. Therefore, these strains represent two subspecies, for which we propose to name them as *Corynebacterium megadyptis* subsp. *megadyptis* subsp. nov. with 3B^T as the type strain and *Corynebacterium megadyptis* subsp. *dunedinense* subsp. nov. with strain 7B^T as the type strain.

DESCRIPTION OF *CORYNEBACTERIUM MEGADYPTIS* SP. NOV.

Corynebacterium megadyptis (me.ga.dyp'tis. N.L. gen. n. *megadyptis* of the penguin genus *Megadyptes*)

Aerobic, acid-fast actinobacterium, catalase-positive, rod-shaped cells. Colonies are cream-white beige in colour, circular and smooth and non-haemolytic on Columbia blood agar (1–2 mm in diameter). Optimal growth observed on DSMZ 535, DSMZ 65 and Columbia blood agar media and at 30 °C. The strains oxidize D-mannose, glycerol, methyl pyruvate, propionic acid, L-aspartic acid, γ-amino-n-butyric acid and α-*keto*-butyric acid. The cell wall is characterized by the presence of *meso*-diaminopimelic acid, and arabinose, galactose, glucose, mannose and ribose sugars. The polar lipids include diphosphatidylglycerol, phosphatidylinositol, phosphatidylglycerol and two unidentified phosphoglycolipids. The predominant fatty acids include $C_{18:1}$ *ω9c* and $C_{16:0}$. The mycolic acid profile is composed of 32–36 carbon atoms.

The type strain is 3B^T (=DSM 111184^T=NZRM 4755^T), isolated from oral lesions of hoiho, yellow-eyed penguin chicks (*Megadyptes antipodes*) in New Zealand. The 16S rRNA gene and genome sequences have been deposited in GenBank under accession numbers MW647632 and PQMV00000000, respectively. The genome size is 2.45–2.46 Mb with G+C content 62.7–62.9mol%.

DESCRIPTION OF *CORYNEBACTERIUM MEGADYPTIS* SUBSP. *MEGADYPTIS* SUBSP. NOV.

Corynebacterium megadyptis subsp. *megadyptis* (me.ga.dyp'tis. N.L. gen. n. *megadyptis* of the penguin genus *Megadyptes*).

The description is as described for the species with following additional characteristics.

Poor growth on GYM and NA agar. Weak growth in presence of lithium chloride and the antimicrobial aztreonam. The strain is able to oxidize dextrin but shows a weak reaction for D-fructose, D-galactose, trehalose, turanose and lithium chloride. Polar lipids include additional unidentified glycolipids and lipid. The main fatty acids are $C_{18:1}$ *ω9c*, $C_{18:0}$ and $C_{16:0}$. The type strain is 3B^T (=DSM 111184^T=NZRM 4755 ^{T}) and was isolated from a lesion in the oral cavity of a yellow-eyed penguin (*Megadyptes antipodes*) chick from the Highcliff region of the Otago Peninsula in New Zealand. The genome is 2.45 Mb in size with G+C content 62.9mol%.

DESCRIPTION OF *CORYNEBACTERIUM MEGADYPTIS* SUBSP. *DUNEDINENSE* SUBSP. NOV.

Corynebacterium megadyptis subsp. *dunedinense* (du.ne.din.en'se. N.L. neut. adj. *dunedinense* pertaining to the major city Dunedin, near the Otago Peninsula in New Zealand, close to the penguin nesting sites).

The description is as described for the species with following additional characteristics.

The strain can grow well on GYM and NA agar as well as in presence of lithium chloride and the antimicrobials aztreonam and nalidixic acid. The strain can oxidize D-fructose, D-galactose, trehalose and turanose, but is weakly positive for utilizing dextrin. The predominant fatty acids are $C_{18:1}$ ω9*c* and $C_{16:0}$.

The type strain, 7B^T (=DSM 111183^T=NZRM 4756 ^{T}), was isolated from a lesion in the oral cavity of a hoiho, yellow-eyed penguin (*Megadyptes antipodes*) chick from the Highcliff region of the Otago Peninsula in New Zealand. The size of genome is 2.46 Mb with the G+C content of 62.7mol%.

Funding Information
This work received no specific grant from any funding agency.

Acknowledgements
We thank Professor Aharon Oren for his advice on nomenclature and help with the new species and subspecies names. This research was undertaken as part of a management project facilitated by the New Zealand Department of Conservation (DOC). In undertaking this research on yellow-eyed penguins/hoiho, we acknowledge DOC's treaty partner, Te Rūnanga o Ngāi Tahu, and their kaitiakitanga. We are thankful to Ms. Gabriele Pöter and Ms. Marlen Jando (Leibniz Institute DSMZ–German Collection of Microorganisms and Cell Cultures, 38124 Braunschweig, Germany) for their help with the growth cultures and Dr David Wilkinson formerly of Massey University for help with genomic analyses.

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

Ethical statement
This study does not involve any animal handling or animal experimentation. The swabs from yellow-eyed penguins (hoiho) were collected as a part of another study (Saunderson *et al*. 2021). In this study, previously isolated *Corynebacterium* strains were characterized using chemotaxonomic, phenotypic and phylogenomic approaches. Therefore, ethical approval to work with animals is not relevant to this manuscript.

References

1. Lehmann KB, NeumannRO. *Lehmann's Medizin, Handatlanten X - Atlas und Grundriss der Bakteriologie und Lehrbuch der speziellen bakteriologischen Diagnostik*. München: Verlag Von J. F. Lehmann; 1907.
2. Janke A. *Allgemeine technische Mikrobiologie: T*. Steinkopff, 1924.
3. Kruse W. Die Mikroorganismen. Leipzig: F. C. W. Vogel; 1886.
4. Lehmann KB, Neumann RO. *Atlas und grundriss der bakteriologie und lehrbuch der speziellen bakteriologischen diagnostik*. München: J. F. Lehmann; 1896.
5. Bernard KA, Funke G, *et al*. Corynebacterium. In: Whitman WB, Rainey F, Kämpfer P, Trujillo M and J C (eds). *Bergey's Manual of Systematics of Archaea and Bacteria*. John Wiley & Sons, Inc; 2015. pp. 1–70.
6. Dover LG, Thompson AR, Sutcliffe IC, Sangal V. Phylogenomic reappraisal of fatty acid biosynthesis, mycolic acid biosynthesis and clinical relevance among members of the genus *Corynebacterium*. *Front Microbiol* 2021;12:802532.
7. Ikeda M, Nagashima T, Nakamura E, Kato R, Ohshita M, *et al*. *In Vivo* roles of fatty acid biosynthesis enzymes in biosynthesis of biotin and α-lipoic acid in *Corynebacterium glutamicum*. *Appl Environ Microbiol* 2017;83:e01322-17.
8. Oliveira A, Oliveira LC, Aburjaile F, Benevides L, Tiwari S, *et al*. Insight of Genus *Corynebacterium*: ascertaining the role of pathogenic and non-pathogenic species. *Front Microbiol* 2017;8:1937.
9. Sangal V, Burkovski A. Insights into old and new foes: pangenomics of *Corynebacterium diphtheriae* and *Corynebacterium ulcerans*. In: Barh D, Soares S, Tiwari S and Azevedo V (eds). *Pan-Genomics: Applications, Challenges, and Future Prospects*. London: Academic Press; 2020. pp. 81–100.
10. Saunderson SC, Nouioui I, Midwinter AC, Wilkinson DA, Young MJ, *et al*. Phylogenomic characterization of a novel *Corynebacterium* species associated with fatal diphtheritic stomatitis in endangered yellow-eyed penguins. *mSystems* 2021;6:e0032021.
11. Alley MR, Hunter SA, Howe LJ, Sijbranda DC, Gartrell BD. Severe mortalities in yellow-eyed penguins, megadyptes antipodes, during the 2012-2013 breeding season. *Kokako* 2013;20:20.
12. Alley MR, Suepaul RB, McKinlay B, Young MJ, Wang J, *et al*. Diphtheritic stomatitis in yellow-eyed penguins (*Megadyptes antipodes*) in New Zealand. *J Wildl Dis* 2017;53:102–110.
13. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 1991;173:697–703.
14. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* 1977;74:5463–5467.
15. Yoon S-H, Ha S-M, Kwon S, Lim J, Kim Y, *et al*. Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int J Syst Evol Microbiol* 2017;67:1613–1617.
16. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004;32:1792–1797.
17. Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol* 2015;32:268–274.
18. Letunic I, Bork P. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res* 2021;49:W293–W296.
19. Montero-Calasanz MDC, Meier-Kolthoff JP, Zhang D-F, Yaramis A, Rohde M, *et al*. Genome-scale data call for a taxonomic rearrangement of *Geodermatophilaceae Front Microbiol* 2017;8:2501.
20. Beye M, Fahsi N, Raoult D, Fournier PE. Careful use of 16S rRNA gene sequence similarity values for the identification of *Mycobacterium* species. *New Microbes New Infect* 2018;22:24–29.
21. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* 2015;25:1043–1055.
22. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014;30:2068–2069.
23. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, *et al*. Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 2015;31:3691–3693.
24. Tange O. GNU Parallel - The Command-Line Power Tool. *login: The USENIX Magazine*;2011:42–47.
25. Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 2013;14:60.
26. Moore WEC, Stackebrandt E, Kandler O, Colwell RR, Krichevsky MI, *et al*. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* 1987;37:463–464.
27. Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, *et al*. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *Int J Syst Evol Microbiol* 2007;57:81–91.
28. Richter M, Rosselló-Móra R. Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci U S A* 2009;106:19126–19131.
29. Sangal V, Goodfellow M, Jones AL, Schwalbe EC, Blom J, *et al*. Next-generation systematics: an innovative approach to resolve the structure of complex prokaryotic taxa. *Sci Rep* 2016;6:38392.
30. Sangal V, Goodfellow M, Jones AL, Seviour RJ, Sutcliffe IC. Refined systematics of the genus *Rhodococcus* based on whole genome analyses. In: Alvarez H (eds). *Biology of Rhodococcus*. Cham: Springer; 2019.
31. MacFaddin JF. *Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria*. Baltimore: Williams and Wilkins; 1985.
32. Gordon RE. The taxonomy of soil bacteria. In: Grey TRG and Parkinson D (eds). *The Ecology of Soil Bacteria*. Liverpool: Liverpool University Press; 1967. pp. 293–321.
33. Vaas LAI, Sikorski J, Hofner B, Fiebig A, Buddruhs N, *et al*. opm: an R package for analysing OmniLog(R) phenotype microarray data. *Bioinformatics* 2013;29:1823–1824.
34. Staneck JL, Roberts GD. Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* 1974;28:226–231.
35. Lechevalier MP, Lechevalier H. Chemical composition as a criterion in the classification of aerobic actinomycetes. *Int J Syst Evol Micr*1970;20:435–443.
36. Minnikin DE, O'Donnell AG, Goodfellow M, Alderson G, Athalye M, *et al*. An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J Microbiol Methods* 1984;2:233–241.
37. Minnikin DE, Goodfellow M. Lipid composition in the classification and identification of nocardiae and related taxa. In: Goodfellow M, Brownell GH and Serrano JA (eds). *The Biology of the Nocardiae*. London: Academic Press; 1976. pp. 160–219.
38. Miller LT. Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids. *J Clin Microbiol* 1982;16:584–586.
39. Kuykendall LD, Roy MA, O'neill JJ, Devine TE. Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. *Int J Syst Evol Micr*1988;38:358–361.
40. Sasser M. MIDI Technical Note 101. Identification of bacteria by gas chromatography of cellular fatty acids. Newark, DE: MIDI; 1990
41. Fernández-Garayzábal JF, Vela AI, Egido R, Hutson RA, Lanzarot MP, *et al*. *Corynebacterium ciconiae* sp. nov., isolated from the trachea of black storks (*Ciconia nigra*). *Int J Syst Evol Microbiol* 2004;54:2191–2195.