Erwinia oleae* sp. nov., isolated from olive knots caused by *Pseudomonas savastanoi* pv. *savastanoi

Chiaraluce Moretti*[1], Taha Hosni*[1], Katrien Vandemeulebroecke[2], Carrie Brady[3], Paul De Vos[2], Roberto Buonaurio[1] and Ilse Cleenwerck[2]

* C. Moretti and T. Hosni contributed equally to this work

[1] Dipartimento di Scienze Agrarie e Ambientali, University of Perugia, Perugia, Italy

[2] BCCM/LMG Bacteria Collection, Ghent University, Ghent, Belgium

[3] LM-UGent, Laboratory of Microbiology, Faculty of Sciences, Ghent University, Ghent, Belgium

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Correspondence: Chiaraluce Moretti, chiaraluce.moretti@unipg.it

The GenBank accession number for the 16S rRNA gene sequence of DAPP-PG 531^T (= LMG 25322^T = DSM 23398^T) is GU810925. The accession numbers for the *atpD*, *gyrB*, *infB* and *rpoB* gene sequences of DAPP-PG 531^T, DAPP-PG 672 (= LMG 25321) and CECT 5264 (= LMG 25328) are GU991653-GU991656, HM439616-HM439619, HM439612-HM439615 respectively.

Summary

Three endophytic bacterial isolates were obtained in Italy from olive knots caused by *Pseudomonas savastanoi* pv. *savastanoi*. Phenotypic tests in combination with 16S rRNA gene sequence analysis indicated a phylogenetic position of these isolates in the genus *Erwinia* or *Pantoea*, and revealed two other strains with highly similar 16S rRNA gene sequences (> 99 %), CECT 5262 and CECT 5264, obtained in Spain from olive knots. Rep-PCR DNA fingerprinting of the five strains from olive knots with BOX, ERIC and REP primers revealed three groups of profiles that were highly similar to each other. Multilocus sequence analysis (MLSA) based on concatenated partial *atpD*, *gyrB*, *infB* and *rpoB* gene sequences, indicated that the strains constitute a single novel species in the genus *Erwinia*. The strains showed general phenotypic characteristic of *Erwinia*, and whole genome DNA-DNA hybridization data confirmed they represent a single novel *Erwinia* species. The strains showed a DNA G+C base composition ranging from 54.7 to 54.9 mol%. They could be discriminated from the phylogenetically related *Erwinia* species by their ability to utilise potassium gluconate, L-rhamnose and D-arabitol, but not glycerol, inositol and D-sorbitol. The name *Erwinia oleae* (type strain DAPP-PG 531^T = LMG 25322^T = DSM 23398^T) is proposed for this new taxon.

Knot formation on olive trees (*Olea europaea* L.) is a serious disease found in many olive producing areas. It is caused by *Pseudomonas savastanoi* pv. *savastanoi* and characterized by outgrowth on trunks and branches, and less frequently leaves and fruits (Sisto *et al*., 2004). Olive knots are ideal niches for bacterial growth, not only of the causal agent of the disease, but also of a number of endophytic *Gammaproteobacteria* such as *Erwinia toletana* (Rojas *et al*., 2004), *Pantoea agglomerans* (Marchi *et al*., 2006; Quesada *et al*., 2007) and other bacteria from the genera *Burkholderia*, *Hafnia*, *Pseudomonas* and *Stenotrophomonas* (Ouzari *et al*., 2008). In the last years, several studies have focussed on the effect of these endophytes in modulating olive knot disease severity (Marchi *et al*., 2006; Hosni, 2010). As such, it has been shown that *P. agglomerans*, frequently isolated from olive knots when inoculated in olive plants together with *P. savastanoi* pv. *savastanoi*, can either depress growth of the pathogen or produce an increase in knot size (Marchi *et al*., 2006).

In the present study, five endophytic strains from olive knots (DAPP-PG 531^T, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264) were investigated using a polyphasic taxonomic approach.

Strains

In September 2003 and May 2007, young knots from branches of diseased olive trees located in orchards at Scanzano in the province of Perugia (Umbria, Central Italy) and Valenzano in the province of Bari (Apulia, South Italy) were collected. Small portions of their internal water-soaked tissue were excised with a scalpel and crushed in a few drops of sterile distilled water. Subsequently, a loopful of these suspensions was streaked onto nutrient agar (NA; Oxoid Ltd, UK) and the plates incubated at 27 ± 1 °C for 2 days. Along with circular (0.5–2.9 mm in diameter), white to pale yellow colonies, resembling 'fried egg', typical for *P. savastanoi* pv. *savastanoi*, another bacterial colony type was frequently isolated. The latter type was selected for further investigation. Pure cultures of this type were obtained by picking up a single colony and streaking it onto NA plates amended with 5 % of sucrose. This way, strains DAPP-PG 531^T and DAPP-PG 537 were obtained in 2003 and strain DAPP-PG 672 in 2007. Initial microbiological characterization of these strains revealed that their colonies were not fluorescent when cultivated on King's medium B (Peptone 20 g l^{-1}, anhydrous K_2HPO_4 1.5 g l^{-1}, $MgSO_4$ 1.5 g l^{-1}, glycerol 10 ml l^{-1}, agar 15 g l^{-1}). It also revealed that their cells were Gram-negative (as they lysed in 3 % KOH; Suslow *et al*., 1982), oxidase negative, catalase positive and facultatively anaerobic suggesting they belong to the family *Enterobacteriaceae*. Additional strains used in this study were obtained from various biological

resource centres, and cultivated following the instructions of the provider. All bacterial strains used in this study are listed in Supplementary Table 1.

16S rRNA gene sequence analysis

Genomic DNA was extracted from strain DAPP-PG 531^T according to the protocol of Niemann *et al*. (1997). Amplification of the 16S rRNA gene was performed with the conserved primers 16F27 (5' AGAGTTTGATCCTGGCTCAG 3') and 16R1522 (5' AAGGAGGTGATCCAGCCGCA 3'). Purification of the amplification product was done with the NucleoFast® 96 PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). Sequencing reactions were performed with the internal primers listed by Coenye *et al*. (1999) using the BigDye® Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Purification of the sequencing reaction products was done using the BigDye® XTerminatorT Purification kit (Applied Biosystems, Foster City, CA, USA). Sequencing was performed using an ABI Prism® 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence assembly was done using the software package BioNumerics (Applied Maths, Belgium). A nearly complete 16S rRNA gene sequence (1494 nt) was obtained for strain DAPP-PG 531^T and compared with 16S rRNA gene sequences deposited at NCBI, using BLAST. This analysis indicated that the strain belonged to the genera *Erwinia* or *Pantoea,* and it revealed two strains with very similar 16S rRNA gene sequences (> 99 % pairwise similarity), '*Pantoea oleae*' CECT 5262 and CECT 5264 that were obtained in Spain from olive knots. Using the software package BioNumerics (Applied Maths, Belgium), the nearly complete 16S rRNA gene sequences of DAPP-PG 531^T, CECT 5262 and CECT 5264 were compared with those of reference strains of the species of *Erwinia*, *Pantoea* and related taxa collected from EMBL. Pairwise similarities were calculated using an open gap penalty of 100 % and a unit gap penalty of 0 %. A neighbour-joining phylogenetic tree (Fig. 1) was constructed using BioNumerics, and the robustness of the branches was evaluated by bootstrap analysis (Felsenstein, 1985). A maximum-likelihood phylogenetic tree was constructed (Supplementary Fig. 1) as described previously (Brady *et al*., 2008). The three strains from olive knots showed more than 99 % 16S rRNA gene sequence similarity among each other, and less than 97 % to the known *Erwinia* and *Pantoea* species. As strains showing less than 97% 16S rRNA gene sequence similarity are not likely to have more than 60 to 70 % DNA-DNA relatedness (Stackebrandt & Goebel, 1994), these similarity values strongly suggested that the strains from olive knots represented at least one novel species in the family *Enterobacteriaceae*.

Rep-PCR DNA fingerprinting

Genomic DNA was extracted from strains DAPP-PG 531^T, DAPP-PG 537, DAPP-PG 672, CECT 5262, CECT 5264 and from *E. toletana* CFBP 6631^T with the GenElute Bacterial Genomic DNA Kit (Sigma Aldrich, St Louis, MO, USA). Rep-PCR fingerprinting was performed with the BOX (Versalovic *et al*., 1994), ERIC (Hulton *et al*., 1991) and REP (Higgins *et al*., 1982; Versalovic *et al*., 1991) primers, according to the method described by Rademaker and de Bruijn (1997). Repeats were performed, and identical results were obtained. The rep-PCR profiles are shown in Supplementary Fig. 2. Irrespective of the primers used, strains DAPP-PG 531^T and DAPP-PG 537 generated the same fingerprints as well as strains CECT 5262 and CECT 5264. The Dice's coefficient between the two groups of strains was 0.88. Strain DAPP-PG 672 had a similarity index of 0.88 with DAPP-PG 531^T and DAPP-PG 537 and 0.94 with CECT 5262 and CECT 5264. *E. toletana* CFBP 6631^T generated different fingerprints and with low similarity (0.36) in comparison with the other tested strains. Based on previous studies (Gevers *et al*., 2001; De Vuyst *et al*., 2008), the rep-PCR data suggested that strains DAPP-PG 531^T, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264 probably constituted a single species.

Multilocus sequence analysis

Multilocus sequence analysis (MLSA) of concatenated partial *atpD, gyrB, infB* and *rpoB* gene sequences enables the differentiation of the phylogenetically related genera *Erwinia*, *Pantoea* and *Tatumella* from each other (Brady *et al*., 2008, 2009a, b, c, 2010). To refine the taxonomic position of the five strains from olive knots, partial sequences of the above-mentioned housekeeping genes were determined for three representative strains, DAPP-PG 531^T, DAPP-PG 672 and CECT 5264, and 12 reference strains from known *Erwinia* species (see Fig. 2 and Supplementary Fig. 3; the gene sequences from accession numbers HM439612 to HM439619, GU991653 to GU991656, Q3953588 to Q393635 were determined in the frame of this study). Partial fragments of the *atpD, gyrB, infB* and *rpoB* genes of these strains were amplified and sequenced using the protocol of Brady *et al*. (2008), for DAPP-PG 531^T, DAPP-PG 672 and CECT 5264 with the following modifications: i) primer atpD 08-R (5'-CCGAGCAGCGCGGAGACTTC-3') was used instead of atpD 04-R ; ii) primer infB 05-F (5'-ACGGBATGRTBACSTTCCTKG-3') was used instead of infB 03-F. The modifications were needed because technical good sequences could not be obtained with the primers atpD 04-R and infB 03-F, probably because they couldn't bind efficiently. Primers atpD 08-R and infB 05-F were designed based on sequences obtained with the primers atpD 03-F and infB 04-R, respectively. Sequence assembly was performed using the software package BioNumerics (Applied Maths, Belgium), and partial nucleotide *atpD, gyrB, infB* and *rpoB* gene sequences were concatenated and aligned with concatenated partial *atpD, gyrB, infB* and *rpoB* gene

sequences of reference strains of *Erwinia*, *Pantoea* and *Tatumella* species taken from EMBL. The software package BioNumerics (Applied Maths, Belgium) was used for this analysis, and neighbour-joining and maximum-liklihood phylogenetric trees (Fig. 2 and Supplementary Fig. 3) were constructed as described for the 16S rRNA gene. MLSA revealed that strains DAPP-PG 531^T, DAPP-PG 672 and CECT 5264 belonged to the genus *Erwinia*, and also suggested that they probably constituted a single novel species.

Phenotypic assays

Strains DAPP-PG 531^T, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264 were subjected to API 20E and API 50CHE systems (bioMérieux), according to the manufacturer's instructions. The results are presented in the species description below. API 50CHE tests were also carried out on type and reference strains of the 12 validly named *Erwinia* species. The strains studied are presented in Supplementary Fig. 4, and the data obtained were numerically analysed to reveal the phenotypic relationship between the five strains from olive knots and the validly named *Erwinia* species. A distant matrix was calculated from similarity matrices generated using the Dice's coefficient (Dice, 1945), and subjected to the unweighted pair-group method with arithmetic average (UPGMA) clustering algorithm using the NTSYSpc software (Exeter Software, New York, USA) version 2.1. A cophenetic value of 0.86 was determined for this matrix, which indicated a high goodness-of-fit. The dendrogram in Supplementary Fig. 4 revealed that strains DAPP-PG 531^T, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264 formed a very homogeneous cluster with an overall similarity of about 93 % well discriminated from the 12 currently recognized *Erwinia* species that each formed a separate cluster. It also showed that the five strains from olive knots had phenotypic features common to the genus *Erwinia*. Table 1 lists a selected number of phenotypic features that permit differentiation of strains DAPP-PG 531^T, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264 from the known *Erwinia* spp. including *Erwinia toletana*, the phylogenetically most closely related *Erwinia* species also isolated from olive knots. Table 1 also reveals that the strains can be discriminated from each *Erwinia* species, including *E. piriflorinigrans* (López *et al*., 2010), by at least two characteristics.

DNA-DNA hybridizations

To confirm whether or not strains DAPP-PG 531^T, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264 truly constituted a single novel *Erwinia* species, DNA-DNA hybridizations were performed. High-molecular mass DNA for DNA-DNA hybridization studies and DNA base composition determination was extracted using the method of Wilson (1987), with minor

modifications (Cleenwerck *et al*., 2002). DNA quantity and quality were determined by measuring the absorptions at 260, 280 and 234 nm, and only high quality DNA with A_{260}/A_{280} and A_{234}/A_{260} ratios of 1.8 – 2.0 and 0.40 - 0.60 was selected for further use. The size of the DNA was estimated by agarose gel electrophoresis. DNA-DNA hybridizations were performed with the strains DAPP-PG 531^T, DAPP-PG 672 and CECT 5264 and the type strain of *Erwinia toletana* LMG 24162^T using a modification (Goris *et al*., 1998; Cleenwerck *et al*., 2002) of the microplate method described by Ezaki *et al*. (1989). The hybridization temperature was 44 °C. Reciprocal reactions (*i. e.* A x B and B x A) were performed, and their variation was generally taken within the limits of this method (Goris *et al*., 1998). The DNA-DNA relatedness values reported are the mean of minimum 6 hybridizations. The strains DAPP-PG 531^T and DAPP-PG 672 and CECT 5264 exhibited high levels of DNA-DNA relatedness (> 80 %) amongst each other, and low levels (< 25 %) with *E. toletana* LMG 24162^T (Table 2). As levels of 60 to70% DNA-DNA relatedness are generally accepted as limit for species delineation (Wayne et al., 1987), the DNA-DNA hybridization results confirmed that the strains from olive knots represented a single novel *Erwinia* species.

The DNA G+C content of strains DAPP-PG 531^T, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264, was determined by HPLC according to the method of Mesbah *et al*. (1989), and varied from 54.7 to 54.9 mol %, which is within the range reported for the genus *Erwinia* (Hauben *et al*., 1998; Mergaert *et al*., 1999; Kim *et al*., 1999; Gardan *et al*., 2004; Rojas *et al*., 2004; Geider *et al*., 2006). The DNA G+C content range was also less than 2 %, the generally accepted range within a species.

In conclusion, based on the genotypic data (from 16S rRNA gene sequence analysis, rep-PCR DNA fingerprinting, MLSA, and DNA-DNA hybridizations) and phenotypic data obtained in this study, we propose to classify the five endophytic bacterial strains DAPP-PG 531^T, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264 from olive knots, caused by *Pseudomonas savastanoi* pv. *savastanoi*, into a novel species. The name *Erwinia oleae* sp. nov. is proposed, with DAPP-PG 531^T (= LMG 25322^T = DSM 23398^T) as the type strain.

Description of *Erwinia oleae* sp. nov.

Erwinia oleae [o´le.ae, L. gen. fem. n. *oleae* of olive (*Olea europaea*), the plant from which the bacterium was isolated].

Strains have all the characteristics of the *Enterobacteriaceae*. Cells are Gram-negative, rods, measuring 0.9 x1.5-3.0 µm; single, pairs, motile; non-spore-forming. After growing for 24-48 h on nutrient agar at 27 ± 1 °C, colonies are light-beige, circular (1-1.2 mm in diameter), convex and with entire margins. They do not produce fluorescent pigment on King's medium B. Growth in Yeast salt and Liquid 523 medium (Shaad *et al*., 2001) occurs at 36 °C, but not at 39 °C. Strains are able to grow in 5 % NaCl. The strains are facultatively anaerobic and oxidase is not produced. Results obtained with API20E (bioMérieux) indicate that strains have β-galactosidase activity, but no arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, tryptophane deaminase, phenylalanine deaminase and gelatinase (except strain DAPP-PG 672). Citrate is not utilised; hydrogen sulfide, indole and acetoin (except strains CECT 5262 and CECT 5264) are not produced. Nitrate is reduced to nitrite. Results obtained with API 50CHE (bioMérieux) indicate that strains utilise the following substrates as sole carbon sources at 27 ± 1 °C within 2 days: L-arabinose, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, N-acetylglucosamine, esculin, D-trehalose, D-arabitol, potassium gluconate and potassium 2-ketogluconate, and arbutin and salicin (except strains DAPP-PG 531^T and DAPP-PG 537). The following carbon sources are not utilised at 27 ± 1 °C within 2 days: glycerol, erythritol, D-arabinose, D-xylose, L-xylose, D-adonitol, methyl *β*-D-xylopyranoside, L-sorbose, dulcitol, inositol, D-sorbitol, methyl *α*-D-mannopyranoside, methyl *α*-D-glucopyranoside, amygdalin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, inulin, D-melezitose, D-raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, potassium 5-ketogluconate. The DNA G+C content of the five strains ranges from 54.7 to 54.9 mol % as determined by the method of Mesbah *et al*. (1989).

The type strain, DAPP-PG 531^T (= LMG 25322^T = DSM 23398^T) and DAPP-PG 537 (= LMG 25323 = DSM 23412) were isolated in Umbria (Italy) from olive knots caused by *Pseudomonas savastanoi* pv. *savastanoi*. Additional strains were isolated in Apulia (Italy) (i.e. DAPP-PG 672 = LMG 25321 = DSM 23411) and Spain (i.e. CECT 5262 = LMG 25327, CECT 5264 = LMG 25328) also from olive knots caused by *Pseudomonas savastanoi* pv. *savastanoi*.

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Table 1. Phenotypic characteristics differentiating strains of *Erwinia oleae* sp. nov. from the other *Erwinia* species.

Species: 1, *E. amylovora* LMG 2024^T; 2, *E. aphidicola* LMG 24877^T; 3, *E. billingiae* LMG 2613^T; 4, *E. mallotivora* LMG 2708^T; 5, *E. papayae* CFBP 5189^T; 6, *E. persicina* LMG 11254^T; 7, *E. psidii* LMG 7034^T; 8, *E. piriflorinigrans*; 9, *E. pyrifoliae* ICMP 14143^T; 10, *E. rhapontici* LMG 2688^T; 11, *E. tasmaniensis* LMG 25318^T; 12, *E. toletana* CFBP 6631^T; 13, *E. tracheiphila* LMG 2707^T; 14, *Erwinia oleae* sp. nov.

Test[a]	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**[c]	**9**	**10**	**11**	**12**	**13**	**14**
Nitrate reduction[b]	-	+	+	-	-	+	-	ND	-	+	-	-	-	+
Growth at 36°C in yeast salt and liquid 523 medium[b]	-	+	-	-	-	+	-	ND	-	-	-	+	-	+
Fermentation of (API 50-CHE):														
L-Arabinose	+	+	+	-	+	+	+	+	+	+	+	+	+	+
D-Mannose	-	+	+	+	+	+	+	-	-	+	-	+	+	+
Esculin	-	+	+	-	+	+	+	-	-	+	-	+	-	+
L-Rhamnose	-	+	+	-	-	+	+	-	-	+	-	-	-	+
D-Arabitol	-	-	+	-	-	-	-	-	-	-	-	+	+	+
Gluconate	-	+	-	-	+	-	-	+	-	-	-	-	-	+
2-Keto-gluconate	-	+	-	-	-	-	-	-	-	-	-	-	-	+
D-Sucrose	+	+	-	+	+	+	+	+	+	+	+	-	+	-
Glycerol	-	+	+	-	-	+	+	+	+	+	+	+	+	-
Inositol	+	+	+	-	-	+	-	+	+	+	+	+	-	-
D-Sorbitol	+	-	+	-	-	+	-	-	+	-	-	-	-	-
Xylitol	-	+	-	-	-	-	-	-	-	+	+	-	-	-

[a]The strains tested for each *Erwinia* species are given in Supplementary Table 1; [b]tests performed according to Schaad *et al*., 2001; [c]Data from López *et al*., 2010; ND, not determined.

Table 2. DNA-DNA relatedness (%) between the strains DAPP-PG 531^T, DAPP-PG 672 and CECT 5264 of *Erwinia oleae* sp. nov. and the type strain of *Erwinia toletana* LMG 24162^T.

	DNA-DNA relatedness (%) with strain*:			
Strain	**1**	**2**	**3**	**4**
1. *E. oleae* DAPP-PG 672	100			
2. *E. oleae* DAPP-PG 531^T	88 ± 6	100		
3. *E. oleae* CECT 5264	88 ± 15	89 ± 16	100	
4. *E. toletana* LMG 24162^T	17 ± 4	14 ± 5	20 ± 5	100

*Each value is the mean of minimum 6 hybridizations ± SD.

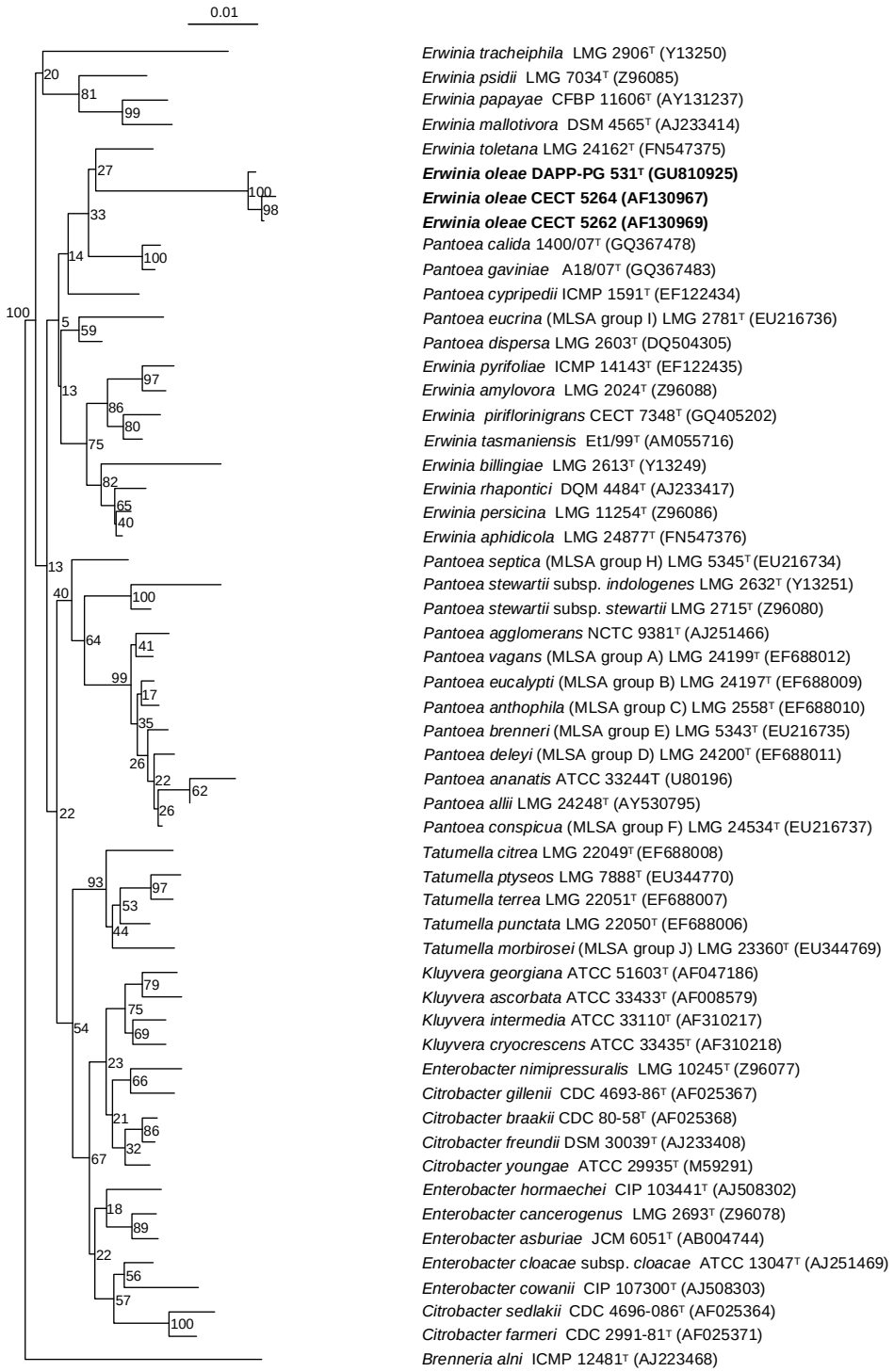

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing the phylogenetic relationship between *Erwinia oleae* sp. nov. and related taxa within the *Enterobacteriaceae* family. *Brenneria alni* ICMP 12481^{T} was used as outgroup. The scale bar indicates 1 % nucleotide substitutions. Numbers at branching points are bootstrap percentage values based on 1000 replications.

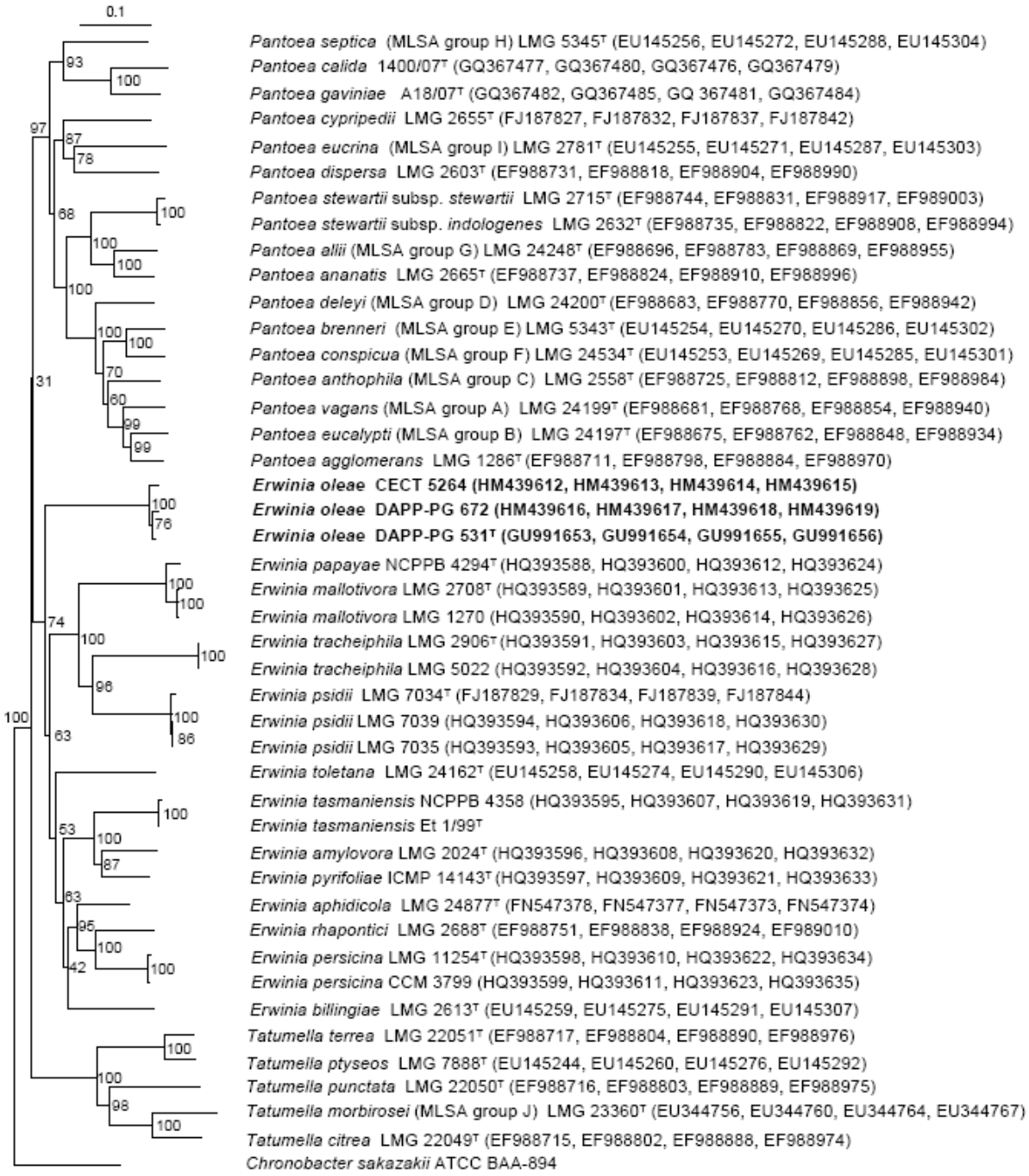

Fig. 2. Neighbour-joining tree based on concatenated partial *atpD, gyrB, infB* and *rpoB* gene sequences showing the phylogenetic relationship between *Erwinia oleae* sp. nov. and related taxa of *Erwinia*, *Pantoea* and *Tatumella*. *Cronobacter sakazakii* ATCC BAA-894 was included as outgroup. The scale bar indicates 10 % nucleotide substitutions. Numbers at branching points are bootstrap percentage values based on 1000 replications.